Pregnancy development from day 28 to 42 of gestation in *postpartum* Holstein cows that were either milked (lactating) or not milked (not lactating) after calving

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Abstract

The objective was to determine if lactation affects fetal and placental development from day 28 to 42 of gestation. *Bos taurus* Holstein cows were assigned to one of the two treatments immediately after parturition (lactating ($n=23$) or nonlactating (dried off immediately after calving; $n=20$)). Cows were inseminated at ~60 days *postpartum* with semen from a single ejaculate. Pregnant cows were slaughtered at 1 of 3 days of gestation (day 28, 35, or 42) and tissues were collected. The interval to first insemination, services per conception, and days to pregnancy were similar for lactating and nonlactating cows. Lactating cows had greater plasma GH and nonesterified fatty acids. Nonlactating cows had greater plasma glucose, insulin, and IGF1. There was no effect of lactation on plasma progesterone or estradiol concentrations. Lactation had a negative effect on the weight of the fetus and placenta (weights were less in lactating cows). Fetuses collected from cows that became pregnant after first insemination were heavier than fetuses collected from cows that became pregnant after second or third insemination. Pregnancy after first insemination was associated with greater blood glucose and IGF1 during the first 30 days *postpartum*. The conclusions were that lactation negatively affects the growth of fetal and placental tissues perhaps through a mechanism that involves hormones and metabolites that are affected by lactation. Fetal growth within cows conceiving at first insemination compared to second or third insemination was more rapid and was associated with greater blood glucose and IGF1 early *postpartum* (before day 30).

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Introduction

Physiological changes that support greater milk production may underlie the progressive decline in fertility experienced by dairy cows over the last half-century (Lucy 2003, Walsh *et al.* 2011). For example, lipid mobilization and nutrient partitioning in early lactation support greater lactation yield but also lead to lower body condition during the breeding period (Bauman & Currie 1980). In cattle, low body condition portends poor reproductive performance (Roche *et al.* 2009). Specific changes in hormone and metabolites that may be either decreased (e.g. glucose, insulin, and IGF1) or increased (e.g. nonesterified fatty acids (NEFA) and ketone bodies) during nutrient mobilization may provide the physiological link between weight loss, lower body condition, and poor reproductive performance *postpartum* (Leroy *et al.* 2008a, 2008b). Ovarian gametogenesis and steroidogenesis are changed during lactation and the observed changes (for example, absence of ovulation or the ovulation of poor-quality gametes) affect

fertility (Walsh *et al.* 2011). For those cows that ovulate a normal oocyte, suboptimal oviductal and uterine environment may provide a second barrier to the establishment of pregnancy (Walsh *et al.* 2011). The high metabolic rate associated with lactation impinges on the system as well. Steroids that are essential for the reproductive process may be more fully metabolized under conditions of high milk production and high dry matter intake (Wiltbank *et al.* 2006). Blood flow to the mammary gland increases *postpartum* so that nutrients such as glucose and NEFA that are used for milk synthesis are efficiently extracted from the circulation (Svennersten-Sjaunja & Olsson 2005). In some case these same nutrients that are depleted in blood during lactation are also required for normal function of reproductive tissues within the nonpregnant animal (Sutton-McDowall *et al.* 2010). During pregnancy, for example, glucose is required by the embryo/fetus and placenta for metabolic energy and the creation of biomass (Bell & Bauman 1997, Cetin & Alvino 2009, Purcell & Moley 2009).

Pregnancy loss occurs when a viable embryo dies before pregnancy term. The majority of pregnancy loss in dairy cows occurs during the first 2 months of pregnancy (Santos *et al.* 2004, Diskin & Morris 2008). There is an initial period of pregnancy loss between day 14 and 20 after fertilization when the elongating embryo is secreting interferon-tau for maternal recognition of pregnancy (Thatcher *et al.* 1995, Berg *et al.* 2010). A second major period of pregnancy loss occurs after this (up until day 60 of gestation). This latter period of pregnancy loss is perhaps more costly because the dam has invested more time in the pregnancy (Santos *et al.* 2004, Diskin & Morris 2008). Embryonic loss during this period appears to be more common in high milk-producing *postpartum* dairy cows than in lower milk-producing cows or nonlactating heifers (Santos *et al.* 2004, Diskin & Morris 2008). Although pregnancy loss in *postpartum* cows has been characterized and possible causal mechanisms have been proposed, few investigators have actually tested the effect of lactation itself on pregnancy development during this time.

