Proposal of a new model for CL regression or maintenance during pregnancy on the basis of timing of regression of contralateral, accessory CL in pregnant cows

Proposal of a new model for CL regression or maintenance during pregnancy based on timing of regression of contralateral, accessory CL in pregnant cows.

Giovanni M. Baez[1, 2], Eduardo Trevisol[1], Rafael V. Barletta[1], Beatriz O. Cardoso[1], Alessandro Ricci[1], Jerry N. Guenther[1], Nicole E. Cummings[1], and Milo C. Wiltbank[1]*

[1]Department of Dairy Science, University of Wisconsin-Madison, 1675 Observatory Drive, Madison, WI, 53706, USA

[2]Universidad Francisco de Paula Santander, Cúcuta, Colombia

*Corresponding author: Milo C. Wiltbank, 1675 Observatory Drive, Department of Dairy Science, Madison, WI 53706; Telephone: (608) 212-8091; FAX: (608) 263-9412;

e-mail: wiltbank@wisc.edu

Short Title: Contralateral CL regression during pregnancy

Abstract

In bovine pregnancy, regression or maintenance of the corpus luteum (CL) is mediated through local communication pathways between embryo, uterus, and ovary with d 16-25 of pregnancy generally recognized as the pivotal period determining either luteolysis or prevention of luteolysis. To evaluate this concept, accessory CL were generated by treating Holstein lactating dairy cows (n=718) with GnRH on D 5 of the first follicular wave to produce an accessory CL on the ovary either contralateral or ipsilateral to the gravid horn. In pregnant cows, 66.2% (86/130) of contralateral CL regressed by d75 of pregnancy, whereas few ipsilateral accessory CL regressed (11.9%; 8/67), based on similar criteria ($P<0.0001$). As hypothesized, some contralateral CL regressions (22/86=25.6%) happened on d 19-25 of pregnancy. However, most contralateral CL regressions (64/86= 74.4%) happened later than expected, from d 33-60 of pregnancy. Later contralateral CL regression was more common in primiparous (84.3%) than multiparous (60.0%; $P = 0.02$) cows. Early accessory contralateral CL regression (d 19-25) may be related to lack of exposure of the contralateral horn to interferon-tau from the elongating embryo since pregnant cows without early accessory CL regression had a smaller uterine volume than non-pregnant cows or pregnant cows that had early accessory CL regression (128.4 ± 3.9 vs. 147.0 ± 3.8 vs. 143.6 ± 10.9 mm^3, respectively; $P = 0.003$). These results indicate that there is a second distinct period for CL protection during bovine pregnancy from d 30-60 and implicate local and not systemic pathways in occurrence or prevention of luteolysis during both the early ($\leq$25d) and later ($\geq$33d) critical periods since accessory contralateral CL regressed while the accessory ipsilateral CL of pregnancy remained.

Key Words: Corpus luteum, luteolysis, pregnancy

1. Introduction

In most mammals, including ruminants, either regression of the corpus luteum (CL) in the non-pregnant animal (luteolysis) or maintenance of the CL during pregnancy (luteolysis avoidance) involves communication between the embryo, uterus, and CL. The period between d 16-20 of pregnancy is designated as maternal recognition of pregnancy or the "decisive" period determining whether CL regression or non-regression will occur in cattle [1,

2]. The uterus produces the first signals for initiation of CL regression in ruminants and some other species, since hysterectomy prolongs the lifespan of the CL in these species [3]. Uterine-induced regression of the CL is mediated by local and not systemic pathways in ruminants, as demonstrated by the finding that unilateral hysterectomy only prolonged the lifespan of the CL when performed ipsilateral to the ovary bearing the CL and not if the uterine horn contralateral to the CL was removed [4]. The pathways for this local action involve diffusion of prostaglandin F- 2α (PGF2α), the uterine-derived luteolytic substance [5], from the uterine vein into the ovarian artery that lies in close apposition to the uterine vein [6-9]. The PGF2α that does not diffuse through this countercurrent system goes into the general circulation and is rapidly metabolized in the lungs of ruminants [10, 11]. Rescue of the CL during pregnancy is also generally viewed as a local process rather than mediated by systemic mechanisms in ruminants. The primary evidence for a local mechanism are based on studies involving surgical anastomosis of the ovarian artery from the gravid to the non- gravid side or vice versa [12]. Cows with isolated contralateral horns bearing CL that had the ovarian artery from the gravid side surgically connected to the opposite ovary, were found to maintain the contralateral CL [12]. Nevertheless, recent

studies have called into question the local mechanism since interferon-tau, the primary conceptus factor involved in rescue of the CL of pregnancy [13], has been shown to exit the uterus and act on the CL causing luteal expression of interferon-stimulated genes [14, 15]. Also, systemic administration of interferon-tau was found to maintain the CL, supporting a systemic route for rescue of the CL [16]. In addition, some studies have implicated local diffusion of prostaglandins E1 and E2 (PGE) from the uterus in pregnancy recognition in ruminants [17]. The evidence for this idea is based on increased uterine PGE secretion during pregnancy [18, 19] and prevention of CL regression using various methods of PGE treatment [20-24]. Thus, maintenance of the CL during pregnancy seems to also be primarily mediated by local mechanisms, potentially involving uterine-secreted PGE, although the importance of changes in PGF2α secretion patterns and of direct systemic actions of interferon-tau on the CL are also being investigated [25, 26]. At all stages of pregnancy, circulating P4 is essential for maintenance of pregnancy and many studies have utilized various strategies to increase circulating P4 in an attempt to increase fertility in cattle [27-29]. One strategy to increase circulating P4 concentrations is to induce an accessory CL by ovulation of a dominant follicle in cows that already have a CL. This strategy has generally been done by treating cows with human chorionic gonadotropin (hCG) or GnRH at 5 to 7 d after breeding in order to ovulate the dominant follicle of the first follicular wave [30, 31]. Studies have generally observed a high percentage of cows with ovulation of the dominant follicle of the first follicular wave and consistent increases in circulating P4 concentrations [32, 33]; however, fertility responses have been variable [34-36]. There is a greater fertility response to induction of an accessory CL in first lactation cows (primiparous) than in multiparous cows [37, 38]. Currently, there are no defined physiological mechanisms

