Using in-line milk progesterone data to characterize parameters of luteal activity and their association with fertility in Holstein cows

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ABSTRACT

Our objectives were to characterize parameters of luteal activity based on milk progesterone concentration (P4c) data from before and after artificial insemination (AI) and to evaluate their potential association with fertility in Holstein cows. Records of AI events ($n = 4,353$) and of milk P4c ($n = 158,961$) obtained through an in-line milk analysis system (Herd Navigator, DeLaval International, Tumba, Sweden) from 1,891 lactations of 1,423 Holstein cows were evaluated. Milk P4c (ng/mL) were measured every 2.2 ± 1.9 d (mean $\pm$ standard deviation) between 23.6 ± 7.3 and 185.3 ± 56.7 d in milk. Variations in milk P4c of consecutive records were used to determine onset of luteal phase (increase in P4c from <5.0 to ≥ 5.0 ng/mL), luteal phase length (period, in days, of P4c ≥ 5.0 ng/mL), cessation of luteal phase (decline from ≥ 5.0 to <5.0 ng/mL, designated as P4c-decline), and pregnancy (AI followed by a luteal phase that remained uninterrupted until 50 d post-AI). The length of the luteal phase preceding AI, the highest P4c (P4c peak) during the luteal phase preceding AI, the lowest P4c preceding AI (P4c pre-AI) that followed a P4c-decline, and the interval between P4c-decline and AI were evaluated, as well as the interval between AI and onset of luteal phase, and P4c at early diestrus (4.5 ± 0.6 d post-AI), mid diestrus (10.0 ± 0.6 d post-AI), and late diestrus (14.1 ± 0.6 d post-AI). Data were analyzed using logistic regressions, and comparisons made based on quartiles and cut-points established by receiver operating characteristic curve analysis. Overall probability of pregnancy was 32.0%. Parameters associated with reduced probability of pregnancy (represented as percentage points decrease in the probability of pregnancy) were (1) luteal phase length >14.4 d (7.6% decrease), (2) P4c peak ≤ 24.7 ng/mL (4.5% decrease), (3) P4c pre-AI >0.5 ng/mL (5.5% decrease),

(4) interval between P4c-decline and AI of >1.6 d (4.0% decrease), (5) interval between AI and onset of luteal phase of <7 or >11 d (9.3 and 12.1% decrease, respectively), and (6) P4c at early diestrus ≤ 0.7 or >3.5 ng/mL (15.2 and 6.7% decrease, respectively), (7) P4c at mid diestrus ≤ 12.4 ng/mL (12.5% decrease), and (8) P4c at late diestrus ≤ 22.7 ng/mL (9.7% decrease). The parameters of luteal activity associated with reduced probability of pregnancy established here could be used as benchmarks while developing recommendations to improve reproductive performance in herds using in-line milk progesterone monitoring.

Key words: luteal phase, ovarian activity, pregnancy, reproductive performance

INTRODUCTION

Reproductive efficiency is one of the main aspects influencing the profitability of dairy operations. An increase in the proportion of cows conceiving soon after the elective waiting period will decrease the proportion of cows with extended lactations; such cows are less profitable in later stages of lactation (Ribeiro et al., 2012). The increase in genetic merit for milk production over the past decades has been associated with an overall decrease in reproductive performance of dairy cows (Lucy, 2001). Although increased milk production per se is not necessarily detrimental to fertility (LeBlanc, 2013), the increased DMI and liver blood flow and consequently the greater metabolic clearance rate in high-producing cows is associated with reduced peripheral concentrations of steroid hormones (Sangsritavong et al., 2002). Reduced concentrations of hormones such as estradiol and progesterone (P4) affect the hypothalamus-pituitary-ovarian axis (Wiltbank et al., 2006) and uterine physiology of the cow (Geisert et al., 1992). For instance, high milk production is associated with reduced concentrations of circulating estradiol and shorter duration of estrus (Lopez et al., 2004). These factors may, at least partially, explain the poor reproductive performance reported in modern dairy herds in North America.

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To overcome poor reproductive performance, protocols for synchronization of ovulation and timed-AI (Pursley et al., 1995), estrus activity monitors (Valenza et al., 2012), and more recently, an in-line milk P4 analysis system (Friggens and Chagunda, 2005; DeLaval International, 2011) have been developed for improved reproductive management of dairy herds. The in-line milk analysis system (**IMAS**) is an electronic tool that automatically samples and quantifies milk P4 concentrations (**P4c**), on average every 2 d, using a biosensor technology. Based on variations in milk P4c, the IMAS identifies both the onset and end of luteal phases, determining estrus and pregnancy status. Although the biomodel used by the IMAS has been mainly designed to identify onset of estrus (Friggens et al., 2008; DeLaval International, 2011), the frequent milk sampling starting approximately 3 wk postpartum and continuing until pregnancy is determined (at approximately 50 d post-AI) provides an opportunity to characterize luteal activity in individual cows and evaluate its associations with fertility.

Using such data, recent studies from our research group examined associations of early postpartum luteal activity (Bruinje et al., 2017a) and pre- and post-AI P4c profiles (Bruinje et al., 2017b) with parity and fertility. Parameters such as delayed commencement of postpartum luteal activity and the occurrence of abnormal luteal phases before first AI are known to be negatively associated with fertility (Lamming and Darwash, 1998; Ranasinghe et al., 2011; Bruinje et al., 2017a). However, relationships between specific variables of luteal function obtained by an automated IMAS (such as length of the luteal phase preceding estrus, interval between variations in P4c and AI, and P4c at different time points) and fertility have not been investigated.

Therefore, the objectives were to characterize and evaluate variables of luteal activity and their associations with fertility in Holstein cows based on milk P4c data obtained before and after AI. Specifically, we examined the associations between (1) length of the luteal phase preceding AI, (2) interval between decline in P4c and AI, (3) interval between AI and onset of subsequent luteal phase, and (4) P4c at different time points before and after AI, and the probability of pregnancy.

MATERIALS AND METHODS

In-Line Milk Progesterone Analysis System and Records Description

Milk P4c records ($n = 195,931$) generated by an automated IMAS (Herd Navigator, DeLaval International, Tumba, Sweden, and Lattec/IS, Hillarød, Denmark) were initially obtained from a total of 2,264 Holstein

cows (3,693 lactations) in 4 commercial dairy herds (A, B, C, and D) in Alberta, Canada, from March 2014 to December 2016. The 4 herds used the IMAS as the primary reproductive management tool, and most AI were performed at spontaneous estrus determined based on P4c profiles, as further described. Herd demographics such as number of AI events and postpartum milk P4c sampling range of the final data set are presented in Table 1. Among the AI data, 49.8% were obtained from herd B, whereas 30.4, 12.8, and 7.0% were obtained from herds A, C, and D, respectively. Overall, 41.9, 28.6, and 29.5% of the AI events were from first, second, and third or greater parity cows, respectively. On average, the first and last postpartum milk P4c were determined at 23.6 ± 7.3 and 185.3 ± 56.7 DIM (mean $\pm$ SD).

Milk P4c measurement started at 21.8 ± 4.4 , 21.3 ± 3.1 , and 21.7 ± 4.7 DIM in herds A, B, and C, respectively. In herd D, milk P4c measurement started at 42.3 ± 2.8 DIM because a management decision to adopt a prolonged elective waiting period was followed. Following first milk P4c measurement, sampling repeated at algorithm-driven intervals on average every 2.2 ± 1.9 d based on a biomodel described in detail by Friggens and Chagunda (2005) and reported elsewhere (Friggens et al., 2008; Tenghe et al., 2015; Bruinje et al., 2017a,b). In brief, the biomodel sampling frequency aims to estimate the day of estrus based on the decline in milk P4c below a default threshold, indicating the cessation of the previous luteal phase. Because of potential differences between batches of dry sticks used for the assay and in temperature/humidity, actual milk P4c are adjusted based on a model that controls for expected random noise (Friggens and Chagunda, 2005). This adjustment allows the biomodel to more accurately distinguish between high and low P4c phases, using a 5.0 ng/mL cut-off value for adjusted P4c.

The adjusted P4c values and cut-off of 5.0 ng/mL were established by the IMAS based on proprietary calculations to identify variations in P4c for each cow. The day of the decline in P4c from above to below the 5.0 ng/mL cut-off for adjusted P4c (referred to as P4c-decline) was considered as the reference point for each P4c profile to determine subsequent sampling frequency. Once a P4c-decline was determined, a notification of estrus occurred in the IMAS software and AI was recommended by the manufacturer to occur within 24 to 36 h (DeLaval International, 2011). After P4c-decline, the sampling frequency was calculated by the biomodel to trigger the subsequent samples. Then, 4 samples were expected to occur at approximately 5, 9, 14, and 18 d after P4c-decline, followed by daily samples between 18 and 25 d, or until the next P4c-decline. If P4c was above 10.0 ng/mL at 25 d after P4c-

Table 1. Summary of AI events, and descriptive statistics of herd performance and DIM to first and last milk progesterone concentration (P4c) record

Item	Herd A	Herd B	Herd C	Herd D	Overall
AI events (no.)	1,322	2,168	558	305	4,353
First P4c record ¹ (DIM)					
Mean $\pm$ SD	21.8 $\pm$ 4.4 ^a	21.3 $\pm$ 3.1 ^a	21.7 $\pm$ 4.7 ^a	42.3 $\pm$ 2.8 ^b	23.6 $\pm$ 7.3
Minimum to maximum	20.2 to 47.6	20.1 to 47.2	20.1 to 48.7	42.0 to 46.9	20.1 to 48.7
Last P4c record ² (DIM)					
Mean $\pm$ SD	171.8 $\pm$ 51.7 ^a	191.9 $\pm$ 54.5 ^b	192.3 $\pm$ 79.4 ^b	191.8 $\pm$ 44.3 ^b	185.3 $\pm$ 56.7
Minimum to maximum	68.3 to 357.4	79.6 to 393.8	80.4 to 493.6	118.7 to 334.9	68.3 to 493.6
First AI (DIM)					
Mean $\pm$ SD	68.6 $\pm$ 12.5 ^a	70.2 $\pm$ 18.8 ^a	69.4 $\pm$ 18.7 ^a	105.1 $\pm$ 17.3 ^b	73.7 $\pm$ 20.4
Minimum to maximum	23.5 to 106.5	33.5 to 179.5	29.5 to 123.5	63.5 to 169.5	23.5 to 179.5
Interval between consecutive AI (d)					
Mean $\pm$ SD	26.6 $\pm$ 6.9 ^a	26.6 $\pm$ 7.6 ^a	26.3 $\pm$ 7.2 ^a	26.8 $\pm$ 7.6 ^a	26.6 $\pm$ 7.3
Minimum to maximum	16.0 to 50.0	15.0 to 50.0	18.0 to 50.0	18.0 to 50.0	15.0 to 50.0
Milk yield ³ (kg/d)					
Mean $\pm$ SD	39.9 $\pm$ 8.7 ^a	37.7 $\pm$ 9.6 ^b	34.6 $\pm$ 8.8 ^c	42.4 $\pm$ 8.3 ^d	38.3 $\pm$ 9.4
Minimum to maximum	17.2 to 62.6	10.5 to 60.4	13.6 to 56.8	19.7 to 59.7	10.5 to 62.6

^{a-d}Different superscripts denote differences ($P \leq 0.05$) of a same variable among herds.

