Effect of progesterone on magnitude of the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows

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ABSTRACT

Ovulation to the first GnRH injection of Ovsynch-type protocols is lower in cows with high progesterone (P4) concentrations compared with cows with low P4 concentrations, suggesting that P4 may suppress the release of LH from the anterior pituitary after GnRH treatment. The objectives of this study were to determine the effect of 1) circulating P4 concentrations at the time of GnRH treatment on GnRH-induced LH secretion in lactating dairy cows and 2) increasing the dose of GnRH from 100 to 200 µg on LH secretion in a high- and low-P4 environment. A Double-Ovsynch (Pre-Ovsynch: GnRH, PGF_{2α} 7 d later, GnRH 3 d later, and Breeding-Ovsynch 7 d later: GnRH, PGF_{2α} 7 d later, and GnRH 48 h later) synchronization protocol was used to create the high- and low-P4 environments. At the first GnRH injection of Breeding-Ovsynch (high P4), all cows with a corpus luteum ≥ 20 mm were randomly assigned to receive 100 or 200 µg of GnRH. At the second GnRH injection of Breeding-Ovsynch (low P4) cows were again randomized to receive 100 or 200 µg of GnRH. Blood samples were collected every 15 min from –15 to 180 min after GnRH treatment, and then hourly until 6 h after GnRH treatment. As expected, mean P4 concentrations were greater for cows in the high- than the low-P4 environment. For cows receiving 100 µg of GnRH, the LH peak and area under the curve (AUC) were greater in the low- than in the high-P4 environment. Similarly, for cows receiving 200 µg of GnRH, the LH peak and AUC were greater in the low- than the high-P4 environment. Cows receiving 100 or 200 µg of GnRH had greater mean LH concentration in the low- than the high-P4 environment from 1 to 6 h after GnRH treatment. On the other hand, when comparing the effect of the 2 GnRH doses in the high- and

low-P4 environments, cows receiving 200 µg of GnRH had a greater LH peak and AUC than cows treated with 100 µg of GnRH both in the high- and low-P4 environments. For the high-P4 environment, mean LH was greater from 1.5 to 5 h after GnRH treatment for cows receiving 200 µg of GnRH than for those receiving 100 µg of GnRH. In the low-P4 environment, mean LH was greater for cows receiving 200 µg of GnRH than for those receiving 100 µg of GnRH from 1 to 2.5 h after GnRH treatment. We conclude that the P4 environment at GnRH treatment dramatically affects GnRH-induced LH secretion, and that a 200-µg dose of GnRH can increase LH secretion in either a high- or a low-P4 environment.

Key words: luteinizing hormone secretion, progesterone, gonadotropin-releasing hormone dose

INTRODUCTION

The Ovsynch protocol uses GnRH and PGF_{2α} to manipulate follicular and luteal function to synchronize ovulation of a follicle and allow for timed AI (TAI; Pursley et al., 1995). Ovulatory response to both GnRH injections of the Ovsynch protocol improves overall synchronization of ovulation and subsequent fertility of TAI services in lactating dairy cows (Chebel et al., 2006; Keskin et al., 2011; Giordano et al., 2012). Ovulation to the first GnRH injection of the Ovsynch protocol is lower in cows that have high circulating progesterone (P4) concentrations, indicative of the presence of a functional corpus luteum (CL), or in cows with a CL detected using ultrasound compared with cows with low P4 or without a CL (Stevenson et al., 2008; Galvão and Santos, 2010; Giordano et al., 2012). Interestingly, in experiments in which cows were pre-synchronized using 2 injections of PGF_{2α}, the presence of a follicle of ovulatory size at the first GnRH injection of Ovsynch does not appear to be the limitation for ovulation to GnRH because most cows have a follicle of ovulatory size at the time of GnRH treatment (Galvão and Santos, 2010). Therefore, it seems likely that the suppression of ovulation observed in cows with a func-

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tional CL treated with GnRH is related to alterations in the mechanisms that trigger the ovulatory process rather than the capacity of follicles to ovulate.

In ruminants, P4 and estradiol play a major role in regulating LH secretion from the anterior pituitary (Goodman and Karsch, 1980; Schoenemann et al., 1985; Nett et al., 2002). Steroids can modulate LH release through indirect mechanisms that regulate hypothalamic GnRH secretion or directly on the pituitary through enhancement or suppression of pituitary response to GnRH (Schoenemann et al., 1985; Baratta et al., 1994; Rispoli and Nett, 2005). During the preovulatory period, an increase in estradiol from the preovulatory follicle increases the sensitivity of the anterior pituitary to GnRH by upregulating GnRH receptor mRNA and protein in gonadotrophs (Schoenemann et al., 1985). In addition, estradiol enhances hypothalamic GnRH secretion, eventually leading to the preovulatory GnRH surge, which triggers the LH surge. Conversely, in the presence of a functional CL, P4 blocks the stimulatory effects of estradiol-17 β (**E2**) at the hypothalamus (Schoenemann et al., 1985; Girmus and Wise, 1992) and possibly at the anterior pituitary (Baratta et al., 1994; Rispoli and Nett, 2005).

Studies using beef heifers and cows support the hypothesis that the magnitude of the GnRH-induced LH surge in cattle depends on the P4 environment at GnRH treatment (Colazo et al., 2008; Dias et al., 2010). A limitation of these studies, however, was that cattle in the low-P4 groups had mean P4 concentrations greater than 1 ng/mL [presence of a controlled internal drug release (**CIDR**) insert or CL in all treatments], a P4 concentration indicative of a functional CL, and a level of circulating P4 that may already be inhibitory to GnRH-induced LH secretion. In contrast, Zolman et al. (1974) reported no differences in LH peak concentrations in response to GnRH treatment in Holstein heifers on either d 15 or 20 of the estrous cycle when P4 concentrations averaged 3.9 and 0.4 ng/mL, respectively. Unfortunately, only LH peak concentrations were reported, a value that does not provide complete information on total GnRH-induced LH secretion. Thus, additional studies are necessary to directly compare the effect of P4 on the magnitude of the LH surge in high- and low-P4 environments. In addition to the biological importance of this research area, our major interest was to compare the GnRH response during experimental conditions that replicate the physiological environments observed during synchronization of ovulation using an Ovsynch protocol in lactating dairy cows. During the Ovsynch protocol, cows have relatively high P4 concentration (≥ 1 ng/mL) at the time of the first GnRH injection (Stevenson et al., 1999; Bello et al.,

2006; Giordano et al., 2012), whereas P4 concentration is usually low (usually below 0.5 ng/mL) at the second GnRH injection (Stevenson et al., 2008; Brusveen et al., 2009; Giordano et al., 2012).