to explain the observed variability between parities or between studies in fertility responses to treatment with hCG or GnRH during the first follicular wave in lactating dairy cows. Treatment with GnRH or hCG on d 5-7 after breeding can produce an accessory CL on the same side as the previous ovulation (ipsilateral) or can produce an accessory CL on the opposite ovary (contralateral). During previous studies, our group has observed accessory CL regression in pregnant lactating dairy cows with contralateral but not ipsilateral CL (JN Guenther

and MC Wiltbank, unpublished results), however, the timing of CL regression during pregnancy or any differences between parities in occurrence of luteolysis of accessory CL has not been previously determined. A previous study [33] found that a new accessory CL was formed in pregnant cows (d 26 to 71 of pregnancy) following treatment with hCG (50% of cows formed an accessory CL) or GnRH (26% of cows formed new CL) and this new accessory CL subsequently regressed in 36.2% of pregnant cows during the next four weeks. Regression of the CL was more likely if the accessory CL was contralateral to the gravid horn rather than ipsilateral [33]. A potentially related recent finding from our laboratory [39] demonstrated that primiparous cows have a smaller uterus compared with older cows, and also, for any given parity, larger uterine size was associated with decreased fertility. It is therefore possible that the contralateralaccessory CL may undergo luteolysis in cows that have a large uterine size, possibly due to inefficient delivery of interferon-tau to the contralateral horn, whereas, the accessory contralateral CL may be maintained in cows with a smaller uterus. This may help explain the parity differences in fertility responses to induction of an accessory CL since contralateral CL regression may occur in older cows with larger uterine size thereby muting the fertility responses to induction of an accessory CL. Thus, embryonic signals maintain the CL that is ipsilateral to the pregnancy but may or may not maintain an accessory CL that is contralateral to the gravid horn. The objective of this research was to determine the percentage of pregnant lactating dairy cows that undergo regression of an accessory CL that has been produced by ovulation of a dominant follicle on the contralateral ovary. We hypothesized that a greater percentage of multiparous cows with a larger uterus would undergo contralateral, accessory CL regression compared to primiparous cows.Thus, we designed an experiment to evaluate the timing of contralateral, accessory CL regression. We hypothesized that contralateral, accessory CL regression in pregnant cows would primarily occur during the time of CL regression that has been typically described, from d 19-25 after AI, probably due to insufficient signals from the embryo reaching the contralateral horn in lactating cows with a large uterus. Thus, we speculated that maternal recognition of pregnancy and rescue of the CL would occur normally in the uterine horn and ovary ipsilateral to thepregnancy but in the contralateral horn, luteolytic mechanisms would develop in some pregnant multiparous lactating dairy cows causing contralateral, accessory CL regression. It should be emphasized that this study was not designed to evaluate the effect of GnRH treatment or anaccessory CL on fertility, as has been performed in previous studies [34-38]. Therefore, wetreated all cows with GnRH on D 5 after breeding in order to maximize the number of pregnantcows with a contralateral CL, which represented the key study animals for testing our hypotheses related to the occurrence and timing of contralateral CL regression. **Materials and methods**

2.1 Animals: Feeding, Housing, and Experimental Protocols

Lactating Holstein dairy cows (n=718) from a commercial dairy in southeast Wisconsin (primiparous n=339, multiparous n=379) were utilized in this experiment. Cows had an average daily milk production of 45.3 ± 9.2 kg/d and were maintained in freestall barns, fed a Total Mixed Ration, and milked three times per d. All cows were synchronized using a Double-Ovsynch protocol as previously described [40]. Briefly, all cows were treated with 100 µ g of gonadorelin acetate (GnRH; GONAbreed®, Parnell Pharmaceutical,

Overland Park, KS), given at a random stage of the estrous cycle from 51-57 d after calving. Cows were treated 7 d laterwith prostaglandin F2α (PGF2α; estroPLAN®, Parnell) and 3 d later with a second dose of GnRH, followed 7 d later by an Ovsynch-56 protocol (GnRH-7d-PGF2α -56h-GnRH) and timed AI at 16 h after the final GnRH. All cows also received an additional treatment with GnRH 5 d after breeding, with the intention of ovulating the dominant follicle or follicles from the first follicular wave after AI in order to generate an accessory CL.

2.2 Ultrasonography

Ovarian structures were monitored by transrectal ultrasonography using a 7.5-MHz, linear-array probe (Ibex Pro, E.I. Medical Imaging, Loveland CO). Two perpendicular diameters of the CL were determined and the volume of the CL was calculated using the formula $V=(4/3)\pi r^3$, as done previously [41]. For CL with a fluid filled cavity, the volume of the cavity was calculated and subtracted from the total CL volume. Ultrasonography was performed before AI on the day of PGF2α treatment to determine the size and location of the dominant pre-ovulatory follicle, and at the time of GnRH injection 5 d after AI to verify ovulation and the size and location of the dominant follicle of the new follicular wave. Cows with double-ovulation (ovulation in both left and right ovaries) at the time of AI were excluded from the experiment while cows with single ovulation or double ovulation in a single ovary continued in the experiment and received the treatment with GnRH on D 5 after breeding. The ovaries were evaluated again on D 11 in order to verify ovulation to the D GnRH, and the location of the accessory CL. Cows with a contralateral accessory CL were evaluated by ultrasound weekly to determine the fate of the accessory CL (d 19, 25, 33, 40, 47, 54, 61, 68, and 75) and to determine pregnancy. Cows with ipsilateral CL were evaluated by ultrasound only on d 32 and 61 to determine pregnancy and presence of the accessory CL. Uterine size was measured on the day of PGF2α injection three days before AI using a method previously described [39]. Briefly, the diameter of the endometrium was measured at the major curvature of the uterine horns using ultrasound (d), and the length of the horns was estimated by rectal palpation (L). The volume (V) was calculated for each uterine horn, using the formula $V= \pi r^2 L$ such that V = volume of the cylinder of each uterine horn, r = radius (based on diameter at greater curvature), and L = horn length estimated by hand palpation and total uterine volume was calculated as the sum of two uterine horns. Uterine size determined in this manner has been previously related to pregnancy per AI (P/AI) in lactating dairy cows [39]. Pregnancy diagnosis was performed at D 32 using ultrasound, by localization of embryonic vesicle, along with an embryo with a heartbeat. Only pregnant cows were scanned at d 33 and later d, and the non-pregnant cows were eliminated from the experiment.

