Use of on-farm milk progesterone information to predict fertility outcomes in dairy cows subjected to timed artificial insemination

B. O. Omontese,^{1*} G. C. Gomes,² A. R. Santos,¹ L. G. Silva,¹ V. R. Merenda,¹ and R. S. Bisinotto^{2†}

¹Department of Veterinary Population Medicine, University of Minnesota, St. Paul 55108

²Department of Large Animal Clinical Sciences, D. H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville 32610

ABSTRACT

The objectives of this study were to evaluate the use of a qualitative on-farm milk progesterone test to predict non-pregnancy in dairy cows. Lactating Jersey cows ($n = 752$) were subjected to the 5-d Cosynch-72 protocol for timed artificial insemination (AI; d -8 GnRH, d -3 and -2 PGF_{2 α} , d 0 GnRH and timed AI). Milk was sampled on d -3 , 0, 7, and 28 relative to timed AI, and progesterone concentrations were assessed using a lateral flow immunochromatographic test. Samples were classified into 3 groups indicative of high (hP4; test line not visible or lighter than reference), intermediate (iP4; test line similar to reference), and low (lP4; test line darker than reference) progesterone concentrations. Blood was sampled from a subset of cows ($n = 50$) on d -3 , 0, 7, and 28 relative to timed AI, and plasma progesterone concentrations were determined by RIA. Cows were observed daily for signs of estrus based on removal of tail paint. Pregnancy was diagnosed by ultrasonography on d 34 and 62 after AI. Plasma progesterone concentrations across all time points were greater for hP4 (3.13 ± 0.20 ng/mL) followed by iP4 (1.12 ± 0.27 ng/mL) and lP4 (0.38 ± 0.23 ng/mL). Cows in lP4 on d -3 had lesser pregnancy per AI (P/AI) compared with iP4 and hP4 (17.4, 38.3, and 37.2%, respectively). For measurements performed on the day of AI (d 0), lP4 cows had greater P/AI compared with hP4 and iP4 (34.8, 0.0, and 15.6%, respectively), and the risk of pregnancy loss tended to be greater for iP4 compared with lP4. Cows in lP4 on d 7 after AI had lesser P/AI than those in iP4 and hP4 (12.0, 34.0, and 37.7%, respectively). Cows classified as lP4 on d 28

had the least P/AI on d 62 followed by iP4 and then hP4 (0.8, 9.2, and 59.4%, respectively) and were at the greatest risk for pregnancy loss (lP4 = 74.6%, iP4 = 8.4%, hP4 = 7.1%). Sensitivity and specificity to predict non-pregnancy on d 62 were 0.86 and 0.32 (d -3), 0.95 and 0.15 (d 0), 0.93 and 0.23 (d 7), and 0.99 and 0.53 (d 28), respectively. On-farm milk progesterone profiling using a lateral flow immunochromatographic test was able to identify cows without functional corpus luteum and to predict fertility outcomes following timed AI.

Key words: corpus luteum, pregnancy, reproduction, synchronization

INTRODUCTION

Reproductive performance in dairy herds that use timed AI relies on adequate response to synchronization protocols, rapid identification of non-pregnant cows, and establishment of pregnancy following reinsemination (Galvão et al., 2013; Giordano et al., 2016). Variants of the Ovsynch protocol encompass a GnRH injection followed 5 or 7 d later by 1 or 2 injections of PGF_{2 α} , a second GnRH injection 48 to 72 h after PGF_{2 α} (i.e., sole treatment in protocols with a single PGF_{2 α} or the first of 2 PGF_{2 α} injections, both defined as PGF_{2 α} injection henceforth), and timed AI 0 to 24 h later (Pursley et al., 1998; Santos et al., 2010; Wiltbank et al., 2015). Optimal pregnancy per AI (P/AI) has been associated with ovulation following the first GnRH, presence of a functional corpus luteum (CL) at PGF_{2 α} , induction of complete luteolysis before timed AI, and synchronized ovulation 8 to 16 h after insemination (Pursley et al., 1998; Bisinotto and Santos, 2011). Despite major progress since publication of the Ovsynch protocol (Pursley et al., 1995), currently available programs are unable to control follicular growth, luteal function, and ovulation in every cow subjected to timed AI (Stevenson et al., 2008; Wijma et al., 2018).

Progesterone concentrations in blood at specific points of the timed AI protocol reflect responses to hor-

Received August 5, 2019.

Accepted February 27, 2020.

*Current address: Department of Food and Animal Sciences, Alabama A&M University, Normal, AL 35762.

†Corresponding author: rsbisinotto@ufl.edu

monal treatments and have been associated with fertility outcomes. Progesterone concentrations at PGF_{2α} are positively correlated with likelihood of pregnancy (Martins et al., 2011), and P/AI in cows with CL at PGF_{2α} detected by ultrasonography is approximately 3-fold greater than that observed in herdmates not bearing a CL (Giordano et al., 2016). This is particularly important given that 10 to 20% of lactating dairy cows lack CL at PGF_{2α} (Ayres et al., 2013; Bisinotto et al., 2015; Giordano et al., 2016). In fact, allocating cows without CL at PGF_{2α} to alternative resynchronization strategies improved P/AI and reduced overall time to pregnancy (Giordano et al., 2016; Wijma et al., 2018). Complete luteolysis following PGF_{2α} is also critical for establishment of pregnancy, and P/AI fell below 10% if progesterone concentration in plasma exceeded 0.7 ng/mL at the time of insemination (Wiltbank et al., 2014; Colazo et al., 2017). Success of timed AI also depends on synchronized induction of ovulation, which leads to formation of a new CL that must be sustained for maintenance of gestation (Thatcher et al., 1984). As a result, cows with plasma progesterone below 1.5 ng/mL on d 7 after timed AI had reduced P/AI compared with counterparts with concentrations above this threshold (Wilsdorf et al., 2016).

Progesterone profiling is an interesting strategy to identify cows that do not respond to hormonal treatments and have poor fertility following timed AI (Wilsdorf et al., 2016). In fact, specificity to predict non-pregnancy based on plasma progesterone concentrations at PGF_{2α}, AI, and 7 d after AI was above 95% (Wilsdorf et al., 2016). Low-fertility cohorts detected at PGF_{2α} or insemination could be resynchronized before timed AI, therefore reducing reproductive costs and shortening interval to pregnancy. Although the benefit of using progesterone profiling to manage reproduction in dairy herds has been demonstrated, currently available blood tests are not streamlined for on-farm use. On the other hand, qualitative assessment of progesterone concentration in milk using a lateral flow immuno-chromatographic test validated for dairy cows has the potential to generate timely information that can be incorporated into routine decision making (Waldmann and Raud, 2016). The objectives of this study were to assess plasma progesterone concentrations, P/AI, and risk of pregnancy loss in cows with different luteal status during the 5-d Cosynch-72 protocol as determined by an on-farm milk progesterone test. We hypothesize that the on-farm milk progesterone test accurately represents hormonal concentrations in plasma and allows for identification of cows that do not respond to synchronization protocols and have reduced likelihood of pregnancy following timed AI.