A potential strategy to enhance GnRH-induced LH release in lactating dairy cows may be to increase the dose of GnRH administered. Studies in cattle have reported diverse results using different doses of GnRH with either enhanced or similar LH release in response to increasing doses of GnRH (Chenault et al., 1990; Yamada et al., 2002; Colazo et al., 2009; Rantala et al., 2009; Souza et al., 2009; Dias et al., 2010). Much of the variation in these results may be attributed to factors associated with the experimental design of these studies, including the type and physiological conditions of the animals used, variations among GnRH products, GnRH doses administered, LH assay methods, parameters used to compare LH secretion, and the steroid milieu at the time of GnRH treatment. From a biological perspective, increased doses of GnRH can provide an indication of whether changes in GnRH-induced LH responses are due to lack of sufficient pituitary LH content. From a practical point of view, increasing the GnRH dose could be a simple method to enhance LH secretion during Ovsynch-type protocols, potentially resulting in enhanced ovulation and increased synchronization to the protocol. Because Ovsynch-type protocols are currently used extensively in the dairy industry (Caraviello et al., 2006; Olynk and Wolf, 2008; Norman et al., 2009), we performed this experiment to assess the effect of P4 concentration and 2 different doses of GnRH during the 2 time points that GnRH is administered during an Ovsynch protocol.

The objectives of this study were to determine the effect of 1) circulating P4 concentrations at the time of GnRH treatment on GnRH-induced LH secretion in lactating dairy cows and 2) increasing the dose of GnRH from 100 to 200 μ g on LH secretion in a high- and low-P4 environment. The specific hypotheses of this experiment were that a high-P4 environment would suppress LH secretion in response to exogenous GnRH treatment and further, that a higher dose of exogenous GnRH would result in greater LH secretion in both a high- and a low-P4 environment.

MATERIALS AND METHODS

This experiment was conducted from November 2009 to December 2009 using cows in the University of Wisconsin–Madison dairy herd. All procedures were approved by the Animal Care and Use Committee for the College of Agriculture and Life Sciences of the University of Wisconsin–Madison.

Cows

Lactating primiparous ($n = 5$) and multiparous ($n = 19$) Holstein ($n = 17$) and crossbred ($n = 7$; 3/4 Holstein, 1/4 Jersey) dairy cows were used for this experiment. During the presynchronization part of the experiment, cows were housed in freestall barns at the Blaine Dairy Cattle Center (Arlington, WI), whereas during the intensive part of the experiment when treatments were administered, cows were housed in a tie-stall barn at the Dairy Cattle Teaching and Research Center at the University of Wisconsin–Madison. During both study periods, cows were fed a TMR diet once daily with ad libitum access to feed and water. The diet was formulated to meet or exceed NRC requirements (NRC, 2001) for high-producing lactating dairy cows. Throughout the experiment, cows were milked twice daily at approximately 12-h intervals, and all cows received s.c. injections of bovine somatotropin (Posilac, 500 mg; Elanco Animal Health, Indianapolis, IN) at 14-d intervals beginning 63 ± 3 d postpartum.

Treatments

High- and low-P4 environments in this experiment were evaluated by enrolling cows in the Double-Ovsynch protocol (Pre-Ovsynch: GnRH, PGF_{2α} 7 d later, GnRH 3 d later, and Breeding-Ovsynch 7 d later: GnRH, PGF_{2α} 7 d later, and GnRH 48 h later; Souza et al.,

2008) with minor modifications (48 instead of 56 h between PGF_{2α} and second GnRH in Breeding-Ovsynch). Cows at various DIM and AI services completed the Pre-Ovsynch portion of the Double-Ovsynch protocol to induce an ovulation and formation of a CL to generate the high-P4 environment at the first GnRH injection of Breeding-Ovsynch. The time of the first GnRH injection of the Breeding-Ovsynch will be referred to as the high-P4 environment (high P4). Seven days after the GnRH injection, cows received a luteolytic dose of PGF_{2α} (500 µg of Estrumate, 250 µg/mL of cloprostenol sodium; Schering-Plough Animal Health, Summit, NJ) to induce luteal regression and generate the low-P4 environment (low P4) to receive the second GnRH injection of Breeding-Ovsynch. At the time of GnRH treatment in both the high- and low-P4 environments, cows ($n = 24$) were paired by lactation number and DIM and were randomly assigned to receive either 100 ($n = 12$) or 200 ($n = 12$) µg of GnRH (Fertagyl, 50 µg/mL of gonadorelin diacetate tetrahydrate; Intervet Animal Health, Millsboro, DE; Figure 1).

Ultrasound Examinations for Determination of Ovulatory Response to GnRH

To determine ovulation after the second GnRH injection of Pre-Ovsynch and ensure the presence of a functional CL at the first GnRH injection of Breeding-

Figure 1. Schematic representation of experimental procedures. Cows were enrolled in a Double-Ovsynch protocol (DO; Pre-Ovsynch: GnRH, PGF_{2α} 7 d later, GnRH 3 d later, and Breeding-Ovsynch 7 d later: GnRH, PGF_{2α} 7 d later, and GnRH 48 h later) to generate high- and low-progesterone (P4) environments. Only cows with confirmed ovulation within 30 to 32 h after the second GnRH injection of Pre-Ovsynch (not shown) and a functional corpus luteum (CL) immediately before the first GnRH injection of Breeding-Ovsynch initiated ($n = 24$) the experiment. Within each P4 environment, cows were paired by lactation number and DIM and were randomly assigned to receive either 100 or 200 µg of GnRH. Blood samples (BS) were collected to measure circulating LH concentrations from -15 min to 6 h after GnRH treatment in both the high- and low-P4 environments. During the first 3 h and 15 min of the blood sampling period, samples were collected every 15 min and then every 1 h from 3 to 6 h after GnRH treatment. Blood samples were also collected at the first GnRH injection, PGF_{2α} injection, 48 h after the PGF_{2α} injection, and 8 d after the second GnRH injection (not shown) of the Breeding-Ovsynch portion of DO to measure P4 concentrations. Ovarian ultrasonography (US) was performed after GnRH treatment to assess ovulatory response to GnRH treatment in the high- and low-P4 environments. Ovarian US was performed at GnRH treatment and then every 2 h beginning 22 h after GnRH treatment until ovulation or 48 h after GnRH treatment, whichever occurred first.