2.3 Determination of contralateral, accessory CL regression

Regression of the CL was determined retrospectively using data from the weekly ultrasound evaluations. When a contralateral CL disappeared or appeared to have a substantial decrease in luteal volume, the previous two ultrasound evaluations were analyzed to determine whether there was a decrease of $\geq$ 20% in luteal volume. The week in which these criteria were met was designated as the week of CL regression;

however all cows with contralateral CL regression were evaluated for subsequent weeks until complete CL disappearance occurred. Day 0 is the day of breeding with weekly ultrasound evaluations used to determine ovulation to Ovsynch-56 (D 5), ovulation to D 5 GnRH (D 11), week of CL regression as weeks 3 (D 19), 4 (D 25), 5 (D 33), 6 (D 40), 7 (D 47), 8 (D 54), 9 (D 61), 10 (D 68), or 11 (D 75). A subset of cows (n = 30) were evaluated on D 180 and no CL regression was observed in any of these cows. Thus, cows without contralateral CL regression by D 75 were assumed to not have contralateral CL regression during pregnancy

2.4 **. Blood sampling and hormonal assays**

2.5 Blood samples were collected from the coccygeal vein or artery to measure P4 concentrations at three days before AI, at the time of PGF2α injection, and on the day before AI at the time of the final GnRH of the Ovsynch-56 protocol. Additional samples were taken on d 19 and 27 after AI to measure concentrations of interferon stimulated genes (ISG) and Pregnancy Specific Protein B (PSPB), respectively. All the blood samples to be analyzed for P4 were collected into evacuated serum tubes (Vacutainer, Becton, Dickinson and Co., Franklin Lakes, NJ), and placed immediately on ice until processed in the laboratory. Blood samples were centrifuged at 1600 x g for 20 minutes at 4°C, and stored at −20°C until analyzed. For P4 determination, non-extracted serum samples were analyzed using an antibody- coated tube RIA kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) with intra-assay and inter-assay coefficients of variation (CV) of 4.6% and 3.6% respectively. PSPB concentrations were determined using the ELISA kit BioPRYN (Biotracking Inc, Moscow, ID). The intra-assay and inter-assay coefficients of variation (CV) were 2.2% and 3.9% respectively.

2.6 **ISG Blood Processing, RNA Extraction, cDNA synthesis, and Quantitative RT-PCR**

Blood samples were collected into vacutainer tubes containing EDTA from all cows on d 19 for isolation of peripheral blood leukocytes (PBL) [42]. Briefly, the tubes were centrifuged at 1200 x g at 4˚C for 12 min, plasma was harvested and the buffy coat fractions were collected by pipet and transferred to 15 ml tubes containing 10 ml of red blood cell (RBC) lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$ and 1 mM EDTA; pH=7). Tubes were inverted several times and incubated at room temperature for 5 min. Samples were centrifuged at 300 x g for 10 min at 4˚C and the supernatant was discarded. The PBL pellet was mixed with 5 ml of RBC lysis buffer, incubated at room temperature for 5 min, centrifuged at 300 x g at 4˚C for 10 min, and the supernatant was discarded. The PBL was washed with ice-cold PBS, centrifuged at 300 x g at 4˚C for 10 min, and the supernatant discarded. The PBL pellet was then suspended with 0.5 ml of Trizol (Molecular Research Center, Inc., Cincinnati, OH), the cells lysed by gentle pipetting, then transferred to 0.6-ml microtubes, and stored at -80˚C. The RNA was then extracted using the manufacturer's protocol (Trizol; Invitrogen). The RNA was quantified by optical density at 260/280 nm with NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). One microgram of mRNA was treated with DNase I and used to synthesize complementary DNA using a cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). The complementary DNA was then used for quantitative real-time PCR. All PCR reactions were arranged as follows: 5 µl of SYBER Green Master Mix, 0.5 µl of each primer (10 µl of primer and 90 µl of dH_20), final volume was brought to 8 µl and then 2.5 µl of cDNA was added. Thermal cycling was done by initially

incubating the mixture at 95˚C for 3 min with subsequent denaturation at 95˚C for 1 min. This was followed by 40 cycles of denaturation, annealing, and amplification (95˚C for 30 sec, 60˚ for 1 min, 72˚C for 30 sec). The reactions were carried out on an Opticon 2 real-time PCR system (Bio-Rad Life Science, Foster City, CA). Melting curve analyses were performed as follows: 95˚C for 1 min, followed by fluorescence measurement performed at one degree increments between 55˚C and For quantitative real-time PCR optimization and validation, the pool of PBL from pregnant cows was utilized at different concentrations and was ultimately utilized at a final dilution of 1:5 for all of the 3 genes used in this study. Three genes were investigated. Based on previous reports of gene expression in response to interferon-tau released during early pregnancy [15, 43], ISG15 ubiquitin-like modifier (ISG15; F = GGTATCCGAGCTGAAGCAGTT, B = ACCTCCCTGCTGTCAAGGT) and MX dynamin like GTPase 2 (MX2; F = CTTCAGAGACGCCTCAGTCG, B = TGAAGCAGCCAGGAATAGTG) were chosen. The Ribosomal protein L19 (RPL19; F = ATCGATCGCCACATGTATCA, B = GCGTGCTTCCTTGGTCTTAG) has been utilized routinely in studies of peripheral blood leukocytes in our laboratory [44] and other laboratories [45, 46] and therefore was chosen as an internal control. A comparative method [47] was used to evaluate these data, using nonpregnant cows from the control group as the calibrator for comparisons. Gene expression data were expressed as fold change relative to the calibrator and after normalization to the endogenous RPL19. All samples were evaluated in duplicate for each cDNA. Efficiencies of qPCR for amplifications were between 90 and 110%. **Statistical Analysis**

Statistical analyses were completed using SAS software (SAS Institute, version 9.4). Differences in P/AI between specific groups were evaluated using Fischer's exact test. Continuous variables were analyzed using Student's t-test for comparison of two means within each parity. For all comparisons, a statistically significant difference was assumed for $P < 0.05$ and a statistical tendency was assumed when $P \geq 0.05$ but $P \leq 0.15$. For analysis of interactions, the logistic procedure of SAS was used.

