Extending the duration of the voluntary waiting period from 60 to 88 days in cows that received timed artificial insemination after the Double-Ovsynch protocol affected the reproductive performance, herd exit dynamics, and lactation performance of dairy cows

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ABSTRACT

This experiment evaluated the reproductive performance, herd exit dynamics, and lactation performance of dairy cows managed with a voluntary waiting period (VWP) of 60 or 88 d. Secondary objectives were evaluating VWP effect on cyclicity status, uterine health, systemic inflammation, and body condition score (BCS) before first service. Lactating Holstein cows from 3 commercial farms in New York State were blocked by parity group and total milk yield in their previous lactation and then randomly assigned to VWP of 60 (VWP60; $n = 1,352$) or 88 (VWP88; $n = 1,359$) days in milk (DIM). All cows received the Double-Ovsynch protocol (GnRH-7 d-PGF_{2α}-3 d-GnRH-7 d-GnRH-7 d-PGF_{2α}-56 h-GnRH-16 to 20 h-timed artificial insemination; TAI) for synchronization of ovulation and TAI. For second and greater artificial insemination (AI), cows received AI after detection of estrus or the Ovsynch protocol (GnRH-7 d-PGF_{2α}-56 h-GnRH-16 to 20 h-TAI) initiated 32 ± 3 d after AI for cows not re-inseminated at detected estrus. Cyclicity status (progesterone concentration), uterine health (vaginal discharge and uterine cytology), BCS, and systemic inflammation (haptoglobin concentration) were evaluated at baseline (33 ± 3 DIM for both treatments), beginning of the Double-Ovsynch protocol, and 10 d before TAI. Effects of treatments were assessed with multivariable statistical methods relevant for each outcome variable. Extending duration of VWP from 60 to 88 DIM increased pregnancies per AI (P/AI) to first service (VWP60 = 41%; VWP88 = 47%). Nonetheless, the greatest benefit of extending VWP on first-service P/AI was for primiparous cows (VWP60 = 46%; VWP88 = 55%), as P/AI did not differ within

the multiparous cow group (VWP60 = 36%; VWP88 = 40%). Physiological status more conducive to pregnancy—characterized by improved uterine health, greater BCS, reduced systemic inflammation, and to a lesser extent more time to resume ovarian cyclicity—explained the increment in P/AI to first service. Our data also indicated that despite having greater P/AI to first service, cows with the longer VWP had delayed time to pregnancy during lactation (hazard ratio = 0.72; 95% confidence interval 0.69–0.98) and greater risk of leaving the herd, particularly for multiparous cows (hazard ratio = 1.34; 95% confidence interval 1.23–1.47). This shift in pregnancy timing led to an overall extension of the lactation length (+13 d), which resulted in greater total milk yield per lactation (+491 kg) but not greater milk yield per day of lactation. In conclusion, data from this experiment highlight the importance of considering the complex interactions between reproductive performance, herd exit dynamics, and lactation performance as well as the effects of parity at the time of defining the duration of the VWP for lactating dairy cows.

Key words: voluntary waiting period, reproductive performance, herd exit dynamics, dairy cow

INTRODUCTION

Timing of pregnancy during lactation affects the profitability of dairy herds by defining calving interval, milk production efficiency, and herd replacement dynamics (De Vries, 2006; Inchaisri et al., 2011; Giordano et al., 2012). Although insemination and conception risk after the end of the voluntary waiting period (VWP) are the 2 major determinants of time to pregnancy during lactation, the duration of the VWP can also influence timing of pregnancy because it determines when cows become eligible for insemination. Traditionally, VWP duration in US dairy farms has been ~60 d (Miller et al., 2007). Recent changes in reproductive performance and management of dairy herds, however, may

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allow the duration of the VWP to be manipulated to optimize reproductive performance and profitability. For example, aggressive management programs that substantially reduce the variation of time to pregnancy may enable dairy farms to select the optimal timing to achieve pregnancy in a substantial proportion of cows.

Extending the duration of the VWP may improve reproductive performance of cows through multiple mechanisms. For example, it may provide more time to recover uterine health (Gilbert et al., 2005; Gautam et al., 2009; Sheldon et al., 2009) through improved immune status, more time to resolve the inflammatory process established immediately after calving, or both (LeBlanc et al., 2011; LeBlanc, 2014). Likewise, a longer VWP may provide cows more time to return to reproductive hormone secretion patterns and a metabolic status that promotes resumption of ovarian cyclicity (Butler, 2003; Kawashima et al., 2012; Cheong et al., 2016). Earlier ovulation postpartum and more estrous cycles before first service were linked to reduced days to first service and greater pregnancies per AI (P/AI; Thatcher and Wilcox, 1973; Butler and Smith, 1989; Darwash et al., 1997). Finally, delaying first service beyond the negative energy balance nadir in early lactation as much as possible may improve reproductive performance by avoiding insemination of cows during severe negative energy balance and provide additional time to recover body tissue reserves. Optimum BCS at the time of insemination is strongly associated with high probability of pregnancy (Souza et al., 2007, 2008; Carvalho et al., 2014).

Despite the potential benefits of extended VWP on reproductive performance, the effect of this management strategy on overall herd performance has not been fully elucidated. Few randomized controlled experiments have evaluated the implications of VWP duration on first-service P/AI and whole-lactation reproductive performance of dairy cows. In an experiment with a limited number of second-lactation cows, Van Amburgh et al. (1997) found no differences in reproductive performance when comparing VWP of 60 versus 150 d. Similarly, Arbel et al. (2001) observed no effect of extending VWP by 60 d on reproductive performance of cows with above-average milk yield under Israeli conditions. In contrast, in an experiment conducted with above- or below-average milk-producing cows, an increment of 13 and 20 percentage points in P/AI was observed when VWP was extended from 77 to 98 or from 56 to 77 DIM for high- and low-producing cows, respectively (Tenhagen et al., 2003). More recently, Gobikrushanth et al. (2014) reported the results of a retrospective cohort study at a commercial farm in Florida that extended VWP duration during summer months only. Cows with the extended

VWP had greater first-service P/AI, more days open, and longer calving intervals. Nonetheless, season of AI might have confounded results as cows with short VWP (57 to 63 d) received first service during summer and fall, whereas cows with long VWP (64 to 121) received first service during fall only. Moreover, the reproductive program used resulted in overlapped DIM at first service for a substantial proportion of cows. Collectively, the ambiguous results and multiple exclusion criteria of previous reports did not allow decisive conclusion that extending the duration of the VWP is beneficial for the performance of dairy herds.

Thus, our primary objective was to evaluate the reproductive performance, herd exit dynamics, and lactation performance of dairy cows managed with a VWP of 60 or 88 DIM. Secondary objectives were evaluating the effect of longer VWP on uterine health, cyclicity status, BCS, and systemic inflammation before first service. We hypothesized that extending VWP duration from 60 to 88 DIM would increase P/AI to first service and improve overall reproductive performance. We further hypothesized that cows with extended VWP would have improved reproductive performance because of improved uterine health, reduced rate of anovulation, improved BCS, and reduced systemic inflammation before first service.

MATERIALS AND METHODS

All procedures performed with cows were approved by the Animal Care and Use Committee of Cornell University.

Farms and Cow Management

Lactating Holstein cows from 3 commercial farms (A, B, and C) in New York State (2 in Cayuga County and 1 in Lewis County) were enrolled in this experiment from March 2014 to March 2015. During the study period the average numbers of milking cows were 1,034, 1,248, and 793 at farms A, B, and C, respectively. Average milk yield per cow per day was 39, 40, and 40 kg for farms A, B, and C, respectively. Data for cow numbers and milk yield were retrieved from the dairy management software (DairyComp305, Valley Ag Software, Tulare, CA) using the ECON\ID command. All farms housed cows in free-stall barns with 4 or 6 rows of stalls, concrete flooring, self-locking headgates in the feedline, and fans and sprinklers in the feedline. On farms A and B, freestall surfaces were covered with either mattresses covered with sawdust or deep sand bedding, whereas on farm C freestall surfaces were covered with deep sand bedding. Farms B and C milked cows thrice daily at approximately 8-h intervals, and

farm A milked cows twice daily at approximately 12-h intervals. Cows were supplemented with recombinant bovine somatotropin (**rbST**; Sometribove zinc, Posilac, Elanco Animal Health, Indianapolis, IN) at all farms. On farm A, cows were supplemented with rbST every 10 or 11 d until dry-off beginning at 120 DIM; on farm B, cows were supplemented with rbST from 110 DIM until dry-off following a 10 and 11 d schedule; and on farm C, cows were supplemented every 10 or 11 d from 65 DIM until dry-off.

