Physiological predictors of ovulation and pregnancy risk in a fixed-time artificial insemination program¹

J. S. Stevenson²

Department of Animal Sciences and Industry, Kansas State University, Manhattan 66506-0201

ABSTRACT

The objective of this study was to determine the relative importance and contribution of several physiological factors as predictors of pregnancy risk in an Ovsynch (GnRH-1 – 7 d – PGF_{2α} – 56 h – GnRH-2 – 16 h – artificial insemination) timed artificial insemination program: (1) age of the corpus luteum (CL; original CL, new CL, or both in response to GnRH-1) and resulting progesterone concentrations as they affected luteolysis, ovulation after GnRH-2, and pregnancy risk; (2) progesterone concentration before GnRH-1 and GnRH-2 on subsequent ovulatory response to GnRH-1 and GnRH-2 as well as pregnancy risk; and (3) a combination of these factors in a multivariable logistic regression model to predict pregnancy risk. Original data from 7 published studies were combined including ovulatory responses to both GnRH injections, blood progesterone concentrations before GnRH-1, before PGF_{2α}, and at 48 h after PGF_{2α}, pregnancy per artificial insemination at d 32 and 60 after artificial insemination, and intervening pregnancy loss. Ovulation outcomes were greater at lesser progesterone concentrations after both GnRH injections despite the fact that pregnancy outcomes were greatest when progesterone exceeded 3 ng/mL before GnRH-1 and PGF_{2α}, suggesting that greater progesterone concentration before GnRH-1 and incidence of ovulation act via different mechanisms to improve subsequent fertility. Ovulation after GnRH-2 and subsequent pregnancy outcome were positively related to lesser concentrations of progesterone at 48 h after PGF_{2α}; however, for maximal pregnancy outcome, progesterone should be <0.5 ng/mL. Cows with a CL that also formed a new CL after GnRH-1 had the greatest pregnancy outcome, whereas cows with only a new CL (i.e., anovulatory cows and those at or

near estrus without a CL at GnRH-1) had the poorest pregnancy outcome. Pregnancy loss was not affected by CL status during the timed artificial insemination program. Receiver operator curves produced cut points of progesterone concentration that predicted ovulation and pregnancy risk. Selected cut points of progesterone concentration 48 h after PGF_{2α} produced true positive risks greater than 90% and false positive risks less than 25%, thus demonstrating the high predictability of ovulation after GnRH-2 and subsequent pregnancy outcome based on progesterone concentration. We conclude that progesterone concentration is highly predictive of ovulation and pregnancy risk in addition to body condition, size of ovulatory follicle, and parity.

Key words: luteolysis, ovulation, progesterone, pregnancy risk

INTRODUCTION

Since the first report of a practical and successful fixed-time artificial insemination (TAI) program (i.e., Ovsynch: GnRH-1 – 7 d – PGF_{2α} – 56 h – GnRH-2 – 16 h – TAI; Figure 1) applied to lactating dairy cows on a commercial dairy farm (Pursley et al., 1997), physiological responses of the hypothalamo-pituitary-ovarian axis to exogenous hormonal interventions of the Ovsynch procedure have been studied. Application of Ovsynch and various adaptations of this program are now extensively used worldwide in the beef and dairy cattle industry (Wiltbank and Pursley, 2014). Preliminary studies formed the foundation for developing bovine TAI programs when GnRH administered to control follicular waves was followed in 6 d (Twagiramungu et al., 1995) or 7 d (Thatcher et al., 1989) by PGF_{2α} to regress functional luteal tissue in a coordinated fashion (Pursley et al., 1997). Lactating dairy cows treated with the Ovsynch program beginning on d 5 through 12 of the estrous cycle had greater incidences of ovulation and pregnancy per AI (P/AI) than cows treated at random stages of the estrous cycle (Vasconcelos et al., 1999).

On the basis of the hypothesis that fertility after a TAI program was related to the stage of the estrous cycle at which it was initiated (i.e., stage of the first

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²Corresponding author: jss@ksu.edu

Figure 1. Standard 7-d Ovsynch timed AI program. GnRH (100 µg) = GnRH-1 or GnRH-2; PGF_{2α} (25 mg); TAI = timed AI. Color version available online.

ovarian follicular wave), presynchronization of estrous cycles resulting in a larger proportion of cows in diestrus at the onset of the TAI program produced greater P/AI than cows initiating the TAI programs at random stages of the estrous cycle (Moreira et al., 2001; Cartmill et al., 2001; El-Zarkouny et al., 2004). Overall, lactating dairy cows exposed to the PGF_{2α}-presynch program have 42% greater odds of pregnancy compared with cows receiving only the TAI program (Bisinotto et al., 2014). Therefore, understanding the limitations and potential enhancements of the standard Ovsynch program is critical to maximizing its success.

At least 3 obvious factors are commonly thought to limit the success of the Ovsynch program including (1) initiating a new follicular wave by inducing ovulation of a dominant follicle in response to GnRH-1; (2) inducing luteolysis of all luteal structures in response to PGF_{2α} as evidenced by concentrations of progesterone <0.4 ng/mL at TAI (Santos et al., 2010; Wiltbank et al., 2014); and (3) timely ovulation of the preovulatory follicle in response to GnRH-2 to coincide with fixed-time semen placement in the uterus (Pursley et al., 1998) to maximize conception.

In the absence of any presynchronization treatment, synchronization of follicle growth by inducing ovulation in response to GnRH-1 of the TAI program occurs in only 45 to 50% of dairy cows (Pursley et al., 1995; Moreira et al., 2001). In addition, approximately 50 to 66% of dairy cows (Pursley et al., 1995; Vasconcelos et al., 1999) at various stages of the estrous cycle ovulate in response to GnRH. Ovulation (Stevenson et al., 2008; Stevenson and Pulley, 2016) and GnRH-induced LH release are suppressed in the presence of a CL and larger concentrations of progesterone in dairy cattle (Giordano et al., 2012; Pulley and Stevenson, 2015).

A second limitation is the inability of a single dose of PGF_{2α} to induce complete regression of all luteal structures depending on the number and age of the CL. Corpora lutea less than 10 d old are often resistant to regression after a standard dose of PGF_{2α} (Momont and Seguin, 1984). Administering PG as a single dose on d 7 or as 2 doses on d 5 and 6 after GnRH-1 (5-d Ovsynch) usually results in 70 to 84% of cows with progesterone <0.3 ng/mL on the day of the TAI (Santos et al., 2010), and likely influences the characteristics

of GnRH-induced LH release when progesterone concentrations are at or near baseline at the final GnRH treatment and subsequent P/AI (Stevenson and Pulley, 2016). Increased dose (Giordano et al., 2013; Wiltbank et al., 2015) or frequency of PGF_{2α} treatments (Brusveen et al., 2009; Santos et al., 2010; Ribeiro et al., 2012) at the end of Ovsynch program has enhanced luteolysis, reduced progesterone concentrations at TAI, and in some cases increased P/AI compared with a single standard dose (Santos et al., 2010; Ribeiro et al., 2012; Wiltbank et al., 2015).

A third limitation is the inability to synchronize ovulation between 24 and 32 h after GnRH-2 (Pursley et al., 1995). Synchronized ovulation averages 85% in dairy cows (Santos et al., 2010) and this percentage seems to decrease when cows are exposed to heat stress because of the supposed deleterious effects of hyperthermia on ovulation (López-Gatius et al., 2005). Multiple studies indicate that timing of GnRH-2 relative to PGF_{2α} and insemination is optimal for pregnancy risk when GnRH-2 is administered at approximately 56 h after PGF_{2α} and insemination occurs 12 to 16 h later (Stevenson, 2016b).

Of the 3 previously defined limitations, 2 are related to the success of ovulation, which is dependent on GnRH-induced LH release and its *in vivo* pre-GnRH steroid milieu (Stevenson and Pulley, 2016). To better understand and define the limitations of the standard Ovsynch program, we combined data from our published studies to examine the interrelationships of these factors. Of these limiting factors, we examined the influence of (1) age of the CL and resulting progesterone concentrations on luteolysis, ovulation response after GnRH-2, synchronization, and pregnancy risks; (2) progesterone concentration before GnRH-1 and GnRH-2 on subsequent ovulatory response to GnRH-1 and GnRH-2 and pregnancy risk; and (3) a combination of these factors in a multivariable logistic regression model used to predict pregnancy risk. Our objective was to determine the relative importance and contribution of these physiological factors as predictors of pregnancy risk in an Ovsynch TAI program.