MATERIALS AND METHODS

Cows and Housing

This study was conducted in 2 commercial dairy herds located near Nicollet, Le Sueur County, Minnesota. The dairies milked approximately 3,000 and 3,200 Jersey cows 3 times daily with yearly rolling herd averages of 7,700 kg. In both herds, cows were housed in cross-ventilated freestall barns equipped with fans. Primiparous and multiparous cows were housed separately. Enrollment extended from September 2016 to January 2017. A total of 789 cows were enrolled initially; however, 37 cows were removed from the study due to receiving wrong injections as part of the synchronization protocol or culling before pregnancy outcomes could be evaluated. Thus, 752 cows, including primiparous (n = 339) and multiparous (n = 413), were available for statistical analyses, out of which 137 were subjected to the first AI postpartum and 615 received subsequent AI. Body condition score was evaluated in all cows using a 1-to-5 scale with 0.25-unit increments described for dairy cows (Edmondson et al., 1989). For statistical analysis, BCS was categorized as low (≤ 2.50) or moderate (≥ 2.75).

Reproductive Management

A combination of estrus detection and timed AI was used in both herds for management of reproduction. After a 35-d voluntary wait period, cows' tailheads were painted daily (Detect-Her tail paint; H&W Products, Salem, OH), and removal of tail paint associated with secondary signs was used as an indication of estrus. Cows detected in estrus were inseminated on the same day and did not receive further hormonal treatments. Complete synchronization protocol for cows inseminated to timed AI for first service postpartum consisted of a presynchronization protocol with 3 injections of PGF_{2α} (Lutalyse Sterile Solution, equivalent to 25 mg of dinoprost; Zoetis, Madison, NJ) administered 14 d apart at 41 ± 3 , 55 ± 3 , and 69 ± 3 DIM. Cows not inseminated in estrus received the 5-d Cosynch-72 protocol starting 12 d after the third PGF_{2α} injection of presynchronization, which consisted of an injection of GnRH (Cystorelin, equivalent to 86 µg of gonadorelin; Merial Ltd., Duluth, GA) followed by injections of PGF_{2α} 5 and 6 d later, and a final injection of GnRH concurrently with timed AI. Cows diagnosed non-pregnant 34 ± 3 d after previous AI were enrolled in the 5-d Cosynch-72 protocol on the same day. The day of timed AI was considered study d 0 (Figure 1). Artificial inseminations were performed by 1 technician

Figure 1. Diagram of activities and the 5-d Cosynch-72 protocol for synchronization of ovulation. Cows receiving the first service postpartum were enrolled in the timed AI protocol 12 d after a presynchronization protocol consisting of 3 PGF_{2α} injections (41 ± 3 , 55 ± 3 , and 69 ± 3 DIM; timed AI at 89 ± 3 DIM). Cows receiving subsequent services were enrolled in the timed AI protocol on the day of non-pregnancy diagnosis (34 ± 3 or 62 ± 3 d after previous AI). Milk P4 = milk sampling for qualitative assessment of progesterone profile by lateral flow immunochromatographic test; PD = pregnancy diagnosis via transrectal ultrasonography.

in herd A using frozen–thawed semen from 6 sires and 2 technicians in herd B using frozen–thawed semen from 9 sires. Dairy personnel oversaw sire selection according to each herd’s reproductive program.

Milk Sampling and Analysis of Progesterone Profile

Composite milk samples (5 mL/cow) were collected by hand stripping during the morning milking on d -3 , 0 , 7 , and 28 d relative to AI (Figure 1). Samples were collected into sterile plastic tubes and placed on ice until analysis. Characterization of milk progesterone profile was performed by immunochromatography using a lateral flow test validated for bovine samples (P4 Rapid; Biomed Diagnostics Inc., White City, OR; Waldmann and Raud, 2016). Within 2 h of collection, milk samples were homogenized by manually inverting the tube. A 500- μ L aliquot was transferred into a borosilicate test tube and exposed to test strips, which remained in contact with milk for 5 min. Color development was assessed after 5 min, and samples were classified into 1

of 3 groups indicative of high (hP4; test line not visible or lighter than reference line), intermediate (iP4; test line similar to the reference line), and low (lP4; test line darker than reference line) milk progesterone concentrations (Waldmann and Raud, 2016; Figure 2).

Pregnancy Diagnosis and Calculation of Reproductive Responses

Pregnancy was diagnosed by transrectal ultrasonography on d 34 ± 3 after AI (referred to as d 34 hereafter). The presence of an amniotic vesicle containing an embryo with a heartbeat was used as the determinant of pregnancy. Pregnant cows were re-examined by transrectal ultrasonography on d 62 ± 3 after AI (referred to as d 62 hereafter). Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant on d 34 and 62 by the number of cows receiving AI. Pregnancy loss was calculated as the number of cows that lost a pregnancy between d 34 and 62 after AI divided by the number of cows diagnosed pregnant on

Figure 2. Example of reading from different milk progesterone profiles evaluated using lateral flow immunochromatographic test. hP4 = high progesterone concentrations (test line not visible or lighter than reference); iP4 = intermediate progesterone concentrations (test line similar to reference); lP4 = low progesterone concentrations (test line darker than reference).

d 34 after AI. Cows detected in estrus before the first pregnancy diagnosis were reinseminated and considered non-pregnant to previous service. Short AI interval was defined as cows reinseminated from d 5 to 17 after the previous insemination.

Analysis of Progesterone Concentrations in Plasma

Blood was sampled from a subset of cows ($n = 50$) on d -3 , 0, 7, and 28 to quantify progesterone concentrations. Samples were collected by puncture of coccygeal vessels into evacuated tubes with K2-EDTA (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Blood tubes were placed on ice immediately after collection and centrifuged at $2,000 \times g$ for 15 min within 6 h of collection. Plasma samples were frozen at -20°C until assayed. Concentrations of progesterone in plasma were evaluated by RIA using a commercial kit (ImmuChem Coated Tube Progesterone RIA Kit, MP Biomedicals, Santa Ana, CA). Plasma harvested from cows in estrus and during diestrus based on the presence of a CL with a diameter larger than 20 mm was incorporated into each assay for quality control. Samples were analyzed in duplicate in 2 assays with average sensitivity of 0.03 ng/mL, calculated as 2 SD below the mean counts per minute at maximum binding. Intra- and interassay coefficients of variation were 10.0 and 20.6%, respectively, for low-progesterone samples and 12.1 and 19.8%, respectively, for high-progesterone controls.