Experimental Treatments

The experimental design was a complete randomized block design with parity (primiparous vs. multiparous) as the blocking factor. Milk yield in the previous lactation for multiparous cows was also used as a stratification factor at enrollment. At each farm a list of all cows eligible for enrollment in the experiment (i.e., all ambulatory cows at 7 ± 3 DIM) was generated weekly and transferred to Excel (Microsoft Corporation, Redmond, WA). Cows were blocked by parity group (primiparous vs. multiparous) and within the multiparous group cows were stratified by total milk yield recorded for the previous lactation. Thereafter, cows were randomly assigned to a VWP of 60 [**VWP60**; $n = 1,352$ (farm A = 575; farm B = 458; farm C = 319)] or 88 [**VWP88**; $n = 1,359$ (farm A = 578; farm B = 462; farm C = 319)] DIM (Figure 1). All cows received the Double-Ovsynch (**DO**) protocol (GnRH-7 d-PGF_{2α}-3 d-GnRH-7 d-GnRH-7 d-PGF_{2α}-56 h-GnRH-16 to 20 h-TAI; Souza et al., 2008) for synchronization of ovulation. Cows received timed AI (**TAI**) at 60 ± 3 or 88 ± 3 DIM in the VWP60 and VWP88 treatments, respec-

tively, as cows were enrolled in the Double-Ovsynch protocol on a weekly basis (i.e., Fridays on all farms: 33 ± 3 and 61 ± 3 DIM for cows in the VWP60 and VWP88 treatments, respectively). For second and greater AI services, cows were submitted for insemination after detection of estrus through visual observation (farms A and C) or a combination of visual observation and physical activity monitoring (farm B) using neck-mounted activity tags (DeLaval Activity Meter System, DeLaval International AB, Tumba, Sweden). In the 3 farms, cows not re-inseminated at detected estrus received TAI after resynchronization of ovulation with the Ovsynch protocol (GnRH-7 d-PGF_{2α}-56 h-GnRH-16 to 20 h-TAI) initiated 32 ± 3 d after AI (**D32-Resynch**). On farm C, cows not having a corpus luteum ≥ 15 mm in diameter at the time of the nonpregnancy diagnosis and PGF_{2α} injection of D32-Resynch received a CIDR-Synch protocol (GnRH⁺CIDR-7 d-CIDR removal+PGF_{2α}-56 h-GnRH-16 to 20 h-TAI) as described in Giordano et al. (2016).

On the 3 farms, GnRH treatments consisted of 100 µg of gonadorelin diacetate tetrahydrate given i.m. and PGF_{2α} treatments consisted of 500 µg of cloprostenol sodium given i.m. On farms A and B, the GnRH product used was Fertagyl (Merck Animal Health, Madison, NJ), whereas on farm C, GonaBREED (Parnell Technologies Pty Ltd., Alexandria, Australia) was used. On farms A and B, the PGF_{2α} product used was Estrumate (Merck Animal Health, Summit, NJ), whereas on farm C, EstroPLAN (Parnell Technologies Pty Ltd.) was used.

Out of 2,711 cows enrolled in the experiment at 7 ± 3 DIM, 186 cows (VWP60, $n = 87$; VWP88, $n = 99$) were excluded because they left the herd or were classified as

Figure 1. Graphical depiction of experimental procedures. Cows were randomly assigned to a voluntary waiting period (VWP) of 60 (VWP60; $n = 1,352$) or 88 (VWP88; $n = 1,359$) DIM. All cows received the Double-Ovsynch (DO) protocol for synchronization of ovulation and timed AI (TAI) at 60 ± 3 or 88 ± 3 DIM. Cyclicality (progesterone concentration in circulation), uterine health (UT; vaginal discharge and uterine cytology), BCS, and systemic inflammation (haptoglobin concentration in circulation) were determined at baseline (33 ± 3 DIM for both treatments), the beginning of the DO protocol (33 ± 3 DIM for VWP60 and 61 ± 3 DIM for VWP88), and 10 d before TAI (50 ± 3 DIM for VWP60 and 78 ± 3 DIM for VWP88). For determination of cyclicality status at baseline and beginning of DO, another sample was collected 7 d before the time point of interest to reduce misclassification of cows as not cyclic due to stage of the estrous cycle at time of sampling. Cows received second and greater AI at detected estrus or after resynchronization of ovulation with the D32-Resynch protocol. BC = blood collection; EDAI = estrus detected AI.

“do not breed” by farm personnel before 30 DIM. Cows were also excluded if TAI occurred outside the DIM range specified for their respective experimental group due to a farm management decision. After removal of these cows, the final number of cows per treatment was 1,265 for VWP60 and 1,260 for VWP88.

Blood Sample Collection and Laboratory Assays

Blood samples (~8 to 9 mL) were collected by puncture of the caudal vein or artery using evacuated tubes containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ) from a subgroup of cows on farms A and B ($n = 684$). After collection, samples were immediately placed in crushed ice until transported to the laboratory for further processing. At the laboratory, blood samples were centrifuged at $2,000 \times g$ for 20 min at 4°C in a refrigerated centrifuge. After centrifugation, 3 aliquots of each sample were placed in Eppendorf storage vials and stored at -20°C until assayed.

A schematic representation of the blood-sample collection schedule is presented in Figure 1. The first 2 samples from cows in both treatments were collected at 26 ± 3 and 33 ± 3 DIM. For cows in the VWP60 treatment, 33 ± 3 DIM was the time of the first GnRH treatment of the Pre-Ovsynch portion of the DO protocol (hereafter referred to as the beginning of the DO protocol). For cows in this treatment, another blood sample was collected at 50 ± 3 DIM, coincident with the first GnRH treatment of the Breeding-Ovsynch portion of the DO protocol or 10 d before TAI (hereafter referred to as 10 d before TAI). For cows in the VWP88 treatment, the third and fourth samples were collected at 54 ± 3 and 61 ± 3 DIM to document the status of cows immediately before the beginning of the DO protocol. Another sample was collected 10 d before first-service TAI at 78 ± 3 DIM, coincident with the first GnRH injection of the Breeding-Ovsynch portion of the DO protocol. Samples collected to represent baseline and the beginning of the DO protocol for both VWP treatments were collected 7 d apart to calculate the proportion of cyclic cows [i.e., circulating concentrations of progesterone (**P4**) ≥ 1 ng/mL] at each of these time points. This approach was used to reduce misclassification of cows as not cyclic due to stage of the estrous cycle at time of sampling.

Blood samples were assayed for P4 at all time points for all cows sampled and for haptoglobin in a subgroup of cows at 33 ± 3 and 50 ± 3 DIM for the VWP60 treatment ($n = 120$), and at 33 ± 3 , 61 ± 3 , and 78 ± 3 DIM for the VWP88 treatment ($n = 120$).

Plasma P4 concentrations were analyzed in duplicate using a commercial solid-phase, no-extraction RIA

(Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA). To assess precision of the assay, control samples with high (4.8 ng/mL) and low (0.4 ng/mL) P4 concentrations were included at the beginning and end of each assay ($n = 20$ assays). Average detection limit of the assay was 0.05 ng/mL. For the high-concentration sample, intraassay coefficient of variation was 6.0% and interassay coefficient of variation was 10.0%. For the low-concentration sample, intraassay coefficient of variation was 14.2% and interassay coefficient of variation was 20.7%.

Plasma haptoglobin was analyzed in duplicate following an enzymatic analysis that quantified haptoglobin-hemoglobin complex by estimated differences in peroxidase activity as described in Bicalho et al. (2014). Concentrations were calculated against a standard curve from 0 to 2.5 mg/mL (Molecular Innovations, Novi, MI). Measurements were conducted using a SpectraMax 190 microplate reader. Intra- and interassay coefficients of variation were 9.3 and 21.3%, respectively.

Evaluation of Reproductive Tract Health

Vaginal discharge and uterine cytology were evaluated in a subgroup of cows ($n = 263$) at the baseline, beginning of DO, and 10 d before TAI for both treatments (Figure 1). Vaginal discharge was examined using a Metrichick device (Simcro, Hamilton, New Zealand), and scored on a 0 to 5 scale (0 = no discharge, 1 = clear mucus, 2 = clear mucus with flecks of pus, 3 = mucopurulent but <50% pus, 4 = mucopurulent with >50% pus, and 5 = foul-smelling discharge) as described in McDougall et al. (2007). Purulent vaginal discharge (**PVD**) was defined as a Metrichick score ≥ 2 . This cut-off was selected based on the minimum value associated with a reduction in P/AI for first service in the current experiment regardless of experimental treatment (data presented in Results section).

Uterine cytology samples were collected through the cytobrush technique to determine the percentage of PMN in the uterine lumen. Samples were collected using a stainless steel gun attached to a sterile brush (Medscand Cytobrush Plus GT, CooperSurgical Inc., Trumbull, CT) following the technique described by Madoz et al. (2013). The smears were air-dried and stained with Dip Quick kit (Jorgensen Laboratories Inc., Loveland, CO). Each slide was evaluated at 400 $\times$ magnification by a single observer. Percentage PMN in each slide was calculated from 2 counts of 100 cells each in 2 different locations of the slide. If the difference between the 2 counts was greater than 10 percentage points, a third count was conducted and the average of the 3 counts was used. Cutoff values to classify cows

with or without cytological endometritis (**CYTO**) were determined using receiver operating characteristic curves generated with MedCalc (version 17.2; MedCalc Software BVBA, Ostend, Belgium). The cutoff selected represented the minimum percentage of PMN that indicated with greatest combined sensitivity and specificity a positive pregnancy outcome at first service (data presented in Results section).