Ovsynch, transrectal ultrasonography (US) of the ovaries was performed in all cows enrolled to record the location and diameter of follicles present. Ovulation in response to GnRH treatment was defined as the disappearance of one or more follicles present on the ovaries within 30 to 32 h after the GnRH injection. Seven days after the second GnRH injection of Pre-Ovsynch, cows that presented an ovulation were evaluated using US to confirm the presence of a CL and only those cows having at least 1 CL with a diameter ≥ 20 mm continued in the study to receive the first GnRH injection of Breeding-Ovsynch ($n = 24$). Throughout this part of the experiment, all US examinations were performed using a portable ultrasound scanner (Easi-Scan; BCF Technology Ltd., Livingston, UK) fitted with a 7.5-MHz linear array transducer.

Ovulatory responses after GnRH treatment in high- and low-P4 environments were determined as described previously for the presynchronization period. At this time, however, an Aloka 500V (Corometrics Medical Systems Inc., Wallingford, CT) ultrasound machine equipped with a transrectal 7.5-MHz linear-array transducer was used to perform the examinations. During all US sessions, all CL and antral follicles ≥ 8 mm present on the ovaries were mapped. For each ovarian structure, 2 diameter measurements, taken at right angles, were recorded on a single image of the apparent maximal diameter of the structure using the digital calipers of the ultrasound machine, and diameters were calculated as the mean of these 2 measurements. Ovarian US sessions were performed at the time of GnRH treatment and then every 2 h starting 22 h after GnRH treatment until ovulation occurred or 48 h after GnRH treatment, whichever occurred first (Figure 1). Ovarian US was performed again 7 d after GnRH treatment in the high-P4 environment and 8 d after GnRH treatment in the low-P4 environment to confirm ovulation.

Blood Sample Collection

Blood samples were collected via puncture of the median caudal vein or artery with 8-mL evacuated tubes (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ) to determine P4 concentrations before GnRH treatment in both the high- and low-P4 environments to confirm that cows had high or low P4 concentrations (cutoff 1 ng/mL), respectively (Figure 1). Blood samples were then collected in the same manner at the time of and 48 h after the PGF_{2 α} injection given 7 d after GnRH treatment in the high-P4 environment to confirm luteal regression (Figure 1). Finally, blood samples for determination of P4 were collected 8 d after GnRH treatment in the low-P4 environment to confirm ovulation.

For determination of LH concentration, blood samples were collected from the jugular vein using Monovette tubes (Sarstedt Inc., Newton, NC) attached to indwelling jugular catheters. Samples were collected every 15 min for 3 h and 15 min starting 15 min before GnRH treatment and then every hour for 3 additional h (total sampling period: 6 h and 15 min) in both the high-P4 and low-P4 environments (Figure 1).

After collection, blood samples were immediately placed on ice for at least 30 min, then removed from the ice for approximately 30 min, and centrifuged ($2,000 \times g$) at 4°C for 25 min. After centrifugation, serum was collected and stored at -20°C in 5-mL scintillation vials until assayed.

P4 and LH Assays

Progesterone concentrations were determined in duplicate using a commercial solid-phase, no-extraction RIA (Coat-a-count; Diagnostic Products Corp., Los Angeles, CA). To assess the precision of the assay, control samples with a high (18 ng/mL) and low (0.5 ng/mL) concentration of P4 were assayed. Average sensitivity for the P4 assay was 0.005 ng/mL. The average intraassay coefficient of variation for the high-concentration serum sample was 1.4%, whereas the coefficient of variation was 5.9% for the low-concentration sample.

Luteinizing hormone concentrations were determined in duplicate samples by RIA as described in Palhao et al. (2009) and modified as described by Hannan et al. (2010). The standard curve ranged from 0.08 to 10 ng/mL of LH. Therefore, samples with percentage binding less than 10% due to high LH concentrations were diluted either 1:4 or 1:20 and reanalyzed to obtain percentage binding in the 30 to 60% range, which corresponded to 1.25 and 0.625 ng/mL standards. To assess the precision of the 5 separate LH assays run, control samples with a low (0.3 ng/mL) and high (1.3 ng/mL) concentration of LH were evaluated in each assay. The average sensitivity of the assays was 0.12 ng/mL. For the low sample, the average intraassay coefficient of variation was 5.5% (range = 1.34 to 9.09), whereas the interassay coefficient of variation was 7.5%. For the high-LH sample, the average intraassay coefficient of variation was 3.2% (range = 1.45 to 4.30) and the interassay coefficient of variation was 5.8%.

Statistical Analyses

Separate statistical analyses were performed to test the first hypothesis of the effect of P4 environment on GnRH-induced LH response and then to test the second hypothesis of the effect of GnRH dose on LH secretion in both high- and low-P4 environments. Before

statistical analyses, normality of the data set for the different variables under consideration [P4 at GnRH, LH peak concentration, time to LH peak, area under the curve (AUC), mean LH concentration, and time to ovulation] was tested using the Shapiro-Wilk statistic and graphical methods (Q-Q plot, probability plot, and histogram) obtained with PROC UNIVARIATE of SAS 9.2 (SAS Institute Inc., Cary, NC). When non-normality of the data was detected, the variables under consideration were transformed accordingly through natural log, square root, or power transformation to ensure normality of the data distribution. For variables in which it was not possible to achieve normality of the data distribution through the previously mentioned transformations, nonparametric analysis for ranked transformed data was performed with the Wilcoxon 2-sample test using the NPARIWAY procedure of SAS. For this analysis, the classification variables were either P4 environment or GnRH dose. For all variables presenting a normal distribution before or after transformations, the effects of P4 environment and GnRH dose on the height of the LH peak, time to the LH peak, and AUC (approximated with the trapezoidal method and reported as arbitrary units) were analyzed by ANOVA with PROC MIXED of SAS. Differences in P4 concentration in the high- and low-P4 environments and for cows receiving 100 versus 200 µg of GnRH were tested similar to the variables associated with the LH response. All original models contained P4 environment (high vs. low) or GnRH dose (100 vs. 200 µg of GnRH), parity (primiparous vs. multiparous), and BCS (high ≥ 2.5 vs. low < 2.5) as classificatory variables, whereas DIM and BW were used as covariates in the model. After running the original models containing all explanatory variables and covariates for each one of the outcome variables under consideration, model selection was performed by backward elimination of those variables with a P -value > 0.10 . In addition, model selection was performed by selecting the model with the lowest value for the Akaike information criterion.