Sample Size Calculation, Statistical Analyses, and Test Characteristics

Sample size calculations were conducted using the Power procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). Based on the report by Wilsdorf et al. (2016), P/AI for cows classified as having an optimal plasma progesterone profile (i.e., d -3 , 0, and 7 relative to timed AI) was 59.3%, whereas P/AI for cows deemed to have nonoptimal profiles was 14.5%. For sample size calculation purposes, we assumed that the difference in P/AI between cows classified as having optimal milk progesterone profile at any time point versus those classified as having non-optimal profiles was less (37 vs. 20%) than that reported by Wilsdorf et al. (2016) to account for potential misclassification of cows based on luteal status inherent to immunochromatography lateral flow test (Waldmann and Raud, 2016). A total of 150 units per group was deemed necessary to detect a 17-percentage-point difference in P/AI between cows with optimal and non-optimal progesterone concentrations during the timed AI protocol (37 vs. 20%; $\alpha = 0.05$; $1 - \beta = 0.90$). The proportion of cows classified

as having non-optimal luteal status in previous studies based on ultrasonography or assessment of progesterone concentrations in plasma was 14 to 18% at the PGF_{2 α} injection of the synchronization protocol (Bisinotto et al., 2010; Giordano et al., 2016), 5 to 12% at timed AI (Bisinotto et al., 2010; Santos et al., 2010; Wilsdorf et al., 2016), 8 to 15% on d 7 after AI (Bisinotto et al., 2010; Wilsdorf et al., 2016), and 30% on d 24 after AI (Bicalho et al., 2008). We assumed that 20% of cows enrolled in the present study would have non-optimal milk progesterone profile at each time point (i.e., d -3 , 0, 7, and 28) based on the estimates presented above and accounting for potential misclassification of cows by immunochromatography lateral flow test (Waldmann and Raud, 2016). Therefore, 750 cows were enrolled to yield 150 cows with a non-optimal progesterone profile at each time point.

Binary responses were analyzed by multivariable logistic regression using the Glimmix procedure of SAS. Initial models included the fixed effects of milk progesterone group (hP4, iP4, or IP4), herd, parity (primiparous or multiparous), BCS category (≤ 2.50 or ≥ 2.75), service number (first AI postpartum or resynchronized AI), sire, AI technician, and 2-way interactions with milk progesterone group. Independent variables were continuously removed if $P > 0.10$ using a backward stepwise elimination method. Milk progesterone group was forced into all final models. Results are presented as adjusted proportions. Plasma concentrations of progesterone were analyzed by ANOVA using the Glimmix procedure of SAS fitting a normal distribution. The fixed effects of milk progesterone group, assay batch, and sampling time (d -3 , 0, 7, and 28) were included in the models as independent variables. Cow was considered a random effect. Tests for normality of residuals and homogeneity of variances were conducted for each dependent variable. Progesterone concentrations are presented as least squares means and associated standard error of the mean. Differences with $P \leq 0.05$ were considered significant, and those with $0.05 < P \leq 0.10$ were considered tendencies. Pairwise comparisons were performed using the Tukey method. Orthogonal contrasts were built to evaluate differences between cows considered to bear a functional CL (hP4 + iP4) and herdmates without CL (IP4) based on milk progesterone profiling and between cows with high (hP4) and intermediate (iP4) concentrations of progesterone in milk.

Test characteristics for the use of milk progesterone profiling to identify cows without functional CL and to predict P/AI were calculated in Microsoft Excel (Microsoft Corp., Redmond, WA). The gold standard to determine presence of functional CL was plasma

progesterone concentrations; cows with concentrations ≥ 1 ng/mL were classified as having functional CL, whereas those with concentrations below this threshold were considered to lack functional CL. Regarding milk progesterone profiling, cows in hP4 and iP4 were classified as having functional CL, whereas those in lP4 were deemed to lack functional CL. The gold standard to determine pregnancy status was transrectal ultrasonography on d 62 after timed AI. Cows were classified as having optimal or non-optimal milk progesterone profile as predictors of P/AI on d -3 (optimal = hP4 and iP4 vs. non-optimal = lP4), d 0 (optimal = lP4 vs. non-optimal = hP4 and iP4), d 7 (optimal = hP4 and iP4 vs. non-optimal = lP4), and d 28 (optimal = hP4 and iP4 vs. non-optimal = lP4) relative to timed AI. For each analysis, a 2×2 table was built containing true-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN) observations. Sensitivity [TP/(TP + FN)], specificity [TN/(TN + FP)], positive predictive value [PPV; TP/(TP + FP)], negative predictive value [NPV; TN/(TN + FN)], and accuracy [(TP + TN)/(TP + TN + FP + FN)] were then calculated. Finally, 95% confidence interval (CI) was calculated for each test characteristic as

$$p \pm 1.96 \times \sqrt{\frac{p \times (1 - p)}{n}},$$

where p is the test characteristic in question and n is its respective denominator.

RESULTS

Milk progesterone data for 1 of the 4 time points were missing from 25 cows due to failed tests ($n = 5$; 1 on d -3, 1 on d 0, and 3 on d 28) and cows missed at the parlor ($n = 20$; 3 on d 7 and 17 on d 28). The proportions of cows categorized in each milk progesterone group at different time points are depicted in Table 1.

Plasma Progesterone Concentrations According to Milk Progesterone Profiling

Progesterone concentrations in plasma were greater ($P < 0.001$) for hP4 compared with iP4 and lP4 and tended to be greater ($P = 0.10$) for iP4 compared with lP4 (3.13 ± 0.20 , 1.12 ± 0.27 , and 0.38 ± 0.23 ng/mL, respectively; Figure 3). Using a threshold of 1 ng/mL

Table 1. Demographics and reinsemination pattern according to milk progesterone profile during the 5-d Cosynch-72 program

Item	Milk progesterone group, ¹ adjusted % (no.)			P-value ²		
	hP4	iP4	lP4	Group	C1	C2
PGF _{2α} (d -3)						
% of total ³	51.8	21.8	26.4			
Rebred on estrus ⁴	52.3 (226)	44.6 (92)	54.8 (155)	0.46	0.31	0.38
Short AI interval ⁵	8.3 (108)	18.4 (49)	29.1 (86)	<0.01	<0.01	0.08
AI (d 0)						
% of total ³	1.6	10.0	88.4			
Rebred on estrus ⁴	56.7 (12)	48.1 (58)	50.8 (402)	0.86	0.88	0.62
Short AI interval ⁵	NA ⁶	40.6 (28)	19.1 (207)	0.02	NA	NA
7 d after AI						
% of total ³	52.5	30.3	17.2			
Rebred on estrus ^{4,7}	52.2 (221)	50.8 (142)	45.3 (106)	0.51	0.27	0.80
Short AI interval ⁵	16.2 (117)	19.4 (73)	31.1 (49)	0.13	0.06	0.60
28 d after AI						
% of total ³	52.9	12.0	35.1			
Rebred on estrus ^{4,8}	19.5 (76)	12.3 (39)	6.7 (131)	0.05	0.05	0.38

¹Classification according to milk progesterone profiling. hP4 = high progesterone concentrations (test line not visible or lighter than reference); iP4 = intermediate progesterone concentrations (test line similar to reference); lP4 = low progesterone concentrations (test line darker than reference).

²Group = overall effect of milk progesterone group; C1 = orthogonal contrast comparing hP4 and iP4 vs. lP4; C2 = orthogonal contrast comparing hP4 vs. iP4.

³Percentage of cows classified in each group at each sampling point out of all cows sampled (includes pregnant and non-pregnant cows).

⁴Cows diagnosed as pregnant on d 34 after AI were not included in the statistical analyses.

⁵Only cows reinseminated in estrus before non-pregnancy diagnosis were included in the statistical analyses.

⁶NA = not assessed; the hP4 group was not included in the statistical analysis because of the small number of cows (only 1 out of 6 cows had a short cycle).