BCS

Body condition score was recorded every time blood was collected. A scale of 1 (emaciated) to 5 (fat) with increments of 0.25 was used (Edmonson et al., 1989). Data for BCS were dichotomized using a threshold BCS of 2.75 units (high ≥ 2.75 , low < 2.75).

Pregnancy Diagnosis

Pregnancy testing was conducted at 39 ± 3 d after AI at the 3 farms. Transrectal palpation of the uterine contents was used on farm A and transrectal ultrasonography on farms B and C. On farm A, rectal palpation was conducted by a trained farm technician whereas transrectal ultrasonography was conducted by veterinarians using an Ibex Pro (Ibex, Loveland, CO) machine on farm B and an Easi-Scan (BCF Technology Ltd., Livingston, UK) machine on farm C. Reconfirmation of pregnancy status in pregnant cows was conducted by transrectal palpation (farm A) or transrectal ultrasonography (farms B and C) at 67 ± 3 , 95 ± 3 , and 109 ± 3 d after AI on farms A, B, and C, respectively. Therefore, pregnancy loss for each farm was estimated from the day of the initial pregnancy diagnosis (39 ± 3 d after AI) until reconfirmation of pregnancy.

Estimation of Daily Milk Yield

Daily milk yield for the entire lactation for each cow was estimated with the MilkBot model (Ehrlich, 2011) using monthly test records retrieved from the dairy management software. Briefly, the model predicts daily milk yield as a function of DIM and 4 MilkBot parameters: scale, ramp, offset, and decay. Tertiles of milk yield, based on the accumulated milk yield up to 30 DIM (**MK30**), were calculated to classify cows in high, medium, and low milk yield groups. To account for the effect of farm and parity on milk yield, cutoff values for milk yield tertiles were obtained for individual farms and parity groups. Cutoff points for MK30 are presented in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13046>). To test if MK30 was representative of milk yield of cows at the approximate DIM for first

service, the correlations between MK30 versus milk yield up to 60 (**MK60**) and 90 (**MK90**) DIM were estimated. Correlations were MK30 versus MK60, $R^2 = 0.997$ ($P < 0.01$) and MK30 versus MK90, $R^2 = 0.973$ ($P < 0.01$). Therefore, we used MK30 instead of MK60 or MK90 to group cows in milk yield groups because the first test date data were available for every cow.

Statistical Analysis

A sample size calculation was performed using the “sample size calculation” option of WinPepi version 11.54 (Abramson, 2011). Based on an expected minimum difference in the proportion of cows pregnant at first TAI of 6 percentage points, assuming 47% P/AI for the VWP88 (approximation based on Souza et al., 2008; Herlihy et al., 2012; Giordano et al., 2013) and a 10% correction factor for cows not expected to have a first service outcome, a total of 1,175 cows per treatment were needed for a 2-tailed test with probability of type I error rate of 5% and probability of type II error rate of 20%.

Dichotomous outcomes [P/AI, pregnancy loss, proportion of cows AI after estrus detection, proportion of cows that were sold, died, or left the herd (sold plus dead), and proportion of cows with $P4 \geq 1$ ng/mL, PVD, CYTO, and $BCS \geq 2.75$] were analyzed by logistic regression using the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Continuous outcomes were analyzed by ANOVA with (monthly milk test) or without (inter-service interval, haptoglobin concentrations, milk yield, lactation length, DIM, and days of gestation at dry-off) repeated measurements using the MIXED procedure of SAS. Time to event outcomes (time to pregnancy and time to herd exit) were analyzed by Cox’s proportional hazards regression using the PHREG procedure of SAS. Kaplan-Meier survival curves generated using the Survival Analysis option of MedCalc were used for illustration of time to pregnancy and herd exit. For time-to-pregnancy analysis, a cow was considered pregnant only if pregnancy was maintained up to 150 d of gestation. Analysis of count data (total number of AI, number of PMN) was performed by Poisson regression using the GENMOD procedure of SAS.

The effect of treatment (VWP60 or VWP88), parity (primiparous vs. multiparous), and treatment by parity interaction were offered as explanatory variables to all models, whereas farm was included as a random effect. In addition, for outcomes related to first service, the effect of season of insemination (cold vs. warm), MK30 (low, medium, and high), treatment by MK30, and treatment by season interactions were offered to the

models. Season of insemination was defined as warm (from June 21 to September 20) or cold (September 21 to June 20) based on the date of first TAI.

For physiological parameters (cyclicity, reproductive tract health, systemic inflammation, and BCS) and time to event data, the predictors were the same as previously described, except that season of first service was removed from the models.

The effect of VWP duration on milk yield measured in monthly milk tests was evaluated with a model that included treatment, monthly milk test number, and the treatment by test number interaction.

Assumptions of normality of residuals, linear relationship, and homoscedasticity for linear regression models were tested by evaluating the normal probability plot (normal Q-Q plot) and plotting residuals versus predicted values. All parameters evaluated by linear regression models met the aforementioned assumptions. For logistic regression models, goodness of fit was evaluated using the Hosmer-Lemeshow test. In all cases, model fit was deemed acceptable based on $P > 0.10$ for the Hosmer-Lemeshow test. For Poisson regression, goodness of fit of all models was evaluated with the Pearson chi-square test. Because overdispersion was present in the 2 Poisson regression models used, standard errors were scaled using the Pearson dispersion factor (Pearson $\chi^2/\text{degrees of freedom}$). The proportional hazard assumption for time to event data analysis was evaluated by graphical examination of the $\log[-\log(\text{survival probability})]$ function obtained from the PROC LIFETEST of SAS. According to this analysis, the assumption of proportional hazards was met for all models. The final model for each binary outcome of interest was selected by backward elimination of explanatory variables with $P > 0.10$ and determination of the lowest value for the Akaike information criterion. Further, the potential effect of confounders with $P > 0.10$ (i.e., milk yield and season of insemination) was evaluated by calculation of the change in the estimate value for the outcome of interest with models containing or not containing the confounder. If inclusion of a confounder changed the estimate for the outcome of interest by more than 30%, it was retained in the model (Dohoo et al., 2014). Treatment and parity were forced in all models. When appropriate, the least significant difference post hoc mean separation test was used to determine differences between least squares means (LSM).

All explanatory variables and their interactions were considered significant if $P \leq 0.05$, whereas $0.05 < P \leq 0.10$ was considered a tendency. Means for binomial outcomes are reported as LSM and the 95% confidence interval obtained with the LSMEANS option of the

PROC GLIMMIX of SAS, whereas for quantitative variables, LSM $\pm$ standard error of the mean obtained with the LSMEANS option of PROC MIXED of SAS are reported. To simplify interpretation, count data evaluated through Poisson regression were reported as arithmetic means generated with PROC MEANS of SAS.

In all tables, results for the effect of VWP treatments are presented for both parity groups combined and for individual parity groups. Superscript letter differences within parity groups are used to report statistically significant differences between VWP treatment groups. This approach to reporting is used because of the known biological and performance differences between parity groups and the observed effects of parity in the data analyses.

RESULTS

Pregnancies Per AI and Pregnancy Loss

At 39 d after first-service TAI, P/AI was greater ($P < 0.01$) for cows in the VWP88 treatment than for those in the VWP60 treatment (Table 1) and greater ($P < 0.01$) for primiparous than multiparous cows (50.4 vs. 38.0%, respectively). Cows with low (45.7%) and medium (46.7%) MK30 had greater ($P = 0.02$) P/AI than cows with high (39.9%) milk yield. The effects of season of AI, treatment by parity, treatment by MK30, and treatment by season interaction were not significant ($P > 0.10$).

Although a treatment by parity interaction for P/AI was not detected, the effect of treatment on P/AI was evaluated for primiparous and multiparous cows separately. For primiparous cows, P/AI at 39 d after TAI was greater ($P < 0.01$) for cows in the VWP88 than the VWP60 treatment, whereas for multiparous cows P/AI was similar ($P = 0.16$) for cows in both treatment treatments (VWP60 = 36.2%, VWP88 = 39.9%; Table 1).

Pregnancy loss for cows pregnant to first-service TAI did not differ ($P = 0.36$) for cows in the VWP60 and VWP88 treatments (Table 1) but was greater ($P = 0.04$) for multiparous (7.2%) than primiparous (4.1%) cows. The interaction between treatment and parity ($P = 0.68$) and the effect of MK30 ($P = 0.57$) were not significant.