2.7 Ovulation to Double-Ovsynch protocol and D 5 GnRH

The cows in this experiment were all synchronized using the Double-Ovsynch protocol, well-established and efficient method for precisely synchronizing the time of ovulation [40, 48]. The total number of animals enrolled at the time of the last PGF2α of Ovsynch-56 was 718 (339 primiparous, 379 multiparous). Cows with P4 concentrations above 0.5 ng/ml on the day of the final GnRH of the Double-Ovsynch protocol (n = 65) were assumed to have not been synchronized correctly, based on the low percentage of P/AI (4.6%) and as previously shown [49], and were removed from the analysis. Ovulation to the final GnRH was determined by the presence of a CL at the ultrasound evaluation that was done 5 d after AI. Five cows (2 primiparous [0.6%] and 3 multiparous [0.8%]) did not ovulate to the protocol. In addition, 12.3% of the cows (88/718) had bilateral double ovulation and therefore had CL on each ovary on D 5 (25/339 primiparous [7.4%], 63/379 multiparous [16.6%]; P = 0.0002). These cows with bilateral ovulations to the Ovsynch-56 protocol could not be used in our experiments due to potential pregnancies in both horns. A number of cows also had double ovulations in the same ovary (ipsilateral) (n = 30; 9 primiparous [4 left, 5 right] and 21 multiparous [6 left, 15 right]) and were used in subsequent analyses. After data refinements

(10 cows met both double-ovulation and high progesterone criteria for being removed), a total of 570 cows were further analyzed. Data on the side of ovulation (right vs. left) are shown in Table 1. Overall, there was a greater percentage of cows with ovulation on the right ovary (57% right, 43% left; P = 0.001) and this difference was consistent in either primiparous or multiparous cows. A total of 402 (70.5%) of the 570 cows ovulated to the treatment with GnRH 5 d after AI, (358 single ovulations and 44 double ovulations, Table 1). Regardless of the side of the original ovulation to the Double-Ovsynch protocol, a greater percentage of the single-ovulating cows 273 ovulated contralateral to the previous ovulation after the D 5 GnRH treatment (65.4% 274 contralateral vs 34.6% ipsilateral; P < 0.0001), with no differences between primiparous and 275 multiparous cows (P = 0.91; Table 1). The remaining 44 cows that double-ovulated after the D 5 276 GnRH treatment, had primarily bilateral (n = 35) and not ipsilateral (n = 9) ovulation. 277 Nevertheless, ovulations to the D 5 GnRH continued to be predominantly on the right (55.0% 278 [221/402]) and not the left (45.0% [181/402]) ovary (P = 0.05). 279 Pregnancies per AI 280 Cows that did not ovulate to the GnRH treatment on D 5 had lower P/AI on D 32 (P = 281 0.002) and D 67 (P = 0.0005) pregnancy diagnoses compared to cows that ovulated to the D 5 282 GnRH (Table 2). Also, in both cases (ovulation or no ovulation) primiparous cows had greater 283 P/AI than multiparous (P = 0.0002 for P/AI at both D 32 and D 67). Pregnancy loss was lower in 284 cows ovulating after the D 5 GnRH (P= 0.04), but was not different due to parity (P = 0.47), or 285 the interaction (P = 0.94) of parity and ovulation (Table 2). Subsequent analyses were performed===286 using data only for cows that ovulated to the D 5 GnRH treatment comparing effects of parity 287 and side of ovulation on P/AI (Table 3). 288 Primiparous cows had a tendency for greater P/AI than multiparous cows at both D 32 289 (57.3% vs 44.9%; P = 0.09) and D 67 (55.3% vs 42.3%; P = 0.07) pregnancy diagnoses (Table 290 3). There tended to be an interaction (P = 0.11on D 32; P = 0.14 on D 67) between parity and 291 side of ovulation (Table 3). There was no effect of parity on P/AI in cows with ipsilateral 292 ovulation (P = 0.97 on D 32; P = 0.82 on D 67) with all parities having high P/AI (54.5 vs. 293 54.2%; primiparous vs. multiparous). However, in cows with contralateral ovulation there was 294 greater P/AI in primiparous than multiparous cows at either D 32 or D 67 pregnancy diagnoses 295 296 (P = 0.003; Table 3). 29

3.3 Regression of the contralateral accessory CL

298 Overall, 66.2% of pregnant cows that had a contralateral accessory CL regressed this 299 accessory CL during pregnancy (86/130; Table 4). There was no difference (P = 0.85) between 300 parities in likelihood of contralateral CL regression in pregnant cows with 65.4% of primiparous 301 and 67.3% of multiparous cows having contralateral CL regression. The likelihood of accessory 302 CL regression was much greater for contralateral than ipsilateral in both primiparous (P < 303 0.0001) and multiparous (P < 0.0001) cows. In pregnant cows with contralateral, accessory CL, 304 cows that had contralateral CL regression were more likely (P = 0.05) to have pregnancy loss 305 between 32 and 67 d of pregnancy (9.3%; 8/86) compared to cows without contralateral CL 306 regression (0.0%; 0/44). 307 The timing of accessory CL regression differed by parity and is detailed in Figure 2. The 308 distribution indicated two peaks of contralateral CL regression, before D 33, henceforth===309 designated as early CL regression and between D 33 and D 68,

henceforth designated as later CL 310 regression. As shown in Table 4, a greater percentage of multiparous cows had early CL 311 regression compared to primiparous cows (40.0% vs. 15.7%; P = 0.02). In contrast, most of the 312 pregnant primiparous cows that had contralateral CL regression were classified as having later 313 CL regression (84.3%; 43/51). Thus, no differences were detected between early and late CL 314 regression for multiparous cows (40.0% early vs 60.0% late, P = 0.24), but in primiparous cows, 315 later CL regression was greater (84.3%) than early CL regression (15.7%, P < 0.0001). Overall 316 for all parities, more pregnant cows with contralateral CL had later rather than early accessory 317 CL regression (74.4% vs 25.6%, P < 0.0001, n = 86). 318 There was no effect of side of ovulation (left vs. right) on occurrence or timing of CL 319 regression. For example, for cows with early CL regression (n = 22), 52.2% of contralateral CL 320 were on the left side and 47.8% were on the right side (P = 0.83). Similarly, for cows with late 321 CL regression (n = 64), 45.3% were on the left side and 54.7% were on the right side (P = 0.45). 32

