REPRODUCTION
RESEARCH

Accessory corpus luteum regression during pregnancy II: reproductive outcomes

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Abstract

The objective of this study was to evaluate the effect of accessory corpus luteum (CL) induction on fertility in dairy cows. On day 5 after artificial insemination (AI), lactating Holstein cows were assigned unequally to receive gonadotrophin-releasing hormone treatment (GnRH) (*n* = 641) or no treatment (control; *n* = 289). Cows had their blood sampled for progesterone (P4), and ovaries were scanned by ultrasound on days 5, 12, 19, 26, 33, 47, and 61 after AI. Pregnancy diagnosis was performed on days 26, 33, 47, and 61. On day 12, cows treated with GnRH were allocated to ipsilateral (*n* = 239) or contralateral (*n* = 241) groups based on the side of accessory CL formation relative to previous ovulation. Accessory CL cows had greater P4 than controls. In total, 52.7% (78/148) of pregnant cows in contralateral group had accessory CL regression earlier (<day 33; 30.8%) or later (days 33–61; 69.2%) in pregnancy with coincident decrease in P4. No cows with ipsilateral accessory CL underwent regression. There was no difference in pregnancy/AI among groups. Cows with contralateral accessory CL that underwent early regression had greater pregnancy loss (30%) than controls (10%), or cows with ipsilateral CL (3%) or contralateral CL with either later or no regression (12%). Cows with ipsilateral accessory CL had lower pregnancy loss than controls. In conclusion, elevating circulating P4 by the induction of accessory CL, particularly ipsilateral CL, increases P4 and reduces pregnancy loss. However, contralateral accessory CL that undergoes regression before day 33 of pregnancy has increased pregnancy loss, possibly due to an abrupt decrease in P4 at a pivotal period of pregnancy (days 26–33).

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Introduction

Dairy cows with high milk production have suboptimal circulating progesterone (P4) concentrations (Sartori *et al.* 2002, 2004) due to greater metabolism of steroid hormones, including P4 (Sangsritavong *et al.* 2002). Increased steroid metabolism in lactating cows occurs because (1) milk production is related to increased dry matter intake (Harrison *et al.* 1990); (2) dry matter intake is directly related ($R^2 = 0.93$) to increased liver blood flow (Ellis *et al.* 2016); and (3) liver blood flow is directly related ($R^2 = 0.85$) to increased steroid metabolism (Parr *et al.* 1993, Sangsritavong *et al.* 2002). Thus, cows with greater milk yield have decreased circulating P4 concentrations, even though they have a larger corpus luteum (CL) than heifers for example (Lopez *et al.* 2005).

It has been known for more than a century that the CL hormone, later identified as P4, is essential for the maintenance of pregnancy (Wiltbank *et al.* 2014) due to multiple effects on the pregnant uterus and uterine environment (Geisert *et al.* 1992, Forde *et al.* 2010, 2012). Although the absolute requirement for P4 during pregnancy is unequivocal, the question of whether increasing P4 concentrations after breeding will increase fertility is less definitive (Wiltbank *et al.* 2014, Yan *et al.* 2016, Lonergan & Sanchez 2020). Decreased circulating P4 concentration after timed-artificial insemination (TAI) was associated with reduced embryonic elongation (Beltman *et al.* 2009, Forde *et al.* 2011), interferon-tau production, and pregnancy per AI (P/AI) (Diskin *et al.* 2006, Wiltbank *et al.* 2006, Parr *et al.* 2012, Monteiro *et al.* 2014, Lonergan & Sanchez 2020); whereas, increased circulating P4 increased embryonic length (Mann *et al.* 2006, Carter *et al.* 2008, Clemente *et al.* 2009), interferon-tau production (Mann *et al.* 2006), and in some cases, increased P/AI (Santos *et al.* 2001, Nascimento *et al.* 2013*b*, Yan *et al.* 2016).

Based on the idea that circulating P4 concentration may be limiting fertility in dairy cows, studies have

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evaluated the effect of increasing circulating P4 after TAI on P/AI. Direct supplementation of P4 after TAI has been done by daily injections of P4 (Wiltbank *et al.* 1956, Johnson *et al.* 1958) or intravaginal treatment with P4 implant (Larson *et al.* 2007, Monteiro *et al.* 2014, 2015). Supplementation has also been done by indirect methods by increasing the size of the ovulatory follicle to produce larger CL (Randi *et al.* 2018), stimulating CL function by luteotrophic treatments such as hCG on day 2 (Sanchez *et al.* 2018) or by inducing accessory CL by treatment with GnRH or hCG on days 5–7 (Santos *et al.* 2001, Nascimento *et al.* 2013*b*, Baez *et al.* 2017). Treatment with GnRH or hCG on days 5–7 after breeding produced accessory CL in 70 to 90% of cows (Nascimento *et al.* 2013*b*, Baez *et al.* 2017) and increased circulating P4 (Nascimento *et al.* 2013*a*). A meta-analysis showed small (+3.0 to 3.5%) but significant effects of hCG treatment on P/AI (Nascimento *et al.* 2013*b*); however, benefits were only in primiparous cows (Santos *et al.* 2001, Nascimento *et al.* 2013*b*). Randomly, accessory CL after hCG treatment may be ipsilateral, or contralateral to pregnancy. The side of accessory CL had an effect on fertility, with multiparous cows having lower fertility than primiparous cows if they had contralateral but not ipsilateral accessory CL (Baez *et al.* 2017). Contralateral accessory CL was also more likely to regress during early pregnancy (<32 days) in multiparous than primiparous cows (Baez *et al.* 2017), although both groups had a similar high overall incidence of accessory CL regression (66.2%). Accessory CL regression during either earlier (days 19–23) or later (>45 days) pregnancy caused ~40% reduction in circulating P4 (Monteiro *et al.* 2021). Thus, ipsilateral CL may be more positive for fertility than contralateral accessory CL, particularly if accessory CL regression occurs during earlier pregnancy. Previous studies evaluated the effect of hCG or GnRH treatment on P/AI (Nascimento *et al.* 2013*b*) or pregnancy per embryo transfer (Niles *et al.* 2019, Garcia-Guerra *et al.* 2020), but previous studies have not evaluated if the occurrence or timing of contralateral accessory CL regression impacts fertility or pregnancy loss.

Thus, the main objective of this study was to evaluate the effect of induction of accessory CL, either contralateral or ipsilateral to pregnancy, on P/AI and pregnancy loss in lactating dairy cows. We hypothesized that induction of ipsilateral accessory CL would increase P/AI and reduce pregnancy loss; however, response to induction of contralateral accessory CL would depend on the timing of accessory CL regression. The first specific hypothesis was that cows with ipsilateral accessory CL would have greater circulating P4, more rapid embryonic development (increased mRNA for interferon-stimulated genes (ISGs), increased pregnancy-specific protein B (PSPB), and larger embryo on day 47 of pregnancy) compared to controls or cows with contralateral accessory CL regression. The second specific hypothesis was that cows with contralateral accessory CL regression during earlier pregnancy would have reduced P4 and PSPB concentrations and smaller embryos compared with cows without accessory CL regression. Finally, our third specific hypothesis was that cows with contralateral accessory CL regression, particularly prior to day 33 of pregnancy, would have greater pregnancy loss than cows without accessory CL regression.

Materials and methods

Animal procedures were performed in accordance with Guide for Care and Use of Agricultural Animals and were approved by Animal Care and Use Committee of College of Agriculture and Life Sciences, University of Wisconsin (Protocol A006314).