⁷Cows reinseminated before or on d 7 were removed from analyses.

⁸Cows reinseminated before or on d 28 were removed from analyses.

to define luteal activity, the proportions of cows in hP4 and iP4 that were misclassified as having a functional CL by milk progesterone profiling were 19.0% (16/84) and 69.0% (29/42), respectively, and the proportion of cows in IP4 misclassified as not having a functional CL was 8.8% (6/68). Sensitivity, specificity, PPV, NPV, and accuracy of using milk progesterone profiling to identify cows without functional CL (i.e., IP4) were 0.58 (95% CI = 0.49–0.67), 0.93 (95% CI = 0.88–0.98), 0.91 (95% CI = 0.84–0.98), 0.64 (95% CI = 0.56–0.73), and 0.74 (95% CI = 0.68–0.80), respectively.

Reinsemination Pattern After Timed AI

Overall, 48.6% of non-pregnant cows were reinseminated before pregnancy diagnosis on d 34 after timed AI (Figure 4). Mean and median intervals between timed AI and reinsemination were 21 and 22 d, respectively. The proportion of cows reinseminated on estrus did not differ among milk progesterone groups evaluated on d –3, 0, and 7 (Table 1). For cows that were not reinseminated by d 28, a greater proportion ($P = 0.05$) of those classified as hP4 and iP4 were reinseminated on estrus before pregnancy diagnosis compared with IP4. The proportion of reinseminated cows that received AI from 5 to 17 d after previous service was smaller ($P < 0.01$) for those classified in hP4 at PGF_{2α}, followed by iP4 and then IP4 (Table 1). Cows classified in iP4 at timed AI were more likely ($P = 0.02$) to be reinseminated from 5 to 17 d after previous service compared with herdmates in IP4. On the other hand, the proportion of cows with short AI intervals tended to be smaller ($P = 0.06$) for hP4 and iP4 on d 7 compared with IP4.

P/AI and Pregnancy Loss

Cows classified as hP4 and iP4 at the injection of PGF_{2α} had more ($P < 0.001$) P/AI on d 34 and 62 compared with those in IP4 (Table 2). The risk of pregnancy loss between 34 and 62 d of gestation did not differ among milk progesterone groups at PGF_{2α}. Based on milk samples collected on the day of timed AI, cows classified in IP4 had more ($P \leq 0.01$) P/AI on d 34 and 62 compared with counterparts in hP4 and iP4. Moreover, cows in iP4 tended to lose ($P = 0.07$) more pregnancies than those in IP4. Based on milk progesterone profiling performed on d 7 after timed AI, P/AI on d 34 was greater ($P < 0.001$) for hP4 and iP4 compared with IP4 and tended to be greater ($P = 0.10$) for hP4 compared with iP4. Pregnancy per AI on d 62 remained greater ($P < 0.001$) for cows in hP4 and iP4 compared with IP4. However, because of differences in pregnancy loss, P/AI on d 62 did not differ between

Figure 3. Progesterone concentration in plasma in cows with different milk progesterone profiles based on lateral flow immunochromatographic test (LSM ± SEM). hP4 = high progesterone concentrations (test line not visible or lighter than reference); iP4 = intermediate progesterone concentrations (test line similar to reference); IP4 = low progesterone concentrations (test line darker than reference). Group means with different lowercase letters (a, b) differed ($P \leq 0.05$). Group means with different uppercase letters (A, B) tended to differ ($P \leq 0.10$).

hP4 and iP4. Pregnancy per AI on d 34 and 62 was greater ($P < 0.001$) for cows classified as hP4 on d 28 after timed AI, followed by herdmates in iP4 and then cows in IP4. Despite the small number of cows available for analyses of pregnancy loss, a greater proportion of IP4 cows lost their pregnancies between d 34 and 62 of gestation compared with hP4 and iP4 cows.

Prediction of Non-Pregnancy Using Milk Progesterone Profiling

As mentioned above, classification of cows in IP4 as not having a functional CL yielded the best predictive value regarding cows' physiological status based on results of progesterone concentration in plasma. Therefore, hP4 and iP4 were clustered together for evaluation of test characteristics. Cows were classified as having optimal milk progesterone profile at PGF_{2α} (hP4 and iP4), timed AI (IP4), and 7 and 28 d after timed AI (hP4 and iP4), whereas the remaining cows were classified as having unfit milk progesterone profile. Sensitivity of using milk progesterone data to predict non-pregnancy on d 62 after timed AI were generally poor and ranged between 0.15 and 0.53 (Table 3). Negative predictive values also varied from 0.36 to

Figure 4. Distribution of cows reinseminated in estrus after timed AI. Light gray bars indicate the frequency of cows not pregnant to timed AI reinsseminated on each day; dark gray bar indicates the frequency of cows that were not detected in estrus before non-pregnancy diagnosis on d 34 after timed AI.

0.51. Conversely, specificity was between 0.86 and 0.99, and PPV ranged from 0.82 to 0.99. To simulate a serial use of milk progesterone profiling, test characteristics were recalculated excluding cows that presented unfit

values on previous sampling points. Removing cows classified as IP4 at $\text{PGF}_{2\alpha}$ from the analysis did not improve sensitivity, specificity, accuracy, PPV, or NPV based on 95% confidence intervals (Table 3). Likewise,

Table 2. Pregnancy per AI (P/AI) and pregnancy loss in lactating dairy cows according to milk progesterone profile during the 5-d Cosynch-72 program

Item	Milk progesterone group, ¹ adjusted % (no.)			P-value ²		
	hP4	iP4	lP4	Group	C1	C2
PGF _{2α} (d -3)						
P/AI d 34	41.0 (389)	44.4 (164)	21.1 (198)	<0.001	<0.001	0.47
P/AI d 62	37.2 (387)	38.3 (159)	17.4 (196)	<0.001	<0.001	0.80
Pregnancy loss	8.0 (161)	7.3 (67)	14.1 (41)	0.43	0.20	0.86
AI (d 0)						
P/AI d 34	0.0 (12)	21.4 (75)	39.1 (664)	<0.01	<0.01	0.30
P/AI d 62	0.0 (12)	15.6 (74)	34.8 (656)	<0.01	0.01	0.53
Pregnancy loss	NA ³	21.3 (16)	8.0 (254)	0.07	NA	NA
7 d after AI						
P/AI d 34	43.3 (392)	36.6 (228)	15.0 (128)	<0.001	<0.001	0.10
P/AI d 62	37.7 (387)	34.0 (225)	12.0 (128)	<0.001	<0.001	0.36
Pregnancy loss	10.6 (166)	3.4 (83)	17.3 (19)	0.09	0.10	0.06
28 d after AI						
P/AI d 34	65.0 (387)	11.3 (87)	3.1 (258)	<0.001	<0.001	<0.001
P/AI d 62	59.4 (379)	9.2 (86)	0.8 (258)	<0.001	<0.001	<0.001
Pregnancy loss	7.1 (246)	8.4 (9)	74.6 (7)	<0.001	<0.001	0.87

¹Classification according to milk progesterone profiling. hP4 = high progesterone concentrations (test line not visible or lighter than reference); iP4 = intermediate progesterone concentrations (test line similar to reference); lP4 = low progesterone concentrations (test line darker than reference).