3. Other measurements

Several other measurements from this experiment are compiled in Table 5. Comparisons 324 were between three groups of cows that all had a contralateral accessory CL: 1) Non-pregnant 325 cows at the D 32 diagnosis, 2) Pregnant cows that regress the accessory CL before D 33, and 3) 326 Pregnant cows that did not regress the contralateral accessory CL before D 33. Uterine volume 327 was greatest for the non-pregnant cows and pregnant cows that regressed the accessory CL 328 before D 33 and smallest for pregnant cows with no contralateral accessory CL regression before 329 D 33 (P = 0.003). In contrast, circulating P4 on D 19 was lowest (P < 0.0001) in the non- 330 pregnant cows, highest in the pregnant cows without CL regression before D 33, and 331 intermediate for pregnant cows with accessory CL regression before D 33. As expected, PSPB===332 was very low for non-pregnant cows compared to pregnant cows (P < 0.0001), but was also 333 lower in pregnant cows with contralateral CL regression before D 33 than in pregnant cows 334 without early CL regression. For the interferon-stimulated genes (ISG15 and Mx2), mRNA 335 concentrations were lowest in non-pregnant cows, highest in pregnant cows with no early CL 336 regression, and intermediate for pregnant cows with CL regression before D 33 (Table 5). 337 Unfortunately, due to the nature of the measurements and the relatively low numbers of animals 338 in the three groups we were not able to divide out these measurements by parity and the reader is 339 340 cautioned that this could bias or confound the results in Table 5.===341

4. Discussion

342 This study provides new insights into the local nature and timing of rescue of the CL during 343 pregnancy in cattle and led us to propose a new working physiological model related to 344 “maternal recognition of pregnancy” and maintenance of the CL during pregnancy (Figure 3). 345 We rejected our first hypothesis that there would be a parity difference in contralateral CL 346 regression, since we observed that first lactation cows had a similar overall rate of contralateral 347 CL regression as older cows (~65%), even though the timing of contralateral CL regression was 348 quite different for first lactation vs. older cows. In addition, we originally hypothesized that CL 349 regression would primarily occur at d 19 to 25 of

pregnancy, the period designated as “maternal recognition of pregnancy”. Although some of the cows, particularly older cows with a larger uterus [39], did have contralateral CL regression at this earlier time, the majority of cows displayed contralateral CL regression between d 33 and 60 of pregnancy. Based on these findings, a physiological model that will need to be tested in the future is proposed with dual periods for maintenance or regression of the CL of pregnancy (Figure 3). Consistent with previous models for maternal recognition of pregnancy, secretion of interferon-tau from the developing bovine conceptus at D 16-25 of pregnancy, causes a local uterine response that allows maintenance of the CL of pregnancy. In most cows, the conceptus produces sufficient interferon-tau to produce a similar local response in the contralateral uterine horn and rescue of the contralateral CL, since the two horns were not surgically separated in our study, as has been done in some previous studies of contralateral CL regression [50, 51]. Of more interest, our results provided unexpected evidence for a second distinct period for rescue of the CL of pregnancy, which occurs after D 30 of pregnancy (Figure 3). Since most accessory CL that were contralateral to a gravid horn regressed from d 30 to 60 of pregnancy, whereas ipsilateral,===accessory CL were maintained, it seems clear that this later pivotal period for a second “rescue of the CL of pregnancy” also involves local and not systemic mechanisms. This is not a phenomena that is limited to lactating dairy cattle or cows with a large uterine size since we have also observed a similarly high contralateral, accessory CL regression rate (54.5%) in pregnant heifers (n = 157) that were recipients of in vitro produced embryos (Garcia-Guerra, Baez, and Wiltbank, unpublished results). An adequate discussion of these concepts will require: 1) Discussion of the physiology and potential limitations of the contralateral CL model, 2) Discussion of the implications of our results for the first period of CL rescue, 3) Speculation on the potential mechanisms producing the second crucial period for maintenance of the ipsilateral but not contralateral CL during the second month of pregnancy, and 4) Discussion of potential practical implications of our findings related to fertility and particularly pregnancy loss in cattle and possibly other species. To test the local mechanisms involved in rescue of the CL of pregnancy, we needed a model in which a pregnancy was present in only one uterine horn but CL were present in both ovaries. We chose to use a well-established method that has been practically used in many previous studies attempting to increase fertility in lactating dairy cattle [34, 37, 38]. Treatment with GnRH during the first follicular wave produced an accessory CL in most cows. One limitation of this model is that the original CL of pregnancy is slightly older (5 d) than the accessory CL, however, the observation that most ipsilateral accessory CL were maintained throughout pregnancy supports the idea that these accessory CL were functional. In addition, this observation supports the view that regression of contralateral, accessory CL was due to local luteolytic mechanisms and not to an inherently inadequate accessory CL. Previous elegant models have been used to study the local nature of both luteolysis and rescue of the CL during===pregnancy in ruminants, such as surgical methods to isolate uterine horns and surgical connection of uterine vessels from the pregnant to the non-pregnant horn [51]. In order to test our hypotheses, we required large numbers of cows and therefore could not use these extremely invasive