The proportion of cows inseminated after a detected estrus for second and greater AI services (Table 2) was similar ($P = 0.11$) for the VWP60 and VWP88 treatments, but was greater ($P < 0.01$) for multiparous (49.9%) than for primiparous cows (42.2%). As expected, the inter-service interval was shorter for

Table 1. Effect of extending duration of the voluntary waiting period (VWP) from 60 to 88 DIM on pregnancies per AI (P/AI) and pregnancy loss after first-service timed AI (TAI) in lactating dairy cows¹

Item	Primiparous		Multiparous		All parities		<i>P</i> -value ²	
	% (CI)		% (CI)		% (CI)			
	VWP60 ³	VWP88 ⁴	VWP60	VWP88	VWP60	VWP88		
P/AI 39 d after TAI	46.0 ^a (41.4–50.6)	55.3 ^b (50.6–59.9)	36.2 (32.6–40.1)	39.9 (36.0–43.8)	41.1 (37.4–44.8)	47.1 (43.2–51.0)	<0.01	0.19
P/AI at pregnancy reconfirmation	43.6 ^a (38.8–48.5)	52.5 ^b (47.5–57.4)	32.9 (29.0–37.0)	36.2 (32.1–40.4)	38.2 (34.3–42.2)	43.6 (39.6–47.8)	0.01	0.22
Pregnancy loss	3.3 (1.4–7.5)	4.7 (2.3–9.4)	6.7 (4.2–10.5)	7.7 (5.1–11.6)	4.9 (3.2–7.4)	6.1 (4.2–8.7)	0.36	0.68

^{a,b}Different superscripts within a row indicate significant differences ($P \leq 0.05$) within the same parity.¹All values are presented as LSM and the 95% CI.²The effect of additional explanatory variables is described in the text. Trt = treatment.³VWP60 = first-service TAI at 60 ± 3 DIM after the Double-Ovsynch protocol.⁴VWP88 = first-service TAI at 88 ± 3 DIM after the Double-Ovsynch protocol.**Table 2.** Effect of extending the duration of the voluntary waiting period (VWP) from 60 to 88 DIM on percentage of cows inseminated at detected estrus, interval between AI services, pregnancies per AI (P/AI) after second and greater AI services, and total number of inseminations up to 350 DIM in lactating dairy cows¹

Item	Primiparous		Multiparous		All parities		<i>P</i> -value ²	
	VWP60 ³	VWP88 ⁴	VWP60	VWP88	VWP60	VWP88	Trt	Parity
	Trt	Parity	Trt	Parity	Trt	Parity	Trt × parity	
EDAI ⁵ [% (CI)]	40.5 (17.2–69.0)	45.8 (20.5–73.5)	48.5 (18.0–80.1)	50.4 (19.2–81.3)	44.5 (17.0–75.8)	47.5 (18.8–78.0)	0.11	<0.01
Inter-service interval ⁶ (d)	33.7 ± 0.6	33.8 ± 0.6	33.3 ± 1.0	33.4 ± 1.0	33.5 ± 0.8	33.6 ± 0.8	0.62	0.11
P/AI [% (CI)]	43.6 ^a (38.2–49.2)	37.2 ^b (31.9–42.8)	31.5 (26.9–36.4)	30.6 (26.0–35.7)	36.7 (31.9–41.8)	34.2 (29.4–39.3)	0.14	<0.01
Total number of services ⁷	2.3 ± 0.05	2.2 ± 0.05	2.8 ± 0.07	2.6 ± 0.08	2.6 ± 0.07	2.4 ± 0.07	0.04	<0.01
								0.72

^{a,b}Different superscripts within a row indicate significant differences ($P \leq 0.05$) within the same parity.¹Values for binomial outcomes are presented as LSM and the 95% CI, values for inter-service interval are presented as LSM ± SEM, and values for total number of services are presented as arithmetic means ± SE.²The effect of additional explanatory variables are described in the text. Trt = treatment.³VWP60 = first-service timed AI at 60 ± 3 DIM after the Double-Ovsynch protocol. Second and greater services after detection of estrus on the D32-Resynch protocol.⁴VWP88 = first-service timed AI at 88 ± 3 DIM after the Double-Ovsynch protocol. Second and greater services after detection of estrus on the D32-Resynch protocol.⁵EDAI = cows inseminated after estrus detection.⁶Inter-service interval = interval between 2 consecutive AI services.⁷Total number of services = total number of AI services received during the lactation (up to 350 DIM).

cows inseminated after estrus detection (24.3 d) than for TAI (42.8 d), but no differences were observed for the overall inter-service interval between VWP60 and VWP88 ($P = 0.62$; Table 2) and between primiparous and multiparous cows ($P = 0.11$).

Pregnancies per AI for all second and greater AI services combined was similar ($P = 0.14$) for the VWP60 and VWP88 treatments and for the warm (37.9%) and cold (34.7%) season ($P = 0.13$), but were greater ($P < 0.01$) for primiparous (40.3%) than for multiparous (30.9%) cows. Inseminations after a detected estrus (37.9%) resulted in greater P/AI ($P < 0.01$) than TAI services (33.0%). Within the primiparous group, P/AI for second and greater AI was greater ($P = 0.04$) for the VWP60 than the VWP88 treatment (Table 2), and for cows inseminated after a detected estrus (47.0%) than for those receiving TAI (34.1%; $P < 0.01$). No effect of treatment ($P = 0.68$) or type of insemination ($P = 0.87$) was observed for P/AI at second and greater AI for the multiparous group.

Total number of AI services up to 350 DIM was greater ($P = 0.04$) for the VWP60 treatment than for the VWP88 (Table 2) treatment and for multiparous than primiparous cows (2.7 vs. 2.2; $P < 0.01$).

Physiological Parameters Before First Service

The proportion of cows with P4 >1 ng/mL (resumed ovarian cyclicity) at baseline was similar ($P = 0.26$) for cows in the VWP60 and VWP88 treatments but was greater ($P < 0.01$) for primiparous than multiparous cows (Table 3). No interaction between treatment and parity was detected ($P = 0.30$). In addition, a lower ($P = 0.02$) proportion of cyclic cows was observed in the high (61.1%) than in the medium (73.1%) and low (68.0%) MK30 groups. At the beginning of DO, more ($P < 0.01$) cyclic cows were observed in the VWP88 than the VWP60 treatment and in the primiparous than the multiparous group ($P < 0.01$). Conversely, 10 d before TAI the proportion of cyclic cows was similar ($P = 0.14$) for both treatments and was not affected by parity ($P = 0.33$), treatment by parity interaction ($P = 0.93$), or MK30 level ($P = 0.11$).

Cows were considered to have PVD if at any time point the Metrichick score was ≥ 2 . This cutoff was selected because cows with a Metrichick score ≥ 2 10 d before TAI had reduced ($P < 0.01$) P/AI after first service as compared with cows with a Metrichick score of 0 and 1 (26.3 vs. 52.6%, respectively; Supplemental Table S2; <https://doi.org/10.3168/jds.2017-13046>). As expected, at baseline the proportion of cows with PVD was similar ($P = 0.51$) for both treatments; it was also

similar for primiparous and multiparous cows ($P = 0.99$). No interaction was observed between treatment and parity ($P = 0.48$), and no effect of MK30 group was observed ($P = 0.75$). Conversely, the proportion of cows with PVD was greater for the VWP60 treatment than for the VWP88 treatment at the beginning of DO ($P = 0.03$) and 10 d before TAI ($P = 0.01$; Table 3).

The percentage of PMN in uterine cytology samples followed a pattern similar to that of PVD. At baseline, no effect was observed of treatment ($P = 0.61$), parity ($P = 0.26$), treatment by parity interaction ($P = 0.70$), or MK30 ($P = 0.20$; Table 3). Conversely, cows in the VWP60 treatment had a greater percentage of PMN than did cows in the VWP88 treatment at the beginning of DO ($P < 0.01$) and 10 d before TAI ($P = 0.02$). No effects ($P > 0.10$) of parity, treatment by parity interaction, and MK30 were detected at these 2 time points.

At baseline, the proportion of cows with BCS ≥ 2.75 was similar ($P = 0.19$) for cows in both treatments, but was affected by parity group ($P < 0.01$) because more primiparous than multiparous cows had BCS ≥ 2.75 (Table 3). No treatment by parity interaction ($P = 0.12$) was detected at this time point. At the beginning of the DO protocol, the proportion of cows with BCS ≥ 2.75 was also similar for both VWP treatments ($P = 0.16$) and was greater for primiparous than multiparous cows ($P < 0.01$), but no treatment by parity interaction was observed ($P = 0.11$). At 10 d before TAI, the proportion of cows with BCS ≥ 2.75 was greater ($P < 0.01$) for the VWP88 treatment and for primiparous than multiparous cows ($P < 0.01$). Milk yield up to 30 DIM did not affect ($P > 0.10$) the proportion of cows with BCS ≥ 2.75 except 10 d before TAI when a greater proportion ($P = 0.02$) of cows had BCS ≥ 2.75 in the low (96.4%) than the medium (92.8%) and high (91.1%) MK30 groups.