Cows, housing, diets, and body condition score

The study was conducted on a large commercial dairy farm in Wisconsin, milking 3200 cows. Lactating Holstein cows (n = 110; primiparous-377; multiparous-678) were housed in free-stall barns equipped with sprinklers and fans and used only at first AI. Cows averaged (mean ± S.D.) 80.0 ± 3.3 days in milk, yielding 43.2 ± 13.3 kg of milk/day, with body condition score (BCS) 2.85 ± 0.24 (Ferguson *et al.* 1994). Primiparous and multiparous cows were housed in different pens. Cows that received the same total mixed ration (TMR) were designed to meet or exceed nutrient requirements for lactating Holstein cows producing 50 kg milk/day with 3.5% fat and 3.1% true protein when dry matter intake was 24 kg/day (NRC 2001). Cows were fed twice and milked thrice daily.

Sample size, reproductive program, and experimental design

The sample size was calculated using POWER procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) using a one-tailed analysis to provide sufficient experimental units and to detect statistical significance ($\alpha = 0.05$; $\beta = 0.20$) when P/AI increased 9 percentage units (e.g. 40 vs 48%) with ipsilateral accessory CL compared to contralateral accessory CL. Approximately, 235 cows per treatment were deemed necessary, or a total of 708. Because of potential issues during a study done on a commercial dairy, a total of 1055 cows were assigned to treatments within parity, as primiparous or multiparous. Using Microsoft Excel version 2016, the list of cows that were inseminated by TAI was sorted by pen and number of the cow. After that, cows were assigned using the sequence of GnRH, GnRH, and control and cows were enrolled during 31 weeks.

All cows were enrolled in Presynch–Ovsynch program (Moreira *et al.* 2001). Briefly, cows received two i.m. treatments of cloprostenol (PGF, 500 µg, estroPLAN, Parnell) on days 45 ± 3 and 59 ± 3 postpartum. On day 71 ± 3 postpartum, all cows received Ovsynch program (Pursley *et al.* 1995) with GnRH treatment (100 µg, GONAbreed, Parnell), 7 days later PGF, and GnRH 56 h later. All cows received TAI 16 h after final GnRH of Ovsynch.

On day 5 after TAI, transrectal ultrasound evaluation of ovaries using 7.5-MHz, linear-array probe (Ibex Pro, EI Medical

Imaging, Loveland, CO) was performed on all cows ($n = 1055$) to determine the size and location of CL. Cows without CL ($n = 43$) or with CL on both ovaries ($n = 82$) were removed from the study. The remaining cows ($n = 930$) were blocked by parity and assigned, unequally, to one of two treatments: GnRH ($n = 641$; 100 µg GnRH on day 5 after TAI to induce accessory CL) or control ($n = 289$; no treatment). Uneven assignment of cows was designed to provide approximately even numbers of cows in three final groups for analysis: untreated, GnRH-treated with ipsilateral CL, and GnRH-treated with contralateral CL. Ovaries were again evaluated by ultrasound on day 12 after TAI to verify ovulation and on day 5 of GnRH treatment and determined the location of accessory CL. Cows without ovulation on day 5 GnRH ($n = 98$), with accessory CL on both ovaries ($n = 55$), or that were not found on day 12 ($n = 8$) were excluded from the experiment (Fig. 1). Remaining cows were evaluated by ultrasound on days 19, 26, 33, 47, and 61 to determine the presence of all CL and pregnancy (33, 47, and 61).

Analysis of progesterone

On days 5, 12, 19, 26, 33, 47, and 61, blood was collected by puncturing coccygeal vein or artery into evacuated tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Immediately after collection, tubes with blood were placed on ice and refrigerated until transported to laboratory for processing within 4–5 h after collection. Tubes were centrifuged at 1500 ***g*** for 15 min at 4°C for serum separation. Aliquots of serum were frozen at −20°C until assayed. Concentrations of P4 were analyzed in serum by RIA using commercial kit (ImmuChem, MP Diagnostics, Burlingame, CA). The sensitivity of the assay and the intraassay CV were 0.02 ng/mL and 1.9%, respectively.

RT-qPCR for interferon-stimulated genes

Blood was sampled by puncturing coccygeal vein or artery into evacuated tubes (Tempus Blood RNA tubes, Applied Biosystems) on day 19 (Fig. 2) from subset of 105 cows randomly selected, 40 control cows, 38 cows with ipsilateral accessory CL, and 27 cows with contralateral accessory CL. Samples were stored at −20°C until RNA extraction. Extraction of RNA and DNAse treatment was performed using a commercial kit (Tempus™, Spin RNA isolation kit, Cat. No. 4380204, Applied Biosystems) following manufacturer's instructions.

Isolated RNA was evaluated for concentration and purity using NanoDrop 2000 spectrophotometer (Thermo Scientific). A total of 250 ng of RNA was reverse-transcribed to cDNA using a commercial kit (iScript™ RT supermix for RT-qPCR, Cat. No. 1708841, BioRad) following manufacturer's instructions. Initial activation was at 60°C for 2 min, denaturation at 95°C for 10 min, and amplification for 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was evaluated in duplicate, and specificity for amplification was verified by melting curve analysis. Four genes were investigated (Table 1): two reference genes, beta-actin (*ACTB*) and ribosomal protein L19 (*RPL19*) and two target genes, interferon-stimulated gene 15-kDa protein (*ISG15*) and Myxovirus resistance gene 2 (*MX2*). The genes were selected based on Baez *et al.* (2017).

Pregnancy-specific protein B assay

Blood samples collected on day 26 (Fig. 2) were assayed for pregnancy-specific protein B (PSPB) using commercial assay validated as a pregnancy test in dairy cattle (BioPRYN®, BioTracking, LLC, Moscow, ID). Intraassay CV for low and moderate PSPB samples averaged 1.8 and 1.4%, respectively. Interassay CV between 12 plates for low and moderate samples was 3.7 and 3.9%, respectively. Receiver operating characteristic (ROC) curve analysis was performed to determine cut-off of circulating PSPB concentration on day 26 after TAI between pregnant and nonpregnant cows.

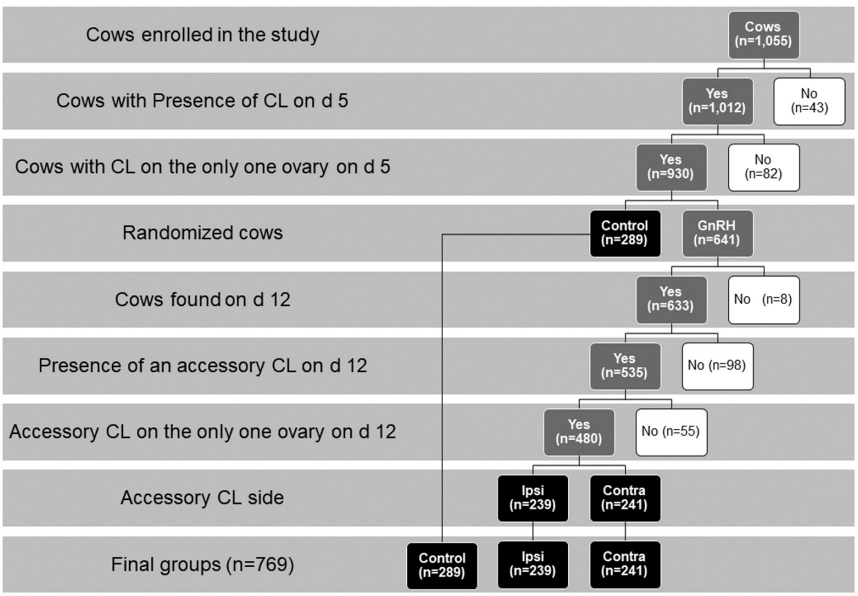

Figure 1 A flow diagram of the number of cows and the premises for them to remain in the study. Open rectangles, cows excluded from the study. Grey rectangles, cows that remained after each premise. Black rectangles, the number total of cows that remained according to the group.