Analysis of differences in mean LH concentration after GnRH treatment was performed using ANOVA with repeated measures with PROC MIXED of SAS. According to the specific analysis being performed, the model contained the fixed effects of P4 environment or GnRH dose, time, and the P4 environment or GnRH dose by time interaction. Parity and BCS were also included as explanatory variables, whereas DIM and BW were included as covariates. Cow within P4 environment or GnRH dose was used as a random effect in the model and was the subject of repeated measures analysis using a spatial power covariance structure to adjust for the nonuniform blood collection time intervals (15 min for the first 3 h after treatment and then

every 1 h until the end of the sampling period). The Kenward-Rogers method was used for the degrees of freedom calculation. Model selection was performed as described previously. Finally, ovulatory response by P4 environment and GnRH dose was analyzed with logistic regression using the PROC GLIMMIX of SAS with a model that contained P4 environment or GnRH dose treatment as explanatory variables.

A significant difference between the levels of a classificatory variable or a covariate was considered when $P < 0.05$, whereas differences between $P \geq 0.05$ and $P \leq 0.10$ were considered a statistical tendency. Further analysis of differences between least squares means was performed with the Tukey-Kramer post hoc mean separation test. For clarity of interpretation data are presented as arithmetic means $\pm$ standard error of the means.

RESULTS

Effect of P4 on LH Secretion

For cows receiving 100 µg of GnRH, the mean P4 concentration was greater ($P < 0.001$) in the high-P4 than in the low-P4 environment (Table 1). Peak LH concentrations after treatment with 100 µg of GnRH were greater ($P < 0.001$), whereas time to the LH peak tended ($P = 0.075$) to be greater for cows in the low- than for cows in the high-P4 environment (Table 1). In addition, the AUC was greater ($P < 0.001$) for cows in the low- than for cows in the high-P4 environment (Table 1).

Mean LH concentrations throughout the sampling period after treatment with 100 µg of GnRH (Figure 2A) were affected by P4 concentration ($P < 0.001$), time ($P < 0.001$), and the P4 concentration by time interaction ($P < 0.001$). In the high-P4 environment, the 100 µg dose of GnRH did not increase mean LH concentrations beyond 5 ng/mL at any time after GnRH treatment. For cows treated with GnRH in the low-P4 environment, mean LH was greater than 5 ng/mL at all times from 0.75 until 3 h after GnRH treatment and was greater than 10 ng/mL from 1.5 to 2.5 h after GnRH treatment. Mean LH concentrations were greater in the low- than the high-P4 environment from 1 to 6 h after GnRH treatment with more than 2-fold greater LH concentrations at all of these times and 5-fold greater differences from 1.75 to 5 h after GnRH treatment.

Ovulatory response to GnRH treatment was recorded, although this study was not designed to validly analyze treatment effects on this binomial response. For cows receiving 100 µg of GnRH, 1 cow treated in the low-P4 environment was eliminated because the only

Table 1. Effect of progesterone concentration at GnRH treatment on LH secretion in lactating dairy cows receiving 100 or 200 µg of GnRH¹

Item	100 µg of GnRH		<i>P</i> -value	200 µg of GnRH		<i>P</i> -value
	High P4 (n = 12)	Low P4 (n = 12)		High P4 (n = 12)	Low P4 (n = 10)	
Progesterone (ng/mL)	3.5 ± 0.2 (2.2 – 4.8)	0.2 ± 0.05 (0.1 – 0.6)	<0.001	3.6 ± 0.4 (1.1 – 5.3)	0.2 ± 0.02 (0.1 – 0.4)	<0.001
LH peak (ng/mL)	3.3 ± 0.3	15.7 ± 2.2	<0.001	8.5 ± 1.7	23.6 ± 1.6	0.001
Time to LH peak (h)	1.3 ± 0.2	1.8 ± 0.1	0.075	1.8 ± 0.2	1.9 ± 0.1	0.850
AUC ² (units)	501.0 ± 42.6	2,186.4 ± 279.7	<0.001	1,177.8 ± 193.3	3,443.2 ± 198.1	0.001

¹All values are means ± SEM; for progesterone, range in parentheses.²Area under the curve. **Figure 2.** Mean (±SEM) LH concentrations in lactating dairy cows treated with 100 µg [panel A; high-progesterone (P4) environment (n = 12) and low-P4 environment (n = 12)] or 200 µg of GnRH [panel B; high-P4 environment (n = 12) and low-P4 environment (n = 10)] in a high- versus a low-P4 environment at the first and second GnRH injections of the Breeding-Ovsynch portion of the Double-Ovsynch protocol, respectively. Asterisks denote a difference (Tukey mean separation test) in mean LH concentration between groups of cows.

follicle of ovulatory size (≥ 10 mm) ruptured during the US examination at GnRH treatment. After elimination of this cow, ovulatory response to the 100-µg dose of GnRH was similar ($P = 0.344$) for cows in the high-P4 (75.0%, 9/12) and the low-P4 (90.9%, 10/11) environments. The mean diameter of the largest follicle of cows in the high-P4 environment was 16.9 mm (range = 14 to 21 mm), whereas it was 17.7 mm (range = 14 to 24 mm) for cows in the low-P4 environment. The diameter of the largest follicle of the 4 cows failing to ovulate in response to GnRH treatment was 17, 18, 18, and 21 mm. The mean time from GnRH treatment to ovulation was similar ($P = 0.256$) for cows receiving the low dose of GnRH both in the high-P4 (29.3 ± 0.5 h; range 28 to 32 h) and the low-P4 (28.6 ± 0.3 h; range 28 to 30 h) environments.