Haptoglobin concentrations in plasma were not affected ($P > 0.10$) by VWP duration, parity group, or the interaction between treatment and parity at baseline or at the beginning of DO (Table 3). At 10 d before TAI, cows in the VWP60 treatment had greater ($P = 0.05$) haptoglobin concentration than cows in the VWP88 treatment, but no effects of parity ($P = 0.14$) or treatment by parity interaction ($P = 0.61$) were observed at this point. A tendency ($P = 0.06$) was observed for MK30 to affect haptoglobin concentration at baseline. No effect of MK30 on haptoglobin concentrations was observed at the beginning of DO ($P = 0.79$) and 10 d before TAI ($P = 0.45$). The effect of cyclicity status, uterine health, and BCS on pregnancies per AI after first service is presented in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-13046>).

Table 3. Effect of extending the duration of the voluntary waiting period (VWP) from 60 to 88 DIM on cyclicity status, uterine health, BCS, and haptoglobin circulating concentrations in lactating dairy cows¹

Item	Primiparous		Multiparous		All parities		<i>P</i> -value ²	
	VWP60 ³	VWP88 ⁴	VWP60	VWP88	VWP60	VWP88	Trt	Parity
P4 ⁵ >1 ng/mL (%; 95% CI)								
Baseline	72.9 (59.4–83.2)	79.8 (67.3–88.4)	56.9 (43.2–69.7)	57.4 (43.4–70.3)	65.4 (52.5–76.3)	69.8 (57.1–80.0)	0.26	<0.01
Beginning DO ⁶	72.9 (58.8–83.5)	91.6 (82.9–96.1)	57.1 (42.7–70.4)	79.1 (67.1–87.5)	65.4 (52.5–76.3)	86.5 (77.4–92.3)	<0.01	<0.01
10 d before TAI ⁷	89.2 (75.9–97.1)	92.6 (81.9–98.3)	87.2 (72.4–96.2)	91.1 (78.7–97.5)	90.2 (75.2–96.5)	93.3 (81.6–97.7)	0.14	0.33
PVD ⁸ (%; 95% CI)								
Baseline	52.2 (37.9–66.1)	43.4 (30.8–57.0)	47.7 (37.5–58.2)	48.0 (36.9–59.3)	50.0 (41.1–58.8)	45.7 (37.1–54.6)	0.51	0.99
Beginning DO	52.8 (36.7–68.4)	32.9 (20.2–48.6)	48.3 (35.7–61.2)	39.8 (27.5–53.6)	50.6 (38.9–62.2)	36.3 (26.0–48.0)	0.03	0.82
10 d before TAI	40.0 (26.8–54.8)	17.7 (9.4–30.6)	34.9 (25.4–45.8)	27.9 (18.6–39.8)	37.4 (29.1–46.6)	22.4 (15.5–31.1)	0.01	0.53
PMN ⁹ (%)								
Baseline	11.0 ± 2.9	11.7 ± 2.4	13.3 ± 2.3	15.4 ± 2.6	12.5 ± 1.8	13.9 ± 1.8	0.61	0.26
Beginning DO	11.0 ± 2.9	6.0 ± 2.1	13.3 ± 2.3	6.0 ± 1.7	12.5 ± 1.8	6.0 ± 1.3	<0.01	0.72
10 d before TAI	5.7 ± 1.4	3.3 ± 1.3	7.4 ± 1.7	3.5 ± 1.1	6.8 ± 1.2	3.4 ± 0.8	0.02	0.55
BCS ≥2.75 (%; 95% CI)								
Baseline	93.1 (87.1–96.5)	97.6 (92.6–99.3)	78.7 (70.9–84.8)	75.4 (66.9–82.4)	87.6 (81.9–91.7)	91.8 (85.4–95.6)	0.19	<0.01
Beginning DO	93.4 (86.1–97.0)	97.7 (92.4–99.3)	79.0 (67.5–87.2)	78.0 (65.9–86.6)	87.9 (79.7–93.1)	92.5 (84.9–96.4)	0.16	<0.01
10 d before TAI	95.0 (— ¹⁰)	100 (— ¹⁰)	79.0 (72.4–84.3)	87.9 (82.0–92.0)	90.8 (85.3–94.4)	95.9 (92.5–97.8)	<0.01	<0.01
HAPTO ¹¹ (mg/mL)								
Baseline	0.51 ± 0.09	0.48 ± 0.08	0.47 ± 0.06	0.51 ± 0.05	0.48 ± 0.05	0.50 ± 0.04	0.50	0.95
Beginning DO	0.51 ± 0.09	0.50 ± 0.07	0.47 ± 0.06	0.59 ± 0.07	0.48 ± 0.05	0.55 ± 0.05	0.15	0.93
10 d before TAI	0.64 ± 0.09	0.46 ± 0.06	0.52 ± 0.06	0.42 ± 0.05	0.56 ± 0.05	0.43 ± 0.04	0.05	0.14

¹Values for binomial outcomes are presented as LSM and the 95% CI, whereas values for quantitative outcomes are presented as LSM ± SEM.²Trt = treatment.³VWP60 = first-service timed AI at 60 ± 3 DIM after the Double-Ovsynch protocol.⁴VWP88 = first-service timed AI at 88 ± 3 DIM after the Double-Ovsynch protocol.⁵P4 = cows with progesterone concentration >1 ng/mL.⁶DO = Double-Ovsynch.⁷TAI = timed artificial insemination.⁸PVD = cows with purulent vaginal discharge (Metricheck score ≥2; Simcro, Hamilton, New Zealand). Scored on a 0 to 5 scale (0 = no discharge, 1 = clear mucus, 2 = clear mucus with flecks of pus, 3 = mucopurulent but <50% pus, 4 = mucopurulent with >50% pus, and 5 = foul-smelling discharge) as described in McDougall et al. (2007).⁹PMN = percentage polymorphonuclear cells in uterine cytology sample.¹⁰No confidence interval because for one of the groups all observations had the same outcome.¹¹HAPTO = haptoglobin concentration.

Time to Pregnancy and Nonpregnant Cows at 350 DIM

The hazard of pregnancy from calving until 350 DIM was greater ($P < 0.01$) for the VWP60 than the VWP88 treatment [hazard ratio (HR) 1.34, 95% CI 1.23 to 1.47; Figure 2A] and greater ($P < 0.01$) for primiparous than multiparous cows (HR 1.48, 95% CI 1.36 to 1.62). Median and mean days to pregnancy were 102 and 132 d for the VWP60 treatment and 128 and 154 d for the VWP88 treatment. The hazard of pregnancy was also affected by milk yield up to 30 DIM ($P < 0.01$), as it was lower for cows with high (HR 0.84, 95% CI 0.76 to 0.94) than medium milk yield. The HR for time to pregnancy did not differ for cows with low and medium milk yield (HR 0.94, 95% CI 0.84 to 1.05) or for cows with high and low milk yield (HR 0.90, 95% CI 0.81 to 1.01). At 350 DIM, the proportion of nonpregnant cows did not differ ($P = 0.28$) for the VWP60 (7.3%) and VWP88 (8.6%) treatments but was greater ($P < 0.01$) for multiparous (12.1%) than primiparous cows (2.5%).

When evaluated within parity group, the hazard of pregnancy was greater for cows in the VWP60 than in the VWP88 treatment both in the primiparous ($P < 0.01$, HR 1.38, 95% CI 1.21 to 1.58; Figure 2B) and multiparous group ($P < 0.01$; HR 1.31, 95% CI 1.19 to 1.50; Figure 2C). For primiparous cows, median and mean days to pregnancy were 85 and 115 d for the VWP60 treatment and 91 and 135 d for the VWP88 treatment. In addition, the proportion of nonpregnant cows at 350 DIM did not differ ($P = 0.51$) between VWP60 (2.3%) and VWP88 (2.9%) within the primiparous group. For multiparous cows, median and mean days to pregnancy, respectively, were 104 and 142 d for the VWP60 treatment and 132 and 163 d for the VWP88 treatment. The proportion of nonpregnant cows at 350 DIM was similar ($P = 0.20$) between treatments (VWP60 = 11.0%, and VWP88 = 13.0%).

On the other hand, the hazard of pregnancy from the end of the VWP until 350 d after the first TAI did not differ ($P = 0.30$) for cows in the VWP60 and VWP88 treatment (HR 0.96, 95% CI 0.88 to 1.14; Figure 3A) but was greater ($P < 0.01$) for primiparous than multiparous cows (HR 1.40, 95% CI 1.28 to 1.53). Median and mean days to pregnancy were 42 and 72 d for the VWP60 treatment and 41 and 66 d for the VWP88 treatment. The hazard of pregnancy was also similar for cows in the VWP60 and VWP88 treatment within both the primiparous ($P = 0.23$; HR 0.92, 95% CI 0.81 to 1.05; Figure 3B) and multiparous group ($P = 0.73$; HR 0.98, 95% CI 0.87 to 1.10; Figure 3C). For primiparous cows, median and mean days to pregnancy were 25 and 55 d for the VWP60 treatment and 0 and 47 d for the VWP88 treatment. For multiparous cows,

Figure 2. (A) Kaplan-Meier survival curves for time to pregnancy after calving. The hazard of pregnancy was greater ($P < 0.001$) for cows in the voluntary waiting period of 60 DIM (VWP60) than the voluntary waiting period of 88 DIM (VWP88) treatment [hazard ratio (HR) 1.34; 95% CI 1.23 to 1.47]. (B) Kaplan-Meier survival curves for time to pregnancy after calving for primiparous cows. The hazard of pregnancy was greater for cows in the VWP60 than the VWP88 treatment ($P < 0.01$, HR 1.38, 95% CI 1.21 to 1.58). (C) Kaplan-Meier survival curves for time to pregnancy after calving for multiparous cows. The hazard of pregnancy was greater for cows in the VWP60 treatment than in the VWP88 treatment ($P < 0.01$; HR 1.31, 95% CI 1.19 to 1.50).