¹Days in milk when the first milk P4c record was obtained in the postpartum period.

²Days in milk when the last milk P4c record was obtained in the postpartum period.

³Average daily milk yield (kg/d) between 10 and 60 DIM.

decline, samples were taken every second day until P4c dropped below 10.0 ng/mL, then samples were taken daily until the next P4c-decline (in the event of non-pregnancy). Once the subsequent P4c-decline occurred, the sampling frequency was re-calculated for the next cycle. By evaluating this sampling biomodel, Friggens et al. (2008) reported 93.3% sensitivity (**Se**) and 93.7% specificity (**Sp**) for detection of estrus when comparing to P4c profiles of estruses at which AI resulted in confirmed pregnancies, although they used a different assay for determining P4c and consequently a different cut-off (4.0 ng/mL) for P4c-decline.

The biomodel was also designed to estimate pregnancy status based on P4c profiles post-AI. If an AI event had been recorded within 5 d after P4c-decline, sampling was standard until 25 d, then was taken every 2 to 3 d between 25 and 30 d, and every 5 d between 30 and 55 d after P4c-decline. If P4c remained uninterrupted above the 5.0 ng/mL cut-off until approximately 55 d, pregnancy was assumed by the IMAS and sampling stopped.

Each P4c record contained corresponding information of herd, cow, parity, sampling date and time, DIM, and actual P4c, adjusted P4c, and slope of P4c among 3 consecutive records. Chronological events were coded using Excel (Microsoft Corp., Redmond, WA) conditional algorithms to identify the first milk P4c record of the lactation, the last milk P4c record of the lactation, the sampling interval between consecutive milk P4c records, and the day of events related to variation in adjusted milk P4c (below vs. above the pre-determined cut-off of 5.0 ng/mL). The day in which

milk P4c increased above the cut-off and was followed by at least one consecutive record above the cut-off was set as the onset of luteal phase. The luteal phase length was defined as the number of days of uninterrupted adjusted P4c values above or equal to 5.0 ng/mL until a P4c-decline to less than 5.0 ng/mL, indicating the cessation of the luteal phase. The interval, in days, between the P4c-decline and onset of subsequent luteal phase was defined as the inter-luteal phase length, and the interval between 2 consecutive P4c-decline events preceding AI was defined as the cycle length.

Filtering Criteria

The total milk P4c records initially obtained included every record available from the IMAS software during the study period. Similar to previous studies evaluating milk P4c data (Friggens et al., 2008; Tenghe et al., 2015), sets of filtering criteria were applied to the data both at a lactation level and at a variable level. The filtering aimed to exclude lactations that had prolonged periods of no P4c measurements (i.e., gaps in sampling) that could occur if a cow had temporarily not been assigned to be sampled by the IMAS for management reasons, or if the system had a temporary breakdown during any time of a lactation period. If such sampling gaps were not considered, they would cause inaccurate estimation of P4c profiles.

At a lactation level, if the first P4c record was obtained later than 50 DIM, or if the outcome of AI was not known, all records from those lactations were excluded. At a variable level: (1) if there was a gap lon-

Figure 1. Milk progesterone concentration (P4c) profile of a hypothetical cow that, based on the standard cut-off value of 5.0 ng/mL for luteal activity, had an onset of luteal phase at 55 DIM (day of milk P4c measurement ≥ 5.0 ng/mL), a P4c-decline at 68 DIM, received an AI at 70 DIM, had an onset of luteal phase at 80 DIM, and had an uninterrupted luteal phase until 115 DIM. Indicators I to IV represent variables before AI: I = luteal phase length (d); II = P4c peak (ng/mL); III = P4c pre-AI (ng/mL); IV = interval between P4c-decline and AI (d). Indicators V to VIII represent variables after AI: V = interval between AI and onset of luteal phase (d); VI = P4c at early diestrus (4.5 ± 0.6 d post-AI); VII = P4c at mid diestrus (10.0 ± 0.6 d post-AI); and VIII = P4c at late diestrus (14.1 ± 0.6 d post-AI).

ger than 8 d during an ongoing luteal phase, or a gap longer than 4 d in the last 3 milk P4c records preceding a P4c-decline, all records from that luteal phase were excluded; (2) if there was a gap longer than 8 d during an inter-luteal phase, all records from that inter-luteal phase were excluded; and (3) if a luteal phase or an inter-luteal phase had only one P4c record, variables respective to those phases were excluded.

The filtering criteria also excluded any AI event (and corresponding P4c records) that did not occur based on IMAS P4c profiles due to management decisions. For instance, some cows in herd A were preassigned by the herd manager to a timed-AI protocol, and reproductive decisions occurred irrespective of the IMAS. Therefore, records of P4c corresponding to P4c profiles before and after AI events that occurred following a timed-AI protocol, or that received any hormonal intervention, were excluded. In addition, AI events and corresponding P4c records were excluded if 2 consecutive AI events occurred within the same inter-luteal phase period, if an AI was not preceded by a P4c-decline within 5 d, or if it was not followed by an onset of luteal phase.

To improve precision in estimating the luteal activity variables, those that were defined based on the interval between consecutive events (i.e., interval between onset of luteal phase and P4c-decline, interval between P4c-decline and AI, and between AI and onset of luteal phase) that showed skewness values less than -2 or

more than 2, had their extreme 1% values considered as outliers and excluded. The final data set contained 4,353 AI events respective to 1,891 lactations of 1,423 cows.

Description of Variables

Luteal Phase Length. The luteal phase length was defined as the number of days of uninterrupted adjusted P4c greater than or equal to 5.0 ng/mL until it declined to less than 5.0 ng/mL followed by AI (indicator I, Figure 1), and determined by subtracting the date of P4c-decline from the date which the luteal phase initiated.

P4c Peak. The P4c peak was defined as the highest actual P4c recorded in the last 8 d of the luteal phase preceding an AI event (indicator II, Figure 1), as the last 8 d of the luteal phase preceding AI was the period with increased sampling frequency (Table 2). Within that 8-d range, 5.6 ± 2.1 (mean $\pm$ SD) P4c records were obtained with an average sampling interval of 1.3 ± 0.6 d between records.

P4c Pre-AI. The P4c pre-AI was defined as the single record of actual P4c less than 5.0 ng/mL preceding AI, that indicated the cessation of the luteal phase preceding AI (indicator III, Figure 1).

Interval Between P4c-decline and AI. The interval, in days, between the time of P4c-decline and the

Table 2. Descriptive statistics including mean $\pm$ SD, minimum and maximum (min. to max.) values, and percentiles (25th, 50th, 75th) of interval in days between consecutive milk progesterone concentration (P4c) records within different stages of P4c profiles

Stage of P4c profile	Sampling interval (d)				
	Mean $\pm$ SD	Min. to max.	25th	50th	75th
Overall ¹ (d)	2.2 $\pm$ 1.9	0.1 to 14.9	0.7	1.3	3.7
From first P4c record to first luteal phase ² (d)	4.4 $\pm$ 2.0	0.2 to 14.0	2.7	4.4	5.9
From first luteal phase to last P4c record ³ (d)	2.1 $\pm$ 1.8	0.1 to 8.0	0.7	1.1	3.4
During luteal phases ⁴ (d)	1.9 $\pm$ 1.6	0.1 to 8.0	0.7	1.0	2.7
During inter-luteal phases ⁵ (d)	2.7 $\pm$ 2.3	0.1 to 8.0	0.7	1.5	5.0
During last 8 d of luteal phase ⁶	1.3 $\pm$ 0.6	0.4 to 3.9	0.8	1.1	1.7
Preceding P4c-decline ⁷ (d)	1.1 $\pm$ 0.6	0.2 to 3.9	0.6	0.8	1.3
From P4c-decline to first subsequent P4c record (d)	6.1 $\pm$ 1.1	1.3 to 7.5	6.0	6.3	6.5
From P4c-decline to second subsequent P4c record (d)	9.0 $\pm$ 2.4	3.7 to 13.1	7.0	7.6	11.4
From P4c-decline to third subsequent P4c record (d)	13.6 $\pm$ 2.4	5.0 to 17.7	12.0	13.0	15.7
From P4c-decline to fourth subsequent P4c record (d)	16.8 $\pm$ 2.1	6.5 to 21.6	16.1	16.7	18.0

¹Includes all data points from all cows from first to last postpartum P4c record.

²From first postpartum P4c record to the onset of first postpartum luteal phase (first increase in adjusted P4c values from a record <5.0 ng/mL to a minimum of 2 records ≥ 5.0 ng/mL).

³From the onset of first postpartum luteal phase to the last postpartum P4c record.