MATERIALS AND METHODS

Source of Data

Data were combined from 7 published studies in which Ovsynch was applied to lactating dairy cows from 2004 through 2014.

Study 1. The first study involved lactating Holstein cows ($n = 635$) located in 6 Midwestern states (Stevenson et al., 2008). Estrous cycles were presynchronized with 2 injections of PGF_{2α} 14 d apart and cows were

enrolled in a 7-d Ovsynch program 14 d after the second PGF_{2α} injection (Presynch 14). Cows with a CL at the time of GnRH-1 served as controls and remaining cows with no CL were alternately assigned to receive a controlled internal drug release (CIDR) progesterone insert for 7 d as part of a 7-d Ovsynch program. The GnRH-2 injection was administered 48 h after PGF_{2α} and cows were inseminated at either 48 or 72 h after PGF_{2α}.

Study 2. Estrous cycles of lactating Holstein cows ($n = 233$) in 1 herd (Stevenson, 2011) were presynchronized with either 2 injections of PGF_{2α} 14 d apart (Presynch) with Ovsynch initiated 14 d after the second presynch PGF_{2α} injection (Presynch-14), Presynch-12, Presynch-10, or with a CIDR insert for 7 d, PGF_{2α} injection at CIDR removal, with CIDR insert removal at either 3 or 10 d before initiating a 7-d Ovsynch. Cows were inseminated and treated concurrently with GnRH-2 at 72 h after PGF_{2α}.

Study 3. Estrous cycles of lactating Holstein cows ($n = 463$) in 1 herd (Stevenson et al., 2012) were presynchronized with either Presynch-10 or with PG-3-G (PGF_{2α} at the same time as the second PGF_{2α} injection in Presynch-10 and GnRH 3 d later). The presynchronization treatments were completed 10 d (Presynch-10) or 7 d (PG-3-G) before cows were enrolled in a 7-d Ovsynch. Cows received GnRH-2 at 56 h after PGF_{2α} and TAI occurred 16 h later.

Study 4. Lactating Holstein cows ($n = 124$) in 1 herd (Pulley et al., 2013) were enrolled in a 7-d Ovsynch and alternately received eCG concurrent with PGF_{2α}. Cows were treated with GnRH-2 at 56 h after PGF_{2α} and inseminated 17 h later.

Study 5. Estrous cycles in lactating Holstein cows ($n = 253$) in 1 herd (Stevenson, 2016a) were presynchronized with either Presynch-11 or Gsynch (GnRH injection administered 7 d before PGF_{2α}, with PGF_{2α} administered at the same time as the second presynch PGF_{2α} injection of Presynch-11). Both presynchronization treatments were administered 11 d before enrolling cows in a 7-d Ovsynch. Cows were treated with GnRH-2 at 56 h after PGF_{2α} and inseminated 16 h later.

Study 6. Lactating Holstein cows ($n = 324$) in 1 herd were diagnosed not pregnant at d 31 since last AI and subsequently treated with PGF_{2α} at the not-pregnant diagnosis and GnRH 3 d later, or served as controls (Pulley and Stevenson, 2015). A 5-d Ovsynch program was then applied to all cows 10 d after the not-pregnant diagnosis with every other control cow receiving a CIDR insert (d 0). All cows received a standard dose of PGF_{2α} on d 5 and 6, GnRH 56 h after the first PGF_{2α} dose, and TAI 16 after GnRH-2.

Study 7. Lactating Holstein cows ($n = 72$) in 1 herd were treated as described previously for cows in Study

3 (Pulley et al., 2015). All cows were inseminated at 72 h after PGF_{2α}, but received GnRH-2 at 56 or 72 h after PGF_{2α}.

Standard Data Collection

Blood Sampling. In all studies, blood was collected before administering GnRH-1 and PGF_{2α} and 48 h after PGF_{2α}. Concentrations of progesterone were measured by radioimmunoassay as described in each study. With few exceptions (Study 2), all blood samples were measured by direct quantitative (nonextracted) radioimmunoassay using Coat-A-Count progesterone kits (Catalog #TKPG; Siemens Medical Solutions Diagnostics, Los Angeles, CA) previously validated for bovine serum as described in those studies.

To assess the effects of progesterone concentration on the occurrence of ovulation and pregnancy risk in blood samples collected immediately before GnRH-1, PGF_{2α}, and at 48 h after PGF_{2α}, values were categorized in 3 concentration ranges to represent different stages of the estrous cycle: (1) < 0.50 ng/mL (proestrus and estrus); (2) 0.5 to 3.00 ng/mL (metestrus and early diestrus); and (3) > 3 ng/mL (mid to late diestrus) as in a previous study (Souza et al., 2008).

To examine more closely the effect of progesterone concentration on eventual pregnancy risk, concentration ranges were further subdivided for samples collected before GnRH-1 (0.00 to 0.49, 0.50 to 0.99, 1.00 to 1.99, 2.00 to 2.99, 3.00 to 3.99, 4.00 to 4.99, 5.00 to 5.99, 6.00 to 6.99, and ≥ 7.00 ng/mL); before PGF_{2α} (0.00 to 0.99, 1.00 to 1.99, 2.00 to 2.99, 3.00 to 3.99, 4.00 to 4.99, 5.00 to 5.99, 6.00 to 6.99, 7.00 to 7.99, and ≥ 8.00 ng/mL); and at 48 h after PGF_{2α} (0.00 to 0.09, 0.10 to 0.19, 0.20 to 0.29, 0.30 to 0.39, 0.40 to 0.49, 0.50 to 0.59, 0.60 to 0.69, and ≥ 0.70 ng/mL).

Detection of Ovulation and Pregnancy. Before GnRH-1, PGF_{2α}, and at 48 h after PGF_{2α}, ovarian structures were measured and mapped by transrectal ultrasonography. Using recorded ovarian maps just before injections of GnRH-1 and PGF_{2α}, luteal status was assessed by determining CL age: new CL resulting from ovulation induced by GnRH-1, original CL present before GnRH-1, and at PGF_{2α}. Cows were categorized in 3 luteal groups as they existed before receiving PGF_{2α}: (1) original CL only; (2) original CL + new CL; or (3) new CL only. Detection of ovulation by transrectal ultrasonography occurred between 48 and 144 h after GnRH-1 or GnRH-2 depending on the study. Based on these categories of luteal structures, concentrations of progesterone, ovulation incidence after GnRH-2, synchronization risk (ovulation after GnRH-2 plus progesterone < 1 ng/mL at 48 h after PGF_{2α}), and subsequent pregnancy outcome were determined.

Pregnancy in 6 studies was diagnosed by transrectal ultrasonography at 32 (30 to 33) and 60 (58 to 62) days after TAI and the intervening pregnancy loss was calculated. In study 6, pregnancy diagnosis occurred at 31 and 78 d after TAI.

Statistical Analyses

Eleven herd-years were created and herd-year was treated as a random testing variable for categories in all models. To test the effect of age of CL on ovulation after GnRH-2, synchronization risk, pregnancy risk at d 32 and 60, and pregnancy loss, data were subjected to binomial logistical regression using procedure GLIMMIX (METHOD = LAPLACE) in SAS (version 9.4, SAS Institute Inc., Cary, NC). The model consisted of the fixed effects of CL age (original CL, original CL + new CL, and new CL), parity (primiparous vs. multiparous), with DIM and BCS treated as covariables, and herd-year as a random effect using the statement RANDOM INT/SUBJECT = HERD-YR. Options used in the model statement included LINK = LOGIT, DIST = BINOMIAL, and the least squares means option of ILINK and DIFF. Two orthogonal contrasts were constructed to test differences in luteal categories: (1) original CL and original CL + new CL versus new CL, and (2) original CL + new CL versus new CL. Least squares means were separated by the Tukey-Kramer option. To determine if differences in ovulatory response to GnRH-1 in the 3 luteal categories were related to diameter of the dominant follicle before GnRH-1, a similar model was employed using procedure GLM in SAS.

Concentrations of progesterone in blood samples collected before GnRH-1, PGF_{2α}, and 48 h after PGF_{2α} were analyzed by ANOVA using procedure MIXED in SAS with the same model as described previously for the binomial variables. Age of CL was tested conservatively by the random effect of age of CL nested within herd-year. Two orthogonal contrasts were constructed to test differences among concentrations ranges: (1) <0.50 versus 0.50 to 3.00 ng/mL; and (2) <0.50 versus >3.00 ng/mL. Individual means were separated by the Tukey-Kramer procedure in procedure MIXED.