procedures in this experiment. One unique aspect of our model was that we left the 391 connection between the two uterine horns intact and yet we still observed the intriguing situation 392 in which the mechanisms involved in maintaining the CL of pregnancy were occurring in the 393 ipsilateral horn and ovary, whereas in the same animal, the contralateral horn and ovary had 394 luteolytic mechanisms occurring. It may be appropriate to use other, more intensive, models in 395 future studies to understand this second pivotal period for rescue of the CL that occurs during the 396 second month of pregnancy. Nevertheless, these studies using intact uterine horns, bilateral CL, 397 and a unilateral pregnancy in a large number of lactating cows provided interesting and, in some 398 ways, unanticipated insights into the whole-animal reproductive physiology involved in 399 maintenance of the CL of pregnancy in ruminants. 400 The first period in which we observed contralateral, accessory CL regression, from D 16 401 to 25 of the estrous cycle or pregnancy, has been extensively studied in ruminants and has been 402 termed maternal recognition of pregnancy (Figure 3). First, the uterus is exposed to elevated 403 circulating progesterone (P4) for multiple d (7-14 d in cattle) and this elevated P4 is thought to 404 produce down-regulation of uterine P4 receptors and subsequent elevation in expression of 405 uterine estradiol (E2) receptors. Activation of these E2 receptors by circulating E2 from the 406 developing dominant follicle causes induction of uterine oxytocin receptors [52]. Oxytocin 407 acting on its receptor then activates phospholipase A2 which releases arachidonic acid from 408 membrane phospholipids and arachidonic acid is converted to PGF2α by cyclooxygenase and 409 PGF2α -synthase enzymes [52, 53]. An elongating conceptus needs to be present by D 16 [1, 2]===410 to initiate the mechanisms that result in rescue of the CL. It is clear that secretion of interferon- 411 tau (INFT) from the elongating conceptus produces changes in endometrial gene expression and 412 subsequent changes in PG secretion patterns (Figure 3). Treatment with exogenous INFT reduces 413 uterine E2 receptor alpha mRNA and protein (ESR1) and reduces expression of oxytocin 414 receptor mRNA and protein, thus blocking initiation of the endometrial luteolytic mechanisms 415 [54]. In our model, early contralateral CL regression is likely to result from a lack of altered PG 416 secretion patterns in the contralateral uterine horn due to insufficient IFNT exposure in the 417 contralateral horn, in spite of rescue of the ipsilateral CL likely due to IFNT-induced alterations 418 in PG secretion patterns from the ipsilateral horn. The observation that contralateral CL 419 regression happened primarily in multiparous cows with a larger uterine size is consistent with 420 our original hypothesis that physical diffusion of IFNT from the ipsilateral to the contralateral 421 horn is the major limiting factor that allowed contralateral CL regression during this early period, 422 corresponding to the normal time of maternal recognition of pregnancy. Thus, local and not 423 systemic mechanisms appear to underlie rescue of the ipsilateral CL, and in most cases also the 424 contralateral CL, during early pregnancy in ruminants [13, 55]. 425 Additional insights into the mechanisms are early contralateral CL regression were 426 provided by comparison of various physiological measurements in cows that were not pregnant 427 at D 32 compared to pregnant cows that had early, contralateral, accessory CL regression (before 428 Day 33) and also compared to pregnant cows without early contralateral CL regression (Table 429 5). Pregnant cows with early, contralateral CL regression had a larger uterine size, similar to the 430 uterine size for cows that were not pregnant (Table 5),

and had lower PSPB concentrations, 431 suggesting a potentially reduced development of placentomes in cows with early contralateral 432 CL regression. In addition, circulating P4 on D 19 tended to be lower in pregnant cows with===433 subsequent contralateral CL regression compared to cows without contralateral CL regression 434 and ISG15 and MX2 expression on D 19 were intermediate between non-pregnant and pregnant 435 cows without early contralateral CL regression. Thus, in addition to uterine size being a risk 436 factor for early, contralateral, accessory CL regression, there may an association with reduced 437 embryo development, measured either by ISG15 or MX2 on D 19, or PSPB on D 27 possibly 438 related to reduced concentrations of circulating P4. 439 The second period of contralateral, accessory CL regression or CL maintenance is more 440 difficult to explain, because this period has not been extensively studied and currently there is 441 not an established model for the mechanisms involved in regression or maintenance of the CL 442 from D 30 to D 60 of pregnancy. It seems likely that INFT is no longer the major factor involved 443 in protecting the ipsilateral CL during this second period, since INFT secretion from the 444 developing conceptus peaks by D 23 of pregnancy and then dramatically decreases during the 445 next few weeks [56, 57]. Indeed, treatment with exogenous INFT maintains the CL of sheep [58] 446 until d 23-28 (native ovine INFT) or d 34-38 (recombinant INFT), and in bovine until d 26-29 447 using recombinant bovine INFT [59, 60]. Therefore, other mechanisms are likely to be involved 448 in the rescue and maintenance of the CL during this second critical period of CL maintenance 449 during pregnancy. Our results are consistent with this second period of ipsilateral CL rescue 450 being mediated by local communication between the ipsilateral uterine horn, embryo, and CL, 451 whereas, on the opposite side, CL regression is occurring in most animals, most likely mediated 452 by signals coming from the contralateral uterine horn to the contralateral CL through local and 453 not systemic mechanisms. 454 Two key questions raised by the results of our studies are: What are the mechanisms that 455 induce regression of the contralateral, accessory CL from d 30-60 of pregnancy in most cows===456 and, alternatively, what are the mechanisms that maintain the ipsilateral CL in spite of 457 contralateral CL regression at this time? It seems likely that the contralateral CL regresses due to 458 PGF2α secretion from the contralateral uterine horn, since ruminant CL do not undergo 459 spontaneous regression with the uterus removed [3, 61]. In addition, ipsilateral CL, either 460 original or accessory CL, do not generally regress during this same time period. If the logical 461 assumption for the involvement of uterine PGF2α in contralateral CL regression is correct, then 462 there seem to be at least three possible physiological explanations for the disparity between sides 463 in CL regression during pregnancy. First, PGF2α production differs between the two sides with, 464 perhaps, a lower PGF2α secretion from the ipsilateral than the contralateral uterine horn. Second, 465 PGF2α secreted from the uterine horn does not reach the ipsilateral CL, however, uterine PGF2α 466 reaches and regresses the contralateral CL. Finally, a third possibility is that PGF2α reaches both 467 ovaries containing CL, but that the responsiveness of the luteal cells to PGF2α are lower for the 468 CL adjacent to the pregnancy than for the contralateral CL. Our results or previous results do not 469 allow us to definitively distinguish between these three potential physiological explanations for 470 lack of PGF2α action in ipsilateral CL, while, complete CL regression occurs in most 471 contralateral, accessory CL

during a continuing pregnancy. In our opinion, the second explanation is the most plausible. This explanation could relate the second period of CL rescue during pregnancy to previous reports of greater blood flow in the uterine artery of the gravid vs. non-gravid horn in pregnant cows [62-64].