⁴Interval between consecutive milk P4c records during luteal phases, defined as periods of uninterrupted milk P4c ≥ 5.0 ng/mL.

⁵Interval between consecutive milk P4c records during inter-luteal phases, defined as periods of uninterrupted milk P4c <5.0 ng/mL between luteal phases.

⁶Interval between consecutive milk P4c records during the last 8 d of luteal phase.

⁷Interval between consecutive milk P4c records during the last 3 milk P4c records that preceded a P4-decline (defined as a decline in P4c from a minimum of 2 records ≥ 5.0 ng/mL to a record <5.0 ng/mL).

time of AI (indicator IV, Figure 1). Milk P4c records included information of date and time of sampling, but only date was available for AI events; thus, the time of AI was established as 1200 h because most of the AI occurred between 0800 and 1600 h.

Interval Between AI and Onset of Luteal Phase. The interval, in days, between the time of AI (d 0) and the time of onset of subsequent luteal phase (i.e., first adjusted P4c greater than or equal to 5.0 ng/mL after AI; indicator V, Figure 1). As the sampling frequency after AI was reduced and usually only 3 samples were expected during the first 15 d following AI, the interval between AI and onset of luteal phase was also categorized as occurring between 3 and 6 d, between 7 and 11 d, or beyond 12 d post-AI.

P4c at Early, Mid, and Late Diestrus. The 3 single P4c records obtained between 3 and 6 (4.5 ± 0.6), between 7 and 11 (10.0 ± 0.6), and between 12 and 15 (14.1 ± 0.6) d post-AI were defined as P4c at early, mid, and late diestrus, respectively (indicators VI, VII, and VIII, Figure 1).

Slopes in P4c (change among 3 consecutive records; ng/mL) automatically calculated by the IMAS were obtained respective to the 3 P4c records preceding AI (P4c slope pre-AI), preceding mid diestrus (P4c slope to mid diestrus), and preceding late diestrus (P4c slope to late diestrus). As no P4c record was expected between the record indicative of P4c pre-AI and the record indicative of early diestrus, the P4c slope to early diestrus

was not estimated. Additional variables evaluated were the sampling interval preceding P4c-decline, defined as the average interval (in days) between consecutive milk P4c records among the last 3 records that preceded the P4c-decline, and milk yield during AI, defined as the average daily milk yield (kg/d) in the 4 d before and after AI.

Once an AI event was entered into the software, the IMAS biomodel adjusted the sampling frequency and was programmed to obtain samples until approximately 50 d post-AI (Friggens and Chagunda, 2005) unless interrupted by a P4c-decline. Thus, similar to previous studies that evaluated IMAS data (Bruinje et al., 2017a,b), the present study determined pregnancy status exclusively based on P4c profiles post-AI. Pregnancy was declared if the post-AI luteal phase remained uninterrupted until 50 d, and nonpregnancy was declared if the luteal phase post-AI was interrupted (P4c-decline) within 50 d. During the study period, herds B, C, and D had ultrasound confirmation by the herd veterinarian at approximately 50 d post-AI in cows assumed pregnant based on P4c profiles post-AI. In herd A, pregnancies were declared exclusively based on the IMAS P4c profiles post-AI. For this herd, the accuracy of determining pregnancy at 50 d post-AI exclusively based on P4c profiles was evaluated in AI events that had the subsequent calving date available ($n = 549$). Based on the expected range of gestation length for Holstein cows (Vieira-Neto et al., 2017), a calving that

occurred 256 to 296 d after AI was retrospectively considered as the gold standard criterion for pregnancy determination. The positive and negative predictive values for pregnancy determination based on P4c profiles at 50 d post-AI was 97.2 and 98.3%, respectively, resulting in accuracy of 97.8%. In the present data set, the interval (mean $\pm$ SD) between AI and the last P4c record measured in pregnant cows was 55.5 ± 5.9 d. The average luteal phase length (mean $\pm$ SD) in cows considered nonpregnant was 13.9 ± 6.4 d.

Statistical Analysis

All analyses were performed with SAS 9.4 (Studio 3.5 platform, SAS Institute Inc., Cary, NC). The MEANS and UNIVARIATE procedures were used to obtain descriptive statistics of herd demographics, sampling frequency, and luteal activity variables. Comparisons of continuous variables respective to herd description were analyzed by mixed effects ANOVA using the GLIMMIX procedure, including cow as a random effect, post-hoc tests performed using Tukey-Kramer adjustment, and values presented as mean $\pm$ standard deviation of the mean. Relationships among continuous variables were assessed by Pearson correlation coefficient tests using the CORR procedure. To evaluate the proportion of total variance of each luteal activity variable attributed to between-animal variance, repeatability estimates were obtained by ANOVA and calculated as

$$\frac{\sigma^2 \text{ between-animal}}{(\sigma^2 \text{ between-animal} + \sigma^2 \text{ within-animal})}.$$

The outcome of interest was pregnancy outcome, which was analyzed as a binary variable (pregnant vs. nonpregnant) using logistic regression models with the GLIMMIX procedure. Event of AI was considered the experimental unit. An initial exploratory model was built including the fixed effects of herd (A, B, C, D), year (2014, 2015, 2016), season at AI [winter (December, January, February), spring (March, April, May), summer (June, July, August), fall (September, October, November)], parity (first, second, third or greater), milk yield during AI, and number of AI as a covariate. Then, multivariable models were built for each continuous variable respective to P4c profiles, which were evaluated for both linear and quadratic effects. Models included the fixed effects of year, season at AI, parity, interaction between luteal activity variable and parity, milk yield during AI, and number of AI as a covariate, in addition to herd as a random effect. Independent variables were then selected based on manual backward stepwise elimination, until all remaining variables had $P \leq 0.10$ in each final model. Odds ratio and confidence interval estimates for continuous variables were as-

sessed using one standard deviation unit offset from the mean. Curves for predicted probability of pregnancy for each luteal activity variable were generated from the respective final mixed models using the BLUP and inverse link functions.

Continuous variables were both categorized into quartiles (below 25th percentile, between 25th percentile and median, between median and 75th percentile, and above 75th percentile), and analyzed against pregnancy outcome to obtain receiver operating characteristic (ROC) curves using the LOGISTIC procedure. The ROC analysis generates a curve that accounts for Se (proportion of AI with the outcome of "pregnant" that was above a cut-point) and Sp (proportion of AI with the outcome of "nonpregnant" that was below a cut-point) to identify the optimal cut-point for each variable that best predict pregnancy. The Youden index (i.e., the point in the ROC curve that had the largest combined Se and $1 - \text{Sp}$) and the area under the curve (AUC) were obtained for each variable, and variables were categorized as below ($\leq$) or above ($>$) the cut-point.

Finally, multivariable logistic regression models were built including the fixed effects previously described, each categorized luteal activity variable, interaction between luteal activity variable and parity, and number of AI as a covariate, with herd kept as a random effect. Independent variables were selected by backward stepwise elimination, until all remaining variables had $P \leq 0.10$ in the final models. The inverse link function was used to obtain estimates on the inverse linked scale for predicted probabilities means of pregnancy, odds ratio, and confidence interval. For all comparisons, $P \leq 0.05$ was considered significant, whereas $0.05 < P \leq 0.10$ was considered a tendency.

RESULTS

Herds, Sampling, and Data Description

Mean ($\pm$ SD) DIM to first AI were similar for herds A, B, and C (68.6 ± 12.5 , 70.2 ± 18.8 , and 69.4 ± 18.7 , respectively) but greater in herd D (105.1 ± 17.3). Summary of AI events and P4c records, and descriptive statistics of herd performance such as sampling range, DIM to first postpartum AI, interval between consecutive AI, and milk yield during AI are presented in Table 1.

As previously described, the IMAS biomodel is designed to take samples for P4c measurement at predetermined intervals according to the reference point of P4c-decline. The intervals between consecutive milk P4c records obtained within different stages of P4c profiles are presented in Table 2.

An interval of greater than 2 d between consecutive P4c records during the last 3 records preceding P4c-decline occurred in 10% of the P4c profiles. All evaluated AI were preceded by a P4c-decline below the cut-off of 5.0 ng/mL for adjusted P4c, and 95% had actual P4c pre-AI less than 1.5 ng/mL.

The mean ($\pm$ SD) interval between P4c-decline and onset of luteal phase (i.e., inter-luteal phase) was 12.0 ± 3.7 d, whereas the luteal phase length averaged 13.7 ± 6.2 d. The average interval between 2 consecutive P4c-decline events that preceded AI, indicating the cycle length, was 25.6 ± 6.6 d, and the 25th, 50th, and 75th percentiles were 21.8, 23.7, and 26.7 d, respectively. Repeatability estimates ($P < 0.01$) for inter-luteal phase, luteal phase, and cycle length were 0.09, 0.16, and 0.12, respectively. Descriptive statistics of luteal activity variables used to evaluate associations with the probability of pregnancy, and respective repeatability estimates, are presented in Table 3.

Initial exploratory analysis showed that herd ($P < 0.001$), year ($P < 0.01$), and parity ($P = 0.04$) were factors influencing the probability of pregnancy. The overall predicted probability of pregnancy was 32.0% and varied ($P < 0.001$) among herds, with 34.4, 19.3, 30.8, and 49.8% probability of pregnancy for herds A, B, C, and D, respectively. The greatest number of AI events were obtained from herd B (49.8%), which had the lowest ($P < 0.001$) probability of pregnancy among herds. The probability of pregnancy was reduced ($P < 0.01$) in 2016 (28.6%) compared with 2015 (33.4%) and 2014

(36.3%). Cows of third or greater parity had reduced ($P = 0.01$) probability of pregnancy (29.9%) than first parity cows (34.5%) and tended to have reduced ($P = 0.06$) probability of pregnancy compared with second parity cows (33.7%). There were no significant effects of season, milk yield during AI, or number of AI on the probability of pregnancy, and no significant interactions between luteal activity variables before or after AI and parity were evident. Pearson correlation coefficients of relationships among variables, including milk yield during AI and lactation number, are presented in Table 4.