Ovulation risk after GnRH-1 was regressed on the 3 progesterone concentration categories described previously (<0.50, 0.50 to 3.00, and ≥3.00 ng/mL) that were assessed before either GnRH-1 or GnRH-2 treatment using procedure LOGISTIC. Fixed effects of progesterone category, parity, size of the dominant follicle at GnRH-1 (<14 mm vs. ≥14 mm; median = 14.0 mm), with DIM and BCS as covariables, and herd-year as a random effect in the model. A final model produced by backward stepwise selection of independent variables

entered or retained in the model was based on a Wald statistic ($P < 0.10$). Adjusted odds ratios (AOR) and 95% confidence intervals are reported in the text. The final model included progesterone categories, 2 dominant follicle categories, BCS as a covariable, and herd-year as a random effect.

A similar model was employed to determine ovulation risk after GnRH-2, but with the substitution of the putative ovulatory follicle diameter identified at 48 h after PGF_{2α} (<14.5 vs. ≥14.5 mm; median size = 14.5 mm) included in the model with the final model determined by backward stepwise selection as described above. The final model consisted of the 3 progesterone categories, 2 ovulatory follicle categories, and herd-year as a random effect.

Pregnancy risk at d 32 also was regressed on the 3 progesterone concentration categories described previously (<0.50, 0.50 to 3.00, and ≥3.00 ng/mL) before GnRH-1, PGF_{2α}, and at 48 h after PGF_{2α} using procedure LOGISTIC. Fixed effects in the model were progesterone category and parity, with DIM and BCS as covariables, and herd-year as a random effect. The same model options were applied as described previously for ovulation risk.

Progesterone data were analyzed and receiver operator curves (ROC) were generated by using procedure LOGISTIC in SAS. The event was the result of the pregnancy or ovulation diagnosis. For purposes of analyses, progesterone concentration was evaluated as a test for predicting subsequent pregnancy or ovulation outcomes. Sensitivity was the proportion of true positive responders (response = 1) that have a positive test result (test = 1) as correctly identified for a given progesterone concentration (cut point). In other words, the number of pregnant cows above (P4 at GnRH-1 or at PGF_{2α}) or below (P4 at 48 h after PGF_{2α}) the selected cut points for pregnancy divided by the total number of pregnant cows. For ovulation detection, it represented the number of ovulating cows below (GnRH-1 or GnRH-2) the selected cut points for ovulation divided by the total number of ovulating cows.

Specificity is the proportion of true negative responders (response = 0) that have a negative test result (test = 0). The false positive rate is equal to 1 – specificity (pregnant cows incorrectly diagnosed as pregnant or ovulating cows incorrectly diagnosed as ovulating). Progesterone concentration for each of the 3 time points for pregnancy (before GnRH-1, before PGF_{2α}, and at 48 h after PGF_{2α}) or for each of 2 time points for ovulation (after GnRH-1 or GnRH-2) were tested independently in the analyses. Herd and lactation were included in the models but were removed because the outcomes were nearly identical to include only the progesterone concentration at each time point. The ROC

plots (not shown) were created by applying a macro described in the SAS Knowledge Base (Sample 25018: Plot ROC curve with cut point labeling and optimal cut point analyses; <http://support.sas.com/kb/25/018.html>). Cut points were selected that had the greatest accuracy in which the greatest sensitivity (true positive rate) and greatest specificity occurred in the ROC plot as predicted by the Youden index.

Factors considered to affect pregnancy risk were modeled in procedure LOGISTIC using both forward and backward stepwise regression to determine the best model. Independent variables in the model included the fixed effects of ovulation risk after GnRH-1 and GnRH-2, ovulatory follicle size (<14.5 vs. ≥ 14.5 mm; median = 14.5 mm) at 48 h after PGF_{2α}, progesterone concentration category (<0.50 , 0.50 to 3.00, and ≥ 3.00 ng/mL) before GnRH-1 and GnRH-2, and at 48 h after PGF_{2α}, with DIM and BCS as covariables, and herd-year as a random effect. The final model produced by either forward or backward stepwise selection of independent variables entered or retained in the model was based on a Wald statistic ($P < 0.10$). Adjusted odds ratios and 95% CI are reported.

To better understand the relationship among the 3 identified limiting factors to pregnancy risk in response to Ovsynch (previously described in the introduction), a multivariable logistic regression model using procedure LOGISTIC was developed to regress d 32 pregnancy risk on ovulation responses to GnRH-1 and GnRH-2, and low progesterone cut points (either <1 or <0.50 ng/mL) at 48 h after PGF_{2α}. The preceding variables were treated as fixed effects with fixed effect of parity and preovulatory follicle size determined at 48 h after

PGF_{2α} (<14.5 vs. ≥ 14.5 mm), with DIM and BCS as covariables, and herd-year as a random effect. Mean P/AI percentages were separated with DIFF option in LOGISTIC when a protected F -test was detected ($P < 0.05$).

RESULTS

Age of CL, Ovulation, and Pregnancy Risk

More than 60% of the cows formed a new CL after GnRH-1 (Table 1). Of that proportion, 41.8% of the cows with at least 1 original CL before GnRH-1 also had a new CL 7 d later. Follicle size tended ($P = 0.079$) to be greater in cows with no CL at GnRH-1, but that formed a new CL after GnRH-1, than in cows having at least 1 original CL before GnRH-1 (15.2 ± 0.5 vs. 14.4 ± 0.2 mm), respectively. Furthermore, an interaction of CL status and progesterone concentration was detected for follicle diameter. No difference in follicle diameter was detected among CL statuses when progesterone >3.0 or <0.5 ng/mL. In contrast, at progesterone concentrations between 0.5 and 3.0 ng/mL, follicle diameters were smaller ($P = 0.023$) in cows ($n = 229$) with at least 1 CL, which failed to ovulate after GnRH-1 (13.4 ± 0.3 mm), than in cows ($n = 300$) with at least 1 CL, which ovulated after GnRH-1 (14.4 ± 0.3 mm). In both of the previous cases, follicles were smaller ($P < 0.001$) in diameter than in cows ($n = 107$) that had no CL before GnRH-1, but formed a new CL after GnRH-1 (16.2 ± 0.4 mm).

Age of CL identified before the PGF_{2α} treatment of Ovsynch influenced progesterone concentrations before

Table 1. Age of corpus luteum (CL) and resulting progesterone concentrations and ovulation response to GnRH in cows subjected to Ovsynch timed AI program

Item	Type of CL ¹			P-value ²	
	Original CL (A)	Original CL + new CL (B)	New CL (C)	C1	C2
Cows per CL type (%)	39.9	41.8	18.4		
Progesterone ³ (ng/mL)					
GnRH-1	4.26 $\pm$ 0.15 ^a	3.59 $\pm$ 0.15 ^b	0.84 $\pm$ 0.18 ^c	0.001	0.001
PGF _{2α}	5.28 $\pm$ 0.30 ^a	5.95 $\pm$ 0.30 ^a	2.90 $\pm$ 0.33 ^b	0.001	0.001
48 h after PGF _{2α}	0.54 $\pm$ 0.08 ^a	0.62 $\pm$ 0.08 ^a	0.65 $\pm$ 0.09 ^a	0.537	0.824
Cows with low progesterone ⁴ [% (no.)]	91.9 ^a (836)	90.1 ^a (874)	84.3 ^b (385)	0.001	0.005
Ovulation after GnRH-2 [% (no.)]	89.1 ^a (836)	91.4 ^a (876)	88.4 ^a (385)	0.450	0.276
Synchronization risk ⁵ [% (no.)]	81.3 ^a (836)	82.1 ^a (876)	74.6 ^b (385)	0.023	0.030

^{a-c}Means within a row with different superscript letters differ ($P < 0.05$).

¹Original CL present before GnRH-1 treatment (A = failed to ovulate to GnRH-1; B = ovulated to GnRH-1). C = new CL formed in response to GnRH-1.

²C1 = A + B versus C. C2 = B versus C.

³Means $\pm$ SE.

⁴Low (<1 ng/mL) progesterone 48 h after PGF_{2α}.

⁵Proportion of cows with progesterone <1 ng/mL 48 h after PGF_{2α} and which subsequently ovulated in response to GnRH-2.