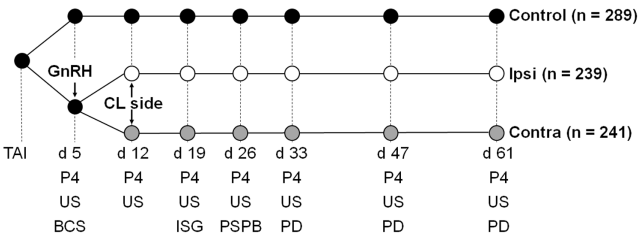

Figure 2 Diagram of activities during the study. Day 0 is the day of timed-artificial insemination (TAI). Control cows did not receive GnRH on day 5; Ipsi, cows that received GnRH (100 µg) on day 5 after TAI and ovulated ipsilateral to the pregnancy; Contra, cows that received GnRH on day 5 after TAI and ovulated contralateral to the pregnancy. A total of 1055 cows were enrolled in this study; however, cows without original CL on day 5 ($n = 43$), with original CL on both ovaries on day 5 ($n = 82$), without accessory CL on day 12 (GnRH treatment; $n = 98$), with an accessory CL on both ovaries (GnRH treatment; $n = 55$) or cows that were not found on day 12 ($n = 8$) were excluded from the analysis. On the designated days, ovaries were evaluated by ultrasound (US), blood samples were collected for PSPB or P4 analyses, and body condition score (BCS) or pregnancy diagnosis (PD) was performed.

Pregnancy diagnosis by ultrasound and calculation of reproductive outcomes

Pregnancy was diagnosed by ultrasonography on day 33 after TAI. The presence of an amniotic vesicle containing an embryo with a heartbeat was a determinant of pregnancy. Pregnant cows on day 33 were reexamined for pregnancy by transrectal palpation on days 47 and 61. The P/AI was calculated by dividing the number of cows diagnosed as pregnant by the number of cows receiving TAI. The proportion of pregnancy loss was calculated as number of cows that lost pregnancy between days 26–33, 33–47, and 47–61 divided by the number of cows diagnosed pregnant.

Embryo and amniotic vesicle size measurements

Cows diagnosed pregnant on day 33 were reexamined by transrectal ultrasonography using a portable ultrasound with 7.5 MHz linear-array transducer (Ibex Pro) on day 47 to determine the volume of amniotic vesicle and fetus, similar to previous studies (Bertolini *et al.* 2002, Garcia-Guerra *et al.* 2020) with some modifications. Each video was recorded for 16 s. Later, videos were analyzed frame by frame by two independent technicians who were blind to treatment. Optimal position and orientation of conceptus were determined to measure embryo crown-rump length and widest longitudinal (LD) and transverse (TD) diameters of the amniotic vesicle. Videos were analyzed using open-source image processing software (ImageJ, NIH; http://rsb.info.nih.gov/ij/index.html), mean and CV from two measurements were calculated for each animal, and CV >20% were reanalyzed by a third evaluator. Amniotic vesicle volume (V) was calculated by using the formula, $V = 4/3 x \pi x (R)^3$. All videos with inaccurate visualization of amniotic vesicle or fetus were eliminated from the analysis.

Statistical analysis

Categorical data, such as P/AI and pregnancy loss, were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) fitting a binary distribution. Models included fixed effects of treatment, parity, and interactions between treatment and parity. Kenward–Roger method was used to calculate denominator degrees of freedom for F tests. Model fitting was evaluated using fit statistics. Estimates were back-transformed using ILINK function of SAS to generate adjusted proportions.

The concentration of circulating P4 was analyzed using GLIMMIX procedure of SAS with models fitting Gaussian distribution. Data were tested for normality of residuals after model fitting using the UNIVARIATE procedure of SAS (SAS/STAT; SAS Institute Inc.) according to the Shapiro–Wilk test. The circulating progesterone concentration did not show normality of residuals; therefore, data were transformed to natural logarithm before statistical analysis. After analysis, the LSM and SE were back-transformed to the original scale. Models included fixed effects of treatment, day of measurement, parity, and interactions between treatment and day, and random effects of cows. As time intervals between measurements were unequal, spatial power covariance structure was used. Model fitting was evaluated using fit statistics. The means were partitioned using the SLICE command in SAS.

The average circulating P4 concentration on days 26 and 33, circulating PSPB concentration, volume of the amniotic vesicle, and crown-rump length were analyzed using the GLIMMIX procedure of SAS with models fitting a Gaussian distribution. All data were tested for normality of residuals and homogeneity of variance after model fitting using the UNIVARIATE procedure of SAS according to the Shapiro–Wilk test. Models included fixed effects of treatment, parity, and interactions between treatment and parity, and random effect of cow. When the F-test for an interaction was significant, means were partitioned using the SLICE command in SAS.

Quantitative PCR data are presented using nonpregnant cows from the control group to reference the relative expression of mRNA abundance and set to a relative value of 1. Delta cycle threshold (ΔC_T) values for each target gene

Table 1 Gene, primer orientation, primer sequence (5′–3′), and National Center for Biotechnology Information (NCBI) accession number and sequence for primers used in RT-qPCR assays.

Gene	Primer sequences (5′–3′)		NCBI sequence
	Forward	Reverse	
ACTB	CTGGACTTCGAGCAGGAGAT	GATGTCGACGTCACACTTC	AY141970
ISG15	GGTATCCGAGCTGAAGCAGTT	ACCTCCCTGCTGTCAAGGT	NM_174366
RPL19	ATTGACCGCCACATGTATCA	GCGTGCTTCCTTGGTCTTAG	NM_001040516
MX2	CTTCAGAGACGCCTCAGTCG	TGAAGCAGCCAGGAATAGTG	NM_173941

were obtained after normalization of C_T value of gene with the geometric mean of C_T values from two reference genes according to Vandesompele *et al.* (2002). Data were analyzed using ΔC_T for day 19 with GLIMMIX procedure of SAS fitting model with fixed effects of treatment, pregnancy on day 26, and interaction between treatment and pregnancy on day 26, and random effect of cow. Relative expression values were obtained by raising PCR amplification efficiency (E=2) to power $\Delta\Delta C_T$ (Yuan *et al.* 2006).

An ROC curve was generated using LOGISTIC procedure of SAS (SAS/STAT; SAS Institute Inc.) to determine the optimal concentration of PSPB on day 26 resulting in the greatest sensitivity and specificity for pregnancy on day 33. For survival analysis, only data from contralateral accessory CL cows that underwent luteolysis of the accessory CL were used. This analysis was performed using the LIFETEST procedure of SAS (SAS/STAT; SAS Institute Inc.). Based on our previous hypothesis that GnRH after TAI would be superior to control, to test the effect of group on P/AI and pregnancy loss, a one-tailed test was used. Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ a tendency.