Variables Before AI Associated with the Probability of Pregnancy

A negative linear relationship ($P < 0.001$) was observed between luteal phase length and the probability of pregnancy (Figure 2a). Based on ROC curve analysis, the cut-point of luteal phase length that best predicted ($P < 0.001$) the probability of pregnancy was 14.4 d (Se: 0.74, Sp: 0.33, AUC: 0.54), and AI following luteal phase of >14.4 d (above the cut-point) or >15.3 d (Q4, mean 21.8 d) resulted in reduced ($P < 0.01$) probability of pregnancy (Table 5).

The P4c peak was positively associated ($P = 0.01$) with the probability of pregnancy (Figure 2b). Although with low accuracy, the cut-point of P4c peak that predicted pregnancy with the largest combined Se and Sp ($P < 0.001$) was 24.7 ng/mL (Se: 0.59, Sp: 0.47, AUC: 0.53). The probability of pregnancy was

Table 3. Descriptive statistics including mean $\pm$ SD, minimum and maximum values (min. to max.), skewness (skew), percentiles (25th, 50th, 75th), and repeatability estimates (rep) of variables associated ($P \leq 0.05$) with the probability of pregnancy

Variable	No.	Mean $\pm$ SD	Min. to max.	Skew	25th	50th	75th	Rep ¹⁰
Luteal phase length ¹ (d)	4,135	13.7 ± 6.2	1.5 to 43.3	1.8	10.0	12.4	15.3	0.16
P4c peak ² (ng/mL)	4,353	24.0 ± 3.6	5.0 to 28.0	-1.7	22.7	25.0	26.5	0.20
P4c pre-AI ³ (ng/mL)	4,353	0.6 ± 0.5	0.1 to 4.6	3.0	0.3	0.5	0.7	0.03
P4c slope pre-AI ⁴ (ng/mL)	4,352	-5.9 ± 2.0	-10.5 to -0.3	0.2	-7.4	-5.9	-4.5	0.19
P4c-decline to AI ⁵ (d)	4,352	1.9 ± 0.6	0.1 to 4.9	-0.1	1.6	1.9	2.3	—
AI to onset of luteal phase ⁶ (d)	4,348	10.1 ± 3.6	3.1 to 33.4	1.7	9.6	10.1	10.7	0.09
P4c at early diestrus ⁷ (ng/mL)	4,313	3.1 ± 4.3	0.1 to 24.9	2.7	0.7	1.5	3.5	0.07
P4c at mid diestrus ⁸ (ng/mL)	4,207	16.0 ± 7.5	0.1 to 28.0	-0.6	10.9	17.4	22.2	0.19
P4c at late diestrus ⁹ (ng/mL)	3,623	20.7 ± 5.9	0.1 to 28.0	-1.4	18.2	22.5	25.0	0.12
P4c slope to mid diestrus ⁴ (ng/mL)	4,040	1.9 ± 1.7	-4.7 to 8.7	0.3	0.8	2.0	2.7	0.07
P4c slope to late diestrus ⁴ (ng/mL)	3,623	5.1 ± 2.2	-4.2 to 10.0	-0.7	3.7	5.4	6.7	0.16

¹Length, in days, of the luteal phase that preceded AI, determined as the period of uninterrupted milk progesterone concentration (P4c) ≥ 5.0 ng/mL until a decline in P4c to <5.0 ng/mL.

²Highest milk P4c recorded in the last 8 d of the luteal phase that preceded AI.

³P4c at the day of first milk P4c record <5.0 ng/mL following cessation of the luteal phase that preceded AI.

⁴Average change in P4c (ng/mL) among the last 3 preceding P4c records.

⁵Interval between cessation of the luteal phase (P4c-decline) and AI.

⁶Interval between AI and onset of subsequent luteal phase (first P4c ≥ 5.0 ng/mL post-AI).

⁷P4c record obtained at 6.1 ± 1.1 d following P4c-decline and 4.5 ± 0.6 d post-AI (range 3 to 6 d).

⁸P4c record obtained at 13.6 ± 2.4 d following P4c-decline and 10.0 ± 0.6 d post-AI (range 7 to 11 d).

⁹P4c record obtained at 16.8 ± 2.1 d following P4c-decline and 14.1 ± 0.6 d post-AI (range 12 to 15 d).

¹⁰All repeatability estimates shown had $P < 0.01$.

Table 4. Pearson correlation coefficient (r) matrix of evaluated variables from a total of 4,353 AI events

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Luteal phase length ¹	—												
2. P4c peak ²	0.19**	—											
3. P4c pre-AI ³	-0.06**	-0.08**	—										
4. P4c slope pre-AI ⁴	-0.41**	-0.62**	0.17**	—									
5. P4c decline to AI ⁵	0.06**	0.01	-0.01	-0.07**	—								
6. AI to onset of luteal phase ⁶	-0.08**	-0.08**	0.04**	0.14**	-0.08**	—							
7. P4c at early diestrus ⁷	0.10**	-0.00	0.02	-0.05**	-0.03	-0.48**	—						
8. P4c at mid diestrus ⁸	0.07**	0.18**	-0.05**	-0.19**	0.04*	-0.50**	0.24**	—					
9. P4c at late diestrus ⁹	0.05**	0.24**	-0.11**	-0.24**	0.02	-0.40**	0.03*	0.43**	—				
10. P4c slope to mid diestrus ⁴	0.10**	0.03*	-0.05**	-0.03*	0.23**	-0.63**	0.42**	0.74**	0.30**	—			
11. P4c slope to late diestrus ⁴	0.04*	0.21**	-0.12**	-0.21**	0.05*	-0.31**	-0.14**	0.66**	0.84**	0.40**	—		
12. Sampling preceding P4c-decline ¹⁰	0.18**	-0.19**	-0.06**	0.00	0.02	-0.12**	0.25**	0.03*	-0.03	0.13**	-0.07**	—	
13. Milk yield during AI ¹¹	-0.04*	-0.19**	-0.05*	0.17**	0.07**	0.18**	-0.18**	-0.20**	-0.19**	-0.16**	-0.16**	0.00	—
14. Lactation number	-0.04*	-0.21**	0.01	0.20**	0.05*	0.18**	-0.16**	-0.21**	-0.19**	-0.16**	-0.17**	-0.01	0.47**

¹Length, in days, of the luteal phase that preceded AI, determined as the period of uninterrupted milk progesterone concentration (P4c) ≥ 5.0 ng/mL until a decline in P4c to <5.0 ng/mL.

²Highest milk P4c recorded in the last 8 d of the luteal phase that preceded AI.

³P4c at the day of first milk P4c record <5.0 ng/mL following cessation of the luteal phase (P4c-decline from ≥ 5 to <5.0 ng/mL) that preceded AI.

⁴Average change in P4c (ng/mL) among the last 3 preceding P4c records.

⁵Interval between cessation of the luteal phase (P4c-decline) and AI.

⁶Interval between AI and onset of subsequent luteal phase (first P4c ≥ 5.0 ng/mL post-AI).

⁷P4c record obtained at 6.1 ± 1.1 d following P4c-decline and 4.5 ± 0.6 d post-AI (range 3 to 6 d).

⁸P4c record obtained at 13.6 ± 2.4 d following P4c-decline and 10.0 ± 0.6 d post-AI (range 7 to 11 d).

⁹P4c record obtained at 16.8 ± 2.1 d following P4c-decline and 14.1 ± 0.6 d post-AI (range 12 to 15 d).

¹⁰Average interval, in days, between last 3 consecutive milk P4c records preceding P4c-decline.

¹¹Average daily milk yield within 4 d before and after AI.

* $P \leq 0.05$; ** $P \leq 0.001$.

Figure 2. Estimated predicted probability of pregnancy of variables before AI obtained from the final mixed models: (a) luteal phase length ($P < 0.001$), defined as the number of days of uninterrupted progesterone concentration (P4c) ≥ 5.0 ng/mL until a decline to < 5.0 ng/mL that preceded AI; (b) P4c peak ($P = 0.01$), defined as the highest P4c recorded in the last 8 d of the luteal phase preceding an AI event; (c) P4c pre-AI ($P < 0.001$), defined as the P4c at the time of P4c-decline, referred to as the first P4c < 5 ng/mL following a luteal phase and preceding AI; and (d) interval between P4c-decline and AI ($P < 0.001$). For ease of interpretation, data points are identified by herd.

decreased ($P \leq 0.05$) when P4c peak was either ≤ 24.7 ng/mL (below the cut-point) or ≤ 22.7 ng/mL [quartile (Q) 1, mean 18.9 ng/mL; Table 5].

A negative linear relationship ($P < 0.001$) between P4c pre-AI and the probability of pregnancy was observed (Figure 2c), as well as a quadratic relationship ($P < 0.001$) between P4c slope pre-AI and the probability of pregnancy (not shown). The ROC curve analysis revealed the largest combined Se and Sp ($P < 0.01$) at 0.5 ng/mL (Se: 0.62, Sp: 0.44, AUC: 0.53), and the probability of pregnancy was reduced ($P \leq 0.001$) when P4c pre-AI was > 0.5 ng/mL (above the cut-off) or > 0.7 ng/mL (Q4; mean 1.2 ng/mL) compared with a P4c pre-AI ≤ 0.5 ng/mL (Table 5).

Considering the above parameters of luteal activity before AI that were associated with improved probability of pregnancy, a total of 19.7% of P4c profiles had a combination of luteal phase length ≤ 14.4 d, P4c

peak > 24.7 ng/mL, and P4c pre-AI ≤ 0.5 ng/mL. The predicted probability of pregnancy of such profiles was greater (41.5 vs. 31.1%; $P < 0.001$) than that of profiles that had luteal phase length > 14.4 d, P4c peak ≤ 24.7 ng/mL, and P4c pre-AI > 0.5 ng/mL (odds ratio: 1.57, 95% CI: 1.32 to 1.87).