Figure 3. (A) Kaplan-Meier survival curves for days to pregnancy after the end of the voluntary waiting period (VWP) for each experimental treatment (end of VWP = d 0). The hazard of pregnancy was similar ($P = 0.30$) for cows in the VWP of 60 DIM (VWP60) and VWP of 88 DIM (VWP88) treatments [hazard ratio (HR) 0.96, 95% CI 0.88 to 1.04]. (B) Kaplan-Meier survival curves for days to pregnancy after the end of the VWP for primiparous cows. The hazard of pregnancy was similar for cows in the VWP60 and VWP88 treatment ($P = 0.23$; HR 0.92, 95% CI 0.81 to 1.05). (C) Kaplan-Meier survival curves for days to pregnancy after the end of the VWP for multiparous cows. The hazard of pregnancy was similar for cows in the VWP60 and VWP88 treatments ($P = 0.73$; HR 0.98, 95% CI 0.87 to 1.10).

median and mean days to pregnancy were 42 and 82 d for the VWP60 treatment and 42 and 74 d for the VWP88 treatment.

Herd Exit Dynamics

The hazard of culling up to 350 DIM was affected by the interaction between treatment and parity group ($P = 0.05$) and milk yield up to 30 DIM ($P < 0.01$). The hazard of culling was similar ($P = 0.56$) for primiparous cows in both treatment treatments (HR 1.12, 95% CI 0.77 to 1.61; Figure 4A) but was greater ($P = 0.03$) for multiparous cows in the VWP88 than the VWP60 treatment (HR 1.21, 95% CI 1.02 to 1.44; Figure 4B). Mean days to culling were 324 and 332 for primiparous cows in the VWP60 and VWP88 treatment, respectively. For multiparous cows, mean days to culling were 302 and 295 for the VWP60 and VWP88 treatments, respectively. The hazard of culling was greater ($P < 0.01$) for cows in the low milk yield group than for cows in the medium (HR 1.72, 95% CI 1.43 to 2.07) and high (HR 2.04, 95% CI 1.69 to 2.47) milk yield groups, but was similar for cows in the medium and high milk yield groups (HR 1.18, 95% CI 0.96 to 1.46; Figure 4C).

The total proportion of cows sold during the experimental lactation (Table 4) was similar ($P = 0.78$) for primiparous cows in both treatments but was greater ($P = 0.04$) for multiparous cows in the VWP88 than the VWP60 treatment. No differences were observed between treatments in the proportion of cows that died during the experimental lactation in the primiparous ($P = 0.45$) or multiparous ($P = 0.87$) group (Table 4). Consequently, the proportion of total cows that left the herd (sold plus died) was similar ($P = 0.58$) between VWP60 and VWP88 treatments for primiparous cows, but it was greater ($P = 0.03$) for multiparous cows in the VWP88 than the VWP60 treatment.

Lactation Performance

The effect of VWP treatment on milk yield during the first 10 monthly milk tests was evaluated for all cows enrolled in the experiment (Figure 5A and 5C) and for cows pregnant at first service only (Figure 5B and 5D). For all primiparous cows enrolled in the experiment (Figure 5A), an interaction was observed between VWP treatment and test number ($P = 0.04$), whereby cows in the VWP88 treatment produced more milk than cows in the VWP60 treatment on tests 8 to 10. No effect of VWP duration on milk yield at dry-off ($P = 0.24$) and on days of gestation at dry-off ($P = 0.17$) was observed for primiparous cows (Table 5). Nevertheless, DIM at dry-off were different ($P < 0.01$) between treatments. For primiparous cows pregnant at

Table 4. Effect of extending the duration of the voluntary waiting period (VWP) from 60 to 88 DIM on herd exit up to 350 d after calving in lactating dairy cows¹

Item	Primiparous		Multiparous		All parities		P-value ²	
	% (95% CI)		% (95% CI)		% (95% CI)			
	VWP60 ³	VWP88 ⁴	VWP60	VWP88	VWP60	VWP88	Trt	Parity
	Trt	Trt × parity						
Sold	7.6 (2.3–22.8)	7.2 (2.1–21.8)	25.5 ^a (20.3–31.7)	30.1 ^b (24.3–36.7)	19.4 (14.3–25.7)	22.1 (16.5–29.0)	0.45	<0.01
Died	1.8 (0.9–3.7)	1.2 (0.5–2.9)	4.1 (2.6–6.4)	4.6 (2.9–7.1)	2.6 (1.6–4.1)	2.6 (1.6–4.2)	0.63	<0.01
Left herd (sold plus died)	8.6 (2.3–27.5)	7.8 (2.1–25.3)	29.9 ^a (25.3–34.9)	35.1 ^b (30.2–40.4)	22.8 (17.8–28.7)	25.7 (20.3–32.0)	0.54	<0.01

^{a,b}Different superscripts within a row indicate significant differences ($P \leq 0.05$) within the same parity.

¹All values are presented as LSM and the 95% CI.

²Trt = treatment.

³VWP60 = first-service timed AI at 60 ± 3 DIM after the Double-Ovsynch protocol. Second and greater services after detection of estrus on the D32-Resynch program.

⁴VWP88 = first-service timed AI at 88 ± 3 DIM after the Double-Ovsynch protocol. Second and greater services after detection of estrus on the D32-Resynch program.

Figure 4. (A) Kaplan-Meier survival curves for time to herd exit up to 350 d after calving for primiparous cows. The hazard of culling was similar for the voluntary waiting period of 60 DIM (VWP60) and voluntary waiting period of 88 DIM (VWP88) treatments [$P = 0.56$; hazard ratio (HR) 1.12, 95% CI 0.77 to 1.61]. (B) Kaplan-Meier survival curves for time to herd exit up to 350 d after calving for multiparous cows. The hazard of culling was greater for the VWP88 treatment than for the VWP60 treatment ($P = 0.03$; HR 1.21, 95% CI 1.02 to 1.44). (C) Kaplan-Meier survival curves for time to herd exit up to 350 d after calving according to milk yield accumulated up to 30 DIM. The hazard of culling was greater for cows in the low milk yield group than for cows in the medium ($P < 0.01$; HR 1.49, 95% CI 1.29 to 1.72) and high (HR 2.04, 95% CI 1.68 to 2.47) milk yield groups, but was similar for cows in the medium and high milk yield groups (HR 1.18, 95% CI 0.96 to 1.46).

Figure 5. Monthly milk yield (kg/d) for all primiparous cows enrolled in the experiment [A; voluntary waiting period of 60 DIM (VWP60): $n = 480$; voluntary waiting period of 88 DIM (VWP88): $n = 471$]; primiparous cows pregnant at first service (B; VWP60: $n = 200$; VWP88: $n = 228$); all multiparous cows enrolled in the experiment (C; VWP60: $n = 791$, VWP88: $n = 794$); and multiparous cows pregnant at first service (D; VWP60: $n = 238$, VWP88: $n = 247$). *Indicates significant difference between the VWP60 and VWP88 treatment based on LSD mean separation test. All values are presented as LSM $\pm$ SEM.

first service only (Figure 5B), there was also a treatment by test number interaction ($P < 0.01$) because cows in the VWP88 treatment produced more milk than cows in the VWP60 treatment from test 6 to 9. Milk yield at dry-off was higher ($P = 0.03$) for the VWP88 than for the VWP60 treatment, and DIM at dry-off was also higher ($P < 0.01$) for the VWP88 than for the VWP60 treatment (Table 5). Days of gestation at dry-off for primiparous cows pregnant at first TAI was similar ($P = 0.48$) between treatments.

For all multiparous cows enrolled in the experiment (Figure 5C), a treatment by test number interaction ($P = 0.03$) was observed whereby cows in the VWP88 treatment produced more milk than did cows in the VWP60 treatment on test 9. The DIM at dry-off were greater ($P < 0.01$) for the VWP88 treatment than the

VWP60 treatment, but milk yield ($P = 0.17$) and days of gestation ($P = 0.12$) were similar between treatments at this time point (Table 5). For multiparous cows pregnant at first service only (Figure 5D), a treatment by test interaction ($P < 0.01$) was also observed whereby cows in the VWP88 treatment produced more milk than cows in the VWP60 treatment on tests 7 to 9. No differences were observed in milk yield ($P = 0.26$) and days of gestation ($P = 0.11$) at dry-off between VWP treatments, but DIM at dry-off was greater ($P < 0.01$) for the VWP88 than the VWP60 treatment (Table 5).