Data from 2 cows receiving 200 µg of GnRH were eliminated from the data set because they had 1.4 and 2.4 ng/mL of circulating P4 at GnRH treatment in the low-P4 environment, indicating lack of complete luteal regression to the PGF_{2α} injection given 2 d earlier. For cows receiving 200 µg of GnRH, the mean concentration of P4 was greater ($P < 0.001$) in the high- than in the low-P4 environment (Table 1). The peak LH concentration after treatment with 200 µg of GnRH was greater ($P = 0.001$), whereas time to the LH peak was similar ($P = 0.850$) for cows with low P4 compared with cows with high P4 concentrations (Table 1). In addition, the AUC was greater ($P = 0.001$) for cows with low P4 than for cows with high P4 concentrations (Table 1).

Mean LH concentrations throughout the sampling period after treatment with 200 µg of GnRH were also affected by the P4 environment ($P < 0.001$), time ($P < 0.001$), and the P4 environment by time interaction ($P < 0.001$; Figure 2B). Unlike cows treated with 100 µg of GnRH in the high-P4 environment, cows receiving 200 µg of GnRH reached mean circulating LH concentrations greater than 5 ng/mL during the maximum secretion period from 1.5 to 2.5 h after GnRH treatment. However, LH concentrations were less than 10 ng/mL at all times for cows receiving 200 µg of GnRH in a high-

P4 environment. By contrast, the pattern of circulating LH concentrations in cows receiving 200 µg GnRH was quite distinct with slowly increasing LH concentrations during the first 30 min and then a dramatic increase in LH concentrations between 45 min and 1 h after GnRH treatment. Circulating LH concentrations remained high from 1 to 3 h after GnRH treatment in cows with low P4, exceeding concentrations of 20 ng/mL during this interval. Mean LH concentrations were greater for cows in the low-P4 environment than the high-P4 environment from 1 to 6 h after GnRH treatment, with more than 3-fold greater LH concentrations from 1 to 2 h after treatment and 2-fold greater LH concentrations until the end of the sampling period.

Finally, ovulatory responses of cows receiving 200 µg of GnRH were similar ($P = 0.979$) for cows in the high-P4 (91.7%, 11/12) and the low-P4 (100%, 10/10) environments. The mean diameter of the largest follicle of cows in the high-P4 environment was 16.7 mm (range = 14 to 21 mm), whereas it was 16.0 mm (range = 12 to 21 mm) for cows in the low-P4 environment. The diameter of the largest follicle of the only cow failing to ovulate after GnRH treatment was 16 mm. At the last US examination, the follicle did not appear to have ruptured; however, P4 concentrations were 1.9 ng/mL 8 d later. The time from GnRH treatment to ovulation was similar ($P = 0.549$) for cows receiving 200 µg of GnRH both in the high- (29.1 ± 0.3 h, range 28 to 30 h) and the low- (28.8 ± 0.3 h, range 28 to 30 h) P4 environments.

Effect of GnRH Dose on LH Secretion

All cows in the high-P4 environment had P4 >1.0 ng/mL at GnRH treatment. Progesterone concentrations were similar ($P = 0.806$) for cows in the 100 and 200-µg GnRH treatments (Table 2).

Cows receiving 200 µg of GnRH had a greater LH peak ($P < 0.001$) and a shorter ($P = 0.040$) time to the LH peak than cows receiving 100 µg of GnRH (Table 2). For LH peak concentration, DIM at the time of treatment was retained in the model. The parameter

estimate for DIM showed a negative relationship between DIM and LH peak concentration (-0.003 ; $P = 0.041$). The AUC for cows receiving 200 µg of GnRH was greater ($P < 0.001$) than for cows receiving 100 µg of GnRH (Table 2). The model for AUC also indicated a negative relationship (-0.002 ; $P = 0.022$) between DIM and AUC.

For cows in the high-P4 environment, mean LH concentrations were affected by GnRH dose ($P < 0.001$), time ($P < 0.001$), and the GnRH dose by time interaction ($P < 0.001$; Figure 3A). Cows receiving the 100- and 200-µg doses of GnRH had mean LH concentrations less than 10 ng/mL at all times after GnRH treatment. Only cows treated with 200 µg of GnRH had mean LH concentrations greater than 5 ng/mL and only from 1.5 to 2.5 h after GnRH treatment when peak LH concentrations occurred. A clear divergence in LH concentrations was observed between the 2 GnRH doses beginning about 1 h after GnRH treatment, with cows receiving the 100-µg dose remaining flat, whereas cows receiving the 200-µg dose having increased LH concentrations. Mean LH concentrations were greater for cows receiving 200 compared with 100 µg of GnRH beginning 1.5 h until 5 h after GnRH treatment, with more than 2-fold greater LH concentrations during this entire period.

The ovulatory response of cows after GnRH treatment in the high-P4 environment was similar ($P = 0.306$) for cows receiving 100 µg (75.0%; 9/12) compared with 200 µg (91.7%; 11/12) of GnRH. For cows treated with GnRH in the high-P4 environment, the time from GnRH treatment to ovulation was similar ($P = 0.799$) for cows receiving 100 µg (29.3 ± 0.5 h; range 28 to 32 h) and 200 µg (29.1 ± 0.3 h; range 28 to 30 h) of GnRH. The mean diameter of the largest follicle for cows receiving 100 µg of GnRH was 16.9 mm (range = 14 to 21 mm) and was 16.7 mm (range = 14 to 20 mm) for cows receiving 200 µg of GnRH.

For the low-P4 environment (after elimination of the 2 cows that had incomplete luteal regression), P4 concentrations were similar ($P = 0.892$) for cows receiving 100 or 200 µg of GnRH (Table 2). Cows with low

Table 2. Effect of 100 versus 200 µg of GnRH on LH secretion in lactating dairy cows with high or low progesterone concentration¹

Item	High progesterone		<i>P</i> -value	Low progesterone		<i>P</i> -value
	100 µg (n = 12)	200 µg (n = 12)		100 µg (n = 12)	200 µg (n = 10)	
Progesterone (ng/mL)	3.5 ± 0.2 (2.2 – 4.8)	3.6 ± 0.4 (1.1 – 5.3)	0.806	0.2 ± 0.05 (0.1 – 0.6)	0.2 ± 0.02 (0.1 – 0.4)	0.892
LH peak (ng/mL)	3.3 ± 0.3	8.5 ± 1.7	<0.001	15.7 ± 2.2	23.6 ± 1.6	0.010
Time to LH peak (h)	1.3 ± 0.2	1.8 ± 0.2	0.040	1.8 ± 0.1	1.9 ± 0.1	0.528
AUC ² (units)	501.0 ± 42.6	$1,177.8 \pm 193.3$	<0.001	$2,186.4 \pm 279.7$	$3,443.2 \pm 198.1$	0.002

¹All values are means $\pm$ SEM; for progesterone, range in parentheses.

