Characterization of luteal dynamics in lactating Holstein cows for 32 days after synchronization of ovulation and timed artificial insemination

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1 **Characterization of luteal dynamics in lactating Holstein cows for 32 daysafter synchronization of**

2 **ovulation and timed artificial insemination**

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6 **ABSTRACT**

7 Approximately 20 to 30% of cows diagnosed not pregnant 32 d after timed artificial insemination (TAI) lack a

8 corpus luteum (CL), and cows submitted to a resynchronization protocol in the absence of a CL have about 10%

9 fewer pregnancies per AI (P/AI) than cows with a CL. An understanding of luteal dynamics after

10 synchronization of ovulation and TAI may help refine strategies for reinseminating cows failing to conceive.

11 Lactating Holstein cows (n = 141) were synchronized for first TAI using a Double-Ovsynch protocol. Thrice

12 weekly from 4 to 32 d after TAI, blood samples were collected for evaluation of plasma progesterone (P4)

13 concentrations, and CL diameter was measured using transrectal ultrasonography. Pregnancy status was de-

14 termined using transrectal ultrasonography 32 d after TAI. Nonsynchronized cows (n = 4) were removed from

15 the study. For cows diagnosed pregnant 32 d after TAI (n = 57), P4 increased from 4 to 15 d and then remained

16 constant until 32 d after TAI, whereas CL volume increased from 4 to 11 d and then remained constant until 32 d

17 after TAI. For cows diagnosed not pregnant 32 d after TAI (n = 80), P4 profiles were evaluated using statistical

18 cluster analysis based on the day after TAI that P4 decreased to <1 ng/mL, resulting in 5 clusters: (1) CL

19 regression 15 d after TAI (1.3%), (2) CL regression 18 to 22 d after TAI (55.0%), (3) CL regression 25 to 27 d

20 after TAI (17.5%), (4) CL regression 29 to 32 d after TAI (5.0%), and (5) CL maintained until 32 d after

21 TAI (21.3%). Plasma pregnancy-associated glycoprotein (PAG) levels at 25 and 32 d after TAI differed among

22 clusters and were below the cut-off value of the assay for the classifica- tion of cows as not pregnant for cows

23 in clusters 2, 3,

24

25

26 and 4, whereas more than half of the cows in cluster 5 had increased plasma PAG levels. We conclude

27 that at least half of the nonpregnant cows that maintained their CL until 32 d after TAI were initially pregnant

28 but underwent early pregnancy loss based on increased plasma PAG levels at 25 and 32 d after TAI.

29 **Key words:** corpus luteum, progesterone, pregnancy loss, pregnancy-associated glycoproteins

30

31 **INTRODUCTION**

32 The use of fertility programs for synchronization of ovulation and timed AI (**TAI**) has increased service

33 rate and pregnancies per AI (**P/AI**) in high-producing lactating dairy cows at first service (Fricke et al., 2015;

34 Santos et al., 2016b). Nonetheless, 50 to 70% of cows fail to conceive after first service (Brusveen et al.,

35 2009; Carvalho et al., 2014; Stevenson et al., 2014). Because of poor detection and expression of estrus in

36 high- producing dairy cows (Lopez et al., 2004), coupling a nonpregnancy diagnosis with a management

37 strategy to rapidly resubmit cows to AI increases reproductive efficiency by decreasing the interval between

38 AI ser-vices (Fricke, 2002; Fricke et al., 2003, 2016).

39   A common reproductive management strategy to decrease the interval between AI services is weekly sub-

40 mission of nonpregnant cows to an Ovsynch protocol for resynchronization of ovulation (**Resynch**) and

41 TAI (Caraviello et al., 2006; Fricke et al., 2014; Santos et al., 2016a). A key factor limiting fertility during

42 the Ovsynch protocol is the response to each of the se- quential hormonal treatments (Giordano et al., 2012b;

43 Carvalho et al., 2015b; Fricke et al., 2015). In this re- gard, initiation of an Ovsynch protocol during early

44 diestrus (d 5–9) results in more P/AI than initiation of an Ovsynch protocol at other stages of the estrous cycle

45 (Vasconcelos et al., 1999). Lactating dairy cows have an estrous cycle length of approximately 23 d (Sartori

46 et al., 2004); therefore, initiation of a Resynch protocol 32 d after AI (i.e., d 9 of the estrous cycle) should

47 resultin more P/AI than initiation of a Resynch protocol

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25 d (i.e., d 2 of the estrous cycle) or 39 d (i.e., d 16 of the estrous cycle) after AI. Several studies have

compared initiation of a Resynch protocol at different intervals after AI and reported no differences in P/AI

for cows initiating a Resynch protocol 25 versus 32 d after insemination (Fricke et al., 2003; Silva et al., 2009;

Bruno et al., 2014) or 32 versus 39 d after insemination (Bilby et al., 2013; Lopes et al., 2013). A consistent and

intriguing observation in all of these studies is that the proportion of cows without a corpus luteum (**CL**) or with

low progesterone (**P4**) concentrations at initiation of a Resynch protocol does not differ between cows ini- tiating

the Resynch protocol at 25 versus 32 d (Fricke et al., 2003; Silva et al., 2009; Bruno et al., 2014) or at 32

versus 39 d (Bilby et al., 2013; Lopes et al., 2013) after insemination and ranges from 20 to 30% of the cows

diagnosed not pregnant.