GnRH-1, PGF_{2α}, and at 48 h after PGF_{2α} (Table 1). Before GnRH-1 (before CL status was determined), cows without a CL, but formed a new CL after GnRH-1, had lesser ($P < 0.001$) progesterone concentration than cows with at least 1 original CL (Table 1). Cows with a CL at GnRH-1, which eventually formed a new CL after GnRH-1, had less ($P < 0.05$) progesterone than original CL-bearing cows that failed to ovulate after GnRH-1 (Table 1). Of the remaining variables in the model, parity and BCS accounted for significant variation in progesterone concentration, but not DIM. Primiparous cows ($n = 936$) had greater ($P = 0.002$) concentrations of progesterone before GnRH-1 than multiparous ($n = 1,162$) cows (3.05 ± 0.11 vs. 2.73 ± 0.10 ng/mL), respectively. Each unit increase in BCS resulted in a 0.44 ± 0.12 ng/mL increase ($P = 0.001$) in progesterone at GnRH-1.

Just before the PGF_{2α} injection, concentrations of progesterone did not differ ($P = 0.282$) between the original CL and the original CL + new CL statuses, but progesterone in those cows bearing original CL was greater ($P < 0.05$) than that in cows bearing only a new CL (Table 1). Primiparous cows ($n = 936$) had greater ($P = 0.009$) concentrations of progesterone before PGF_{2α} than multiparous ($n = 1,162$) cows (4.88 ± 0.2 vs. 4.54 ± 0.2 ng/mL), respectively. Neither BCS nor DIM, however, had any effect on progesterone concentration at PGF_{2α}.

Progesterone decreased to <1 ng/mL at 48 h after PGF_{2α} and did not differ among CL statuses. Although cows having only a new CL did not have less progesterone than cows bearing original CL at 48 h, a smaller ($P < 0.001$) proportion of new CL cows had concentrations <1 ng/mL than cows bearing original CL (Table 1). Although primiparous cows ($n = 936$) had slightly greater ($P = 0.046$) concentrations of progesterone at 48 h after PGF_{2α} than multiparous ($n = 1,162$) cows (0.65 ± 0.05 vs. 0.56 ± 0.05 ng/mL), respectively, neither BCS nor DIM had any effect on progesterone

concentration at 48 h after PGF_{2α} or on the percentage of cows with progesterone <1 ng/mL at the same time period.

Ovulatory response to GnRH-2 did not differ among CL statuses, but synchronization risk was less ($P = 0.023$) in new CL-bearing cows than in cows bearing original CL at PGF_{2α} and those that had original CL + new CL (Table 1). Neither parity, BCS, nor DIM affected ovulation response to GnRH-2 or synchronization risk. Ovulation response to GnRH-2 was greater in cows that had the ovulatory follicles >14.5 mm compared with cows having follicle <14.5 mm (92 vs. 88%).

Pregnancy risk at d 32 after TAI was greater ($P = 0.002$) in original-CL-bearing cows than in cows bearing only a new CL and was greater ($P = 0.002$) in original CL + new CL cows than in cows with only a new CL (Table 2). Pregnancy risk at d 32 also tended ($P = 0.06$) to be greater in original-CL-bearing cows with a new CL than in their herd mates with only an original CL at PGF_{2α}, which had failed to ovulate after GnRH-1. Primiparous cows had ($n = 936$) greater ($P = 0.025$) P/AI than multiparous ($n = 1,162$) cows (39.2 vs. 34.3%), respectively. For each unit increase in BCS, P/AI at d 32 increased ($P = 0.015$) by $6.4 \pm 2.6\%$.

Pregnancy risk in synchronized cows was greater ($P = 0.052$) in cows with original CL compared with cows with only a new CL and was greater ($P = 0.013$) in original-CL-bearing cows that also had a new CL compared with cows with only a new CL (Table 2). Neither parity nor DIM affected P/AI in synchronized cows, but for each unit increase in BCS, P/AI increased ($P = 0.002$) by $9.6 \pm 3.0\%$. In contrast, no differences in P/AI were detected among CL statuses for cows not synchronized.

Pregnancy risk at d 60 followed the same patterns as those for d 32, with the difference between original-CL-bearing cows with or without a new CL also approaching significance ($P = 0.055$). Primiparous cows ($n = 936$) had greater ($P = 0.048$) pregnancy risk at d

Table 2. Age of corpus luteum (CL) and resulting pregnancy per AI (P/AI) in cows subjected to Ovsynch timed AI program

Item [% (no.)]	Type of CL ¹			P-value ²	
	Original CL (A)	Original CL + new CL (B)	New CL (C)	C1	C2
Pregnancy per AI (d 32)	37.0 ^{ab} (839)	42.4 ^{a,A} (876)	31.1 ^b (385)	0.002	0.002
Synchronized ³	43.0 ^{ab} (666)	47.8 ^a (707)	38.9 ^b (278)	0.052	0.013
Not synchronized	14.6 ^a (170)	19.0 ^a (167)	11.4 ^a (107)	0.203	0.106
Pregnancy per AI (d 60)	33.1 ^{ab} (839)	38.5 ^{a,A} (876)	27.6 ^b (385)	0.003	0.001
Pregnancy loss	9.9 ^a (311)	8.2 ^a (317)	10.2 ^a (117)	0.702	0.524

^{a,b}Means within a row with different superscript letters differ ($P < 0.05$).

^ATended ($P = 0.06$) to differ from original CL (A).

¹Original CL present before GnRH-1 treatment; new CL formed in response to GnRH-1.

²C1 = A + B versus C. C2 = B versus C.

³Proportion of cows with progesterone <1 ng/mL at 48 h after PGF_{2α} and which subsequently ovulated in response to GnRH-2.

60 than multiparous ($n = 1,162$) cows (35.0 vs. 30.9%), respectively.

Pregnancy losses between d 32 and 60 did not differ among CL statuses (Table 2). Although no other single factor influenced pregnancy loss, a tendency ($P = 0.057$) for an interaction between CL status and parity was detected. Pregnancy loss tended to not differ or be less in primiparous ($n = 388$) than multiparous cows ($n = 410$) with an original CL (6.3 vs. 10.5%) compared with original CL + new CL status (10.8 vs. 9.1%), respectively, except for cows in the new CL status in which loss was nearly 3-fold greater in primiparous than multiparous cows (16.9 vs. 6.0%), respectively.

Progesterone Concentrations and Ovulation Risk

Ovulation risk was related to pre-GnRH-1 and GnRH-2 concentrations of progesterone (Figure 2). As progesterone concentration increased from ≤ 0.50 to > 0.50 ng/mL, ovulation risk after GnRH-1 decreased ($P < 0.01$) by almost 14 percentage points, and further decreased ($P < 0.01$) another 21 percentage points when progesterone exceeded 3.0 ng/mL. Ovulation tended (AOR = 1.20; 0.97 to 1.47) to be more likely in cows bearing a dominant follicle ≥ 14 mm ($n = 957$) than for cows with smaller-size follicles ($n = 948$; 70.1 vs. 67.5%), respectively. As BCS increased ($P = 0.05$) by 1 unit, ovulation risk increased by $5.9 \pm 2.6\%$.

Although the magnitude of ovulation risk was greater after GnRH-2 than after GnRH-1, ovulation risk also was compromised ($P < 0.01$) at greater concentrations of progesterone at 48 h after PGF_{2α} (Figure 2). Cows ($n = 1,035$) with ovulatory follicles ≥ 14.5 mm were more (AOR = 1.51; 95% CI = 1.14 to 2.00) likely to ovulate after GnRH-2 than cows with follicles < 14.5 mm ($n = 1,016$; 87.4 vs. 82.4%), respectively.

Ovulation risk after GnRH-1 was 60.1%. Area under the ROC for a progesterone test for ovulation was 0.55; the cut point occurred at 3.66 ng/mL with a sensitivity of 70.2% and a false positive rate of 59% (Table 3). In other words, in cows with progesterone greater than the selected cut point, 70.2% of the ovulating cows would be diagnosed correctly. The same cut point would incorrectly diagnose 59% of nonovulating cows as ovulating. At the selected cut point, it was 1.6 times more ($P < 0.05$) likely that ovulation risk of cows diagnosed correctly as ovulating was greater than the ovulation risk of nonovulating cows (Table 3).