There was a negative linear relationship ($P < 0.001$) of interval between P4c-decline and AI with the probability of pregnancy (Figure 2d). For this variable, the largest combined Se and Sp to predict pregnancy ($P < 0.001$) was 1.6 d (Se: 0.37, Sp: 0.73, AUC: 0.57), and AI occurring beyond 1.6 d after P4c-decline resulted in reduced ($P = 0.03$) probability of pregnancy. Multiple comparisons among quartiles revealed reduced ($P < 0.01$) probability of pregnancy when the interval between P4c-decline and AI was > 2.3 (Q4; mean 2.5 d) compared with ≤ 1.6 d (Q1; mean 1.1 d; Table 5). A total of 7.2% of AI events occurred within 1 d after

Table 5. Associations between variables before AI and the predicted probability of pregnancy at AI (P/AI) for a total of 4,353 AI events

Categorized variable ¹	ROC		P/AI, % (total no.)	Odds ratio	95% CI	P-value
	AUC (Se, Sp) ²	P-value				
Luteal phase length ³ (d)						
Q1, 8.0 ^a			36.8 (1,101)	1.57	1.29 to 1.92	<0.001
Q2, 11.2 ^a			34.4 (966)	1.41	1.15 to 1.74	0.001
Q3, 13.7 ^a			33.6 (1,018)	1.36	1.11 to 1.67	0.003
Q4, 21.8 ^b			27.1 (1,050)	Referent		
Cut-point: 14.4	0.54 (0.74, 0.33)	<0.001				
≤14.4			35.4 (2,839)	1.42	1.22 to 1.67	<0.001
>14.4			27.8 (1,296)	Referent		
P4c peak ⁴ (ng/mL)						
Q1, 18.9 ^a			29.7 (1,095)	0.86	0.70 to 1.06	0.15
Q2, 24.0 ^{ab,x}			32.1 (1,075)	0.96	0.79 to 1.17	0.70
Q3, 25.8 ^{b,y}			35.8 (1,117)	1.14	0.94 to 1.38	0.19
Q4, 27.1 ^{ab}			33.0 (1,066)	Referent		
Cut-point: 24.7	0.53 (0.59, 0.47)	<0.001				
≤24.7			30.4 (2,010)	0.82	0.71 to 0.94	0.004
>24.7			34.9 (2,343)	Referent		
P4c pre-AI ⁵ (ng/mL)						
Q1, 0.3 ^a			33.8 (1,063)	1.22	1.00 to 1.49	0.05
Q2, 0.4 ^a			35.2 (1,064)	1.30	1.07 to 1.59	0.001
Q3, 0.5 ^{ab}			32.6 (1,167)	1.16	0.95 to 1.41	0.14
Q4, 1.2 ^b			29.5 (1,059)	Referent		
Cut-point: 0.5	0.53 (0.62, 0.44)	<0.01				
≤0.5			35.1 (2,537)	1.28	1.11 to 1.49	<0.001
>0.5			29.6 (1,816)	Referent		
P4c slope pre-AI ⁶ (ng/mL)						
Q1, -8.4 ^a			29.1 (1,054)	0.89	0.72 to 1.09	0.25
Q2, -6.6 ^b			34.9 (1,108)	1.16	0.95 to 1.41	0.14
Q3, -5.2 ^b			34.9 (1,080)	1.16	0.96 to 1.41	0.13
Q4, -3.3 ^{ab}			31.6 (1,110)	Referent		
Cut-point: -7.3	0.51 (0.78, 0.26)	0.35				
P4c-decline to AI ⁷ (d)						
Q1, 1.1 ^{a,x}			35.9 (1,080)	1.40	1.12 to 1.75	0.003
Q2, 1.8 ^{ab,x}			32.4 (1,092)	1.19	0.97 to 1.47	0.10
Q3, 2.0 ^{ab}			32.1 (1,078)	1.18	0.96 to 1.46	0.12
Q4, 2.5 ^{b,y}			28.6 (1,102)	Referent		
Cut-point: 1.6	0.57 (0.37, 0.73)	<0.001				
≤1.6			35.3 (1,263)	1.20	1.02 to 1.41	0.03
>1.6			31.3 (3,089)	Referent		
Sampling interval to P4c-decline ⁸ (d)						
Q1, 0.5 ^a			34.5 (1,018)	1.42	1.15 to 1.75	0.001
Q2, 0.7 ^a			35.3 (1,167)	1.48	1.21 to 1.79	<0.001
Q3, 1.0 ^a			32.7 (1,059)	1.31	1.07 to 1.60	0.01
Q4, 2.0 ^b			27.0 (1,109)	Referent		
Cut-point: 1.0	0.54 (0.66, 0.44)	<0.001				
<1.0			34.7 (2,850)	1.39	1.19 to 1.61	<0.001
≥1.0			27.7 (1,503)	Referent		

^{a-c}Different superscripts denote differences ($P \leq 0.05$) in P/AI of multiple comparisons among quartiles of same variable.

^{x-z}Different superscripts denote comparisons among quartiles of same variable tended ($P \leq 0.10$) to differ in P/AI.

¹Variables were categorized based on quartiles and receiver operating characteristic (ROC) curve analysis cut-points (below vs. above). Q1 = below 25th percentile; Q2 = between 25th and 50th percentiles; Q3 = between 50th and 75th percentiles; Q4 = above 75th percentile (Table 3). Mean value for each quartile is indicated.

²Area under the curve (AUC), sensitivity (Se), and specificity (Sp) respective to each cut-point obtained through ROC curve analysis that best predicted ($P \leq 0.05$) pregnancy. Only variables that showed a significant ROC cut-point ($AUC \geq 0.50$ and $P \leq 0.05$) to predict the probability of pregnancy were compared among cut-point groups.

³Length of the luteal phase that preceded AI, determined as the period of uninterrupted milk progesterone concentration (P4c) ≥ 5.0 ng/mL until a decline in P4c to <5.0 ng/mL.

⁴Highest progesterone concentration (P4c) recorded in the last 8 d of the luteal phase that preceded AI.

⁵P4c at the day of first milk P4c record <5.0 ng/mL following cessation of the luteal phase that preceded AI.

⁶Average change in P4c (ng/mL) among the last 3 preceding P4c records.

⁷Interval between cessation of the luteal phase (P4c-decline) and AI.

⁸Average interval between last 3 consecutive milk P4c records preceding P4c-decline.

P4c-decline, 87.1% occurred between 1.0 and 2.5 d after P4c-decline, and 5.7% occurred beyond 2.5 d after P4c-decline. The predicted probabilities of pregnancy were no different ($P = 0.30$) after an interval between P4c-decline and AI of ≤ 1.0 versus an interval of 1.0 to 2.5 d (36.0 vs. 33.0%; odds ratio: 1.14, 95% CI: 0.87 to 1.47). However, the predicted probability of pregnancy after an interval between P4c-decline and AI of 1.0 to 2.5 d was greater ($P < 0.001$) than an interval > 2.5 d (36.0 vs. 20.9%; odds ratio: 1.87, 95% CI: 1.32 to 2.65).

The ROC cut-point for the average interval, in days, among the last 3 milk P4c records preceding P4c-decline that best predicted pregnancy ($P < 0.001$) was 1.0 (Se: 0.66, Sp: 0.44, AUC: 0.54). The probability of pregnancy was decreased ($P < 0.001$) from 34.7 to 27.7% when the average sampling interval exceeded 1.0 d. Likewise, the probability of pregnancy was lower ($P \leq 0.01$) for Q4 (> 1.3 d; mean 2.0 d) compared with Q1, Q2, and Q3 (≤ 1.3 d; Table 5).

Variables After AI Associated with the Probability of Pregnancy

Predicted probability curves of pregnancy for interval between AI and onset of luteal phase and for P4c at early, mid, and late diestrus are presented in Figure 3. There was a quadratic association ($P < 0.001$) of the interval between AI and onset of luteal phase with the probability of pregnancy (Figure 3a). The greatest ($P < 0.001$) probability of pregnancy (36.3%) was observed when the onset of luteal phase (i.e., rise in P4c to greater than or equal to 5.0 ng/mL) occurred between 7 and 11 d post-AI, compared with an early (between 3 and 6 d; 27.0%) or delayed (beyond 12 d; 24.2%) onset of luteal phase post-AI (Table 6).

A quadratic association ($P < 0.01$) between P4c at early diestrus and the probability of pregnancy was observed (Figure 3b). Multiple comparisons among quartiles showed that P4c at early diestrus between 0.7 and 3.5 ng/mL (Q2 and Q3) was associated ($P < 0.01$) with increased probability of pregnancy compared with P4c ≤ 0.7 (Q1) or > 3.5 ng/mL (Q4; Table 6). The P4c at early diestrus was correlated ($P < 0.001$) with the interval between AI and onset of luteal phase ($r = -0.48$; Table 4).

A positive association ($P < 0.001$) was observed between P4c at mid diestrus and the predicted probability of pregnancy (Figure 3c). The cut-point with the largest combined Se and Sp that predicted pregnancy ($P < 0.001$) was 12.4 ng/mL, and the probability of pregnancy was reduced ($P < 0.001$) when P4c at mid diestrus was below versus above the cut-point (23.6 vs. 36.1%). Multiple comparisons among quartiles showed that the probability of pregnancy was reduced ($P <$

0.001) when P4c was ≤ 10.9 ng/mL (Q1; mean 6.2 ng/mL) compared with > 10.9 ng/mL. Nonetheless, either a low (Q1, mean -0.2 ng/mL) or a high (Q4, mean 3.8 ng/mL) P4c slope to mid diestrus was associated ($P < 0.01$) with reduced probability of pregnancy compared with a P4c slope to mid diestrus between 2.0 and 2.7 ng/mL (Q3, mean 2.4 ng/mL; Table 6).