Results

Accessory CL model information

Cows enrolled on day 5 were 930, with 289 control cows and 641 cows treated with GnRH. A total of 84.5% (535/633) of GnRH cows ovulated to treatment. Overall, there was greater ($P < 0.01$) proportion of cows that ovulated on the right than left ovary after the breeding GnRH (59.7 (459/769) vs 40.3% (310/769), respectively) and after day 5 GnRH ($P < 0.01$; right-57.9 (220/380) left-42.1% (160/380)). There was no difference ($P = 0.93$) in the percentage of cows that ovulated ipsilateral or contralateral to previous CL after GnRH on day 5 after TAI (49.8 (239/480) vs 50.2% (241/480), respectively).

Only pregnant cows on day 26 had undergone CL confirmation and blood sampling after that time and up to day 61 after TAI. Thus, a total of 164, 130, and 148 cows were pregnant on day 26 for control, ipsilateral, and contralateral accessory CL groups, respectively. Only pregnant cows that were treated with GnRH on day 5 after TAI could be analyzed for accessory CL regression. There was a difference ($P < 0.01$) in the proportion of ipsilateral and contralateral accessory CL that regressed with no ipsilateral accessory CL regression in cows (0/130) and 52.7% (78/148) of cows with contralateral accessory CL having accessory CL regression. Proportion of cows with contralateral accessory CL regression during earlier pregnancy (<day 33; 30.8% (24/78)) was less ($P < 0.01$) than during later pregnancy (days 33–61; 69.2% (54/78)). Based on survival analysis, the timing of accessory CL regression differed by parity, with multiparous cows having earlier accessory CL regression ($P = 0.03$) than primiparous cows (Fig. 3).

Circulating progesterone

Overall, treatment ($P < 0.01$), day of pregnancy ($P < 0.01$), and interaction of treatment and day of pregnancy ($P < 0.01$) affected circulating P4 concentrations. From days 12 to 61 after TAI, both accessory CL groups had greater ($P < 0.01$) circulating P4 concentrations than controls. However, on day 61, there was also a detectable difference between ipsilateral and contralateral accessory CL cows with greater ($P = 0.03$) circulating P4 in ipsilateral than contralateral cows (Fig. 4).

Another analysis of circulating P4 concentration was performed using only cows with contralateral accessory CL (Fig. 5). In this analysis, there was an effect of treatment ($P < 0.01$), day of pregnancy ($P < 0.01$), and interaction of treatment by day ($P = 0.01$). Cows without contralateral CL regression had greater ($P < 0.01$) circulating P4 than cows with accessory CL regression either during earlier or later (second month) pregnancy. Also, cows with accessory CL regression during the second month of pregnancy had greater ($P < 0.01$) circulating P4 compared with cows with earlier accessory CL regression. In contrast, cows with later contralateral accessory CL regression had lower ($P < 0.01$) circulating P4 concentrations on days 47 and 61 compared with cows without contralateral accessory CL regression.

Analysis of average circulating P4 concentration on days 26 and 33 was performed to compare four groups (control vs ipsilateral vs contralateral earlier regression vs contralateral later or no regression of accessory CL) and parity (Fig. 6A). There was the effect of group ($P < 0.01$) and parity ($P = 0.03$). The group of cows that underwent

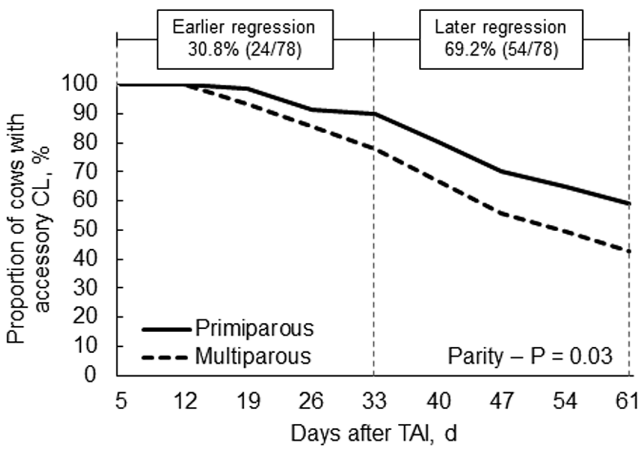

Figure 3 Kaplan–Meier survival curves comparing parity for days of gestation when regression of the contralateral accessory CL occurred. Cows received GnRH on day 5 after TAI, ovulated contralateral to the pregnancy, with detection of a contralateral accessory CL on day 12. Multiparous cows regressed the contralateral accessory CL earlier ($P=0.03$) than primiparous cows. Overall, 52.7% (78/148) of cows with contralateral accessory CL underwent luteolysis with no difference between parities (60.8 ± 6.5% vs 50.3 ± 7.1% ($P=0.31$), for multiparous and primiparous, respectively).

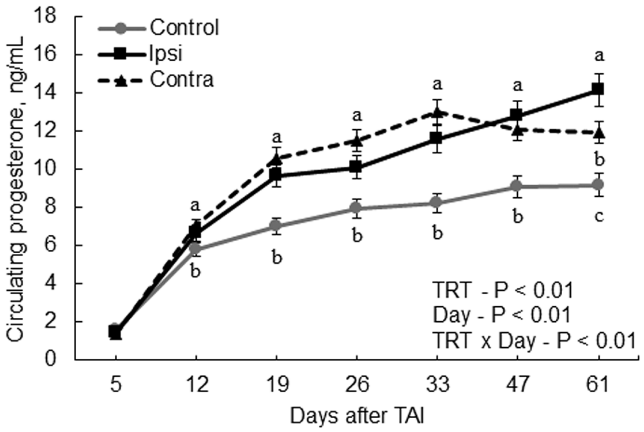

Figure 4 Circulating P4 concentrations until day 61 of pregnancy based on treatment group. Control ($n = 29$) cows did not receive GnRH on day 5 after TAI; Ipsi ($n = 30$) cows received GnRH on day 5 after TAI and ovulated ipsilateral to the pregnancy; Contra ($n = 47$) cows received GnRH on day 5 after TAI and ovulated contralateral to the pregnancy. The mean P4 concentrations for groups were 6.2 ± 0.2[b], 7.9 ± 0.3[a], and 8.1 ± 0.2[a] for control, ipsi, and contra, respectively; [c]difference detected between all three treatmnets (day 61).

earlier luteolysis had lower circulating P4 concentration than the other groups. Thereby, control had lower circulating P4 than ipsilateral and contralateral later or no regression. No difference in average circulating P4 concentration on days 26 and 33 was detected between ipsilateral and contralateral later or no regression. In the same analysis, primiparous had greater ($P = 0.03$) average circulating P4 concentration on days 26 and 33 than multiparous (10.6 ± 0.6 vs 9.0 ± 0.4 ng/mL).