Ovulation risk after GnRH-2 was 87.6%. Area under the ROC for a progesterone test for pregnancy was 0.55, the cut point occurred at 1.18 ng/mL with a sensitivity of 91.7% and a false positive rate of 24.2% (Table 3). Therefore, the cut point diagnosed correctly 91.7% of the ovulating cows as ovulating and the same cut

Figure 2. Ovulatory responses to GnRH-1 and GnRH-2 of the Ovsynch timed AI program on the basis of progesterone concentrations assessed just before GnRH-1 or 48 h after PGF_{2α} (0 to 24 h before GnRH-2). Bars within GnRH category having different letters differ ($P < 0.01$). Values within bars represent the number of observations. Color version available online.

point would incorrectly diagnose 24.2% of nonovulating cows as ovulating. At the selected cut point, it was 34.6 times more ($P < 0.05$) likely that the ovulation risk of cows diagnosed correctly as ovulating was greater than the ovulation risk of nonovulating cows (Table 3).

Progesterone Concentrations and Pregnancy Risk

Pregnancy risk was related to progesterone concentrations before GnRH-1, before PGF_{2α}, and those at 48 h after PGF_{2α} (Figure 3). Subsequent P/AI was greater ($P < 0.05$) in cows when progesterone exceeded 3 ng/mL before GnRH-1 than when progesterone was ≤ 3 ng/mL.

Pregnancy risk also was greater ($P < 0.05$) at larger pre-PGF_{2α} progesterone concentrations and increased as average progesterone concentration exceeded 0.5 ng/mL (Figure 3). At 48 h after PGF_{2α}, pregnancy risk increased ($P < 0.01$) as progesterone progressively decreased from > 3.0 to < 0.5 ng/mL (Figure 3). Cows ($n = 1,016$) having smaller-size ovulatory follicles (< 14.5 mm) at 48 h after PGF_{2α} were less (AOR = 1.23; 1.02 to 1.47) likely to be pregnant at d 32 than cows ($n = 1,035$) with larger follicles (≥ 14.5 mm; 26.0 vs. 30.1%), respectively.

At each time point when progesterone was related to subsequent pregnancy risk, primiparous cows ($n = 922$) were more ($P < 0.05$; AOR ranged from 1.19 to 1.28) likely to be pregnant at d 32 than multiparous cows ($n = 1,129$). Furthermore, at all 3 time points in the

Table 3. Receiver operator-derived true positive and false positive risks for serum progesterone (P4) before GnRH-1 and 48 h after PGF_{2α} used to predict ovulation risk¹

Item [no./no. (%)]	Accuracy ² (%)	True positive ³	False positive ⁴	Ovulation risk	
				No ovulation ⁵	Ovulation ⁶
P4 at GnRH-1	58.5	884/1,260 (70.2)	494/838 (59.0)	376/720 (52.2)	884/1,378 (64.2)
		67.6–72.7 ⁷	55.6–62.3 ⁷	Referent	1.64 ⁸ (1.36–1.97)
P4 at 48 h after PGF _{2α}	89.7	1,683/1,835 (91.7)	63/260 (24.2)	152/349 (43.6)	1,683/1,746 (96.4)
		90.5–93.0 ⁷	19.0–29.4 ⁷	Referent	34.6 ⁸ (24.9–48.1)

¹Overall ovulation after GnRH-1 = 838/2,098 = 60.1% and after GnRH-2 = 1,835/2,095 = 87.6%.

²Correct classification rate is the proportion of observations for which the test result and actual response agree.

³True positive (sensitivity). Number of ovulating cows below (after GnRH-1 or GnRH-2) the selected cut points for progesterone divided by the total number of ovulating cows. Selected cut points for progesterone were 3.66 and 1.18 ng/mL for the 2 time points, respectively.

⁴False positive (1 – specificity). Number of nonovulating cows below (GnRH-1 or GnRH-2) the selected cut points for progesterone divided by the total number of ovulating cows. Selected cut points for progesterone were 3.66 and 1.18 ng/mL for the 2 time points, respectively.

⁵Ovulation risk for cows not ovulating based on the selected cut point.

⁶Ovulation risk for cows ovulating based on the selected cut point.

⁷95% confidence intervals for true positive or false positive values.

⁸Odds ratio and 95% confidence interval for comparison of ovulation risk.

Ovsynch program, cows with greater BCS were 1.26 to 1.34 times more ($P < 0.05$) likely to be pregnant at d 32 than those with poorer body condition.

These relationships between progesterone concentrations at GnRH-1, PGF_{2α}, and at 48 h after PGF_{2α} and subsequent pregnancy risk are illustrated further when concentrations before GnRH-1, PGF_{2α}, and at 48 h after PGF_{2α} were further subdivided, producing multiple coefficients of determination (R^2) exceeding 0.78 (Figure 4). With the pre-GnRH-1 progesterone concen-

trations, a linear relationship was detected ($y = 30.3 + 1.71x$; $R^2 = 0.84$) between progesterone concentration and P/AI (Figure 4A). The relationship between pre-PGF_{2α} progesterone concentrations and pregnancy risk produced a third-order polynomial ($y = 9.25 + 18.74x - 3.55x^2 + 0.21x^3$; $R^2 = 0.83$; Figure 4B). Progesterone concentrations 48 h after PGF_{2α} also were related to subsequent P/AI in a decreasing curvilinear manner ($y = 33.28 + 6.38x + 0.91x^2$; $R^2 = 0.78$; Figure 4C).

Area under the ROC for a progesterone test before GnRH-1 for pregnancy was 0.55; the cut point occurred at 2.93 ng/mL with a sensitivity of 58.9% and a false positive rate of 50% (Table 4). This means that at the cut point selected 58.9% of all pregnant cows would be diagnosed correctly and the same cut point would incorrectly diagnose 50% of nonpregnant cows as pregnant. At this cut point, the odds of the false positive proportions did not differ ($P = 0.423$) from 0.5, indicating a poor predictability of progesterone before GnRH-1 for subsequent pregnancy risk. At the selected cut point, however, it was 1.4 times more ($P < 0.05$) likely that the pregnancy risk of cows diagnosed correctly as pregnant was greater than the pregnancy risk of nonpregnant cows (Table 4).

Area under the ROC for a progesterone test before PGF_{2α} for pregnancy was 0.55; the cut point occurred at 3.53 ng/mL with a sensitivity of 71.0% and a false positive rate of 37.2% (Table 4). At the cut point selected, 71% of the pregnant cows would be diagnosed correctly and the same cut point would incorrectly diagnose 37.2% of nonpregnant cows as pregnant. At the selected cut point, it was 4.1 times more ($P < 0.05$) likely that the pregnancy risk of cows diagnosed cor-

Figure 3. Pregnancy per AI at d 32 post-AI on the basis of progesterone concentrations assessed just before GnRH-1 or PGF_{2α} treatments, or at 48 h after PGF_{2α}. Bars within treatment category having different letters differ ($P < 0.05$). Values within bars represent the number of observations. Color version available online.

Figure 4. Pregnancy per AI d 32 post-AI on the basis of progesterone concentrations assessed just before GnRH-1 (A; $P = 0.0323$), PGF_{2α} (B; $P = 0.002$), or at 48 h after PGF_{2α} (C; $P < 0.001$) treatments. Values within bars represent the number of observations. Color version available online.

rectly as pregnant was greater than the pregnancy risk of nonpregnant cows (Table 4).

Area under the ROC for a progesterone test before PGF_{2α} for pregnancy was 0.57; the cut point occurred at 0.79 ng/mL with a sensitivity of 93% and a false positive rate of 21.4% (Table 4). At the cut point selected, 93% of all pregnant cows would be diagnosed correctly and the same cut point would incorrectly diagnose 21.4% of nonpregnant cows as pregnant. At the selected cut point, it was 48.7 times more ($P < 0.05$) likely that the pregnancy risk of cows diagnosed correctly as pregnant was greater than the pregnancy risk of nonpregnant cows (Table 4).

Multifactorial Factors Related to Pregnancy Risk

Pregnancy risk was regressed on the combined effects of both ovulation response to GnRH-2 and concentrations of progesterone 48 h after PGF_{2α} (i.e., synchronization risk). The model revealed that both variables were nearly equal contributors to the variation in pregnancy risk and no interaction was detected. The subsequent original multifactorial logistic model contained 10 variables with the best model describing pregnancy risk identified by both backward and stepwise selection. The model excluded DIM, ovulation after GnRH-1, and dominant follicle before GnRH-1 resulting in retention of 7 significant variables (Table 5). The 7 significant variables are listed in order by the proportion of variation accounted for by each variable after adjusting for all other variables in the model (Table 5). Three variables accounting for more than 78% of variation in pregnancy risk were, in order of importance, ovulation after GnRH-2, progesterone concentration 48 h after PGF_{2α}, and progesterone concentration before PGF_{2α}. The remaining 4 variables accounted for the residual proportion of variation in pregnancy risk (progesterone concentration before GnRH-1, BCS, size of the ovulatory follicle 48 h after PGF_{2α}, and parity; Table 5).