An analysis of 42,252 cows in 159 herds across England and Wales reported that the distribution of

interservice intervals in lactating dairy cows is longer than the expected 18 to 24 d, with a significant pro-

portion of cows having extended interservice intervals (Remnant et al., 2015). Longer interservice intervals,

particularly in high-producing dairy cows, may result from successful conception and maintenance of the CL

followed by early pregnancy loss and luteal regres- sion, thereby delaying return to estrus (Diskin et al., 2011).

Pregnancy-associated glycoproteins (**PAG**) be- long to a large family of aspartic proteinases expressed by the

placenta of domestic ruminants including cows, ewes, and goats (Haugejorden et al., 2006; Wallace et al.,

2015), and assays for detecting PAG levels in blood and milk have been developed and commercialized to determine pregnancy status in cattle (Ricci et al., 2015). Further, circulating PAG levels are dramatically affected by pregnancy loss early during pregnancy and have been used to define pregnancy loss in dairy cows (Giordano et al., 2012; Ricci et al., 2015).

A better understanding of luteal dynamics after synchronization of ovulation and TAI may help refine strategies for reinseminating cows failing to conceive. Thus, the objective of our study was to evaluate P4 concentrations and luteal dynamics from 4 to 32 d after TAI and PAG levels at 25 and 32 d after TAI. Our hypothesis was that luteal regression after TAI among cows diagnosed nonpregnant would not be synchronous and that some cows would have extended luteal phases and increased plasma PAG levels.

MATERIALS AND METHODS

All animal handling procedures were approved by the Animal Care and Use Committee of the College of Agricultural and Life Sciences at the University of Wisconsin–Madison.

Synchronization of Ovulation and Timed AI

Cows were housed at the University of Wisconsin– Madison Dairy Cattle Research   Center (Arlington, WI) in loose housing with headlocks. Cows were fed ad libitum a TMR formulated to meet or exceed NRC (2001) requirements for high-producing dairy cows. Primiparous cows were housed separately from mul- tiparous cows. The rolling herd average was 13,880 kg and average daily milk production was 41.6 kg/cow per day with 3.8% fat and 3.2% protein for the herd during the study period. Lactating Holstein cows (n = 141; 41 primiparous and 100 multiparous) from 53 ± 3 DIM were synchro- nized for first TAI using a Double-Ovsynch protocol (Souza et al., 2008). Prostaglandin $F_{2\alpha}$ (25 mg/dose of dinoprost tromethamine; Lutalyse) and GnRH (100 μg/dose of gonadorelin hydrochloride; Factrel) were from Zoetis (Madison, NJ). Briefly, cows received the first GnRH treatment of the Presynch portion of the Double-Ovsynch protocol at 53 ± 3 DIM, followed by treatment with $PGF_{2\alpha}$ 7 d later and a GnRH treatment 72 h after $PGF_{2\alpha}$. Seven days later, cows received an Ovsynch-56 protocol (GnRH at 70 ± 3 DIM, $PGF_{2\alpha}$ 7 d later, GnRH 56 h after $PGF_{2\alpha}$, and AI 16–20 h later), and all cows received a TAI at 80 ± 3 DIM. Three experienced AI technicians performed all inseminations using sires with high genetic merit and proven fertility.

Blood Sampling, P4, and PAG Assays

Blood samples were collected thrice weekly (Monday, Wednesday, and Friday) from 4 to 32 d after TAI (Fig-ure 1) for analysis of plasma P4 concentrations and at 25 and 32 d after TAI for evaluation of plasma PAG levels. Samples were collected by venipuncture of the median coccygeal artery or vein into 10-mL evacu-ated plasma collection tubes (Vacutainer; BD, Franklin Lakes, NJ). Blood samples were immediately placed on ice and were centrifuged (1,600 × g; 4°C) for 20 min, and plasma was harvested and stored at −20°C in 2-mL Safe-Lock tubes (Eppendorf AG, Hamburg, Germany). Plasma P4 concentrations were assayed using a solid- phase, no-extraction RIA (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA). The average sensi-tivity for the 3 assays was 0.027 ng/mL. The average intra-assay coefficient of variation was 5.61%, and the interassay coefficient of variation was 6.91% based on a quality control sample (2.50 ng/mL of P4) that was