For all cows enrolled in the experiment (Table 5), total milk yield during the experimental lactation was greater ($P = 0.01$) for the VWP88 than the VWP60 treatment. Primiparous cows in the VWP88 treatment produced more milk ($P < 0.01$) than those in

Table 5. Effect of extending the duration of the voluntary waiting period (VWP) from 60 to 88 DIM on lactation parameters and performance in lactating dairy cows¹

Item	Primiparous			Multiparous			All parities			P-value ²	
	VWP60 ³	VWP88 ⁴		VWP60	VWP88		VWP60	VWP88		Trt	Parity
Milk dry-off (kg/d)											
All cows ⁵	32.4 ± 0.3	32.9 ± 0.3		32.5 ± 0.3	31.9 ± 0.3		32.4 ± 0.2	32.4 ± 0.2		0.82	0.22
Preg first AI ⁶	31.8 ± 0.5 ^a	33.1 ± 0.4 ^b		33.9 ± 0.4	33.1 ± 0.4		32.9 ± 0.3	33.1 ± 0.3		0.59	0.02
DIM at dry-off (d)											
All cows	325.4 ± 2.8 ^a	344.7 ± 2.8 ^b		326.4 ± 2.5 ^a	346.9 ± 2.6 ^b		325.9 ± 1.9	345.8 ± 1.9		<0.01	0.56
Preg first AI	283.9 ± 0.8 ^a	312.3 ± 0.8 ^b		284.4 ± 0.8 ^a	309.7 ± 0.8 ^b		284.1 ± 0.6	311.0 ± 0.5		<0.01	0.20
DG ⁷ at dry-off (d)											
All cows	223.7 ± 0.9	222.6 ± 0.9		221.6 ± 0.8	220.5 ± 0.8		222.7 ± 0.6	221.1 ± 0.6		0.11	0.13
Preg first AI	223.8 ± 0.8	224.4 ± 0.8		224.4 ± 0.8	223.9 ± 0.8		224.1 ± 0.6	223.1 ± 0.5		0.20	0.22
Milk (kg/lactation)											
All cows	10,831 ± 689 ^a	11,648 ± 689 ^b		12,249 ± 677	12,413 ± 677		11,540 ± 671	12,031 ± 671		0.01	<0.01
Preg first AI	9,464 ± 293 ^a	10,785 ± 291 ^b		12,001 ± 290 ^a	12,914 ± 292 ^b		10,732 ± 279	11,849 ± 279		<0.01	<0.01
Lactation length (d)											
All cows	319.4 ± 14.0 ^a	341.3 ± 14.0 ^b		300.7 ± 13.7	305.0 ± 13.7		310.0 ± 13.6	323.1 ± 13.5		<0.01	<0.01
Preg first AI	283.9 ± 0.8 ^a	312.3 ± 0.8 ^b		284.4 ± 0.8 ^a	309.7 ± 0.8 ^b		284.1 ± 0.6	311.0 ± 0.5		<0.01	0.20
Milk (kg/d)											
All cows	33.2 ± 0.7	33.6 ± 0.7		40.5 ± 0.6	40.1 ± 0.6		36.9 ± 0.6	36.9 ± 0.6		0.94	<0.01
Preg first AI	33.2 ± 0.7 ^a	34.5 ± 0.7 ^b		42.1 ± 0.7	41.6 ± 0.7		37.6 ± 0.7	38.0 ± 0.7		0.31	<0.01

^{a,b}Different superscripts within a row indicate significant differences ($P \leq 0.05$) within the same parity.¹All values are presented as LSM ± SEM.²Trt = treatment.³VWP60 = first-service timed AI at 60 ± 3 DIM after the Double-Ovsynch protocol. Second and greater services after detection of estrus or the D32-Resynch protocol.⁴VWP88 = first-service timed AI at 88 ± 3 DIM after the Double-Ovsynch protocol. Second and greater services after detection of estrus or the D32-Resynch protocol.⁵All cows = all cows enrolled in the study (n = VWP60: 1,265; VWP88: 1,260).⁶Preg first AI = only cows pregnant at first service that did not abort or exit the herd during the lactation (421 in VWP60 and 425 in VWP88).⁷DG = days of gestation at dry-off.

the VWP60 treatment (~ 817 kg difference), but no significant differences ($P = 0.50$) were observed within the multiparous group (~ 164 kg difference). Accordingly, length of the lactation was also greater ($P < 0.01$) for primiparous cows in the VWP88 than the VWP60 treatment, but no differences ($P = 0.47$) were observed within the multiparous group. Nonetheless, lactation length was longer ($P < 0.01$) for multiparous cows in the VWP88 treatment when only cows that did not leave the herd were included in the analysis (data not shown). No differences ($P = 0.94$) were observed for daily milk yield (kg/d) between cows in the VWP60 and VWP88 treatments.

For cows pregnant at the first service only (Table 5), milk yield during the experimental lactation was greater ($P < 0.01$) for cows in the VWP88 treatment than in the VWP60 treatment ($\sim 1,117$ kg difference). This difference in favor of the VWP88 treatment was present in both primiparous ($P < 0.01$; $\sim 1,321$ kg difference) and multiparous ($P < 0.01$; ~ 913 kg difference) cows. Lactation length was also different ($P < 0.01$) between VWP60 and VWP88 by ~ 27 d, and reflected the 28-d difference in DIM at first service. Daily milk yield (kg/d) was greater ($P < 0.01$) for primiparous cows in the VWP88 than for those in the VWP60 treatment (~ 1.3 kg/d difference), but no difference ($P = 0.38$) was observed for multiparous cows.

DISCUSSION

Reproductive Performance and Markers of Physiological Status Before First Service

In support of our main hypothesis, extending the duration of the VWP from 60 to 88 DIM after synchronization of ovulation with the Double-Ovsynch protocol increased P/AI after first service in lactating dairy cows. Nevertheless, most of the observed difference could be attributed to the greater P/AI of primiparous cows in the VWP88 treatment. Our overall results are in agreement with previous studies, which documented improved P/AI after extending the duration of the VWP (Tenhagen et al., 2003; Gobikrushanth et al., 2014). Nevertheless, direct comparisons between studies are difficult because of differences in experimental design and interactions between treatments and other confounders. For example, a 21 d longer VWP increased P/AI 20 and 13 percentage points for cows with low and high milk yield submitted to TAI after the Ovsynch protocol, respectively (Tenhagen et al., 2003). In a retrospective cohort study, a 23 d extension (range = 1 to 64 d) of the VWP for cows that calved during summer (AI in fall) increased overall P/AI by

6 percentage points when compared with cows with shorter VWP (AI in summer and fall; Gobikrushanth et al., 2014). In contrast, Arbel et al. (2001) reported similar P/AI for cows with above-average milk yield inseminated at detected estrus after a 60 d extension of the VWP (from 90 and 60 DIM in primiparous and multiparous cows, respectively), and Van Amburgh et al. (1997) reported no benefit of extending the duration of the VWP from 60 to 150 d on P/AI for cows in their second lactation inseminated at detected estrus. Thus, in spite of substantial variation across studies, the collective results of the current experiment and others (Tenhagen et al., 2003; Gobikrushanth et al., 2014) conducted under conditions more similar to ours (i.e., using TAI and less difference in VWP duration) suggest that extending VWP duration increases P/AI to first service. The magnitude of the increment in P/AI, however, may be affected by parity, method of insemination, season, milk yield level, and the magnitude and timing of the extension of the VWP.

The observed difference in first-service P/AI between parity groups was expected because it has been extensively documented (Souza et al., 2008; Herlihy et al., 2012; Giordano et al., 2013). In part, it can be explained by the greater incidence of anovulation and greater proportion of cows with low BCS at different time points in the multiparous group (no difference for other parameters). Nonetheless, the reason for the different response to treatments by parity is unclear at the moment because both groups presented a fairly similar physiological response to the extension of the VWP. Differences between parities in metabolic status, health, or both not captured by the parameters monitored in this experiment may explain such a discrepancy. For example, whereas primiparous cows received first service when milk yield per day was still increasing in both VWP treatments, milk yield was increasing at a greater rate in the VWP60 than the VWP88 treatment. Conversely, milk yield per day was already declining for multiparous cows from both VWP treatments. It is also possible that the 28 d extension of the VWP was insufficient for multiparous cows to attain a physiological status substantially better than that of cows in the VWP60 group. To some extent this was supported by data for PVD because the difference in proportion of cows with PVD 10 d before TAI was of only ~ 7 percentage points for multiparous, whereas the difference was of ~ 22 percentage points for primiparous. Likewise, the difference in haptoglobin levels 10 d before TAI was ~ 0.10 mg/mL for multiparous cows, whereas it was ~ 0.18 mg/mL for primiparous cows. This reasoning, however, did not apply to data for cyclicity status, uterine cytology, and BCS.