Examining only 3 variables commonly thought to affect pregnancy risk: ovulation response to GnRH-1 and GnRH-2, and progesterone concentration at 48 h after PGF_{2α}, as evidence for complete luteolysis, pregnancy risk ($>42\%$) was consistently greatest (79.3% of cows) when progesterone was <1 ng/mL 48 h after PGF_{2α} and when ovulation occurred after GnRH-2 (i.e., synchronization risk = 1), regardless of whether ovulation occurred after GnRH-1 (Table 6). Reduced pregnancy risk ($<19.5\%$) in the remaining 20.7% of cows occurred at all other combinations of the 3 variables (Table 6).

When similar comparisons were made, but with progesterone at 48 h after PGF_{2α} at lesser cut point concentration (<0.5 ng/mL), a different pattern was detected. The most likely pregnancy risk ($>43\%$) again

Table 4. Receiver operator-derived true positive and false positive risks for serum progesterone (P4) before GnRH-1, PGF_{2α}, and 48 h after PGF_{2α} predicting pregnancy per AI¹

Item [no./no. (%)]	Accuracy ² (%)	True positive ³	False positive ⁴	Pregnancy per AI	
				Not pregnant ⁵	Pregnant ⁶
P4 at GnRH-1	48.4	470/798 (58.9)	654/1,300 (50.3)	328/974 (33.7)	470/1,124 (41.8)
		55.5–62.3 ⁷	47.6–53.0 ⁷	Referent	1.42 ⁸ (1.18–1.69)
P4 at PGF _{2α}	65.9	566/797 (71.0)	484/1,301 (37.2)	231/1,048 (22.0)	566/1,050 (53.9)
		67.9–74.2 ⁷	34.6–39.8	Referent	4.14 ⁸ (3.42–5.0)
P4 at 48 h after PGF _{2α}	84.2	742/798 (93.0)	278/1,300 (21.4)	56/1,078 (5.2)	742/1,020 (72.7)
		91.2–94.7 ⁷	19.2–23.6	Referent	48.7 ⁸ (36.0–65.9)

¹Overall pregnancies per AI (P/AI) = 798/2,098 = 38.0%.²Correct classification rate is the proportion of observations for which the test result and actual response agree.³True positive (sensitivity). Number of pregnant cows above (P4 at GnRH-1 or at PGF_{2α}) or below (P4 at 48 h after PGF_{2α}) the selected cut points for progesterone divided by the total number of pregnant cows. Selected cut points for progesterone were 2.93, 3.53, and 0.78 ng/mL for the 3 time points, respectively.⁴False positive (1 – specificity). Number of nonpregnant cows above (P4 at GnRH-1 or at PGF_{2α}) or below (P4 at 48 h after PGF_{2α}) the selected cut points for progesterone divided by the total number of pregnant cows. Selected cut points for progesterone were 2.93, 3.53, and 0.78 ng/mL for the 3 time points, respectively.⁵Pregnancy per AI for cows diagnosed nonpregnant based on the selected cut point.⁶Pregnancy per AI for cows diagnosed pregnant based on the selected cut point.⁷95% confidence intervals for true positive or false positive values.⁸Odds ratio and 95% confidence interval for comparison of pregnancy risk.

occurred when progesterone <0.5 ng/mL and ovulation occurred after GnRH-2 (60.4% of cows), regardless of the ovulatory response to GnRH-1 (Table 6). In contrast, a secondary range of pregnancy risk (33 to 35%; 27.6% of cows) occurred with the greater risk occurring

in cows that ovulated after GnRH-2, regardless of the response to GnRH-1, but all had progesterone ≥0.5 ng/mL 48 h after PGF_{2α} (Table 6). These 2 responses were followed by 2 groups of cows (6.8% of cows), which had pregnancy risk that did not differ from the latter

Table 5. Best-fit multifactorial logistic model accounting for pregnancy per AI (P/AI) in dairy cows subjected to Ovsynch timed AI program

Item	no.	P/AI (%)	Wald χ^2	<i>P</i> <	AOR ¹	Lower CI ²	Upper CI ²
Ovulation after GnRH-2							
No	244	14.3	40.0	0.001	Referent		
Yes	1,805	41.4			3.46	2.35	5.09
Progesterone 48 h after PGF (ng/mL)			26.9	0.001	Referent		
<0.50	1,378	42.2			0.61	0.49	0.76
0.50 to 3.0	603	31.8			0.31	0.16	0.63
≥3.0	68	14.7					
Progesterone at PGF _{2α} (ng/mL)			11.8	0.003	Referent		
<0.50	89	23.6			1.84	1.05	3.22
0.50 to 3.0	503	34.4			2.30	1.37	3.87
≥3.0	1,457	40.4					
Progesterone at GnRH-1 (ng/mL)			7.9	0.019	Referent		
<0.50	363	31.1			1.05	0.76	1.45
0.50 to 3.0	612	35.1			1.39	1.02	1.88
≥3.0	1,074	42.3					
BCS	2,049	38.2	5.1	0.024	1.27	1.03	1.57
Ovulatory follicle, mm			4.4	0.035	Referent		
<14.5	1,016	35.9			1.23	1.01	1.50
≥14.5	1,035	40.0					
Parity			3.7	0.053	Referent		
Primiparous	922	41.6			0.82	0.67	1.00
Multiparous	1,127	35.4					

¹Adjusted odds ratio.²95% confidence intervals.

Table 6. Pregnancy per AI (P/AI) based the ovulatory responses to GnRH-1 and GnRH-2, at 2 progesterone (P4) cut points (<0.5 or <1 ng/mL) at 48 h after PGF_{2α} in dairy cows subjected to Ovsynch timed AI program

Response					Response				
Ovulation after GnRH-1	Ovulation after GnRH-2	P4 (ng/mL)	n	P/AI (%)	Ovulation after GnRH-1	Ovulation after GnRH-2	P4 (ng/mL)	n	P/AI (%)
Yes	Yes	<1	974	45.2 ^a	Yes	Yes	<0.5	721	45.6 ^a
No	Yes	<1	653	42.8 ^a	No	Yes	<0.5	519	43.4 ^a
Yes	No	<1	90	19.5 ^b	Yes	Yes	≥0.5	380	35.3 ^b
Yes	Yes	≥1	127	18.1 ^b	No	Yes	≥0.5	187	33.6 ^b
No	No	<1	94	17.1 ^b	Yes	No	<0.5	66	25.0 ^b
No	Yes	≥1	53	15.5 ^b	No	No	<0.5	74	18.9 ^{bc}
Yes	No	≥1	44	4.4 ^b	No	No	≥0.5	36	5.5 ^c
No	No	≥1	16	0.0 ^{ab}	Yes	No	≥0.5	68	4.4 ^{cd}

^{a-d}Means within a column with different superscripts differ ($P \leq 0.05$).

2 groups of cows (19 to 25%), but had progesterone <0.5 ng/mL, did not ovulate after GnRH-2, regardless of the response to GnRH-1 (Table 6). A last group of cows (5.1% of total) with pregnancy risk <6% had progesterone >0.5 ng/mL and failed to ovulate after GnRH-2, regardless of their response to GnRH-1.

DISCUSSION

Results of the present study identified the relative importance of factors that facilitate luteolysis, ovulation, and pregnancy risk, and provide potential testable hypotheses that may increase pregnancy risk in lactating dairy cows subjected to TAI programs. Furthermore, by applying the ROC analyses, specific cut points in progesterone concentrations were identified, some of which were highly predictive of pregnancy and ovulation risks.