replicated within each assay. Plasma PAG levels were assayed for samples collect- ed 25 and 32 d after TAI. After completion of sample collection at the end of the experiment, frozen plasma samples were shipped overnight in a cooled container by courier from the University of Wisconsin to Idexx Laboratories (Westbrook, ME) for analysis of plasma PAG levels using a commercial ELISA assay (Idexx Bovine Pregnancy Test, Idexx Laboratories). Trained technicians, who were blind to the pregnancy outcomes of the individual cows, performed the plasma PAG ELISA assays according to the manufacturer's instruc- tions. Briefly, a microtiter plate format was configured by coating an anti-PAG monoclonal antibody onto the plate. The PAG monoclonal antibody was raised against the PAG-55 protein fraction comprising PAG-4, PAG-6, PAG-9, PAG-16, PAG-18, and PAG-19 (Nagap- pan et al., 2009). After incubation of the diluted test sample in the coated well, captured PAG was detected with a PAG-specific antibody (detector solution) and horseradish peroxidase conjugate. Unbound conjugate was washed away, and 3,3□,5,5□-tetramethylbenzidine substrate was added to the wells. Color development was proportional to the amount of PAG in the sample and was measured using a spectrophotometer. Results were calculated from the optical density (**OD**) of the sample corrected by subtraction of the reference wavelength OD of the sample (**S**) minus the OD of the negative control (**N**) at 450 nm (with both values corrected by subtraction of the reference wavelength OD of the negative control), which resulted in an S − N value. Each microplate included negative and positive controls. Pregnancy outcomes from plasma PAG assays for in- dividual cows were determined based on cut-off values set by the PAG ELISA manufacturer. For the plasma PAG ELISA, when the S − N value was <0.300, the cow was classified as not pregnant; when the S − N value was >0.300 to <1.000, the cow was classified as recheck; and when the S − N value was ≥1.000, the cow was classified as pregnant.

Ultrasound Evaluation of CL Volume and Pregnancy Diagnosis

Ovarian structures were evaluated using a portable scanner (Ibex Pro, E. I. Medical Imaging, Loveland, CO)

equipped with a 7.5-MHz linear-array transducer thrice weekly (Monday, Wednesday, and Friday) from 4 to 32 d after TAI to measure CL diameter (Figure 1). The ultrasound image was frozen when the CL was visualized to be at its maximal size, and CL diameter was determined by taking the mean of 2 perpendicular diameter measurements [length (L) and width (W)] obtained using the built-in digital calipers of the ultrasound scanner. When present, CL cavities were mea- sured, mapped, and recorded. Total CL volume was calculated by the formula $V = 4/3 \times \pi \times R^3$ using a radius (R) obtained with the formula $R = (L/2 + W/2)/2$ (Carvalho et al., 2015c). When a CL cavity was present, the volume of the cavity was calculated using the same formula, and the volume of the cavity was subtracted from the total CL volume to estimate total luteal tissue volume. Pregnancy diagnosis was performed 32 d after TAI for all cows using transrectal ultrasonography using the same scanner and transrectal probe used for evaluation of ovarian structures. A positive pregnancy diagnosis was based on visualization of a CL on the ovary ipsilateral to the fluid-filled uterine horn containing an embryo with a heartbeat.

Statistical Analyses

All statistical analyses were performed using SAS computational software (version 9.3 for Microsoft Win- dows; SAS Institute Inc., Cary, NC). Four cows were re- moved from all analyses because they failed to ovulate after the final GnRH treatment of the Double-Ovsynch protocol. For individual cows diagnosed nonpregnant 32 d after TAI, the day of CL regression was defined as the day P4 first decreased to <1.0 ng/mL. The Cluster procedure of SAS was used to hierarchically cluste
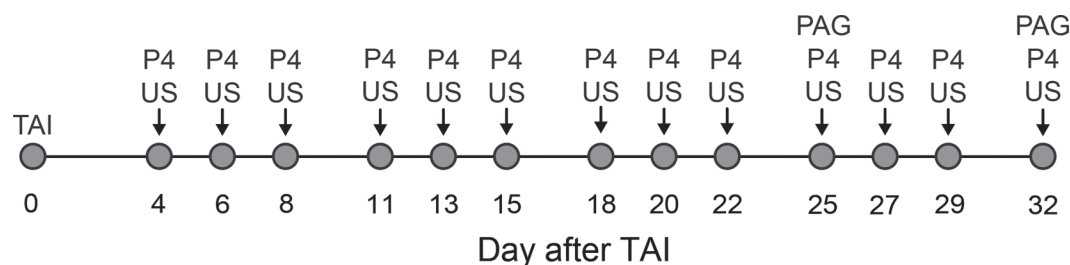
Figure 1. Schematic representation of the sample collection period. Lactating Holstein cows were submitted to a Double-Ovsynch protocol for synchronization of ovulation for first timed AI (TAI). Blood samples were collected for analysis of plasma progesterone (P4) levels, and ovaries were evaluated using transrectal ultrasonography (US) thrice weekly from 4 to 32 d after TAI. Pregnancy-associated glycoprotein (PAG) levels were assessed at 25 and 32 d after TAI.

the observations. The Cluster procedure segregated nonpregnant cows into 5 clusters (Figure 2); however, because cluster 1 included only 1 cow that underwent CL regression 15 d after TAI, this cow was removed from all subsequent analyses. Cows diagnosed pregnant 32 d after TAI were analyzed as a separate cluster.