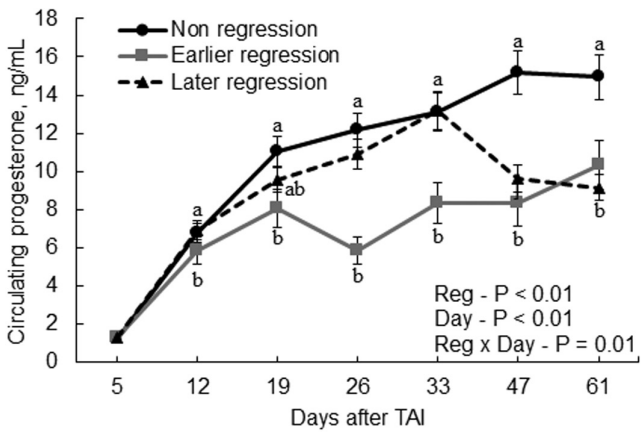

Figure 5 Circulating P4 concentration for the cows with contralateral accessory CL based on occurrence or timing of CL regression. This analysis included only cows that received GnRH on day 5 after TAI, ovulated contralateral to the pregnancy, and were pregnant on day 61. Non-regression ($n = 19$), cows that did not regress the accessory CL by day 61; earlier regression ($n = 7$), cows that regressed the accessory CL by day 33; late regression ($n = 21$), cows that regressed the accessory CL between days 33 and 61 after TAI. Mean P4 concentration between groups was 8.6 ± 0.3[a], 5.9 ± 0.4[c], and 7.3 ± 0.3[b] for nonregression, earlier regression, and late regression, respectively.

Pregnancy/artificial insemination and pregnancy loss

As shown in Table 2, P/AI on day 26 after TAI did not differ ($P = 0.12$) among groups. In contrast, the side of accessory CL (ipsilateral vs contralateral) tended to differ for P/AI on day 26 of pregnancy. Cows with contralateral accessory CL tended to have greater ($P = 0.10$) P/AI on day 26 than ipsilateral cows. The P/AI on days 33, 47, and 61 after TAI did not differ among groups and there was no effect of the side of accessory CL (Table 2).

Pregnancy loss between days 26 and 33 after TAI tended to differ ($P = 0.06$) among groups. In addition, the side of accessory CL had an effect on pregnancy loss between days 26 and 33. Cows with contralateral accessory CL had greater ($P = 0.02$) pregnancy loss than cows with ipsilateral accessory CL (Table 2). Interestingly, there was an effect of treatment ($P = 0.02$) and side of accessory CL ($P = 0.01$) even when cows with contralateral accessory CL regression were excluded from the analysis. Pregnancy loss between days 26 and 33 was 7.5 ± 2.1[B] (12/164), 3.1 ± 1.5[bC] (4/130), and 13.4 ± 0.3%[aA] (10/70), for control, ipsilateral accessory CL, and contralateral accessory CL without regression, respectively. Pregnancy loss from days 33 to 47 and between days 47 and 61 did not differ among groups ($P = 0.43$ and $P = 0.26$, respectively), and there was no effect of the side of accessory CL ($P = 0.94$ and $P = 0.50$, respectively). Overall pregnancy loss from days 26 to 61 ($P = 0.06$) was 13.7 ± 2.9[B] (24/164), 6.6 ± 2.3[A] (9/130), and 14.0 ± 3.0%[B] (22/148), for control, ipsilateral accessory CL, and contralateral accessory CL, respectively. In addition, there was an effect of the side of accessory CL on overall pregnancy loss. Cows with a contralateral accessory CL had greater ($P = 0.03$) overall pregnancy loss than cows with ipsilateral accessory CL (Table 2). When only cows with contralateral accessory CL that did not regress were included in the analysis, there was a tendency for an effect of group ($P = 0.06$) and side of accessory CL ($P = 0.06$) on overall pregnancy loss. Pregnancy loss from days 26 to 61 was 13.7 ± 2.9[A] (24/164), 6.6 ± 2.3[B] (9/130), and 15.4 ± 4.5%[A] (11/70), for control, ipsilateral, and contralateral accessory CL.

Another analysis was performed to evaluate the effect of groups in primiparous and multiparous cows on pregnancy loss from days 26 to 33 and from days 26 to 61. There was no effect of group on pregnancy loss in either period for primiparous cows. However, multiparous cows with contralateral accessory CL had greater ($P = 0.02$) pregnancy loss from days 26 to 33 compared with multiparous cows with ipsilateral accessory CL (Fig. 7A). In addition, multiparous cows with contralateral accessory CL tended ($P = 0.06$) to have greater pregnancy loss than control multiparous cows from days 26 to 33. For overall pregnancy loss (Fig. 7B), from days 26 to 61, multiparous cows with contralateral accessory CL had

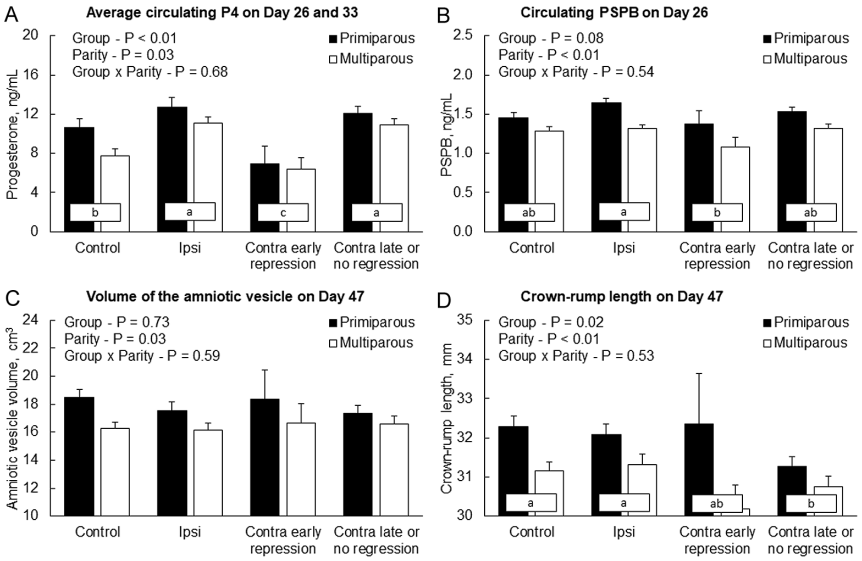

Figure 6 Average circulating progesterone (P4) on days 26 and 33 of pregnancy (A; control – $n = 29$, ipsi – $n = 30$, contra earlier regression – $n = 7$, and contra later or no regression – $n = 40$), circulating pregnancy specific protein B (PSPB) concentrations on day 26 (B; control – $n = 164$, ipsi – $n = 130$, contra earlier regression – $n = 24$, and contra later or no regression – $n = 124$) and fetal measurements (C and D; control – $n = 112$, ipsi – $n = 95$, contra earlier regression – $n = 11$, and contra later or no regression – $n = 91$) on day 47 according to groups. Control, cows that did not receive GnRH on day 5 after TAI; Ipsi, cows that received GnRH (100 µg) on day 5 after TAI and ovulated ipsilateral to the pregnancy; Contra earlier regression, cows that received GnRH on day 5 after TAI, ovulated an accessory CL contralateral to the pregnancy, and the accessory CL underwent luteolysis by day 33 of pregnancy; Contra later or no regression, cows that received GnRH on day 5 after TAI, ovulated an accessory CL contralateral to the pregnancy, and the accessory CL underwent luteolysis between days 33 and 61 of pregnancy or did not undergo luteolysis by day 61 of pregnancy. Different lowercase letters mean $P \leq 0.05$ among groups.

greater ($P = 0.03$) pregnancy loss than multiparous cows with ipsilateral accessory CL. A tendency ($P = 0.07$) for an effect of group was observed. Cows with ipsilateral accessory CL tended to have lower pregnancy loss than control cows. No difference was observed between control and contralateral accessory CL cows.