Luteal status at GnRH-1 and its resulting progesterone concentration were related to subsequent ovulatory and pregnancy risks that followed TAI. Ovulatory response to GnRH-1 (60%) in the present study was consistent with other reports of approximately 45 to 70% in cows exposed to Ovsynch at random stages of the estrous cycle (Pursley et al., 1995; Vasconcelos et al., 1999). The percentage of cows that ovulated after GnRH-1 in response to various PGF_{2α} Presynch programs varied from 45 to 70% (Galvão et al., 2007; Souza et al., 2008; Stevenson et al., 2012), but were less than approximately 80% in studies employing both GnRH and PGF_{2α} as part of Presynch programs (Souza et al., 2008; Stevenson et al., 2012; Ayres et al., 2013). Cows with the greatest average concentration of progesterone before GnRH-1 were less likely to ovulate and form a new CL consistent with previous observations (Giordano et al., 2012; Stevenson and Pulley, 2016). The cut point concentration of progesterone at 3.66 ng/mL provided good sensitivity for predicting ovula-

tion after GnRH-1 (70.2% true positives), but the false positive risk was quite high at 59%. This high false positive risk may be partially explained by the lack of an LH sensitive and responsive follicle at GnRH-1. Although it is clear that GnRH-induced LH release is compromised and ovulation response is reduced at greater progesterone concentrations (Stevenson and Pulley, 2016), elevated progesterone was not solely responsible for inhibiting or reducing ovulation risk. Reports of progesterone concentration before GnRH-1 in cows exposed to GnRH-PGF_{2α} Presynch programs compared with PGF_{2α} Presynch programs indicated that progesterone in the mid-range between 0.5 and ≤3.0 ng/mL was one factor associated with increased pregnancy risk for cows exposed to Double Ovsynch (Ayres et al., 2013).

Another limiting factor to ovulation after GnRH-1 was the stage of the estrous cycle (i.e., stage of follicle dominance) for cows classified in the original CL status having greater progesterone concentrations. Dominant follicles were smaller in diameter in cows having a CL at GnRH-1, which failed to ovulate when progesterone concentrations were between 0.5 and 3.0 ng/mL, which concentration also was below the selected cut point. Despite adequate LH release, ovulation is unlikely for cows during early follicular-wave development in which a follicle with functional LH receptors capable of ovulation is not present in the ovaries (Sartori et al., 2001). In the present study, ovulation after GnRH-1 was more likely when follicles were >14.5 mm in diameter.

Although ovulation risk was always greater when progesterone concentrations were least in response to both GnRH injections (Figure 1), accuracy of predicting true positive ovulators after GnRH-2 was almost 90% at the selected progesterone cut point of 1.18 ng/mL. The false positive risk was also relatively small at 24.2%. It is important to note the selected cut point was based on progesterone concentrations assessed at 48 h after

PGF_{2α}, and for cows bearing an original CL. Regardless of their ovulation status after GnRH-1, more than 90% of the cows had progesterone concentrations <1 ng/mL at 48 h. Furthermore, one would expect those concentrations to have decreased further 24 h later at the time of AI. Ovulation outcomes were greater at lesser progesterone concentrations after both GnRH injections despite the fact that pregnancy outcomes were greatest when progesterone exceeded 3.0 ng/mL before GnRH-1 and PGF_{2α}. It seems that progesterone concentration at GnRH-1, and not ovulation incidence, is a limiting factor in improving subsequent fertility. Because ovulatory response to GnRH-1 was eliminated from the multivariable logistic regression model and progesterone concentration before PGF_{2α} was one of 3 most important predictors of pregnancy risk, progesterone concentration after GnRH-1 is the direct key predictor, which may or may not require successful ovulation to GnRH-1. Improved fertility resulting from greater progesterone concentration during follicular development after GnRH-1 may be partially explained by decreased LH pulsatility and its effect on improving competency of the dominant follicle, quality of the ovulated oocyte (Mihm et al., 1994; Revah and Butler, 1996; Pursley and Martins, 2011), and progesterone-enhanced uterine environment (Bridges et al., 2013). Furthermore, reduced fertility in dairy cows occurs when the preovulatory follicle developed in a reduced progesterone environment despite similar fertilization risk and only minor changes in embryo quality (Cerri et al., 2011a,b).

The fact that more than 90% of cows with at least 1 original CL at GnRH-1 had progesterone concentrations <1 ng/mL by 48 h after PGF_{2α} indicates more complete luteolysis in these cows compared with cows having only a new, younger CL. This finding is consistent with previous observations in which younger CL (<d 10 of the estrous cycle) are less vulnerable to the luteolytic effects of exogenous PGF_{2α} (Momont and Seguin, 1984). Furthermore, that more cows with both older and new CL had complete luteolysis than cows with only a new younger CL is consistent with the report that hCG-induced CL on d 10 of the estrous cycle were responsive to PGF_{2α} when they were less than 5 d old when in the presence of an older CL (Howard and Britt, 1990). In that study, the authors concluded that CL induced by treatment with human chorionic gonadotropin (hCG) on d 10 of the estrous cycle regress after exogenous PGF_{2α} by 2 d after ovulation, whereas spontaneously formed CL respond to PGF_{2α} only after 5 d. Furthermore, the d 5 CL in nonlactating dairy cows is equally resistant to two 25-mg (administered 8 h apart on d 5) or one 50-mg dose of PGF_{2α} (administered on

d 5; Nascimento et al., 2014). Alternatively, structures induced by exogenous hCG may not be CL, but luteinized follicles, which may respond to differently to exogenous PGF_{2α} when compared with a mature CL.

Results of the present study and those of the previously cited study (Howard and Britt, 1990) are intriguing because high affinity PGF_{2α} receptors were identified in CL recovered from plasma membranes of CL of heifers on d 2, 4, 6, or 10 (d 0 = ovulation), suggesting that the reported lack of responsiveness to PGF_{2α} in the early CL was not attributable to a deficiency of high-affinity PGF_{2α} receptors (Wiltbank et al., 1995). Yet in the presence of an older CL, a younger CL is responsive to PGF_{2α}. More complete luteal regression occurred in recent studies in which 2 standard doses of PGF_{2α} administered to lactating dairy cows on d 7 and 8 of a 7-d Ovsynch program preceded by either a nonbreeding Ovsynch (Double Ovsynch) or GnRH alone before first postpartum AI (Wiltbank et al., 2015). Although improvement in pregnancy risk at TAI averaged only 3 percentage points in 12 herds, the greatest enhancement in fertility occurred in multiparous cows and cows having the greatest progesterone concentration before PGF_{2α} and not in cows with low progesterone (<2 ng/mL) before the first of the 2 PGF_{2α} treatments. These observations are consistent with greater progesterone concentration in cows of the present study that had an original CL + new CL for both luteal regression and greater pregnancy risk compared with cows with lower progesterone before PGF_{2α} and likely poorer complete luteal regression and poorer pregnancy risk (Table 2). The present findings may also indicate that CL response to PGF_{2α} and complete luteolysis is essential to improve fertility, as well as the uterine response to these greater progesterone concentrations before PGF_{2α}-induced luteolysis.

In contrast, the greatest improvement in CL regression in response to 2 PGF_{2α} treatments was observed in cows with low progesterone in the former report (Wiltbank et al., 2015) indicates a potential nonluteolytic role for the second PGF_{2α} injection. A recent study (Ambrose et al., 2015) consisting of 2 experiments demonstrated increased pregnancy risk in lactating dairy cows exposed to 10, but not 5 mg of PGF_{2α} administered at the time of an Ovsynch TAI. Apart from its primary luteolytic role, PGF_{2α} has multiple actions on the female reproductive system in cattle (Weems et al., 2006) including altered oviductal and uterine motility in addition to known post-LH surge effects of PGF_{2α} in the ovary and preovulatory follicle such as lysosomal enzyme activity, contraction of ovarian smooth muscle, and increased follicular pressure (Espey, 1994). Similar improvements in complete luteolysis were reported for

cows in which ovulation was resynchronized after a negative pregnancy diagnosis when 2 standard doses of PGF_{2α} (24 h apart) were administered compared with 1 single dose (Carvalho et al., 2015), but pregnancy risk was not improved significantly.

Pregnancy risk improved with increasing concentrations of progesterone assessed before GnRH-1 and PGF_{2α}. Cows with concentrations >3.0 ng/mL before GnRH-1 and PGF_{2α} had the greatest pregnancy risk, but at those greater concentrations, ovulatory response to GnRH-1 was significantly reduced. The true positive risk of predicting pregnancy risk for the selected cut point of progesterone before GnRH-1 was only 59% and the false positive risk was 50%. The cut point of 2.93 ng/mL for pregnancy risk was less than that for ovulation risk (3.66 ng/mL). Therefore, the utility of using progesterone concentration before GnRH-1 to predict subsequent pregnancy risk at TAI was less accurate than its ability to predict ovulation success to GnRH-1.