Progesterone and CL volume were analyzed using the Mixed procedure of SAS. The model contained the fixed effects of cluster, time, parity (primiparous vs. multipa- rous), and cluster × time interaction. Circulating PAG levels were also analyzed using the Mixed procedure of SAS. The model contained the fixed effects of cluster, parity (primiparous vs. multiparous), and cluster × parity interaction. A contrast statement was used to compare pregnant and nonpregnant cows. Continuous data (P4, CL volume) are presented as least squares means ± standard error of the mean, whereas binomial data are presented as percentages. A significant dif- ference between levels of a classification variable was considered when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a statistical tendency.

RESULTS AND DISCUSSION

Luteal Dynamics from 4 to 32 d After TAI

Overall, 41.6% (57/137) of cows were diagnosed preg- nant 32 d after TAI, and pregnant cows exhibited plasma
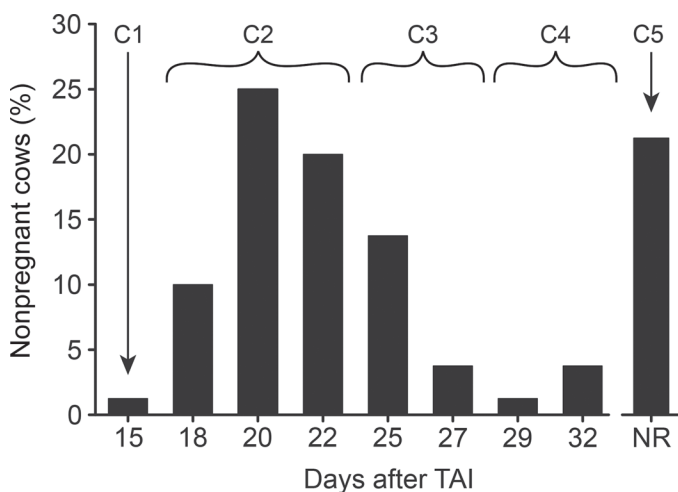

Figure 2. Day of corpus luteum (CL) regression for cows (n = 80) diagnosed nonpregnant 32 d after timed AI (TAI). Cows were submit- ted to a Double-Ovsynch protocol for synchronization of ovulation for first TAI. Day of CL regression was determined based on the day progesterone first decreased to <1.0 ng/mL. Cows were segregated into 5 clusters (C1–C5) based on a statistical cluster analysis of the day of CL regression. Cluster 1, n

= 1; cluster 2, n = 44; cluster 3, n = 14; cluster 4, n = 4; cluster 5, n = 17. NR = cows that did not regress their original CL by 32 d after TAI.
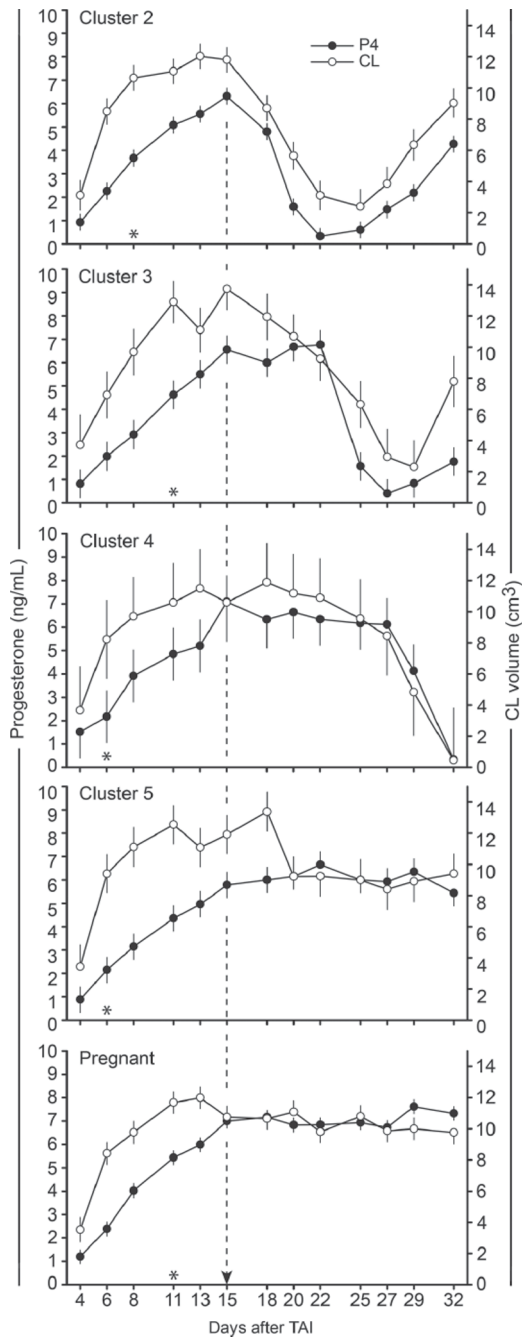