²Area under the curve.

Figure 3. Mean ($\pm$ SEM) LH concentrations for lactating dairy cows in high- [panel A; 100 μ g of GnRH ($n = 12$) and 200 μ g of GnRH ($n = 12$)] or low- [panel B; 100 μ g of GnRH ($n = 12$) and 200 μ g of GnRH ($n = 10$)] progesterone (P4) environments treated with 100 versus 200 μ g of GnRH. The high- and low-P4 environments corresponded to the first and second GnRH injections of the Breeding-Ovsynch portion of the Double-Ovsynch protocol, respectively. Asterisks denote a difference (Tukey mean separation test) in mean LH concentration between groups of cows.

P4 that received 200 μ g of GnRH had a greater LH peak than cows receiving 100 μ g of GnRH ($P = 0.010$), whereas time to the LH peak was similar ($P = 0.528$) for both groups of cows (Table 2). The AUC for cows receiving 200 μ g of GnRH was greater ($P = 0.002$) compared with cows receiving 100 μ g of GnRH (Table 2).

For cows in the low-P4 environment, mean LH concentrations were affected by GnRH dose ($P < 0.001$), time ($P < 0.001$), and the GnRH dose by time interaction ($P < 0.001$; Figure 3B). Unlike the results for the high-P4 environment, the analysis for cows with low P4 included parity and BCS, but all other covariates in-

cluded in the original models were not significant or did not improve model fit and were, therefore, eliminated from all statistical models.

For cows with low P4, both doses of GnRH elicited similar increases in LH concentrations during the first 45 min of sampling (Figure 3B). Between 45 min and 1 h after GnRH treatment, a more dramatic increase in LH concentrations occurred in cows that received 200 μ g compared with 100 μ g of GnRH, although increases in circulating LH concentrations continued in both groups until about 2 h after GnRH treatment. Unlike the high-P4 environment, when neither GnRH dose elicited mean LH concentrations greater than 10 ng/mL, both groups peaked above 10 ng/mL of circulating LH; however, cows treated with 200 μ g of GnRH in a low-P4 environment had an LH peak greater than 20 ng/mL. Cows in the low-P4 environment treated with 100 μ g of GnRH had mean LH concentrations greater than 10 ng/mL from 1.25 to 2.5 h after treatment, whereas cows in the low-P4 environment receiving 200 μ g of GnRH maintained mean LH concentrations greater than 15 ng/mL during the same time period. Differences between groups were observed from 1 to 2.5 h after treatment, with cows receiving 200 μ g of GnRH maintaining greater mean LH concentrations than cows receiving 100 μ g of GnRH.

Ovulatory response to GnRH treatment for cows in the low-P4 environment was similar ($P = 0.979$) for cows receiving 100 μ g (90.9%; 10/11) or 200 μ g of GnRH (100.0%; 10/10). The mean diameter of the largest follicle of cows receiving 100 μ g of GnRH was 17.7 mm (range = 14 to 24 mm), whereas it was 16.0 mm (range = 12 to 21 mm) for cows receiving 200 μ g of GnRH. The diameter of the largest follicle of the only low-P4 cow failing to ovulate in response to GnRH was 16 mm. For cows in the low-P4 environment, the time from GnRH treatment to ovulation was similar ($P = 0.686$) for cows receiving 100 μ g (28.6 ± 0.3 h, range 28 to 30 h) compared with 200 μ g (28.8 ± 0.3 h, range 28 to 30 h) of GnRH.

Individual Cow LH Responses to GnRH by P4 Concentration

Figure 4 shows the relationship between P4 and AUC (panel A) and peak LH (panel B) concentration for individual cows after the 2 doses of GnRH. For both AUC and LH peak, it is possible to distinguish clear patterns due to dose of GnRH, but particularly due to P4 concentration. In general, cows with P4 concentrations greater than 1 ng/mL had low total LH secretion (AUC <1,000 units) and LH peak [78% (18/23) peaked below 5 ng/mL] compared with cows with lower P4 concentrations. Most cows with P4 >1.0 ng/mL receiving

Figure 4. Relationship between progesterone concentration at GnRH treatment, area under the curve (AUC; arbitrary units; panel A), and peak LH concentration (panel B) in lactating dairy cows. Filled squares and circles denote individual cows that failed to ovulate in response to GnRH treatment.

200 µg of GnRH had LH concentrations only slightly greater than cows receiving 100 µg of GnRH, except for a few cows that had dramatically greater LH responses than the rest of the cows. One cow receiving 200 µg of GnRH and having P4 concentration slightly greater than 1 ng/mL had an LH response similar to that of cows with much greater circulating P4 concentrations.

On the other hand, most cows with P4 concentration less than 1 ng/mL had dramatically greater LH responses and, in general, more variation than cows with high P4 concentrations. In this case, the greatest variation in LH response was observed for cows receiving 100 µg of GnRH. Indeed, although most cows had LH responses similar to that of cows receiving 200

µg of GnRH in the low-P4 environment, a few low-P4 cows receiving 100 µg of GnRH had much lower LH responses. Except for 1 low-P4 cow that failed to ovulate and had an AUC of 1,650 units and an LH peak of 12 ng/mL, most cows failing to ovulate had an LH surge of low magnitude with AUC of less than 700 units and an LH peak less than 5 ng/mL (Figure 4).

DISCUSSION

This experiment was designed to evaluate the effect of circulating P4 on response to 2 different doses of GnRH observed during protocols used to synchronize ovulation for TAI in lactating dairy cows. Therefore, we chose to evaluate the magnitude of LH response to GnRH at a time when P4 was elevated, such that occurs in cows at the first GnRH injection of the Ovsynch protocol, compared with the GnRH response at a time when P4 was low, such that occurs in cows at the time of the second GnRH injection of the Ovsynch protocol. The most important observation in this experiment was the consistently lower response to GnRH when cows were in a high-P4 environment, supporting our first hypothesis that P4 concentrations affect the magnitude of a GnRH-induced LH surge. With either dose of GnRH, a 3 to 5-fold increase in the peak of the LH surge or AUC for LH occurred when cows were treated with GnRH in the low- compared with the high-P4 environment. Our second hypothesis, that increasing the dose of GnRH would increase the magnitude of the LH surge regardless of P4 environment, was also supported by our results. Our results provide a better understanding of key physiology related to practical reproductive management programs that use GnRH for synchronization of ovulation and have important biological and practical implications for future reproductive research.