Thus in this scenario, high uterine blood flow would not allow efficient transfer of uterine-secreted PGF2α from the uterine vein to the ovarian artery on the ipsilateral side but efficient PGF2α transport would continue to occur on the contralateral side, potentially due to reduced uterine blood flow [62]. Further evidence for this explanation comes from the classical studies of bovine placentome development [65] showing attachment===and placentome development by D 30 in the gravid horn, but placentome development was still not observed by D 38 in the non-gravid horn. Thus, differences in placentome development in the two uterine horns, might be the key determinant of uterine blood flow and, therefore, CL regression from d 28-60, as illustrated in Figure 3B. In addition to the results from this study providing a rationale for testing a new biological model for CL maintenance during pregnancy, they may also have important practical implications for fertility in lactating dairy cows [66-68]. Although fertilization is high in lactating dairy cows that are not exposed to heat stress [69, 70] and, theoretically, 100% in recipients of an IVF embryo, pregnancy loss can occur at various stages of pregnancy [71-73]. Embryonic death during the first 7 d of pregnancy is high in lactating dairy cows [69, 70], however these degenerate embryos would not be expected to alter the timing of CL regression since they do not undergo elongation. In contrast, suboptimal embryonic elongation or INFT production could result in pregnancy loss during maternal recognition of pregnancy (16 to 25 d after breeding). Finally, about 12% of pregnancies are lost between 30 and 60 d of pregnancy and this can vary, possibly due to specific physiologic, nutritional, or management conditions [68, 72, 74]. Our observation of increased fertility in cows that ovulated to D 5 GnRH treatment are consistent with previous findings [37, 38] and may help to explain the parity differences that were previously observed in fertility responses to hCG treatment [37, 38]. Although, both primiparous and multiparous cows had increased P/AI in cows with ipsilateral ovulation (Table 3), contralateral ovulation did not improve P/AI in multiparous cows perhaps because of regression of CL during early pregnancy in many multiparous cows, which did not occur in primiparous cows. Similarly, a previous analysis of 601 pregnant lactating dairy cows, reported that cows with an additional CL were 8 times less likely to lose their pregnancy between d 38===and 90 after AI [75], although, no information was provided regarding the location of the additional CL (ipsilateral or contralateral). Pregnancy loss can be initiated by death of the embryo or alternatively by regression of the CL [76, 77]. It seems possible, and perhaps likely, that pregnancy loss occurring from d 28-60 of pregnancy is related to similar mechanisms that are producing regression of the contralateral, accessory CL in our studies; although, we do not fully understand those mechanisms at this time. Thus, we postulate that a thorough analysis of the mechanisms involved in contralateral CL regression in pregnant ruminants could provide important insights into the mechanisms that underlie increased pregnancy loss during specific stages of pregnancy in ruminants and potentially other species. In conclusion, we found that most accessory CL that are contralateral to the gravid

uterine horn regressed by the end of the second month of pregnancy. However, the timing of CL 513 regression in these cows was somewhat unexpected with most happening after 32 d and before 514 67 d of pregnancy. Early regression of the contralateral, accessory CL (before D 32) was 515 associated with older cows with a larger uterus, whereas, even primiparous cows regressed most 516 of the remaining contralateral, accessory CL during the second month of pregnancy. Based on 517 these observations, a new physiological model is proposed with two different critical periods 518 when the CL of pregnancy must be protected from luteolysis, the first during D 19-25 and the 519 520 521 second from D 32-60**.**

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GnRH treatment.

Group	Right Ovulation after Double-Ovsynch	No ovulation after D 5 GnRH	Contralateral Ovulation after D 5 GnRH 533
rimiparous	7.9% (162/280) = 0.01	8.2% 1/287)	4.9% (122/188) < 0.0001 535
ultiparous	5.2% (150/267)	0.7%	5.9% (112/170) 8)
P = 0.04 (87/283) P < 0.0001 537 Parity P-Value		0.73	0.52 0.91 538 Overall 57.0% (312/547)
29.5% 65.4% (234/35 539		P = 0.001 (168/570)	P < 0.0001 540===541 542 543 544 545 546 547

[1]Single ovulations only. Comparisons are whether there is a difference from expected 50% right and 50% left ovulations using Goodness of Fit analysis.

[2]Comparison of percentage of ovulations that were contralateral vs. ipsilateral, as compared to an expected 50% contralateral ovulation using Goodness of Fit analysis.

[3]Cows with double ovulation in both ovaries were eliminated from this column.===548

Table 2. Fertility (pregnancies/AI at pregnancy diagnosis on D 32 and D 67 and pregnancy loss) in cows that received a treatment of GnRH 5 d after AI and that ovulated or did not ovulate in response to this D 5 GnRH treatment.

No Ovulation after D 5 GnRH	Ovulation after D 5 GnRH	Effects, P-Value[1] Parity	Ovulation
0.0002	0.002===0.0002	0.0005===0.47	0.04

a, b = different letters indicate significant differences (P < 0.05) within a row.

[1]Only the main effects of parity and ovulation are shown. The interactions were not significant (P > 0.50) for any of the categories.===557

	Primiparous	Multiparous	Primiparous	Multip
Pregnant on D 32	48.1%[ab] (39/81)	26.4%[c] (23/87)	57.3%[a] (118/206)	44.9 (88/1
Pregnant on D 67	43.2%[ab] (35/81)	23.0%[c] (20/87)	55.3%[a] (114/206)	42.3 (83/1
Pregnancy Loss (D 32-67)	10.3%[b] (4/39)	13.0%[b] (3/23)	3.4%[a] (4/118)	5.7% (5/8

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Table 3. Effect of side of ovulation to D 5 GnRH, in relation to previous ovulation after

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Double-Ovsynch, on fertility measurements (P/AI at 32 and 67 d pregnancy diagnoses and

559

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pregnancy loss between 32 and 67 d after AI).