Figure 3. Progesterone (P4) and corpus luteum (CL) volume pro- files (LSM ± SEM) of cows (n = 137) diagnosed nonpregnant 32 d after insemination in clusters 2 to 5 and cows diagnosed pregnant 32 d after insemination from 4 to 32 d after timed AI (TAI). Cows were submitted to a Double-Ovsynch protocol for synchronization of ovula- tion for first TAI. Cluster 2 = cows undergoing CL regression from 18 to 22 d after TAI, n = 44; cluster 3 = cows undergoing CL regression from 25 to 27 d after TAI, n = 14; cluster 4 = cows undergoing CL regression from 29 to 32 d after TAI, n = 4; cluster 5 = cows that did not undergo CL regression by 32 d after TAI, n = 17; pregnant = cows diagnosed pregnant 32 d after TAI, n = 57. Asterisks (*) above the X-axis in each panel denote the day when CL reached the last statisti- cal increase before CL regression. The arrow with broken line indicates the day when P4 concentrations reached the last statistical increase before CL regression

P4 profiles and CL volume consistent with maintenance of CL function until 32 d after TAI (Figure 3). By contrast, the statistical cluster analysis hierarchically clustered the nonpregnant cows (58.4%; 80/137) into 5clusters based on the day P4 concentrations decreased to <1.0 ng/mL (Figure 2).

Cluster 1 included only 1 cow (0.73%; 1/137) that underwent CL regression 15 d after TAI (data not shown). Short luteal phases can and do occur in lactat- ing dairy cows. The occurrence of short luteal phases is associated with cyclicity status at the time of sub- mission to an Ovsynch protocol, with 6% of cycling cows and 23% of anovular cows having a luteal phase of <11 d (Gümen et al., 2003). When first postpartum ovulations were excluded from the analysis in another experiment, only 0.5% of cycling cows had a short lu- teal phase (Opsomer et al., 1998). In the present study, all cows were submitted to a Double-Ovsynch protocol, and cows submitted to a Double-Ovsynch protocol had more CL (94 vs. 68%) at initiation of the Breeding- Ovsynch portion of the protocol than cows submitted to a Presynch-Ovsynch protocol (Ayres et al., 2013). Nonetheless, the cow in cluster 1 ovulated to the last GnRH treatment of the Double-Ovsynch protocol but underwent CL regression earlier than all other nonpreg-nant cows.

Cows in cluster 2 included nonpregnant cows (32%; 44/137) that underwent CL regression from 18 to 22 d after TAI, and cows in cluster 3 included nonpregnant cows (10%; 14/137) that underwent CL regression from 25 to 27 d after TAI (Figure 3). Differences in timing of luteal regression between cows in clusters 2 and 3 may be attributable to differences in follicular wave patterns among cows. The number of follicular waves per cycle affects estrous cycle length and timing of luteolysis in both lactating dairy cows and dairy heifers (Townson et al., 2002; Ginther and Bashir, 2013; Ginther et al., 2013). Townson et al. (2002) reported that lactating dairy cows with 3 follicular waves had longer estrous cycles (24.5 vs. 21.2 d) and luteal phases (19.3 vs. 17.6 d) than cows with 2 follicular waves. Similarly, Ginther and Bashir (2013) reported that Holstein heifers with 3 follicular waves had longer estrous cycles (23.0 vs. 20.4 d) and luteal phases (20.0 vs. 17.8 d) than heifers with 2 follicular waves, and Sartori et al. (2004) reported longer estrous cycles (23.1 vs. 20.7 d) and luteal phases (19.1 vs. 17.4 d) in Holstein heifers with 3 versus 2 follicular waves.

Interovulatory interval and timing of ovulation rela- tive to CL regression can vary widely among dairy heif- ers and lactating dairy cows. Sartori et al. (2004) com- pared ovarian function and circulating steroids during the estrous cycle of Holstein heifers and lactating dairy

cows. Lactating cows with typical cycles had an estrous cycle length of approximately 23 d. Interestingly, some cows were classified as having atypical estrous cycles. Cows with atypical cycles underwent CL regression at the expected time of spontaneous luteolysis; however, ovulation occurred approximately 14

d after luteolysis, whereas cows with typical cycles ovulated approxi- mately 4.9 d after luteolysis (Sartori et al., 2004). In the present study, 74% (59/80; i.e., cows in clusters 1, 2, and 3) of nonpregnant cows would be classified as having typical cycles after TAI according to Sartori etal. (2004).

Cows in cluster 4 included cows (3%; 4/137) that underwent CL regression from 29 to 32 d after TAI, and cows in cluster 5 included cows (12%; 17/137) that maintained the CL formed by the last GnRH treatment of the Double-Ovsynch protocol until 32 d after TAI (Figure 3). These cows (26%; 21/80 of the nonpregnant cows—i.e., cows in clusters 4 and 5), however, did not exhibit typical or atypical cycles as defined by Sartori et al. (2004). Rather, cows in cluster 4 had extended lu- teal phases and underwent delayed CL regression (Sar- tori et al., 2004; Ginther and Bashir, 2013), whereas cows in cluster 5 had not undergone CL regression by32 d after TAI (Figure 3).