²Group = overall effect of milk progesterone group; C1 = orthogonal contrast comparing hP4 and iP4 vs. lP4; C2 = orthogonal contrast comparing hP4 vs. iP4.

³NA = not applicable. Pregnancy loss could not be calculated for hP4 because no cow in this cohort was diagnosed as pregnant on d 32 after AI (C1 and C2 could not be estimated).

Table 3. Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV; 95% CI in parentheses) of milk progesterone profile to predict non-pregnancy on d 62 after AI

Sample	Sensitivity	Specificity	Accuracy	PPV	NPV
PGF _{2α} (d -3)	0.32 (0.28–0.36)	0.86 (0.81–0.90)	0.50 (0.46–0.54)	0.82 (0.77–0.88)	0.38 (0.34–0.42)
AI (d 0)	0.15 (0.12–0.18)	0.95 (0.92–0.98)	0.41 (0.38–0.45)	0.86 (0.79–0.93)	0.36 (0.32–0.39)
AI, optimal on d -3 ¹	0.17 (0.13–0.21)	0.95 (0.92–0.98)	0.47 (0.43–0.51)	0.84 (0.75–0.93)	0.41 (0.37–0.46)
7 d after AI	0.23 (0.19–0.26)	0.93 (0.90–0.97)	0.46 (0.42–0.49)	0.88 (0.82–0.93)	0.37 (0.33–0.41)
7 d, optimal on d 0 ²	0.24 (0.20–0.28)	0.93 (0.90–0.96)	0.48 (0.45–0.52)	0.86 (0.80–0.93)	0.40 (0.36–0.44)
28 d after AI	0.53 (0.48–0.57)	0.99 (0.98–1.00)	0.68 (0.65–0.71)	0.99 (0.98–1.00)	0.51 (0.46–0.55)
28 d, optimal on d 7 ³	0.52 (0.47–0.57)	0.99 (0.98–1.00)	0.69 (0.66–0.73)	0.99 (0.98–1.00)	0.55 (0.50–0.60)
28 d, not rebred ⁴	0.50 (0.44–0.56)	0.99 (0.98–1.00)	0.73 (0.69–0.77)	0.99 (0.97–1.01)	0.63 ⁵ (0.58–0.68)

¹Only cows with high (hP4) and intermediate (iP4) milk progesterone at PGF_{2α} (d -3) were included in the calculation of test characteristics.

²Only cows with low milk progesterone (lP4) at AI (d 0) were included in the calculation of test characteristics.

³Only cows with high (hP4) and intermediate (iP4) milk progesterone on d 7 after AI (d 7) were included in the calculation of test characteristics.

⁴Cows reinseminated on estrus before d 28 were removed from the calculation of test characteristics.

⁵Test characteristic improved compared with analyses within the same sampling point that included all cows.

excluding cows with hP4 and iP4 at timed AI from the evaluation performed on d 7 as well as excluding lP4 from the evaluation performed on d 28 had no effect on test characteristics. An increase in NPV of using milk progesterone profiling on d 28 after timed AI to predict non-pregnancy was observed when only cows not reinseminated at that time were included in the calculation.

DISCUSSION

Pregnancy rate is positively associated with the economic success of dairy operations and is used widely to monitor reproductive performance (Giordano et al., 2012; Galvão et al., 2013). As pregnancy rate is a function of insemination rate and risk of pregnancy per service, development of programs that improve P/AI and strategies to reinseminate non-pregnant cows early after unsuccessful AI are critical. Measurement of progesterone concentrations in blood is commonly used to assess luteal function in cattle and has been applied to predict pregnancy outcomes following timed AI (Wilsdorf et al., 2016). Despite its predictive value, evaluation of blood progesterone concentrations is lengthy and involves laboratory procedures such as ELISA or RIA, and thus provides only limited information to support decision making in dairy herds. Milk progesterone profiling by lateral flow immunochromatography allowed for on-farm assessment of luteal status in lactating dairy cows, particularly for identification of animals lacking functional CL at the time of sampling. Based on milk progesterone information, 26.4, 11.6, 17.2, and 35.1% of cows subjected to the 5-d Cosynch-72 protocol were classified as having unfit luteal status at the injection of PGF_{2α} 3 d before timed AI, on the day of insemination, and 7 and 28 d after timed AI, respectively. Using milk progesterone profiling to predict non-pregnancy on d 62

after timed AI resulted in specificity ranging between 0.86 and 0.99 and PPV between 0.82 and 0.99. These results support the use of milk progesterone profiling at key time points during the timed AI program to identify low-fertility cohorts of cows that are eligible for early resynchronization.

Despite extensive advancement of GnRH/PGF_{2α}-based timed AI programs, currently available protocols are unable to synchronize follicular development, luteolysis, and ovulation in every enrolled cow (Bisinotto and Santos, 2011). For instance, 10 to 20% of cows do not have CL at the injection of PGF_{2α} and proceed to have fewer P/AI compared with herdmates with CL (Bisinotto et al., 2013; Giordano et al., 2016). In agreement with previous studies conducted using ultrasonography, cows classified as not having functional CL at PGF_{2α} based on milk progesterone profiling had fewer P/AI at 34 and 62 d after insemination compared with counterparts bearing CL. In the study by Giordano et al. (2016), P/AI in cows without CL or with CL smaller than 10 mm in diameter at the injection of PGF_{2α} was 3.1-fold lesser compared with herdmates with CL larger than this threshold. Likewise, P/AI for cows without functional CL at the injection of PGF_{2α} determined by milk progesterone profiling in the present study was 2.6-fold lesser compared with cows with functional CL. The low-fertility cohort of cows without CL at PGF_{2α} is a combination of cows without CL at the initiation of the timed AI program that fail to ovulate in response to the first treatment with GnRH and cows in late diestrus that undergo spontaneous luteolysis before PGF_{2α} injection (Stevenson et al., 2008; Bisinotto et al., 2013). Early studies of ovarian responses in cows enrolled in a timed AI program at various stages of the estrous cycle observed greater incidence of premature ovulation in cows expected to undergo spontaneous luteolysis before the injection of PGF_{2α}, which creates an asynchrony

between sperm and oocyte availability (Vasconcelos et al., 1999). Moreover, cows without CL throughout development of the ovulatory follicle have inadequate progesterone concentrations in blood to support the establishment of pregnancy (Bisinotto et al., 2015). Although the exact mechanism by which fertility in cows lacking functional CL during follicular growth remains elusive (Santos et al., 2016), insufficient exposure to progesterone before ovulation has been linked to reduced embryonic survival and upregulation of luteolytic machinery after ovulation (Cerri et al., 2011a,b; Rivera et al., 2011). Altogether, P/AI in cows without CL at the injection of PGF_{2α} is 2- to 3-fold lesser compared with counterparts with CL (Bisinotto et al., 2013; Giordano et al., 2016). Definitive conclusions regarding synchrony of ovulation in response to the final GnRH injection and luteal lifespan in the cycle subsequent to timed AI cannot be drawn from our data. Nevertheless, the proportion of cows reinseminated from 5 to 17 d after timed AI was greater among IP4 cows at PGF_{2α} compared with hP4 and iP4 counterparts. Such observation can be explained, at least in part, by a combination of cows undergoing short luteal phases after insemination and cows in proestrus to metestrus at the time of PGF_{2α}.