Cows treated with GnRH in the high-P4 environment had average P4 concentrations greater than 3 ng/mL, similar to P4 concentrations in many cows 7 d after an induced ovulation. Such physiological conditions are usually observed in cows presynchronized with protocols like Double-Ovsynch (Souza et al., 2008; Giordano et al., 2012) and Presynch-Ovsynch (Moreira et al., 2001). Moreover, this high-P4 environment occurs in cycling cows that start the Ovsynch protocol in the presence of a functional CL (Stevenson et al., 1999; Bello et al., 2006; Giordano et al., 2012). By contrast, cows treated with GnRH in a low-P4 environment had circulating P4 of only 0.2 ng/mL, similar to concentrations in cows with complete luteal regression in response to a luteolytic dose of PGF_{2α} (Brusveen et al., 2009; Giordano et al., 2012). All cows with P4 concentrations less than 0.5 ng/mL had an LH peak greater than 5 ng/mL and almost all cows with low P4 (86%) had an LH peak

greater than 10 ng/mL. By contrast, only 13% of cows with P4 greater than 1 ng/mL had an LH peak greater than 10 ng/mL with the average LH peak of only 3.3 ng/mL for cows receiving 100 µg of GnRH and 8.5 ng/mL for cows receiving 200 µg of GnRH. Attenuation of the GnRH-induced LH surge in a high-P4 environment has been reported in beef heifers and cows (Cline, 2002; Colazo et al., 2008; Dias et al., 2010). Moreover, a similar suppressive effect of P4 on LH secretion has been reported for cattle receiving a GnRH injection during the luteal phase of the estrous cycle compared with cows treated during metestrus or proestrus (Schams et al., 1974; Atkins et al., 2008). The mechanisms that underlie the decrease in GnRH-induced LH secretion have not yet been fully elucidated.

The gonadal steroids E2 and P4 are key regulators of gonadotropin secretion in cattle (Nett et al., 2002; Clarke and Pompolo, 2005; Rispoli and Nett, 2005). This can occur through regulation of the hypothalamus by altering frequency or amplitude of GnRH pulses as well as by direct regulation of GnRH responsiveness at the anterior pituitary (Goodman and Karsch, 1980; Schoenemann et al., 1985; Nett et al., 2002). In the present study, we observed a decreased responsiveness of the pituitary gland to exogenous GnRH treatment in the presence of high circulating concentrations of P4. During the follicular phase of the estrous cycle, the absence of P4 and the presence of increased E2 and GnRH pulses stimulates expression of GnRH receptor mRNA in pituitary cells (Turzillo and Nett, 1995). The subsequent increase in GnRH receptors on the cell membrane of gonadotrophs allows for a robust preovulatory LH surge in response to the preovulatory GnRH surge (Schoenemann et al., 1985; Turzillo and Nett, 1995; Turzillo et al., 1998). This scenario likely corresponds to the low-P4 environment in our experiment. Conversely, during the luteal phase of the estrous cycle when circulating P4 concentrations are high, the number of GnRH receptors on the pituitary cells are decreased either due to direct suppressive effects of P4 or lack of stimulatory effects of E2 on pituitary GnRH receptor numbers (Turzillo et al., 1998; Rispoli and Nett, 2005). Our experimental design does not allow us to separate the effects of changes in E2 from the effects of P4. It is tempting to speculate that the decrease in GnRH-induced LH release is due to a decrease in pituitary GnRH receptors because fewer receptors are present during the luteal phase (Rispoli and Nett, 2005) and an association exists between the amplitude of LH pulses after exogenous GnRH and pituitary GnRH receptors (Wise et al., 1984). Nevertheless, we are not able to distinguish whether the decreased GnRH-induced LH surge is due to decreased numbers of GnRH receptors or a decreased responsiveness within pituitary cells to

activation of GnRH receptors (i.e., decreased activation of second messengers). Another possibility is that a decreased quantity of releasable LH in the pituitary of cows exists in the high-P4 environment; however, previous studies have not found decreased pituitary LH content after P4 treatment (Moss et al., 1981; Baratta et al., 1994). In addition, our observation that a doubling of the GnRH dose caused a concomitant doubling in LH release indicates that more releasable LH was present than was released when cows were treated with 100 µg of GnRH in either the high-P4 or low-P4 environment.

Besides ovarian steroids, GnRH itself plays an important role in the regulation of its own receptor with greater GnRH release upregulating the GnRH receptor in the pituitary (Nett et al., 2002). Thus, inhibition of GnRH pulses by P4 might decrease the number of GnRH receptors and, therefore, the responsiveness of pituitary cells through this indirect pathway. Interestingly, one experiment in sheep concluded that the suppressive effect of P4 on LH release was not related to number of GnRH receptors (Girmus et al., 1996). Actions of P4 directly on the pituitary are clearly possible because receptors for P4 are present in the ovine (Girmus et al., 1996) and bovine (Jacobs and Smith, 1981; Baratta et al., 1994) anterior pituitary and may mediate the direct effects of P4 on LH release (Batra and Miller, 1985; Girmus and Wise, 1992). Furthermore, acute actions of P4 on the anterior pituitary may involve membrane-bound nongenomic receptors for P4 (Ashley et al., 2006; Ashley et al., 2009) rather than classical P4 receptors, which act as transcription factors.

There are many practical implications of the suppression of a GnRH-induced LH surge. As previously mentioned, the physiological conditions of the high-P4 environment, with consequent suppression of ovulation, are usually observed in lactating dairy cows that are either presynchronized or initiate the Ovsynch protocol during diestrus (Stevenson et al., 2008; Galvão and Santos, 2010; Giordano et al., 2012). Ovulation after GnRH treatment is necessary to ensure the initiation of a new follicular wave to maximize the chances of a synchronized ovulation around the TAI. Moreover, some studies have reported a decreased ovulatory response to GnRH in cows receiving an intravaginal progesterone releasing (CIDR) insert at GnRH administration (Galvão et al., 2004; Stevenson et al., 2008). In lactating dairy cows receiving a CIDR device, circulating P4 concentrations increased within 15 min after treatment with a new or used CIDR insert (Cerri et al., 2009). Thus, we speculate that in addition to the long-term effect (days) of P4 to suppress ovulation, an acute effect of P4 is also possibly mediated through an acute sup-

pression of GnRH-induced LH secretion. In addition, previous difficulties in inducing ovulation with GnRH in cows with a functional CL, such as pregnant cows or nonlactating heifers (unpublished observations), even in the presence of growing dominant follicles, may be related to decreased GnRH-induced LH secretion rather than the inability of follicles to ovulate.