Differences might exist between the present study and previous studies (Sartori et al., 2004; Ginther and Bashir, 2013) that evaluated the length of the luteal phase. In our study, all cows were inseminated, whereas the studies by Sartori et al. (2004) and Ginther and Bashir (2013) used lactating cows and heifers that were not inseminated. Nonetheless, the luteal regression distribution of cows in our experiment that included cows with extended luteal phases (Figure 2) is similar to the interservice interval distribution from a large epidemiological data set of dairy cows in the United Kingdom (Remnant et al., 2015).

PAG 25 and 32 d After TAI

Pregnancy-associated glycoproteins belong to a large family of aspartic proteinases expressed by the placenta of domestic ruminants, including cows, ewes, and goats (Haugejorden et al., 2006; Telugu et al., 2010; Wallace et al., 2015), and circulating PAG levels in blood can be used as an indirect method for pregnancy diagnosis (Sasser et al., 1986; Fricke et al., 2016). For pregnant cows, PAG levels begin to increase in circulation by approximately 25 d in gestation, with an early peak occurring 7 d later at 32 d after TAI (Giordano et al., 2012a; Ricci et al., 2015; Carvalho et al., 2017). In ad- dition, between 7 and 14 d is required for circulating PAG and pregnancy-specific protein B (**PSPB**) levels

in cows undergoing induced or spontaneous pregnancy loss to return to baseline levels similar to nonpregnant cows (Giordano et al., 2012a; Ricci et al., 2015).

Plasma PAG levels differed among clusters at both 25 ($P = 0.03$) and 32 ($P < 0.01$) d after TAI (Figure 4). At 25 d after TAI, 95% (54/57) of pregnant cows had detectable plasma PAG levels [37% (21/57) and 58% (33/57) of the pregnant cows were categorized as re- check and pregnant, respectively, by the assay], whereas by 32 d after TAI all pregnant cows had plasma PAG levels above the threshold cut-off set

for a positive preg- nancy outcome for this ELISA. Although no cows in clusters 1, 2, 3, or 4 had detectable plasma PAG levels at 25 or 32 d after TAI, 41 and 58% of cows in cluster 5 had detectable plasma PAG levels at 25 and 32 d after TAI, respectively. Based on plasma PAG levels, more than half of the cows in cluster 5 maintained their original CL because they became pregnant and then underwent early pregnancy loss. In addition, it is likely that some or most cows in cluster 5 without detectable
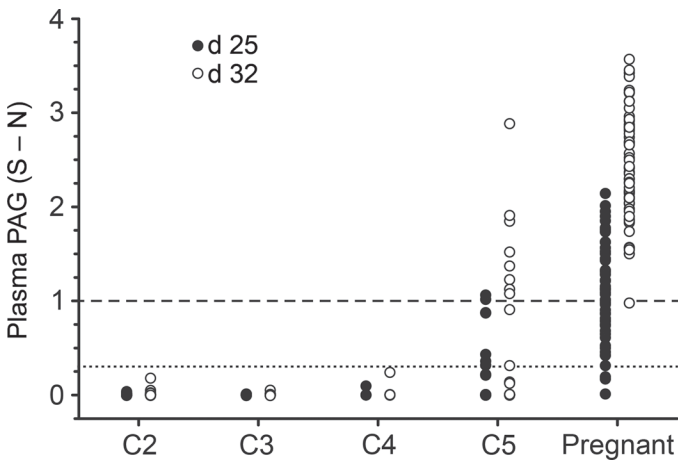

Figure 4. Scatter plot distribution of relative pregnancy-associated glycoprotein (PAG) levels in plasma at 25 and 32 d after insemination for individual cows (n = 137). Cows were submitted to a Double- Ovsynch protocol for synchronization of ovulation for first timed AI (TAI). Cows were segregated into clusters based on a statistical clusteranalysis of the day of corpus luteum (CL) regression. Cluster 2 (C2) = cows undergoing CL regression from 18 to 22 d after TAI, n = 44; cluster 3 (C3) = cows undergoing CL regression from 25 to 27 d after TAI, n = 14; cluster 4 (C4) = cows undergoing CL regression from 29 to 32 d after TAI, n = 4; cluster 5 (C5) = cows that did not undergo CL regression by 32 d after TAI, n = 17; pregnant = cows diagnosed pregnant 32 d after TAI, n = 57. Horizontal lines denote the cut-off values of the assay for the classification of cows as not pregnant when S − N values are <0.300 (dotted line) or pregnant when S − N values are ≥1.000 (dashed line). Cows were classified as recheck when the S − N value was >0.300 to <1.000. Results were calculated from the optical density (OD) of the sample corrected by subtraction of the ref- erence wavelength OD of the sample (S) minus the OD of the negative control (N) at 450 nm (with both values corrected by subtraction of the reference wavelength OD of the negative control), which resulted in an S − N value.

PAG levels 25 or 32 d after TAI could have undergone early pregnancy loss shortly after maternal recognition of pregnancy (Wijma et al., 2016), resulting in enough time to clear residual PAG from circulation before 25 to 32 d after TAI. Nonetheless, not all cows in cluster 5 had detectable PAG levels at 25 or 32 d after TAI in the present experiment.