There was no difference in pregnancy loss from days 33 to 61 when only cows without contralateral accessory CL were compared with cows with later accessory CL regression (after day 33). Therefore, subsequent analyses used data from cows with contralateral accessory CL that had no regression combined with cows that had later contralateral accessory CL regression for comparison with cows with earlier (<day 33) contralateral accessory CL regression, ipsilateral accessory CL, and controls (Fig. 8). Cows with earlier contralateral accessory CL regression had greater pregnancy loss from days 26 to 33 ($P = 0.03$) and from days 26 to 61 ($P < 0.01$) compared with control, ipsilateral, and cows with contralateral accessory CL without regression or with later regression, combined. In addition, cows with ipsilateral CL had less pregnancy loss from days 26 to 61 than controls (Fig. 8).

Table 2 Pregnancy per AI and pregnancy loss between days 26 and 61 for the three groups.

	Treatment[1]			*P*-value	
	Control	Ipsi	Contra	TRT	Ipsi × Contra
Pregnancy/AI, % (*n*/*n*)					
Day 26[2]	57.3 ± 3.0 (164/289)	55.2 ± 3.3 (130/239)	62.8 ± 3.2 (148/241)	0.12	0.10
Day 33[3]	52.8 ± 3.0 (152/289)	53.4 ± 3.3 (126/239)	56.6 ± 3.3 (133/241)	0.34	0.49
Day 47[3]	49.6 ± 3.0 (141/289)	51.5 ± 3.3 (121/239)	54.7 ± 3.3 (128/241)	0.27	0.50
Day 61[3]	49.4 ± 3.0 (140/289)	51.5 ± 3.3 (121/239)	53.8 ± 3.3 (126/241)	0.31	0.63
Pregnancy loss, % (*n*/*n*)					
Days 26 to 33	7.5 ± 2.1^{A} (12/164)	3.1 ± 1.5^{B} (4/130)	9.6 ± 2.5^{A} (15/148)	0.06	0.02
Days 33 to 47	4.4 ± 2.2 (11/152)	3.2 ± 1.7 (5/126)	3.0 ± 1.6 (5/133)	0.43	0.47
Days 47 to 61	0.7 (1/141)	0.0 (0/121)	1.6 (2/128)	0.26	0.25
Days 26 to 61	13.7 ± 2.9^{A} (24/164)	6.6 ± 2.3^{B} (9/130)	14.0 ± 3.0^{A} (22/148)	0.06	0.03

$^{A, B}0.10 \geq P > 0.05$. [1]Control – cows did not receive GnRH on day 5 after TAI; Ipsi – cows received GnRH on day 5 after TAI and ovulated ipsilateral to the pregnancy; Contra – cows received GnRH on day 5 after TAI and ovulated contralateral to the pregnancy. [2]Pregnancy diagnosis was performed by circulating PSPB (pregnancy-specific protein B) concentration on day 26. [3]Pregnancy diagnosis was performed by ultrasound evaluation.

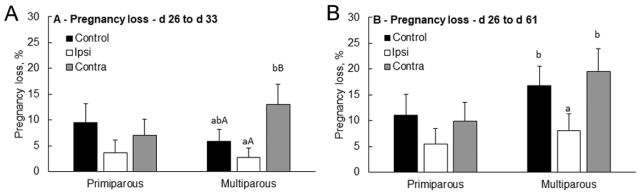

Figure 7 Pregnancy loss between days 26 and 33 (A) or between days 26 and 61 (B) based on groups and parity. Control ($n = 63$ and $n = 101$, for primiparous and multiparous, respectively), cows that did not receive GnRH on day 5; Ipsi ($n = 56$ and $n = 74$, for primiparous and multiparous, respectively), cows that received GnRH (100 µg) on day 5 after TAI and ovulated ipsilateral to the pregnancy; Contra ($n = 71$ and $n = 77$, for primiparous and multiparous, respectively), cows that received GnRH on day 5 after TAI and ovulated contralateral to the pregnancy. Different lowercase and uppercase letters mean $P \leq 0.05$ and $0.10 \geq P > 0.05$, respectively.

Embryo development

Interferon-stimulated genes expression in leukocytes

On day 19 after TAI, mRNA concentrations in PBMC for ISG15 ($P = 0.63$) and MX2 ($P = 0.51$) were not influenced by group. Regardless of group, cows that were pregnant on day 26 had greater ($P < 0.01$) mRNA concentrations for ISG15 and MX2 on day 19 compared with nonpregnant cows (Fig. 9). No interaction was observed between groups and pregnancy status (day 26) for mRNA concentrations for ISG15 ($P = 0.72$) or MX2 ($P = 0.21$).

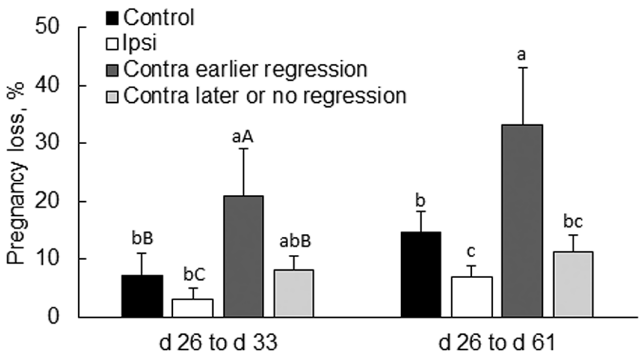

Figure 8 Pregnancy loss between days 26 and 33 and between days 26 and 61 according to groups and timing of accessory CL regression. Control ($n = 164$), cows that did not receive GnRH on day 5; Ipsi ($n = 130$), cows that received GnRH (100 µg) on day 5 after TAI and ovulated ipsilateral to the pregnancy; Contra earlier regression ($n = 24$), cows that received GnRH (100 µg) on day 5 after TAI, ovulated an accessory CL contralateral to the pregnancy, and the accessory CL underwent luteolysis by day 33 of pregnancy; contra later regression ($n = 124$), cows that received GnRH (100 µg) on day 5 after TAI, ovulated an accessory CL contralateral to the pregnancy, and the accessory CL underwent luteolysis between days 33 and 61 of pregnancy; no regression, cows that received GnRH (100 µg) on day 5 after TAI, ovulated an accessory CL contralateral to the pregnancy, and the accessory CL had not undergone luteolysis by day 61 of pregnancy. Different lowercase and uppercase letters mean $P \leq 0.05$ and $0.10 \geq P > 0.05$, respectively.

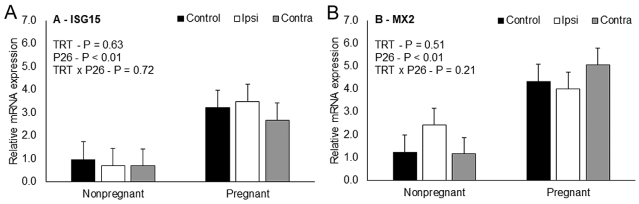

Figure 9 Relative abundance of mRNA for IFN-stimulated genes (ISG), interferon-stimulated gene 15-kDa protein (ISG15; A), and Myxovirus resistance gene 2 (MX2, B) expression in leukocytes isolated on day 19 after FTAI from cows that did not receive GnRH on day 5 (control, 18 nonpregnant and 22 pregnant on day 26), received GnRH (100 µg of gonadorelin acetate, GONAbreed, Parnell, Alexandria, New South Wales, Australia) on day 5 after FTAI and ovulated an accessory corpus luteum ipsilateral (ipsi, 17 nonpregnant and 21 pregnant on day 26) or contralateral (contra, 14 nonpregnant and 13 pregnant on day 26) to the pregnancy. Control nonpregnant cows were the reference group for depicting relative mRNA expression.