As timing of pregnancy in lactating dairy cow herds is determined by the combined effect of all AI services rather than first service only, evaluating the pattern of pregnancy creation during the entire lactation is essential to truly determine the effect of VWP duration on reproductive performance. In this regard, cows in the VWP60 treatment became pregnant at a faster rate after calving than cows in the VWP88 treatment. The reduced P/AI to first service for cows in VWP60 was fully compensated by the creation of more pregnancies at earlier DIM due to more and earlier opportunities for re-insemination. The faster rate of pregnancy creation did not result, however, in a reduced proportion of nonpregnant cows at 350 DIM. In agreement, 2 other studies reported the same pattern of pregnancy creation and similar proportion of nonpregnant cows in late lactation (Tenhagen et al., 2003; Gobikrushanth et al., 2014). Collectively, these data suggest that the greatest consequence of longer VWP is shifting timing of pregnancy toward later lactation rather than generating a greater proportion of pregnant cows.

Previous data are ambivalent about the effect of milk yield on fertility and suggest that the interaction between milk yield and fertility is complex (Faust et al., 1988; López-Gatius et al., 2006; Bello et al., 2013). In our experiment, not all parameters of reproductive performance were affected equally by milk yield level. Whereas first-service P/AI was reduced for cows in the high milk yield group, the hazard of pregnancy for high-producing cows was lower than for cows in the medium but not the low milk yield group. At least in part, the discrepancy for the effect of milk yield on P/AI and time to pregnancy can be explained by the outcomes measured and the influence of others factors on these outcomes.

Our results for the multiple markers of physiological status support the hypothesis that a longer VWP would lead to an improved uterine environment, reduced anovulation, improved BCS, and reduced systemic inflammation before first service. Our data are also in agreement with many previous reports that documented reduced P/AI at first service in cows with PVD, CYTO, BCS <2.75 , and elevated circulating haptoglobin levels (Barlund et al., 2008; Souza et al., 2008; Dubuc et al., 2011; Huzzey et al., 2015). The effect on uterine health of extending VWP duration was evident, as fewer cows were considered to have PVD and CYTO at the beginning of DO and 10 d before TAI. As PVD and CYTO alone or combined reduce P/AI of dairy cows (Gilbert et al., 2005; Barlund et al., 2008; Dubuc et al., 2010), the reduction in proportion of cows affected likely contributed to the greater P/AI for the VWP88 treatment. The longer VWP also re-

sulted in more cows with a BCS ≥ 2.75 , which has been associated with greater first-service P/AI (Souza et al., 2007, 2008; Carvalho et al., 2014). Assuming that most cows lost body reserves after calving, our data suggest that the longer interval from calving to first service for cows in the VWP88 treatment allowed recovery of more body reserves. Collectively, these observations for physiological markers and overall metabolic status help explain, at least in part, the greater P/AI for cows with longer VWP and suggest that providing cows more time to recover before first service is a feasible strategy to promote a physiological status more conducive to pregnancy.

Finally, the greater proportion of cyclic cows at the beginning of the DO protocol reflected the effect of additional time for resumption of cyclicity, whereas the similar proportion of cyclic cows 10 d before TAI reflected the efficacy of the DO protocol to resolve anovulation. This was expected because previous studies have demonstrated that GnRH-based presynchronization protocols are effective for reducing the proportion of anovular cows before TAI (Souza et al., 2008; Herlihy et al., 2012; Ayres et al., 2013). Thus, in our experiment any potential benefit of extending the VWP on reducing the incidence of anovulation must have been neutralized by the induction of ovulation in early lactation for cows in the VWP60 treatment.

The effect of extending the VWP from 60 to 88 DIM on first-service P/AI and subsequent reproductive performance in the current experiment may be specific to the use of all TAI after synchronization of ovulation with a GnRH-based fertility protocol. Compared with reproductive management programs designed to submit all cows for AI at detected estrus or through a combination of insemination at detected estrus with TAI, all TAI with a GnRH-based synchronization protocol may have conditioned the effect of extending the VWP. For example, using all TAI for first service results in a narrow range of DIM to first service regardless of the ability of cows to display estrous behavior, reducing variation of not only DIM to first service but also for second and greater AI services. By resolving anovulation, proper synchronization of ovulation, and optimization of the endocrine environment before insemination in most cows (Souza et al., 2008; Herlihy et al., 2012; Giordano et al., 2013); GnRH-based protocols may also offset the detriment of shorter VWP on P/AI to a greater extent than programs not including synchronization of ovulation or synchronization of ovulation with PGF_{2α}-based protocols. Thus, the method of submission to first service and the type of synchronization of ovulation protocol, if any is used, are important considerations at the time of defining the duration of the VWP.

Herd Exit Dynamics and Lactation Performance

Cow parity and pregnancy status are major determinants of the herd exit dynamics in dairy farms. Pregnant cows and cows in lower parity groups have lesser risk of removal from the herd (De Vries et al., 2010; Pinedo et al., 2010). The smaller proportion of primiparous than multiparous cows leaving the herd and similar herd exit dynamics across VWP treatments for primiparous cows reflected the protective effect for culling of early pregnancy and younger age. Conversely, for multiparous cows the greater proportion of cows exiting the herd from the VWP88 treatment as lactation progressed reflected the compounded effect of delayed pregnancy and greater culling pressure in older cows. Evidently milk yield level also played a role and added another layer of complexity because nonpregnant cows with medium and high milk yield had lower culling pressure than cows with low milk yield.

A direct consequence of the different herd exit dynamics across parities was a smaller difference between treatments in lactation length and milk yield per lactation for multiparous cows when all cows (i.e., culled and not culled) in the experiment were included in the calculations. Whereas the difference in lactation length and milk yield per lactation for multiparous cows was 4 d and 164 kg, respectively, the differences for primiparous cows were 22 d and 817 kg, respectively. Cows that left the herd earlier in lactation contributed shorter lactations and less milk yield per lactation than cows that left the herd at later DIM or stayed in the herd. Indeed, when only cows that were not culled were evaluated, the differences between treatments for multiparous cows (21 d and 790 kg) better reflected the effect of delayed pregnancy on lactation length and milk yield (i.e., longer lactation and more milk per lactation). For primiparous cows the differences remained unchanged (21 d and 807 kg) because fewer cows left the herd. Therefore, the collective results for herd exit, timing of pregnancy, and effect of milk yield level on herd exit dynamics suggest that multiple, complex interactions are present that should be accounted for when defining the duration of the VWP for a dairy herd.

The shift in overall timing of pregnancy toward later DIM in the VWP88 treatment resulted in extension of average lactation length. This was clearly due to delayed pregnancy, as cows were dried off at the same days in gestation. Longer lactations coupled with high milk yield persistency at later DIM resulted in greater accumulated milk yield for cows in the VWP88 treatment, primarily due to greater milk yield in primiparous cows. This is relevant because milk yield per lactation is one the major factors affecting cow profitability (Van Amburgh et al., 1997; Arbel et al., 2001; Österman and

Bertilsson, 2003). Nonetheless, it is important to note that not only total milk yield but also efficiency of milk yield during lactation influences cow profitability (Pec-sok et al., 1994; Ferguson and Galligan, 1999; Britt et al., 2003). Thus, lactation performance data from this experiment should be interpreted with caution because the data are insufficient to determine the true economic effect of different durations of the VWP.

The effect of extending the VWP and shifting timing of pregnancy toward later DIM affected the pattern of milk yield in mid to late lactation. This effect was more evident in primiparous cows and cows pregnant to first service. As for the other lactation parameters, the observed pattern of milk yield for each treatment was also affected by the herd exit dynamics. Cows leaving the herd at earlier DIM, which were more likely to be low-producing cows, reduced the difference between VWP treatments when all cows were included in the calculation. In contrast, for cows pregnant at first service the greater milk yield in late lactation for cows in the VWP88 treatment may be explained by the effect of gestation on milk yield. It is well known that after the fifth month of pregnancy, milk yield declines more steadily (Olori et al., 1997; Van Amburgh et al., 1997; Roche, 2003). This was evident in our data, as cows pregnant at first service in the VWP60 treatment had lower milk yield than cows in the VWP88 starting at the sixth (primiparous) and seventh (multiparous) monthly test, which was the approximate time at which cows were starting or completing the fifth month of gestation. At the same monthly tests, cows in the VWP88 treatment were approximately 1 mo earlier in gestation.

CONCLUSIONS

In conclusion, extending the duration of the VWP from 60 to 88 DIM in lactating dairy cows that received TAI after synchronization of ovulation with the Double-Ovsynch protocol increased P/AI to first service. Nonetheless, the greatest gain in P/AI was observed in primiparous cows. Cows with the extended VWP had a physiological status more conducive to pregnancy characterized by improved uterine health, greater BCS, reduced systemic inflammation, and (to a lesser extent) reduced anovulation before insemination. In spite of increasing P/AI to first service, extending the VWP delayed time to pregnancy during lactation and increased the risk of leaving the herd, in particular during late lactation. This overall extension of lactation length resulted in greater total milk yield per lactation but not greater milk yield per day of lactation. Thus, data from the current experiment highlight the importance of considering the complex interactions between reproductive performance, herd exit dynamics, and

lactation performance when defining VWP duration for lactating dairy cows.

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