Although pregnancy loss in lactating dairy cows can occur throughout gestation, the greatest incidence of pregnancy loss occurs during early gestation (Santos et al., 2004; Fricke et al., 2016; Wiltbank et al., 2016). Wiltbank et al. (2016) reported a 20 to 50% pregnancy loss rate during the first week of gestation and a 25 to 41% pregnancy loss rate between 8 and 27 d after AI compared with a 12% pregnancy loss rate between 28 and 60 d and an approximately 2% pregnancy loss rate from 60 d until calving. Similarly, Santos et al. (2004) reported a 34% pregnancy loss rate between 2 and 8 d after AI and a 20% pregnancy loss rate between 6 and 28 d after AI compared with a 12.5% pregnancy loss rate between 28 and 42 d, an 8.5% pregnancy loss rate between 42 and 90 d, and a 6% pregnancy loss rate from 90 d

until calving. Cows undergoing early pregnancy loss have extended luteal phases (>27 d; Wijma et al.,[11] 2016). In addition, cows undergoing late embryonic loss have increased interferon-τ stimulated genes 18 to 22 d after AI and increased PSPB concen- trations in circulation beginning 24 d after AI, whereas cows undergoing early pregnancy loss have increased interferon-τ stimulated genes 18 to 22 d after AI but may not have increased PSPB concentrations (Wijma et al., 2016). In the present study, nonpregnant cows in cluster 5 that maintained their CL until 32 d after TAI would result in a 23% (17/74) rate of early pregnancy loss that occurs before pregnancy diagnosis is routinely conducted on commercial dairy farms.

Luteal Dynamics for Pregnant and NonpregnantCows from 4 to 15 d After TAI

To compare luteal dynamics before spontaneous lu- teolysis among clusters, plasma P4 concentrations and CL volume were statistically analyzed from 4 to 15 d after TAI (Table 1). Overall, P4 concentrations did not differ ($P = 0.18$) among clusters (Table 1) or parities ($P = 0.22$) from 4 to 15 d after TAI; however, pregnant cows tended ($P = 0.06$) to have greater P4 concentra- tions from 8 to 13 d after TAI (Table 1). Interestingly, mean plasma P4 concentrations reached maximum concentrations at 15 d after TAI for all clusters (Figure 3). Overall, CL volume did not differ ($P = 0.92$) among clusters ($P = 0.25$) or parities from 4 to 15 d after TAI. In addition, CL volume did not differ ($P = 0.96$) btween pregnant and nonpregnant cows (Table 1). The day of maximal CL volume occurred earlier (fro6 to11 d after TAI; Figure 3) than the day of maximal P4 concentrations (15 d after TAI; Figure 3).

There is an unequivocal requirement for P4 dur- ing early pregnancy to support embryo development and for establishment and maintenance of pregnancy (Spencer et al., 2016). Observational studies have con- sistently reported that pregnant cows have greater P4 concentrations after AI than nonpregnant cows (Sterry et al., 2009; Parr et al., 2012). In this regard, supple- mentation with exogenous P4 increases circulating P4 concentrations and induces changes in the endometrial transcriptome (Forde et al., 2009; Carter et al., 2010), leading to increased embryo elongation between 13 and 16 d of gestation (Carter et al., 2008; Clemente et al., 2009). By contrast, decreasing P4 concentrations after AI decreases expression of interferon-τ stimulated gene 15 at 20 d after AI, PSPB concentrations from 25 to 67 d after AI, and embryonic crown–rump length 46 d after AI (Carvalho et al., 2017). Results from field trials evaluating the effect of supplementation with exogenous P4, however, are equivocal, with some re- porting an increase (Monteiro et al., 2014), no change (Stevenson et al., 2007; Monteiro et al., 2015), or a decrease (Parr et al., 2014) in P/AI. In addition, de- creasing P4 concentrations after AI did not affect P/AIor pregnancy loss (Carvalho et al., 2017).

Implications for Reinsemination Strategies

Accurate identification of cows returning to estrus from 18 to 24 d after AI is an early, simple, and inex-pensive method for identification and reinsemination of nonpregnant cows (Fricke et al., 2016). Data from the present study highlight several issues that limit this strategy as the only method for reinseminating nonpregnant cows. First, estrous cycle duration varies widely among individual cows, with many cows exhib-iting extended interservice intervals (Remnant et al., 2015). Data from the present study support that 26.3% of inseminated cows would be expected to exhibit ex-tended luteal phases and have a delayed return to estrus beyond 24 d. Second, only 51.5% of eligible cows were detected in estrus and inseminated in a study in which detection of estrus was performed through continuous monitoring with an activity monitoring system from AI until a pregnancy diagnosis conducted 32 d after AI (Giordano et al., 2015). A phenomenon referred to as the phantom cow syndrome occurs in seasonally calv-ing herds in Australia and New Zealand in which cows inseminated on the mating start date fail to be detected in estrus within 24 d after AI and are subsequently diagnosed not pregnant 35 d after AI (Cavalieri et al., 2003). Data from the present study support that 21.3% of nonpregnant cows maintained their original CL until 32 d after insemination. Thus, around 1 in 5 insemi-nated cows would not be expected to return to estrus within 32 d after insemination despite the most ac-curate and aggressive strategies for detection of estrus. Data on return to estrus after insemination support the need for a resynchronization strategy to reinsemi-nate nonpregnant cows failing to be detected in estrus within 32 d after insemination. A key aspect for fertility to a Resynch protocol is the presence or absence of a CL at the onset of the protocol (Carvalho et al., 2014