Circulating PSPB

Overall, on day 26 after TAI, circulating PSPB concentrations were not influenced by treatment group ($P = 0.43$); or side of accessory CL ($P = 0.36$; 1.37 ± 0.04, 1.44 ± 0.04, and 1.38 ± 0.04 ng/mL, for control, ipsilateral, and contralateral, respectively).

In the analysis comparing circulating PSPB concentration on day 26 among four groups (control, ipsilateral, contralateral earlier regression, and contralateral later or no regression), there was a tendency ($P = 0.08$) for group effect. Ipsilateral tended to have more circulating PSPB on day 26 than contralateral earlier regression. Control and contralateral later regression or no regression did not differ from ipsilateral and contralateral earlier regression. There was the effect

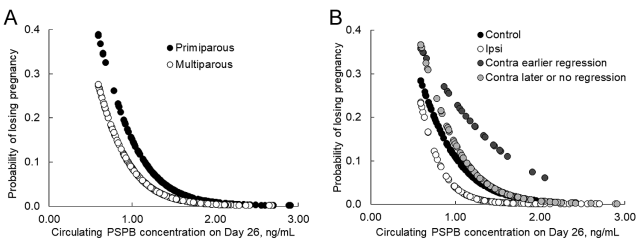

Figure 10 Logistic regression analysis of the relationship between circulating pregnancy-specific protein B (PSPB) concentration at day 26 of pregnancy with pregnancy loss between days 26 and 33 according to parity (A; primiparous ($n = 190$; $P < 0.01$) and multiparous ($n = 250$; $P < 0.01$) and groups (B). Control ($n = 164$; $P < 0.01$), cows that did not receive GnRH on day 5 after TAI; Ipsi ($n = 130$; $P = 0.02$), cows that received GnRH (100 µg) on day 5 after TAI and ovulated ipsilateral to the pregnancy; Contra earlier regression ($n = 24$; $P = 0.31$), cows that received GnRH on day 5 after TAI, ovulated an accessory CL contralateral to the pregnancy, and the accessory CL underwent luteolysis by day 33 of pregnancy; contra later or no regression ($n = 124$; $P < 0.01$), cows that received GnRH on day 5 after TAI, ovulated an accessory CL contralateral to the pregnancy, and the accessory CL underwent luteolysis between days 33 and 61 of pregnancy or had not undergone luteolysis by day 61 of pregnancy.

of parity on circulating PSPB on day 26 with primiparous cows having greater ($P < 0.01$) PSPB than multiparous (1.50 ± 0.05 vs 1.25 ± 0.04, respectively; Fig. 6B).

Logistic regression analysis demonstrated an overall effect in primiparous ($P < 0.01$) and multiparous ($P < 0.01$) of circulating PSPB concentration on day 26 on pregnancy loss, with increased circulating PSPB associated with reduced pregnancy loss from days 26 to 33 (Fig. 10A). Logistic regression of the four groups (Fig. 10B) demonstrated decreased pregnancy loss from days 26 to 33 as PSPB increased in controls ($P < 0.01$), ipsilateral ($P = 0.01$), and contralateral later or no regression accessory CL ($P < 0.01$) groups. However, no effect ($P = 0.31$) was detected in the contralateral earlier regression accessory CL group.

Embryo measurements

On day 47 after TAI, the volume of the amniotic vesicle was not influenced by group ($P = 0.78$; 17.2 ± 0.36, 17.0 ± 0.37, and 16.9 ± 0.39 cm^3, for controls, ipsilateral, and contralateral, respectively) or side of accessory CL ($P = 0.36$). In a separate analysis evaluating only cows with earlier contralateral accessory CL regression, there was no effect of group on the volume of the amniotic vesicle (Fig. 6C). In contrast, in this analysis, there was a difference between primiparous and multiparous cows. Primiparous cows had a greater volume of amniotic vesicle than multiparous cows ($P = 0.03$; 17.9 ± 0.58 vs 16.4 ± 0.40 cm^3, respectively; Fig. 6C).

In contrast, crown-rump length on day 47 was affected by group ($P < 0.01$). Cows with contralateral accessory CL had smaller ($P < 0.01$) crown-rump length than cows with ipsilateral accessory CL or control cows (31.2 ± 0.2^b, 32.0 ± 0.2^a, and 32.0 ± 0.2^a mm, respectively). In a separate analysis evaluating only cows with earlier contralateral accessory CL regression, a difference ($P = 0.02$) was detected between treatment groups in crown-rump length on day 47 (Fig. 6D). In the same analysis, there was an effect of parity. Primiparous had greater crown-rump length than multiparous ($P < 0.01$; 32.0 ± 0.34 vs 30.9 ± 0.19, respectively; Fig. 6D).

Discussion

Many researchers have attempted to improve reproductive outcomes by increasing circulating P4 concentrations after breeding. One common method for increasing P4 is by induction of an accessory CL (Santos *et al.* 2001, Nascimento *et al.* 2013*b*, Baez *et al.* 2017); however, substantial unexplained variability in the fertility response to induction of accessory CL has been observed. For example, in a large study on six farms, treatment with hCG on day 5 after TAI increased P/AI by 10% in primiparous cows compared with primiparous enrolled in the control group, but had no effect on multiparous cows (Nascimento *et al.* 2013*b*). Although the present study does not explain all of the variability in responses, the results provide evidence that the side of ovulation as well as the occurrence and timing of regression of the induced accessory CL are key factors in determining whether there is a positive reproductive outcome from induction of an accessory CL. As expected, treatment on day 5 after TAI with GnRH caused ovulation of a follicle and production of an accessory CL in most cows. Unfortunately, cows that did not ovulate by the GnRH needed to be excluded from the study due to the absence of an accessory CL. Since similar control cows do not exist and, therefore, cannot be excluded from the analysis, the reader needs to be aware of this potential bias in our final P/AI and pregnancy loss results. Notwithstanding, the accessory CL increased circulating P4 by 40% (8.3 vs 11.6 ng/mL, for control and ipsilateral accessory CL on day 33, respectively), compared with control cows; however, if this accessory CL subsequently underwent regression, the positive effect of GnRH treatment on circulating P4 concentrations in pregnant cows was eliminated. Only cows with accessory CL that were contralateral to the pregnancy had accessory CL regression (~52.7%) consistent with the idea that local utero-ovarian communication is involved in both luteolysis and maintenance of the CL during pregnancy. The ~40% decrease in circulating P4 after regression of contralateral accessory CL is similar to what is being reported in our companion paper (Monteiro *et al.* 2021), which also describes a rapid decrease in circulating P4 after lysis of the accessory CL (during ~24 h). Similar to Baez *et al.* (2017) and the companion manuscript (Monteiro *et al.* 2021), the present study found two pivotal periods when the contralateral accessory CL underwent regression; one during the first month of pregnancy (30.8% of cows had earlier accessory CL regression) and the other during the second month (69.2% of cows had later accessory CL regression) of pregnancy. The timing of accessory CL regression not only had effects on circulating P4 concentrations but also had effects on pregnancy loss. Thus, this study provides the first valid analysis of the effects of the side of accessory CL and the occurrence and timing of accessory CL regression on reproductive outcomes in lactating dairy cows. Two limitations that are inherent in the experimental design and that should be considered by readers of the manuscript are that outcomes could have been unequally affected by the response to GnRH and by parity distribution within final experimental groups that were analyzed.