Positive linear relationships ($P < 0.001$) of P4c at late diestrus (Figure 3d) and P4c slope to late diestrus (not shown) with the probability of pregnancy were observed. Significant ROC cut-points ($P < 0.001$) were determined for P4c at late diestrus (cut-point: 22.7 ng/mL, Se: 0.56, Sp: 0.54, AUC: 0.56) and for P4c slope to late diestrus (cut-point: 4.7 ng/mL, Se: 0.71, Sp: 0.41, AUC: 0.58). Multiple comparisons among quartiles showed reduced probability of pregnancy for P4c at late diestrus < 22.5 ng/mL ($P \leq 0.03$) and for P4c slope to late diestrus < 6.7 ng/mL ($P \leq 0.02$; Table 6).

A total of 21.4% of P4c profiles had a combination of interval between AI and onset of luteal phase between 7 and 11 d, P4c at early diestrus between 0.7 and 3.4 ng/mL, P4c at mid diestrus > 12.4 ng/mL, P4c at late diestrus > 22.7 ng/mL, P4c slope to mid diestrus > 0.4 ng/mL, and P4c slope to late diestrus > 4.7 ng/mL. The predicted probability of pregnancy of such "optimal" P4c profiles post-AI was greater (44.4 vs. 30.8%; odds ratio: 1.80, 95% CI: 1.50 to 2.16; $P < 0.001$) than that of profiles that had interval between AI and onset of luteal phase < 7 or > 11 d, P4c at early diestrus < 0.7 or > 3.4 ng/mL, P4c at mid diestrus ≤ 12.4 ng/mL, P4c at late diestrus < 22.7 ng/mL, P4c slope to mid diestrus ≤ 0.4 ng/mL, and P4c slope to late diestrus ≤ 4.7 ng/mL.

The overall prevalence of categories determined based on quartiles or ROC cut-points that were associated with reduced ($P \leq 0.05$) probability of pregnancy are presented in Figure 4. The most prevalent parameter was the interval between P4c-decline and AI > 1.6 d, which occurred in 71.0% of P4c profiles. After AI, the most prevalent parameters were P4c at late diestrus ≤ 22.7 ng/mL and P4c slope to late diestrus ≤ 4.7 ng/mL, evident in 51.4 and 38.7% of the P4c profiles evaluated, respectively.

DISCUSSION

Characterization of In-Line Milk Progesterone Profiles

The primary function of the IMAS biomodel is to estimate the day of estrus based on the decline in milk P4c, indicating the cessation of the luteal phase. The increased sampling frequency before P4c-decline observed indicates a relatively high precision of the biomodel in

Figure 3. Estimated predicted probability of pregnancy of variables before AI obtained from the final mixed models: (a) interval between AI and onset of luteal phase ($P < 0.001$), defined as the interval, in days, between the day of AI and the first progesterone concentration (P4c) ≥ 5.0 ng/mL post-AI; (b) P4c at early diestrus ($P = 0.003$), defined as the P4c record obtained at 4.5 ± 0.6 d post-AI; (c) P4c at mid diestrus ($P < 0.001$), defined as the P4c record obtained at 10.0 ± 0.6 d post-AI; and (d) P4c at late diestrus ($P < 0.001$), defined as P4c record obtained at 14.1 ± 0.6 d post-AI. For ease of interpretation, data points are identified by herd.

estimating the day of P4c-decline. A reduced sampling frequency before P4c-decline was observed in a small proportion of the P4c profiles evaluated, with only 10% of the profiles having an average interval of greater than 2 d between consecutive records before P4c-decline. In such cases, the increased interval between consecutive samples could have resulted in a reduced precision of estimating the day of P4c-decline and, consequently, subsequent variables. The final data set was based on filtering criteria applied to account for inconsistencies in the sampling frequency, as described elsewhere, that would have otherwise affected the precision of estimating the variables.

The day on which adjusted P4c values declined to below the cut-off of 5.0 ng/mL was the IMAS standard reference point for monitoring P4c profiles. Roelofs et al. (2006) reported that 5.0 ng/mL of milk P4c was comparable to a 2.0 ng/mL of plasma P4c as cut-offs of

decline in P4c to anticipate time of ovulation, and the overall correlation between milk and plasma P4c was high ($r = 0.62$). In that study, the 5.0 ng/mL cut-off for milk P4c resulted in less variation in the interval between decline in P4c and ovulation when compared with either a higher (15.0 ng/mL) or lower (2.0 ng/mL) cut-off (Roelofs et al., 2006). Nonetheless, the IMAS cut-off used in this data set seemed to have estimated the cessation of luteal phase with reasonably high precision, as the actual P4c at P4c-decline (i.e., P4c pre-AI) averaged 0.6 ± 0.5 ng/mL, with 95% of the observations being less than or equal to 1.5 ng/mL. Furthermore, the P4c change among the last 3 P4c records that preceded P4c-decline (i.e., slope pre-AI) averaged -5.9 ± 2.0 ng/mL (Table 3).

The luteal phase length (mean $\pm$ SD) preceding AI was 13.7 ± 6.2 d, similar to previous reports of 12.9 ± 5.0 (Lamming and Darwash, 1998) and 13.0 ± 11.5

d (Tenghe et al., 2015). The inter-luteal phase length averaged 12.0 ± 3.7 d and was greater than the 7.8 ± 4.3 and 9.3 ± 8.5 d reported by Lamming and Darwash (1998) and Tenghe et al. (2015), respectively. Repeat-

ability estimates for luteal and inter-luteal lengths were 0.16 and 0.09, respectively, which were greater than that of Tenghe et al. (2015), who reported estimates close to zero for both variables. The variation in inter-

Table 6. Associations between variables after AI and the predicted probability of pregnancy at AI (P/AI) for a total of 4,353 AI events

Categorized variable ¹	ROC		P/AI, % (total no.)	Odds ratio	95% CI	P-value
	AUC (Se, Sp) ²	P-value				
Interval between AI and onset of luteal phase ³ (d)						
3 to 6 d ^a			27.0 (741)	1.16	0.89 to 1.52	0.26
7 to 11 d ^b			36.3 (2,872)	1.79	1.46 to 2.20	<0.001
≥12 d ^a			24.2 (735)	Referent		
Cut-point: 13.1	0.50 (0.90, 0.17)	0.84				
P4c at early diestrus ⁴ (ng/mL)						
Q1, 0.5 ^a			24.3 (1,092)	0.72	0.59 to 0.89	0.002
Q2, 1.1 ^b			39.5 (1,117)	1.47	1.22 to 1.78	<0.001
Q3, 2.3 ^b			37.5 (1,028)	1.35	1.11 to 1.64	0.003
Q4, 8.8 ^c			30.8 (1,076)	Referent		
Cut-point: 5.9	0.48 (0.89, 0.16)	0.01				
P4c at mid diestrus ⁵ (ng/mL)						
Q1, 6.2 ^a			23.5 (942)	0.55	0.45 to 0.69	<0.001
Q2, 14.3 ^b			34.1 (1,083)	0.94	0.77 to 1.13	0.50
Q3, 19.8 ^b			36.5 (1,095)	1.04	0.86 to 1.25	0.71
Q4, 24.5 ^b			35.6 (1,087)	Referent		
Cut-point: 12.4	0.56 (0.80, 0.30)	<0.001				
≤12.4			23.6 (1,158)	0.55	0.46 to 0.65	<0.001
>12.4			36.1 (3,049)	Referent		
P4c at late diestrus ⁶ (ng/mL)						
Q1, 12.2 ^a			26.2 (902)	0.55	0.44 to 0.69	<0.001
Q2, 20.5 ^b			31.8 (905)	0.72	0.58 to 0.89	0.002
Q3, 23.9 ^c			37.0 (901)	0.91	0.74 to 1.12	0.36
Q4, 26.2 ^c			39.3 (915)	Referent		
Cut-point: 22.7	0.56 (0.56, 0.54)	<0.001				
≤22.7			28.9 (1,862)	0.65	0.56 to 0.75	<0.001
>22.7			38.6 (1,761)	Referent		
P4c slope to mid diestrus ⁷ (ng/mL)						
Q1, -0.2 ^a			26.4 (1,008)	0.78	0.63 to 0.97	0.03
Q2, 1.5 ^{bc}			34.7 (1,058)	1.16	0.95 to 1.41	0.15
Q3, 2.4 ^b			38.2 (931)	1.35	1.10 to 1.65	0.004
Q4, 3.8 ^c			31.5 (1,043)	Referent		
Cut-point: 0.4	0.53 (0.44, 0.88)	<0.01				
≤0.4			21.7 (760)	0.51	0.41 to 0.62	<0.001
>0.4			35.4 (3,280)	Referent		
P4c slope to late diestrus ⁷ (ng/mL)						
Q1, 2.0 ^a			24.4 (885)	0.45	0.36 to 0.57	<0.001
Q2, 4.6 ^b			32.5 (926)	0.68	0.55 to 0.84	<0.001
Q3, 6.1 ^b			35.8 (914)	0.79	0.64 to 0.97	0.02
Q4, 7.6 ^c			41.5 (898)	Referent		
Cut-point: 4.7	0.58 (0.71, 0.41)	<0.001				
≤4.7			26.4 (1,402)	0.58	0.50 to 0.68	<0.001
>4.7			38.1 (2,221)	Referent		

^{a-c}Different superscripts denote differences ($P \leq 0.05$) in P/AI of multiple comparisons among quartile of same variable.

¹Variables were categorized based on quartiles and receiver operating characteristic (ROC) curve analysis cut-points (below vs. above). Q1 = below 25th percentile; Q2 = between 25th and 50th percentiles; Q3 = between 50th and 75th percentiles; Q4 = above 75th percentile (Table 3). Mean value for each quartile is indicated.

²Area under the curve (AUC), sensitivity (Se), and specificity (Sp) respective to the cut-point obtained through ROC curve analysis that best predicted ($P \leq 0.05$) pregnancy. Only variables that showed a significant ROC cut-point ($AUC \geq 0.50$ and $P \leq 0.05$) to predict the probability of pregnancy were compared among cut-point groups.

³Interval between AI and onset of subsequent luteal phase [first progesterone concentration (P4c) ≥ 5.0 ng/mL post-AI], categorized as occurring between 3 and 6 d post-AI, between 7 and 11 d post-AI, or beyond 12 d post-AI.