The second hypothesis of this experiment was that an increased GnRH dose would increase LH secretion. Increasing the GnRH dose from 100 to 200 μg increased both the peak of the LH surge and the AUC. In addition, LH dynamics differed according to the dose of GnRH with no clear peak in LH for cows receiving 100 μg of GnRH in a high-P4 environment, whereas cows receiving 200 μg of GnRH produced a clear LH peak, even in a high-P4 environment. These observations in the high-P4 environment indicate that the greater dose of GnRH partially overcame the suppressive effect of circulating P4 on GnRH-induced LH release.

For cows in the low-P4 environment, both doses of GnRH induced a major LH surge with only 1 cow having a LH peak less than 5 ng/mL and AUC less than a 1,000 units. Overall, treatment with 200 μg of GnRH enhanced LH secretion, resulting in greater LH peak, mean LH, and total LH secreted (AUC) compared with cows receiving 100 μg of GnRH. The total amount of LH released after GnRH was 1.6 times greater in cows treated with 100 compared with 200 μg of GnRH.

In contrast with these results, Chenault et al. (1990) reported no differences in serum LH concentration after increasing the dose of gonadorelin from 100 to 250 μg in Holstein heifers with concentrations of P4 characteristic of mid to late diestrus (d 8 to 16 of the estrous cycle). In the latter study, AUC and peak LH concentration were numerically greater for heifers treated with the higher GnRH dose; however, the difference did not reach statistical significance. In the same study, a difference in total LH secretion was only detected when heifers were treated with 500 μg of gonadorelin, supporting the notion that greater GnRH doses generate greater LH responses. The discrepancies between the present study and that of Chenault et al. (1990) regarding LH secretion in response to 100 versus 250 μg of GnRH may be partially due to the desensitization of the pituitary reported for some of the doses and analogs used. The 48-h interval between GnRH injections in Chenault et al. (1990) may have precluded a full pituitary response to GnRH in heifers previously receiving a desensitizing dose of some of the GnRH analogs. It is also intriguing that a lack of difference was observed in LH secretion in Holstein heifers receiving 100 compared with 500 μg of gonadorelin 24 h after a luteolytic dose of a synthetic PGF_{2 α} analog (Rantala et al., 2009). Conversely, in agreement with our results, Dias et al. (2010) reported

that beef heifers treated with 200 μg of gonadorelin when mean P4 concentration was 6 to 7 ng/mL had greater mean LH concentration at 1 and 2 h after treatment than heifers receiving 100 μg of GnRH. Dias et al. (2010) also reported greater mean LH concentration in a lower-P4 environment when comparing the same GnRH doses as in the present study. Our results are also in partial agreement with those reported by Colazo et al. (2009) for ovariectomized nonlactating Holstein cows treated with 50, 100, or 250 μg of gonadorelin after an induced follicular phase. Cows receiving the highest GnRH dose had greater mean LH concentrations and duration of the LH surge but not a greater LH peak when compared with cows receiving the lower GnRH doses.

Taken together, results of these previous studies and the current experiment suggest that increasing the GnRH dose from 100 to 200 μg may be a practical method for increasing GnRH-induced LH secretion in lactating dairy cows in a high-P4 environment. Clearly, the LH surge in response to the recommended GnRH dose of 100 μg for cattle is attenuated by P4 and it seems likely that some cows will have an insufficient LH surge to induce ovulation. This may be particularly important to maximize ovulatory response to the first GnRH injection of Ovsynch in protocols that include presynchronization of ovulation before initiation of the Ovsynch protocol either for first service (Moreira et al., 2001; Bello et al., 2006; Souza et al., 2008) or for cows resynchronized for second and subsequent AI services (Dewey et al., 2010; Giordano et al., 2012). Likewise, the 200- μg dose of GnRH may be used to increase the release of pituitary LH in a low-P4 environment. In this case, however, an LH peak of greater magnitude may not be as critical as under a high-P4 environment given the acceptable LH surges observed for cows treated with 100 μg of GnRH.

Despite the dramatic differences in LH secretion in the high- and low-P4 environment for cows treated with 100 and 200 μg of GnRH, no differences were observed for ovulatory response. Overall, ovulatory response in this study was high, which could be explained, at least in part, because cows were selected throughout the presynchronization part of the protocol and only those cows that responded adequately to the previous injections, had a functional CL, and a follicle of ovulatory size remained in the experiment. In addition, the number of cows used in this study may have precluded the detection of statistical differences regardless of some clear trends in ovulatory response. In general, more cows treated with 200 μg of GnRH ovulated both in the high- and the low-P4 environment. Interestingly, a similar percentage of cows ovulated to the 200- μg dose of GnRH in the high-P4 environment than cows

treated with the 100- μ g dose in the low-P4 environment. As expected, the lowest ovulatory response was observed for cows treated with 100 μ g of GnRH in the high-P4 environment because they had an LH surge of low magnitude. Indeed, most cows (3 out of 5) that failed to ovulate in this experiment received the low dose of GnRH in the high-P4 environment and had some of the lowest LH responses with low AUC and LH surge peaks. The fourth cow that failed to ovulate in the high-P4 environment received the 200- μ g dose of GnRH but the magnitude of the LH surge was comparable to that of cows receiving 100 μ g of GnRH in the high-P4 environment.

CONCLUSIONS

Results from the present experiment indicate that P4 concentrations at GnRH treatment dramatically suppress GnRH-induced LH release in lactating dairy cows receiving 100 or 200 μ g of GnRH. Cows receiving either dose of GnRH in the high-P4 environment had an LH surge of much lower magnitude than cows treated with the same doses of GnRH in the low-P4 environment. In addition, treatment with 200 μ g of GnRH enhanced pituitary LH release when compared with the recommended dose of 100 μ g of GnRH for cattle both in the high- and the low-P4 environment.

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