Our central hypothesis was that induction of an accessory CL, particularly ipsilateral to the original CL, would increase circulating P4 and improve reproductive outcomes. Although previous studies have evaluated the effects of induction of accessory CL on P/AI (Santos *et al.* 2001, Howard *et al.* 2006, Nascimento *et al.* 2013*b*), only one study assessed the side of ovulation and P/AI. That study, however, did not have a control group

without GnRH treatment (Baez *et al.* 2017) making it problematic for determining the effect of the side of accessory CL induction on fertility. In this study, the increase in circulating P4 after induction of accessory CL was similar for contralateral (51.0%) and ipsilateral (37.2%) accessory CL on day 19 of pregnancy. Although there was no detectable effect of the side of accessory CL on P/AI, there was an effect on pregnancy loss. This study evaluated losses that occurred during 35 days of pregnancy (days 26–61; 5 weeks) and observed more than half of losses in the first week of evaluation (days 26–33; 56.4%). Having ipsilateral accessory CL reduced losses to 3.1% compared to 7.5% in controls. Cows with contralateral accessory CL had similar pregnancy loss during this earlier pregnancy period (9.6%) as controls with more than three-fold greater losses compared to cows with ipsilateral accessory CL. During the entire period of evaluation (days 26–61), pregnancy loss for cows with ipsilateral accessory CL remained much lower than either cows with contralateral accessory CL or controls. A previous study (Cerbito *et al.* 1994*b*) demonstrated differences in P4 concentration during the luteal phase between uterine horn tissues ipsilateral vs contralateral to CL. Thus, our central hypothesis was partially supported as cows with ipsilateral accessory CL had reduced pregnancy loss, possibly due to effects of increasing circulating P4 and possibly effects of local (ipsilateral) P4 concentrations.

An important observation in explaining the lack of improvement in cows with contralateral accessory CL was provided by evaluating cows with earlier regression of contralateral CL, before day 33 of pregnancy, compared with cows having either later or no regression of contralateral CL. Cows with earlier contralateral CL regression had reduced circulating P4, particularly during the first pivotal period of pregnancy loss in this study, days 26–33. Average circulating P4 on days 26–33 was 6.6 ng/mL in cows with earlier contralateral CL regression, lower ($P = 0.04$) than controls (9.2 ng/mL) and lower than cows without earlier contralateral CL regression (11.5 ng/mL, $P < 0.01$) or cows with ipsilateral accessory CL (11.9 ng/mL; $P < 0.01$). In addition, cows with earlier contralateral CL regression had lower circulating PSPB concentration than cows with ipsilateral accessory CL. This study and other studies (Gabor *et al.* 2016, Garcia-Guerra *et al.* 2020) have associated lower circulating PSPB during the first month of pregnancy with increased pregnancy loss. Pregnancy loss for cows with earlier contralateral CL regression was over 20% from days 26 to 33, significantly greater than controls or cows with ipsilateral accessory CL. In addition, pregnancy loss during the entire period was 33.3% for cows with earlier accessory CL regression, different from controls (14.6%), cows with ipsilateral accessory CL (6.9%), as well as cows with contralateral accessory CL that did not regress before day 33 of pregnancy (11.3%). Cows with contralateral accessory CL that did not have earlier regression were intermediate between controls and cows with ipsilateral accessory CL and not significantly different from either of these groups. Thus, the major reason that cows with contralateral accessory CL did not have an improvement in pregnancy loss, in spite of increased circulating P4, was the dramatic increase in pregnancy loss in cows with earlier contralateral CL regression. Some differences in concentration of P4 (Cerbito *et al.* 1994*b*), prostaglandin E2 (Cerbito *et al.* 1994*a*), and oxytocin (Cerbito *et al.* 1997) between uterine horn tissues in relation to CL side during luteal phase have been described in non-pregnant cows. The ipsilateral uterine horn to CL had greater P4 concentration, prostaglandin E2, and oxytocin than contralateral horn. In addition, in non-pregnant cows, concentrations of oxytocin and oxytocin receptors in uterine tissue were greater in ipsilateral than contralateral horn. However, in early pregnant cows, there was the opposite effect. Oxytocin and oxytocin receptors were lower in ipsilateral than contralateral horn (Cerbito *et al.* 1997). Whether local P4 effects were responsible for improved pregnancy maintenance by ipsilateral accessory CL compared to contralateral accessory CL that did not have early regression remains an intriguing and researchable hypothesis.

Parity of lactating dairy cows has been an important determinant of reproductive outcomes. In the present study, although there was no effect of parity in the proportion of cows that regressed the accessory CL, multiparous cows had CL regression earlier than primiparous. Some factors might explain differences in results between parity. Differences in the size of uterus (Baez *et al.* 2016), ISG expression on day 19 of pregnancy (Ribeiro *et al.* 2014), circulating PAG (Mercadante *et al.* 2016) or PSPB (Toledo *et al.* 2017) concentrations, and volume of the amniotic vesicle (Toledo *et al.* 2017) between primiparous and multiparous cows have been described by previous studies. Baez *et al.* (2016) reported that primiparous cows have a smaller uterine horn diameter and length than multiparous. As mentioned previously, IFN secreted by the elongating embryo during days 17–25 of pregnancy prevents luteolysis. Indirectly, ISG expression in PBMC may reflect the amount of embryonic ISG. Ribeiro *et al.* (2014) reported greater ISG15 expression in primiparous compared with multiparous cows. Similarly, primiparous had greater circulating PAG or PSPB concentration than multiparous cows (Mercadante *et al.* 2016, Toledo *et al.* 2017). Thus, smaller uterine size and earlier/greater embryo elongation may clarify why primiparous cows had later contralateral accessory CL regression than multiparous. Although there was no difference in ISG expression on day 19 of pregnancy between primiparous and multiparous, there were differences in other embryo development markers, such as greater circulating PSPB concentration on day 26 and larger amniotic vesicle volume and crow-rump length on day 47 in primiparous cows. Greater circulating P4 concentration from days 26

to 33 likely contributed to faster embryo development in primiparous cows. Thus, primiparous cows tended ($P = 0.09$) to have lower pregnancy loss than multiparous cows from days 26 to 61 of pregnancy.

In summary, it has been confirmed that accessory CL undergoes regression only when the CL is on the contralateral side from the pregnancy. As shown in Monteiro *et al*. (2021; companion paper), there are two distinct pivotal periods for contralateral accessory CL regression. Cows with regression of accessory CL experienced reduced circulating P4 but no difference in P/AI. However, earlier accessory CL regression increased pregnancy loss particularly during the choriovitelline placental period from days 26 to 33, with ipsilateral accessory CL reducing pregnancy loss compared to controls in the same period. Interestingly, multiparous cows had regression of contralateral accessory CL earlier than primiparous cows, seemed to have slower embryo development, and greater pregnancy loss than primiparous cows.

Declaration of interest

The authors declare that there is no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

P L J M designed and performed the experiment, performed the progesterone assay, analyzed the results, and wrote the manuscript; C A G, R S G, A B P, R V B, and P G D performed the experiment, A M O C, performed the experiment and the RT-qPCR; R S and M C W designed the experiment, analyzed the results, wrote the manuscript, and acquired funding.

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