Ipsilateral			Contralateral		P-value		
	imiparous	ultiparous	imiparous	ultiparous	arity	de	XS
egnant per AI	4.5%[abA]	4.2%[abA]	3.6%[a]	0.9%[bB]			
aD 32	6/66)	2/59)	2/140)	6/137)	09	38	11
egnant per AI	4.5%[a]	2.5%[abA]	5.7%[a]	3.0%[bB]			
a D 67	6/66)	1/59)	8/140)	2/137)	07	21	14
egnancy Loss	0%[aA]	1%[a]	9%[a]	1%[aB]			
32-67)	/36)	/32)	/82)	/56)	38	15	89

a, b = different letters indicate significant differences (P < 0.05) within a row.

A, B = different capital letters indicate tendencies (P < 0.15) for differences within a row.===565

Table 4. Regression of the accessory CL by D 67 in cows that were pregnant at D 32 pregnancy diagnosis.

Incidence of regression			Timing of contralateral regression		
Ipsilateral	Contralateral	P-value	Early (≤ 33)	Late (> 33)	P-value
i parous 8.3% (3/36)					
Multiparous 16.1%					
(5/31)					
65.4% (51/78)	67.3% (35/52)				
<0.0001===<0.0001					
15.7% (8/51)	40.0% (14/35)				
84.3% (43/51)	60.0% (21/35)				
<0.0001===0.24					
P-value		0.46	0.85	0.02	0.0
Overall 11.9% (8/67)					
66.2% (86/130)					
<0.0001	25.6% (22/86)				
74.4% (64/86)					
<0.0001===570					

Table 5. Comparison of various measures for cows with contralateral, accessory CL that were either not pregnant (left column) or pregnant at the 32 pregnancy diagnosis and were found on D 33 to have regressed (early CL regression) or not regressed the contralateral CL (right columns). Not pregnant Pregnant ANOVA

Not pregnant Regression No regression Overall===Uterine Volume (mm^3) P4 D 19 (ng/ml) PSPB D 27 (ng/ml) ISG15 MX2 D 19 at D 32 147.0 ±3.8[a] (135) 6.8±0.5[a] (107) 0.39±0.08 [a] (74) 4.4±1.2 [a] 3.6±0.9 [a] (78) before D 33 143.6±10.9 [abA] (24) 10.2±0.9 [bA] (20) 1.27±0.13 [b] (19) 7.1±1.7 [ab] 6.4±1.1 [ab] (16) before D 33 128.4±3.9 [bB] (111) 12.1±0.6[bB] (82) 1.49±0.08 [c] (71) 10.4±1.5[b] 9.2±1.1 [b] (61) P-value 0.003===< 0.0001===< 0.0001===0.0004 < 0.0001

Means ± SEM are shown with number of cows evaluated for each measure done on D 32 and at all subsequent US examinations

(n). 576 Letters (a,b) within rows, between columns, indicates statistical difference (P < 0.05). 577 Capital letters (A, B) indicate a tendency (0.05 < P < 0.15) for difference between pregnant cows 578 with early contralateral CL regression (regression before D 33) vs. cows with no early 579 contralateral CL regression.===580 **Figure 1.** Protocol used with lactating cows. All cows were synchronized with the Double- 581 Ovsynch protocol before timed AI (TAI). All cows then had ovarian structures determined using 582 transrectal ultrasonography (US) and blood samples (BS) were taken at the indicated times 583 between D 5 and 68 of pregnancy. Pregnancy Check

(PCHK) was

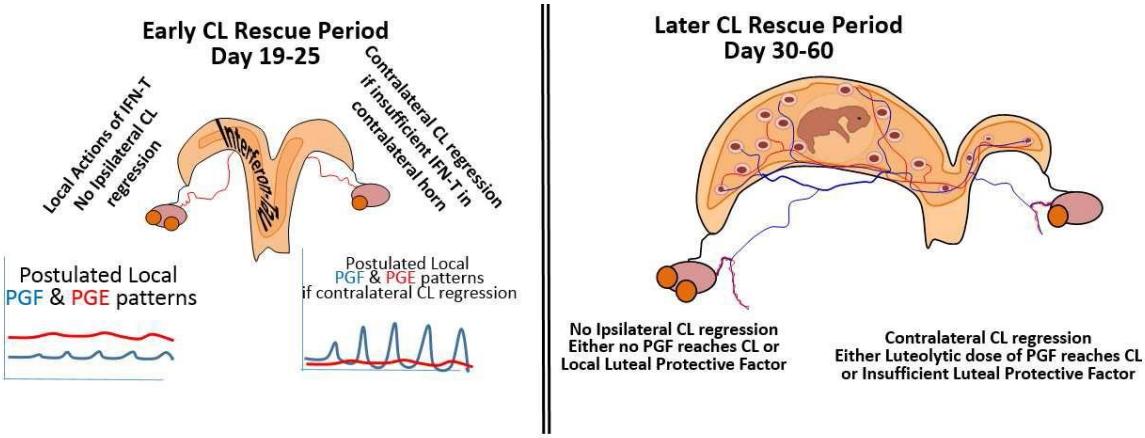

Figure 2. Distribution of contralateral CL regression in Experiment 1 for primiparous and multiparous lactating dairy cows.===595

Figure 3. Proposed physiological models for mechanisms involved in regression or

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maintenance of ipsilateral and contralateral CL regression during the early (D 19-25) andlater (D 30-60) crucial periods.===601

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Highlights for Baez et al.

Proposal of a new model for CL regression or maintenance during pregnancy based on timing of regression of accessory, contralateral CL in pregnant cows.

1. Accessory CL, contralateral to a pregnancy, mostly (66.2%) regressed by d75 of pregnancy.
2. Most (77.4%) accessory, contralateral CL regression occurred after d33 of pregnancy.
3. Early accessory CL regression (d19-25) occurred more in multiparous than primiparous cows.
4. A new model is proposed with two critical periods for CL regression/maintenance.