⁴P4c record obtained at 6.1 ± 1.1 d following P4c-decline and 4.5 ± 0.6 d post-AI (range 3 to 6 d).

⁵P4c record obtained at 13.6 ± 2.4 d following P4c-decline and 10.0 ± 0.6 d post-AI (range 7 to 11 d).

⁶P4c record obtained at 16.8 ± 2.1 d following P4c-decline and 14.1 ± 0.6 d post-AI (range 12 to 15 d).

⁷Average change in P4c (ng/mL) among the last three preceding P4c records.

luteal phase lengths among studies might be explained by the different sampling frequency during P4c profiles and, consequently, different criteria in estimating this variable. In the present study, the greater inter-luteal phase length might have occurred because of the reduced sampling frequency during inter-luteal phases.

The inter-luteal phase length determined based on milk P4c profiles alone was longer than expected when considering the actual interval between luteolysis and ovulation. For instance, the interval between decline in either milk or plasma P4c and confirmation of ovulation using transrectal ultrasonography averaged 3 (Roelofs et al., 2006) to 5 d (Sartori et al., 2004), whereas the average inter-luteal phase length in the present study was 12 d. This suggests that the increase in milk P4c indicative of luteal activity occurs much later, at least 7 d after ovulation. However, such an assumption does not consider cows that could have had a delayed ovulation, which would result in increased inter-luteal phase length, as demonstrated by Sartori et al. (2004). Consequently, the late increase in milk P4c in relation to day of ovulation would explain the overall short luteal phase length observed in studies evaluating milk P4c profiles (Lamming and Darwash, 1998; Tenghe et al., 2015), when compared with the expected corpus luteum (CL) lifespan of approximately 18 d (Sartori et al., 2004).

The P4c obtained at early diestrus averaged 3.1 ± 4.3 ng/mL and was similar to the milk P4c values reported by Stronge et al. (2005) at 4 d post-AI. In the present study, P4c increased to 16.0 ± 7.5 and to 20.7 ± 5.9

ng/mL at mid diestrus and late diestrus, respectively. The P4c at early, mid, and late diestrus were determined by single P4c record available at each time point (at 4.5 ± 0.6 , 10.0 ± 0.6 and 14.1 ± 0.6 d post-AI, respectively), which varied considerably, particularly at early diestrus. Such variations in milk P4c could be attributed to components that were not evaluated here, such as milk fat (Pope et al., 1976), although Stronge et al. (2005) found no associations between milk components (fat, protein, lactose) and milk P4c at 4 to 7 d post-AI. The determination of P4c peak in the present study was based on the highest P4c obtained during the 8 d that preceded P4c-decline, when 5.6 ± 2.1 P4c records were available, on average. This likely reduced potential variations in P4c that could be attributed to components not assessed here (such as milk fat) if only a single P4c record had been used to determine P4c peak. The P4c peak averaged 24.0 ± 3.6 ng/mL and was similar to previous reports evaluating milk P4c in dairy cows (Pope et al., 1976; Roelofs et al., 2006; Bruinje et al., 2017b).

The average cycle length was 25.6 ± 6.6 d, with a repeatability estimate of 0.12 ($P < 0.01$). Similarly, Tenghe et al. (2015) reported a cycle length of 26.2 ± 13.3 d based on P4c profiles obtained by the same IMAS, but with no significant repeatability estimate. Although these data are based on P4c profiles alone, they indicate a greater variability in the estrous cycle length over the general assumption of 18 to 24 d in length (Savio et al., 1990). Such greater variation in the estrous cycle length could be associated with increased

Figure 4. Prevalence (%) of parameters of luteal activity associated ($P \leq 0.05$) with reduced probability of pregnancy among the progesterone concentration (P4c) profiles evaluated, as indicated in Tables 4 and 5.

incidence of delayed ovulations (Lamming and Darwash, 1998; Sartori et al., 2004) and the unexpectedly large variations reported in the inter-service interval (Remnant et al., 2015, 2016).

Variables Before AI Associated with the Probability of Pregnancy

Evaluations of luteal phase length in previous studies were often based on pre-determined classifications (Opsomer et al., 1998; Ranasinghe et al., 2011) or based on the general assumption that the bovine estrous cycle length ranges from 18 to 24 d (Savio et al., 1990). This latter assumption often resulted in estimates of high prevalence (of approximately 50%) of abnormal cycle length in postpartum dairy cows (Opsomer et al., 1998; Hommeida et al., 2004). Although the cut-point of 14.4 d of luteal phase length obtained through ROC analysis in the present study had low accuracy to predict the outcome of AI, it can be used as a reference point to identify prolonged luteal phases in herds monitoring P4c profiles that reduce the probability of pregnancy.

Factors reported to be associated with increased luteal phase length, such as delayed resumption of estrous cyclicity postpartum and postpartum uterine disorders (Ranasinghe et al., 2011), were also associated with reduced fertility (Ranasinghe et al., 2011; Bruinje et al., 2017a). It is possible that a prolonged luteal phase indicates an impaired or delayed spontaneous luteolysis that could be associated with health complications that were not evaluated here. For instance, Ranasinghe et al. (2011) reported that cows undergoing postpartum uterine complications arising from dystocia, retention of fetal membranes, or conditions such as endometritis, metritis, or pyometra were 5 times more likely to have a prolonged luteal phase (greater than 20 d in length). Another possibility is that a prolonged luteal phase might interfere with the biomodel's precision to detect the day of P4c-decline. Beyond a certain day of the cycle (i.e., 25 d after the previous P4c-decline), the sampling frequency is decreased. If the cessation of a prolonged luteal phase occurs during a period with reduced sampling frequency, the day of P4c-decline would be determined with reduced precision, resulting in less precise timing of AI and consequently reduced probability of pregnancy.

The importance of increased P4c during the development of the ovulatory follicle and its positive association with fertility has been studied. In this regard, Bisinotto et al. (2013) reported that cows having increased plasma P4c from 7 to 3 d preceding timed-AI either by the presence of a CL (3.4 ng/mL) or via exogenous P4 (2.7 ng/mL) had greater pregnancy per AI compared with cows with low plasma P4c (0.5 ng/mL) during

that period. In the present study, we only evaluated cows that had a luteal phase before AI and that had increased sampling frequency during the last 8 d of the luteal phase, to characterize to what extent the P4c peak during the luteal phase preceding AI could be associated with fertility.

We observed that 46.2% of the luteal phases had a P4c peak ≤ 24.7 ng/mL before AI, which was associated with decreased probability of pregnancy. Although this suggests that increased P4c in the cycle preceding AI might increase fertility, Bisinotto et al. (2015) evaluated cows that had a CL before AI and did not find improvement in fertility when plasma P4c was increased (7.4 vs. 6.2 ng/mL) from 9 to 3 d preceding timed-AI through additional supplemental P4. Although the latter study evaluated P4c in plasma rather than in milk, the correlation between P4c in plasma and milk is high (Pope et al., 1976; Roelofs et al., 2006), and intravaginal P4 supplementation increases P4c in both plasma and milk (van Cleeff et al., 1992). Similarly, Colazo et al. (2013) reported no improvements in fertility in pre-synchronized cows that received supplemental P4 during the synchronization protocol preceding AI compared with cows that were subjected to the same protocol but not supplemented with P4. These studies (Colazo et al., 2013; Bisinotto et al., 2015) suggest that P4 supplementation does not affect fertility in cows with high P4c in the cycle preceding AI.

It is possible that lower P4c peak during the luteal phase preceding AI might have caused altered luteolytic signal resulting in premature luteolysis in the subsequent luteal phase post-AI, as previously reported (Cerri et al., 2011), reducing the probability of pregnancy. In this regard, we observed a positive, but weak relationship ($P < 0.001$) between P4c peak and luteal phase length ($r = 0.19$; Table 4).

All AI events evaluated herein were preceded by a P4c-decline below 5.0 ng/mL, which indicated a spontaneous cessation of the previous luteal phase. Regardless, elevated P4c pre-AI (>0.5 ng/mL) was associated with reduced probability of pregnancy. Reasons for elevated P4c following a spontaneous cessation of luteal phase is unclear; however, it is possible that an incomplete luteolysis would drop the P4c below the cut-off value of 5.0 ng/mL yet maintain a slightly high P4c around the time of AI. Previous reports demonstrated that more than 20% of cows might experience an incomplete or inadequate luteolysis during GnRH-based synchronization protocols (Wiltbank et al., 2012), which might cause elevated circulating P4c near time of AI and consequently reduced fertility (Ambrose et al., 2015; Colazo et al., 2017). The above studies were based on cows subjected to synchronization treatments; therefore, investigating the actual incidence of incom-

plete luteolysis in spontaneously cycling cows is warranted. However, based on our results that 41.7% of AI were preceded by P4c pre-AI >0.5 ng/mL (Figure 4) and associated with reduced probability of pregnancy (Table 5), we speculate that incomplete luteolysis could be at least partially contributing to such association. Another potential contributing factor to elevated P4c pre-AI being negatively associated with the probability of pregnancy is the biomodel sampling frequency. The sampling frequency before P4c-decline was, on average, increased to daily samples (Table 2). However, 10% of the profiles had a sampling interval greater than 2 d between consecutive samples before P4c-decline, which could have contributed to an inaccurate (i.e., late) determination of the day of P4c-decline. This could result in cows having elevated P4c pre-AI, and AI occurring after ovulation. We evaluated the overall association between the sampling interval preceding P4c-decline and the P4c pre-AI, which turned out to be very weak ($r = -0.06$, $P < 0.001$). Further research is required to examine the prevalence of incomplete luteolysis based on the current IMAS biomodel, and to re-examine the standard 5.0 ng/mL cut-off to detect cessation of the luteal phase, as proposed by Adriaens et al. (2018). Increasing the sampling frequency toward the end of the luteal phase until the time of AI is likely to improve the precision of AI.