Identification of cows without CL at the PGF_{2α} injection of resynchronization and implementation of alternative protocols can be executed rather easily in herds that use transrectal ultrasonography for non-pregnancy diagnosis (Giordano et al., 2016; Wijma et al., 2018). Nevertheless, adoption of similar strategies in herds that identify non-pregnant cows via rectal palpation or laboratory assays (i.e., pregnancy-associated glycoproteins and pregnancy-specific protein B) along with cows subjected to the first service postpartum requires additional animal handling, labor from trained technicians, and equipment. Results from the present study support the application of milk progesterone profiling as a potential alternative for identification of such low-fertility cohort. The proportion of cows classified as not having CL at PGF_{2α} in the present study using lateral flow immunochromatographic tests was comparable with previous reports in which cows were evaluated using ultrasonography during first postpartum and resynchronized timed AI (Bisinotto et al., 2013). In addition, the decrease in P/AI observed in cows classified as not having functional CL at PGF_{2α} compared with herdmates with CL in the present study was similar to that reported previously in studies in which cows were evaluated using ultrasonography (Giordano et al., 2016).

Complete CL regression before insemination is a critical step in timed AI protocols. Recent studies show that reducing progesterone concentration in plasma

below 1 ng/mL, which is a commonly used threshold to classify CL as functional, is insufficient to improve fertility responses. Instead, optimal P/AI is observed when concentrations of progesterone in plasma at AI are below 0.4 ng/mL (Brusveen et al., 2009; Bisinotto et al., 2010; Colazo et al., 2017). Average concentration of progesterone in plasma for IP4 cows according to lateral flow immunochromatographic test was 0.38 ± 0.23 ng/mL, suggesting that this strategy is conducive to predicting fertility responses when applied on the day of insemination. In fact, P/AI for cows without functional CL at timed AI was 2.4-fold greater compared with herdmates that bore functional CL. Despite a large difference in P/AI, the benefits of screening animals on the day of insemination based on milk progesterone were diminished given that only 11.6% of cows enrolled in the synchronization protocol were classified as having a functional CL on the day of timed AI. Because bovine CL are refractory to PGF_{2α} in the first 4 d after ovulation (Tsai and Wiltbank, 1998), cows synchronized using 5-d Cosynch protocols receive 2 injections of PGF_{2α} 5 and 6 d after the first GnRH (Santos et al., 2010). Brusveen et al. (2009) reported that less than 5% of cows treated with 2 PGF_{2α} injections 7 and 8 d after the initial GnRH had high progesterone concentrations in plasma at timed AI using a threshold similar to that achieved using lateral flow immunochromatographic test (i.e., <0.4 ng/mL). Although only 8.8% of cows in IP4 had functional CL based on plasma concentrations of progesterone, it is possible that misclassification explains, at least in part, the greater proportion of cows with adverse milk progesterone profiling at timed AI observed in our study. In contrast to 5-d synchronization programs, 15 to 20% of cows enrolled in 7-d protocols and treated with a single injection of PGF_{2α} fail to undergo complete luteolysis using 0.4 to 0.5 ng/mL as a threshold (Brusveen et al., 2009; Martins et al., 2011). The effect of using milk progesterone profiling on the day of timed AI during a 7-d protocol with a single injection of PGF_{2α} remains to be determined. However, having a larger proportion of cows with incomplete luteolysis at timed AI would likely increase the benefit of milk progesterone profiling on reproductive performance and economic return.

Timely ovulation in response to the final GnRH injection is critical for establishment of pregnancy following timed AI because it controls the synchrony between oocyte and sperm cells' capacity for fertilization (Pursley et al., 1998). However, 10 to 15% of lactating dairy cows subjected to 7-d Ovsynch-56 or 5-d Cosynch-72 programs fail to ovulate within 48 h of the final GnRH (Santos et al., 2010; Bisinotto et al., 2015). Although ovulation in response to the final GnRH injection was not assessed in the present study, a

comparable proportion of cows (i.e., 17.2%) were classified as not having functional CL on d 7 after timed AI by the lateral flow immunochromatographic test. Cows that respond to each injection within a synchronization protocol are expected to bear a preovulatory follicle responsive to the LH surge triggered by the final GnRH and start a new estrous cycle after timed AI (Vasconcelos et al., 1999). Therefore, return to estrus after d 19 following timed AI is expected in most non-pregnant cows that responded to the synchronization protocol (Sartori et al., 2004). The proportion of cows detected in estrus between 5 and 17 d of timed AI tended to be greater among those lacking functional CL on d 7 in the present study, which provides further support for our conclusion that milk progesterone profiling can be used to identify cows with asynchronous ovulation in response to the timed AI program. Besides impaired synchrony of ovulation, cows that undergo a slow increase in plasma progesterone concentration following ovulation are likely contributors to the pool of animals classified as IP4 on d 7 after timed AI in the present study. Previous studies reported an average difference of 22 h between ovulation and plasma concentrations of progesterone greater than 1 ng/mL comparing cows classified as having early versus late increases (Starbuck et al., 2006). Although a steeper increase in circulating concentrations of progesterone after ovulation may be associated with larger preovulatory follicles and smaller likelihood of pregnancy (Vasconcelos et al., 1999), such a slope has been positively associated with early embryo development in dairy cows (Green et al., 2005). As a result of absent or delayed ovulation around the final GnRH and slow increase in plasma progesterone concentrations after timed AI, cows without functional CL on d 7 after timed AI as identified by the lateral flow immunochromatographic test had fewer P/AI and increased risk of pregnancy loss compared with hP4 and iP4 counterparts.

As opposed to earlier sampling points, classification of cows based on luteal function on d 28 after timed AI lateral flow immunochromatography was designed primarily as a non-pregnancy test rather than a strategy to predict pregnancy outcomes. Despite elevated specificity and PPV to diagnose non-pregnant cows on d 28, overall accuracy of progesterone profiling ranged between 0.68 and 0.73 due to marginal sensitivity and NPV. Such results were anticipated because cows that fail to respond to the synchronization protocol and those that undergo embryonic mortality past 22 d of gestation may bear functional CL 28 d after timed AI in the absence of pregnancy (Wijma et al., 2016). Chemical pregnancy tests based on proteins directly associated with pregnancy, such as pregnancy-associated glycoproteins and pregnancy-specific protein B, have

been validated for use between 24 and 28 d after insemination in dairy cattle (Mayo et al., 2016; Reese et al., 2018; Middleton and Pursley, 2019). Such methods include a quick-read ELISA test with similar incubation time as described for lateral flow immunochromatography that can be performed on farm (Mayo et al., 2016). Successful strategies to shorten reinsemination interval based on assessment of luteal function for non-pregnancy diagnosis have been evaluated using B-mode ultrasonography combined or not with Doppler flow measurement of CL perfusion in cattle (Kelley et al., 2016; Pugliesi et al., 2019). Such approaches are also affected by maintenance of functional CL in a portion of non-pregnant females past 21 d after previous insemination, thus requiring subsequent examination of those not deemed open upon the initial assessment. Furthermore, ultrasound-based approaches required additional equipment and trained technicians compared with the use of lateral flow immunochromatographic tests evaluated in the present study.