Table 1. Plasma progesterone concentrations and corpus luteum (CL) volume from 4 to 15 d after timed AI (TAI) in lactating Holstein cows[1]

| Item | CL cluster | | | | | P-value | |
	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Pregnant	Cluster	C1
n	44	14	4	17	57		
Progesterone (ng/mL)							
d 4	0.9 ± 0.3	0.8 ± 0.5	1.4 ± 0.9	0.8 ± 0.5	1.2 ± 0.3	0.39	0.25
d 6	2.3 ± 0.3	1.9 ± 0.5	2.1 ± 0.9	2.1 ± 0.5	2.3 ± 0.3	0.56	0.14
d 8	3.7 ± 0.3	2.9 ± 0.5	3.8 ± 0.9	3.1 ± 0.5	4.0 ± 0.3	0.11	0.06
d 11	5.0 ± 0.3	4.6 ± 0.5	4.8 ± 0.9	4.3 ± 0.5	5.5 ± 0.3	0.22	0.05
d 13	5.5 ± 0.3	5.5 ± 0.5	5.1 ± 0.9	4.9 ± 0.5	6.0 ± 0.3	0.24	0.05
d 15	6.3 ± 0.3	6.5 ± 0.5	7.0 ± 0.9	5.7 ± 0.5	7.1 ± 0.3	0.42	0.23
CL volume (cm³)							
d 4	3.3 ± 1.1	3.7 ± 1.9	3.7 ± 2.8	3.4 ± 1.4	3.5 ± 0.8	0.95	0.56
d 6	8.5 ± 0.8	6.9 ± 1.5	8.2 ± 2.5	9.4 ± 1.2	8.4 ± 0.7	0.73	0.84
d 8	10.6 ± 0.8	9.7 ± 1.5	9.7 ± 2.5	11.1 ± 1.3	9.8 ± 0.7	0.71	0.39
d 11	11.1 ± 0.8	12.9 ± 1.3	10.6 ± 2.5	12.6 ± 1.2	11.7 ± 0.7	0.83	0.82
d 13	12.0 ± 0.8	11.1 ± 1.4	11.5 ± 2.5	11.1 ± 1.2	12.0 ± 0.7	0.99	0.84
d 15	11.8 ± 0.8	13.7 ± 1.3	10.6 ± 2.5	11.9 ± 1.2	10.8 ± 0.7	0.27	0.13

[1]Cluster 2 = cows undergoing luteal regression 18 and 22 d after TAI; cluster 3 = cows undergoing luteal regression 25 and 27 d after TAI; cluster 4 = cows undergoing luteal regression 29 and 32 d after TAI; cluster 5 = cows that did not undergo luteal regression by 32 d after TAI; C1 = preplanned contrast between pregnant and nonpregnant cows (clusters 2, 3, 4, and 5).

Bisinotto et al., 2015; Carvalho et al., 2015a). Further, fertility of cows lacking a CL at the onset of a Resynch protocol can be increased by administering exogenous P4 during the Resynch protocol (Bilby et al., 2013; Bisinotto et al., 2015). An initial strategy to optimize fertility to Resynch protocols was to empirically determine the optimal interval after TAI to initiate Resynch based on assumptions regarding the physiology of the estrous cycle (Fricke et al., 2003). Assuming an estrous cycle duration of 21 to 23 d, initiation of a Resynch protocol 32 d after TAI should ensure that the first GnRH treatment of the Resynch protocol occurs at d 9 to 11 of the estrous cycle, a stage of the cycle when a CL should be present. Nonetheless, the proportion of nonpregnant cows lacking a CL ranges from 20 to 30% among studies (Fricke et al., 2003; Silva et al., 2009; Bilby et al., 2013; Lopes et al., 2013; Bruno et al., 2014). Data from the present study in which P4 profiles and luteal dynamics were defined from 4 to 32 d after TAI support that implementation of a Resynch protocol based on whether or not a nonpregnant cow has a CL is a better strategy for increasing fertility to TAI than basing initiation of a Resynch protocol on a specific day after AI due to variability in CL regression among groups of cows. Nonpregnant cows without a CL can then be submitted to a protocol with exogenous P4 to increase fertility to TAI. We conclude that at least half of the nonpregnant cows that maintained their CL until 32 d after TAI were initially pregnant but under- went early pregnancy loss based on increased plasma PAG levels at 25 and 32 d after TAI.

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