Studies have evaluated the optimal time of AI in relation to onset of estrus based on mounting or stepping activity (Dransfield et al., 1998; Stevenson et al., 2014) or in relation to expected ovulation time in cows subjected to synchronized ovulation (Pursley et al., 1998). However, the optimal interval between P4c-decline and AI is yet to be determined to maximize AI success in herds monitoring in-line P4c profiles. Bleach et al. (2004) reported an interval of approximately 3 d from spontaneous luteolysis (detected by ultrasonography) to estrus, and Roelofs et al. (2006) reported an interval of approximately 80 h between spontaneous decline in milk P4c to less than 5.0 ng/mL and ovulation, with a high variability (range 54 to 98 h) among cows.

The manufacturer's recommendation for herds using the IMAS is to inseminate cows between 24 and 36 h (i.e., 1.0 to 1.5 d) after a P4c-decline (DeLaval International, 2011). In the present study, the interval between P4c-decline and AI greater than 1.6 d resulted in reduced probability of pregnancy. It is noteworthy that the present study did not aim to test different intervals between P4c-decline and AI, and the exact time of which AI was performed was not available. Besides, the variability in the interval between P4c-decline and AI was small, and 87.1% of the AI events evaluated occurred between 1.0 and 2.5 d after P4c-decline.

Therefore, a short or long interval between P4c-decline and AI and its association with the probability of pregnancy should be cautiously interpreted. Interestingly, however, the predicted probabilities of pregnancy were not different ($P = 0.30$) whether the interval between P4c-decline and AI was ≤ 1.0 or between 1.0 and 2.5 d (36.0 vs. 33.0%).

The probability of pregnancy was decreased from 34.7 to 27.7% when the average sampling interval before P4c-decline exceeded 1.0 d. This suggests that less frequent sampling at the end of the luteal phase may be determining the day of P4c-decline later than the actual day of luteolysis. As previously discussed, this could happen if a prolonged luteal phase occurs, as the sampling frequency is reduced beyond the expected cessation of the luteal phase (i.e., beyond approximately 23 d after AI). We observed a positive association ($P < 0.001$) between luteal phase length and sampling interval preceding P4c-decline ($r = 0.18$; Table 4), meaning that a reduced sampling frequency in cows with prolonged luteal phase could contribute to reduced precision of timing of AI, consequently affecting the probability of pregnancy.

Variables After AI Associated with the Probability of Pregnancy

Increasing circulating P4c following AI is essential to prepare the uterine environment and nurture embryo development (Garrett et al., 1988); thus, lower P4c during mid and late diestrus were expected to be associated with reduced fertility. In the present study, a quadratic association of the interval between AI and onset of luteal phase with the probability of pregnancy was observed (Figure 3a). A previous study reported a negative relationship of milk P4c at 4 d post-AI with embryo survival, but a positive relationship of P4c at 5, 6, and 7 d post-AI with embryo survival (Stronge et al., 2005). Comparisons between rise in P4c occurring at 4, 5, 6, and 7 d post-AI and the probability of pregnancy were not possible here, as sampling frequency was automatically reduced during the early post-AI period. A rapid increase in P4c post-AI, which could happen when AI is delayed, would lower the chances of fertilization (Valenza et al., 2012) and result in increased P4c early post-AI. Another possibility is the occurrence of an incomplete luteolysis preceding AI, which could reduce P4c to less than 5.0 ng/mL (indicating a P4c-decline), yet result in elevated P4c near time of AI (e.g., pre-AI), and consequently a rapid increase in P4c early post-AI. A very weak association ($r = 0.04$, $P = 0.001$) between P4c pre-AI and interval between AI and onset of luteal phase was observed in the present study,

possibly due to the high variability among P4c profiles for both variables.

Lamming and Darwash (1998) analyzed milk P4c profiles in over 1,600 dairy cows and reported that 12.9% of cycles had a delayed onset of luteal phase, which was defined as an inter-luteal phase greater than or equal to 12 d long. We found a similar prevalence (16.9%) of delayed onset of luteal phase, which was negatively associated with the probability of pregnancy (Figure 3a; Table 6). A delayed onset of luteal phase post-AI will result in lower P4c at early diestrus, whereas a short interval between AI and onset of luteal phase might result in greater P4c at early diestrus (i.e., between 3 and 6 d post-AI). Both factors were associated with reduced probability of pregnancy. Lower P4c at early diestrus could be associated with the accelerated hepatic clearance rate of P4 reported in high-producing dairy cows (Sangsritavong et al., 2002), as a negative relationship between milk yield during the time of AI and milk P4c at 4 d post-AI was also reported (Stronge et al., 2005). In the present study, milk yield during AI was negatively associated with P4c at early ($r = -0.18$), mid ($r = -0.20$), and late diestrus ($r = -0.19$; Table 4).

Prevalence of Parameters Associated with Reduced Fertility and Potential Strategies to Improve Fertility

Most variables of luteal activity evaluated in the present study had significant associations with the probability of pregnancy. The cut-points obtained by ROC curve analysis had low accuracy to predict pregnancy. Nonetheless, they were used as reference points to categorize parameters negatively associated with fertility, which were often highly prevalent (Figure 4). Based on the reference points that resulted in increased probability of pregnancy, only 5.6% (182/3,234) of P4c profiles had a combination of “optimal” luteal activity parameters (i.e., within categories that were associated with improved probability of pregnancy) both before and after AI. The predicted probability of pregnancy for such P4c profiles was greater (52.2 vs. 33.3%; $P < 0.001$) than that of P4c profiles that had a combination of categories associated with reduced probability of pregnancy. However, inferences from such a comparison should be drawn cautiously due to the limited number of P4c profiles with the so called “optimal” parameters both before and after AI.

In total, 31.3% of the evaluated P4c profiles had a prolonged luteal phase (>14.4 d), and 46.2% had a P4c peak ≤ 24.7 ng/mL, both factors negatively associated with the probability of pregnancy. Evaluating specific mechanisms of these parameters affecting the probab-

ility of pregnancy was outside the scope of the present study. However, it can be hypothesized that inducing CL regression through administration of exogenous PGF_{2 α} (Pursley et al., 1995) in cows that were not inseminated in the antecedent cycle but had a luteal phase exceeding 14.4 d in length preceding AI could improve the probability of pregnancy.

In all, 41.7% of the AI events was preceded by elevated P4c pre-AI (between 0.5 and 5.0 ng/mL), and 17.0% of AI was followed by a rapid increase in P4c between 3 and 6 d post-AI, both factors associated with reduced probability of pregnancy. These factors could be related to an imprecision of the IMAS biomodel in detecting the actual day of luteolysis, affecting the precision of AI. This could occur in cows that do not follow the P4c profile expected by the biomodel (e.g., prolonged estrous cycle length or different individual thresholds of milk P4c indicative of luteal activity). Elevated P4c pre-AI and a rapid increase in P4c post-AI could also occur if a cow had an incomplete luteolysis. If elevated P4c pre-AI is at least a partial manifestation of incomplete luteolysis, it may be hypothesized that giving exogenous PGF_{2 α} when P4c pre-AI is >0.5 ng/mL will decrease P4c near the time of AI, thereby improving fertility. In support of this hypothesis, Ambrose et al. (2015) observed improved conception rates in cows that received a low dose of PGF_{2 α} (10 mg of dinoprost) concurrent with AI following a synchronization protocol. Conversely, Sauls et al. (2018) evaluated the effects of a similar PGF_{2 α} treatment administered at timed-AI and found no effects on pregnancy per AI.

In the present study, 16.9% of AI was followed by a delayed onset of luteal phase beyond 12 d post-AI, which lowered P4c at early diestrus and was negatively associated with the probability of pregnancy. In view of this finding, another potential strategy is to administer exogenous GnRH near the time of AI to synchronize ovulation (Pursley et al., 1995), to reduce the incidence of delayed onset of luteal activity post-AI and improve fertility.

Based on the ROC cut-points used to characterize variables associated with reduced fertility in the present study, the prevalence of sub-optimal P4c at mid (≤ 12.4 ng/mL) and late diestrus (≤ 22.7 ng/mL) were 27.5 and 51.4%, respectively. A moderate positive relationship ($P < 0.001$) existed between P4c at both mid and late diestrus ($r = 0.43$). Among the P4c profiles that had a sub-optimal P4c at mid diestrus, 77.0% also had sub-optimal P4c at late diestrus; both factors were associated with reduced fertility. Interestingly, the parameter that had the greatest probability of pregnancy (41.5%) was P4c slope to late diestrus of >6.7 ng/mL (Q4, Table 6), indicating that a rapid increase

rate in P4c between early and late diestrus (between approximately 5 and 14 d post-AI) is highly beneficial to fertility.

Several studies have investigated the effects of supplementing P4 post-AI on fertility and reported inconsistent results, such as positive (Garcia-Ispuerto and López-Gatius, 2017), negative (Parr et al., 2014), or no association (Colazo et al., 2013). Although the conditions among studies varied (i.e., breed, P4 dose, day of cycle when P4 was administered), our results provide an insight that cows that benefited from P4 supplementation in previous studies might be those that had sub-optimal P4c at different time points after AI. In this regard, monitoring real-time P4c profiles provides an opportunity to strategically supplement P4 in cows with sub-optimal P4c post-AI. Future studies should explore factors associated with sub-optimal P4c and evaluate potential benefits associated with strategic hormonal interventions. Moreover, refinement of the IMAS biomodel to improve its precision will also be of value to optimize reproductive performance in herds monitoring in-line milk P4c profiles.

In conclusion, we characterized parameters of luteal activity associated with reduced probability of pregnancy that could be used as benchmarks in herds monitoring in-line milk P4c profiles in dairy cows. Such parameters include prolonged luteal phase preceding AI, delayed onset of luteal phase post-AI, and sub-optimal P4c before and after AI. Recommendations such as targeted hormonal interventions remain to be tested in cows with luteal activity parameters that are negatively associated with the probability of pregnancy. The in-line milk P4 analysis system, a relatively new tool for reproductive management of dairy herds, warrants further evaluations to optimize the precision of sampling frequency and timing of AI to improve reproductive performance.

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