CONCLUSIONS

Milk progesterone profile using a lateral flow immunochromatographic test provides a valuable opportunity to tailor reproductive management practices to cows' physiological status. On-farm testing allowed for the identification of cows without CL with elevated specificity and PPV. Furthermore, classification of cows according to luteal function using milk progesterone profiling identified low-fertility cohorts both before and after insemination ranging from 12.6 to 35.1% of cows at various time points. Although overall sensitivity was poor, specificity to predict non-pregnancy on d 62 after timed AI ranged between 0.86 and 0.99 depending on specific sampling points.

ACKNOWLEDGMENTS

The authors thank the owners and staff of Davis Family Dairies (Nicollet, Le Sueur, MN) for the use of their cows and facilities as well as their invaluable assistance during the experimental procedures. Milk progesterone tests and financial support for this project were provided by Biomed Diagnostics Inc. (White City, OR). The authors have not stated any conflicts of interest.

REFERENCES

- Ayres, H., R. M. Ferreira, A. P. Cunha, R. R. Araujo, and M. C. Wiltbank. 2013. Double-Ovsynch in high-producing dairy cows: Effects on progesterone concentrations and ovulation to GnRH treatments. *Theriogenology* 79:159–164. <https://doi.org/10.1016/j.theriogenology.2012.10.001>.

- Bicalho, R. C., K. N. Galvão, C. L. Guard, and J. E. P. Santos. 2008. Optimizing the accuracy of detecting a functional corpus luteum in dairy cows. *Theriogenology* 70:199–207. <https://doi.org/10.1016/j.theriogenology.2008.03.015>.
- Bisinotto, R. S., L. O. Castro, M. B. Pansani, C. D. Narciso, N. Martinez, L. D. Sinedino, T. L. Pinto, N. S. Van de Burgwal, H. M. Bosman, R. S. Surjus, W. W. Thatcher, and J. E. P. Santos. 2015. Progesterone supplementation to lactating dairy cows without a corpus luteum at initiation of the Ovsynch protocol. *J. Dairy Sci.* 98:2515–2528. <https://doi.org/10.3168/jds.2014-9058>.
- Bisinotto, R. S., E. S. Ribeiro, F. S. Lima, N. Martinez, L. F. Greco, L. F. Barbosa, P. P. Bueno, L. F. Scagion, W. W. Thatcher, and J. E. P. Santos. 2013. Targeted progesterone supplementation improves fertility in lactating dairy cows without a corpus luteum at the initiation of the timed artificial insemination protocol. *J. Dairy Sci.* 96:2214–2225. <https://doi.org/10.3168/jds.2012-6038>.
- Bisinotto, R. S., E. S. Ribeiro, L. T. Martins, R. S. Marsola, L. F. Greco, M. G. Favoreto, C. A. Risco, W. W. Thatcher, and J. E. P. Santos. 2010. Effect of interval between induction of ovulation and artificial insemination (AI) and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. *J. Dairy Sci.* 93:5798–5808. <https://doi.org/10.3168/jds.2010-3516>.
- Bisinotto, R. S., and J. E. P. Santos. 2011. The use of endocrine treatments to improve pregnancy rates in cattle. *Reprod. Fertil. Dev.* 24:258–266. <https://doi.org/10.1071/RD11916>.
- Brusveen, D. J., A. H. Souza, and M. C. Wiltbank. 2009. Effects of additional prostaglandin F_{2α} and estradiol-17β during Ovsynch in lactating dairy cows. *J. Dairy Sci.* 92:1412–1422. <https://doi.org/10.3168/jds.2008-1289>.
- Cerri, R. L., R. C. Chebel, F. Rivera, C. D. Narciso, R. A. Oliveira, M. Amstalden, G. M. Baez-Sandoval, L. J. Oliveira, W. W. Thatcher, and J. E. P. Santos. 2011a. Concentration of progesterone during the development of the ovulatory follicle: II. Ovarian and uterine responses. *J. Dairy Sci.* 94:3352–3365. <https://doi.org/10.3168/jds.2010-3735>.
- Cerri, R. L., R. C. Chebel, F. Rivera, C. D. Narciso, R. A. Oliveira, W. W. Thatcher, and J. E. P. Santos. 2011b. Concentration of progesterone during the development of the ovulatory follicle: I. Ovarian and embryonic responses. *J. Dairy Sci.* 94:3342–3351. <https://doi.org/10.3168/jds.2010-3734>.
- Colazo, M. G., I. Lopez Helguera, A. Behrouzi, D. J. Ambrose, and R. J. Mapletoft. 2017. Relationship between circulating progesterone at timed-AI and fertility in dairy cows subjected to GnRH-based protocols. *Theriogenology* 94:15–20. <https://doi.org/10.1016/j.theriogenology.2017.02.004>.
- Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. *J. Dairy Sci.* 72:68–78.
- Galvão, K. N., P. Federico, A. De Vries, and G. M. Schuenemann. 2013. Economic comparison of reproductive programs for dairy herds using estrus detection, timed artificial insemination, or a combination. *J. Dairy Sci.* 96:2681–2693. <https://doi.org/10.3168/jds.2012-5982>.
- Giordano, J. O., A. S. Kalantari, P. M. Fricke, M. C. Wiltbank, and V. E. Cabrera. 2012. A daily herd Markov-chain model to study the reproductive and economic impact of reproductive programs combining timed artificial insemination and estrus detection. *J. Dairy Sci.* 95:5442–5460. <https://doi.org/10.3168/jds.2011-4972>.
- Giordano, J. O., M. J. Thomas, G. Catucumbamba, M. D. Curler, M. Masello, M. L. Stangaferro, and R. Wijma. 2016. Reproductive management strategies to improve the fertility of cows with a sub-optimal response to resynchronization of ovulation. *J. Dairy Sci.* 99:2967–2978. <https://doi.org/10.3168/jds.2015-10223>.
- Green, M. P., M. G. Hunter, and G. E. Mann. 2005. Relationships between maternal hormone secretion and embryo development on day 5 of pregnancy in dairy cows. *Anim. Reprod. Sci.* 88:179–189. <https://doi.org/10.1016/j.anireprosci.2004.12.007>.
- Kelley, D. E., L. Ibarbia, R. Daetz, J. H. Bittar, C. A. Risco, J. E. P. Santos, E. S. Ribeiro, and K. N. Galvão. 2016. Combined use of progesterone inserts, ultrasonography, and GnRH to identify and resynchronize nonpregnant cows and heifers 21 days after timed artificial insemination. *Theriogenology* 85:230–237. <https://doi.org/10.1016/j.theriogenology.2015.09.052>.
- Martins, J. P., R. K. Policelli, L. M. Neuder, W. Raphael, and J. R. Pursley. 2011. Effects of cloprostenol sodium at final prostaglandin F_{2α} of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. *J. Dairy Sci.* 94:2815–2824. <https://doi.org/10.3168/jds.2010-3652>.
- Mayo, L. M., S. G. Moore, S. E. Pooch, W. J. Silvia, and M. C. Lucy. 2016. Technical note: Validation of a chemical pregnancy test in dairy cows that uses whole blood, shortened incubation times, and visual readout. *J. Dairy Sci.* 99:7634–7641. <https://doi.org/10.3168/jds.2016-11224>.
- Middleton, E. L., and J. R. Pursley. 2019. Short communication: Blood samples before and after embryonic attachment accurately determine non-pregnant lactating dairy cows at 24 d post-artificial insemination using a commercially available assay for pregnancy-specific protein B. *J. Dairy Sci.* 102:7570–7575. <https://doi.org/10.3168/jds.2018-15961>.
- Pugliesi, G., D. Z. Bisinotto, B. P. Mello, F. C. Lahr, C. A. Ferreira, G. D. Melo, M. R. Bastos, and E. H. Madureira. 2019. A novel strategy for resynchronization of ovulation in Nelore cows using injectable progesterone (P4) and P4 releasing devices to perform two timed inseminations within 22 days. *Reprod. Domest. Anim.* 54:1149–1154. <https://doi.org/10.1111/rda.13475>.
- Pursley, J. R., M. O. Mee, and M. C. Wiltbank. 1995. Synchronization of ovulation in dairy cows using PGF_{2α} and GnRH. *Theriogenology* 44:915–923. [https://doi.org/10.1016/0093-691X\(95\)00279-H](https://doi.org/10.1016/0093-691X(95)00279-H).
- Pursley, J. R., R. W. Silcox, and M. C. Wiltbank. 1998. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. *J. Dairy Sci.* 81:2139–2144. [https://doi.org/10.3168/jds.S0022-0302\(98\)75790-X](https://doi.org/10.3168/jds.S0022-0302(98)75790-X).
- Reese, S. T., M. H. C. Pereira, J. L. Edwards, J. L. M. Vasconcelos, and K. G. Pohler. 2018. Pregnancy diagnosis in cattle using pregnancy associated glycoprotein concentration in circulation at day 24 of gestation. *Theriogenology* 106:178–185. <https://doi.org/10.1016/j.theriogenology.2017.10.020>.
- Rivera, F. A., L. G. Mendonca, G. Lopes Jr., J. E. P. Santos, R. V. Perez, M. Amstalden, A. Correa-Calderon, and R. C. Chebel. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. *Reproduction* 141:333–342. <https://doi.org/10.1530/REP-10-0375>.
- Santos, J. E. P., R. S. Bisinotto, and E. S. Ribeiro. 2016. Mechanisms underlying reduced fertility in anovular dairy cows. *Theriogenology* 86:254–262. <https://doi.org/10.1016/j.theriogenology.2016.04.038>.
- Santos, J. E. P., C. D. Narciso, F. Rivera, W. W. Thatcher, and R. C. Chebel. 2010. Effect of reducing the period of follicle dominance in a timed artificial insemination protocol on reproduction of dairy cows. *J. Dairy Sci.* 93:2976–2988. <https://doi.org/10.3168/jds.2009-2870>.
- Sartori, R., J. M. Haughian, R. D. Shaver, G. J. Rosa, and M. C. Wiltbank. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. *J. Dairy Sci.* 87:905–920. [https://doi.org/10.3168/jds.S0022-0302\(04\)73235-X](https://doi.org/10.3168/jds.S0022-0302(04)73235-X).
- Starbuck, G. R., C. G. Gutierrez, A. R. Peters, and G. E. Mann. 2006. Timing of follicular phase events and the postovulatory progesterone rise following synchronisation of oestrus in cows. *Vet. J.* 172:103–108. <https://doi.org/10.1016/j.tvjl.2005.02.006>.
- Stevenson, J. S., D. E. Tenhouse, R. L. Krisher, G. C. Lamb, J. E. Larson, C. R. Dahlen, J. R. Pursley, N. M. Bello, P. M. Fricke, M. C. Wiltbank, D. J. Brusveen, M. Burkhart, R. S. Youngquist, and H. A. Garverick. 2008. Detection of anovulation by heat-mount detectors and transrectal ultrasonography before treatment with progesterone in a timed insemination protocol. *J. Dairy Sci.* 91:2901–2915. <https://doi.org/10.3168/jds.2007-0856>.