As previously pointed out, studies in which cows were exposed to Double Ovsynch versus Presynch-Ovsynch demonstrated that more Double Ovsynch cows had mid-range progesterone concentrations between 1 and 3 ng/mL before GnRH-1 and fewer had concentrations <1 ng/mL (Ayres et al., 2013). Finding that progesterone was more mid-range in concentration is consistent with greater ovulatory responses after GnRH-1 and may be part of the mechanism by which greater pregnancy risk is facilitated in cows exposed to presynchronization protocols that include both GnRH and PGF_{2α} (Souza et al., 2008; Herlihy et al., 2012; Stevenson and Pulley, 2012). Furthermore, more cows, subjected to Double Ovsynch, had progesterone concentrations >3 ng/mL and fewer in the mid range from 1 to 3 ng/mL at the time of PGF_{2α} administration before the breeding week (Ayres et al., 2013). This is consistent with the results from the present study in which pregnancy risk was greatest in cows with progesterone >3.0 ng/mL before PGF_{2α} administration (Figure 3) and with the ROC selected cut point of 3.53 ng/mL. At that cut point, the true positive risk of identifying pregnant cows was 71%, with a false positive risk of 37.2% (Table 4). In addition, when progesterone was at lesser concentrations (<0.5 ng/mL) by 48 h after PGF_{2α} administration during the breeding week, fertility of cows was maximized in the present study. The importance of this lesser progesterone concentration after PGF_{2α} administration corroborates with other reports (Santos et al., 2010; Pursley and Martins, 2011; Wiltbank et al., 2014). The selected cut point of 0.78 ng/mL at 48 h after PGF_{2α} correctly identified 93% of the pregnant cows with a false positive risk of only 20.4% (specificity = 78.6%). This selected cut point of 0.78 ng/mL in the

present study is greater than the ROC-predicted cut point of 0.24 ng/mL reported for cows (n = 1,165) at 72 h after PGF_{2α} (time of AI; Santos et al., 2010). The sensitivity for their cut point was 89.7% for predicting pregnancy risk at d 38 after AI, whereas the specificity was 37.3%. The greater accuracy of our results relative to the previous report is partly related to a greater sample size (n = 2,098 vs. 1,165). The greatest accuracy of predicting both ovulation and pregnancy risk was found in the progesterone concentration assessed at 48 h after PGF_{2α}. In both cases, the accuracies were between 84 and 89%, and they shared almost equally in the amount of variation they explained in pregnancy risk, which lends further support to the fact that both factors are essential to accurately predict the success of TAI.

In the current experiment, pregnancy risk at TAI tended to be greater in cows bearing an original CL, and that ovulated after GnRH-1, compared with cows with an original CL that failed to ovulate after GnRH-1. Improved pregnancy risk tended to be related to increased synchrony of luteolysis and subsequent ovulation after GnRH-2 as assessed by improved synchronization risk in the present study. The surprise in these results arose in the multiple logistic regression models in which the best fit model accounting for variation in pregnancy risk eliminated not only DIM and dominant follicle size before GnRH-1, but also ovulation response after GnRH-1. That ovulatory response to GnRH-1 was not found to explain a significant proportion of variation in pregnancy risk could be interpreted to be inconsistent with the results in Tables 1 and 2, if one assumes that increased pregnancy risk is solely dependent on successful ovulation after GnRH-1 as suggested in previous studies (Bello et al., 2006; Chebel et al., 2006; Galvão and Santos, 2010). These conclusions fail to consider the increase in progesterone resulting from cows that ovulate after GnRH-1 in the face of also having a CL at GnRH-1. Therefore, the explanation for the associated increased pregnancy risk in cows in the present study and in other studies for cows with an original CL and a new CL is confounded by the fact that these cows also have greater progesterone before PGF_{2α}.

Recent studies may unravel this confounding of greater progesterone and ovulation response to GnRH-1. When progesterone was supplemented to cows without a CL before first postpartum AI at the onset of either a 5- (Bisinotto et al., 2013) or 7-d Ovsynch program (Stevenson et al., 2008; Bisinotto et al., 2015), subsequent TAI pregnancy risk was enhanced compared with non-CL control cows such that P/AI in progesterone-supplemented cows equaled that of cows starting the program in diestrus having a CL. This improvement

in P/AI occurred in the supplemented non-CL cows compared with the nonsupplemented non-CL cows despite ovulation risk after GnRH-1 in both groups being similar (61 to 67%) and even less than that detected in the diestrous cows (47 to 52%; Bisinotto et al., 2013, 2015). One can therefore conclude from these results that the greater concentrations of progesterone explain the subsequent increase in P/AI independent of the ovulatory response to GnRH-1.

The multiple logistic regression approach of the present study ranked ovulation outcome after GnRH-1 to be of much lesser importance (Tables 5 and 6) than several other factors. In support of the present findings are results of a prospective experiment in which increased doses of GnRH at GnRH-1 increased ovulation risk, but did not increase subsequent pregnancy risk (Giordano et al., 2013). The lesser importance of ovulatory response to GnRH-1 also was supported by the critically essential combination of both low progesterone concentrations (<0.5 ng/mL) by 48 h after PGF_{2 α} and the high ovulatory response to GnRH-2. None of the permutations of the latter 2 variables plus the ovulation response to GnRH-1 showed strong evidence that ovulation after GnRH-1 was critically important to the subsequent pregnancy outcome (Table 6). The strength of these interpretations lie in the accuracy of assessing multiple factors together and examining their individual contributions to account for the total variation in the response measured (i.e., ovulation and pregnancy risks). This interpretation is further reinforced in the present study by the fact that the greatest increase in pregnancy risk (Table 2) was associated with cows that had achieved both basal progesterone concentration by 48 h after PGF_{2 α} and successful ovulation after GnRH-2. Therefore, increased progesterone in those cows before PGF_{2 α} was critical to pregnancy outcome, and overall, all cows with progesterone >3 ng/mL at PGF_{2 α} were more likely to conceive than cows with less progesterone. This conclusion is further supported by the true positive predictability of pregnancy in cows with progesterone at PGF_{2 α} greater than the cut point of 3.53 ng/mL of 93% (Table 4).

The 3 variables accounting for more than 78% of variation in pregnancy risk were ovulation after GnRH-2, progesterone concentration 48 h after PGF_{2 α} , and progesterone concentration before PGF_{2 α} . The first 2 variables are consistent with findings of many studies that indicate that cows whose ovulation is synchronized (complete luteolysis and ovulation after GnRH-2) have the greatest pregnancy risk (see Bisinotto et al., 2014). Furthermore, greater progesterone concentrations before PGF_{2 α} improved pregnancy risk in cows inseminated at estrus compared with other treatments that

did not produce as great of progesterone concentrations before PGF_{2 α} (Folman et al., 1984).

CONCLUSIONS

Ovulation outcomes were greater at lesser progesterone concentrations after both GnRH injections despite the fact that pregnancy outcomes were greatest when progesterone exceeded 3 ng/mL before GnRH-1 and PGF_{2 α} , suggesting that greater progesterone concentration before GnRH-1 and incidence of ovulation act via different mechanisms to improve subsequent fertility. Ovulation after GnRH-2 was related positively to lesser concentrations of progesterone at 48 h after PGF_{2 α} , as was pregnancy outcome, but for maximal pregnancy outcome, progesterone must be <0.5 ng/mL. Cows with a CL before GnRH-1, who also formed a new CL after GnRH-1, had the greatest pregnancy outcome, whereas cows with only a new CL (i.e., anovulatory cows and those at or near estrus without a CL at GnRH-1) had the poorest pregnancy outcome. Pregnancy loss was not affected by CL status during the timed AI program. Receiver operator curves produced cut points in progesterone concentration that predicted ovulation and pregnancy risk. Cut points for progesterone before GnRH-1 and PGF_{2 α} produced true positive risks for pregnancy in the range of 59 to 71% with false positive risks of 37 to 50%. Similar results were detected for predicting ovulation risk after GnRH-1 with true positive risk of 71%, but with a high false positive risk of 59%. In contrast, selected cut points of progesterone concentration 48 h after PGF_{2 α} produced true positive risks greater than 90% and false positive risks less than 25%, thus demonstrating the high predictability of ovulation after GnRH-2 and subsequent pregnancy outcome based on progesterone concentration.

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