- Thatcher, W. W., F. F. Bartol, J. J. Knickerbocker, J. S. Curl, D. Wolfenson, F. W. Bazer, and R. M. Roberts. 1984. Maternal recognition of pregnancy in cattle. *J. Dairy Sci.* 67:2797–2811.
- Tsai, S. J., and M. C. Wiltbank. 1998. Prostaglandin $F_{2\alpha}$ regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. *Biol. Reprod.* 58:346–352. <https://doi.org/10.1095/biolreprod58.2.346>.
- Vasconcelos, J. L., R. W. Silcox, G. J. Rosa, J. R. Pursley, and M. C. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology* 52:1067–1078. [https://doi.org/10.1016/S0093-691X\(99\)00195-8](https://doi.org/10.1016/S0093-691X(99)00195-8).
- Waldmann, A., and A. Raud. 2016. Comparison of a lateral flow milk progesterone test with enzyme immunoassay as an aid for reproductive status determination in cows. *Vet. Rec.* 178:260. <https://doi.org/10.1136/vr.103605>.
- Wijma, R., M. M. Perez, M. Masello, M. L. Stangaferro, and J. O. Giordano. 2018. A resynchronization of ovulation program based on ovarian structures present at nonpregnancy diagnosis reduced time to pregnancy in lactating dairy cows. *J. Dairy Sci.* 101:1697–1707. <https://doi.org/10.3168/jds.2017-13489>.
- Wijma, R., M. L. Stangaferro, M. M. Kamat, S. Vasudevan, T. L. Ott, and J. O. Giordano. 2016. Embryo mortality around the period of maintenance of the corpus luteum causes alterations to the ovarian function of lactating dairy cows. *Biol. Reprod.* 95:112. <https://doi.org/10.1095/biolreprod.116.142075>.
- Wilsdorf, L. J., D. H. Keisler, S. E. Poock, W. R. Lamberson, R. C. Escalante, and M. C. Lucy. 2016. Short communication: Test for nonpregnancy in dairy cows based on plasma progesterone concentrations before and after timed artificial insemination. *J. Dairy Sci.* 99:5858–5865. <https://doi.org/10.3168/jds.2016-10864>.
- Wiltbank, M. C., G. M. Baez, F. Cochrane, R. V. Barletta, C. R. Trayford, and R. T. Joseph. 2015. Effect of a second treatment with prostaglandin $F_{2\alpha}$ during the Ovsynch protocol on luteolysis and pregnancy in dairy cows. *J. Dairy Sci.* 98:8644–8654. <https://doi.org/10.3168/jds.2015-9353>.
- Wiltbank, M. C., A. H. Souza, P. D. Carvalho, A. P. Cunha, J. O. Giordano, P. M. Fricke, G. M. Baez, and M. G. Diskin. 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. *Animal* 8(Suppl. 1):70–81. <https://doi.org/10.1017/S1751731114000585>.