We designed a study to investigate the direct effect of lactation on hormone and metabolic profiles as well as pregnancy development for cows that were first inseminated at ~60 days *postpartum*. Primiparous Holstein cows that calved were either milked twice daily (lactating group) or were dried off immediately after calving (nonlactating group). By using a contemporary group of cows that were not milked, we controlled for *postpartum* interval (identical for lactating and nonlactating) and eliminated possible confounding effects that arise when heifers (virgin reproductive tract; Sartori *et al.* 2004) or alternative sources of nonlactating cows (typically having long *postpartum* intervals; De la Sota *et al.* 1993) are used for comparison with early lactation cows. We retrospectively stratified the cows into whether they conceived or did not conceive at first insemination. Plasma hormone and metabolites were analyzed *postpartum*. Once pregnant, cows were slaughtered at 28, 35, or 42 days of pregnancy to assess the effect of lactation on the weight of the fetus, placenta, and corpus luteum (CL).

Results

BW, body condition, and milk production

There was an effect of treatment by week for BW ($P<0.001$). Lactating and nonlactating cows were similar for BW at week 1 *postpartum* (582 ± 13 and 576 ± 13 kg respectively). During the subsequent 3 week, lactating cows lost BW (549 ± 11 kg at week 4) whereas nonlactating cows maintained BW (573 ± 11 kg at week 4). Cows in both treatments gained BW afterward but the gain in BW was less for lactating (570 ± 12 kg at 12 week) compared to nonlactating (645 ± 12 kg at 12 week) cows.

There was an effect of treatment by week for body condition score (BCS; $P<0.001$; Fig. 1A). Lactating cows had a decrease in BCS from week 1 (2.9 ± 0.1) to week 4 (2.6 ± 0.1) and subsequently remained unchanged (2.7 ± 0.1 at week 12). Nonlactating cows were similar for BCS from week 1 (3.1 ± 0.1) to week 4 (3.1 ± 0.1) but then increased in BCS (3.8 ± 0.1 at week 12). There appeared to be greater BCS loss in lactating cows that did not conceive at first insemination (Fig. 1A). The treatment by status by week interaction was not significant in the full model ($P=0.68$) but the status by week interaction was significant ($P<0.02$) in an analysis that included lactating cows alone. There was a status by week interaction for milk production in lactating cows ($P<0.001$; Fig. 1B). Cows that did not become pregnant at first insemination produced less milk during the first 6 week *postpartum*. Milk production was equivalent thereafter and averaged 31 ± 1 kg/day for all cows at 12 week *postpartum*.

Figure 1 Least squares means and s.e.m. (bar) for BCS (A: 5-point scoring system; 1 (thin) to 5 (obese)) and milk production; (B: lactating cows only) for *Bos taurus* primiparous Holstein cows that were either lactating (Lact) or nonlactating (Nonlact; dried off immediately after calving) and were either pregnant or not pregnant to first insemination.

General reproductive characteristics

The interval from calving to first ovulation (based on a sustained increase in plasma progesterone) was ~10 days lesser in nonlactating compared to lactating cows (Table 1). The percentage of lactating cows that had not ovulated by the time of the first GnRH was 13 and 43% for cows that were pregnant or not pregnant after first insemination. All of the nonlactating cows had ovulated by the time of the first GnRH injection of the synchronization program. Days to first insemination (60 ± 1 days), first insemination conception rate (63%), second insemination conception rate (53%), services per conception (1.5 ± 0.1), and days from calving to conception (74 ± 4) were similar ($P > 0.10$) for lactating and nonlactating cows. The overall pregnancy rate before slaughter was 98% (42 of 43 cows) but two cows did not have a viable pregnancy (dead embryo found) at the time of slaughter (lactating, $n=1$; nonlactating, $n=1$; 40 out of 43 cows were truly pregnant, 93%). The one cow that never became pregnant was nonlactating. The maximum number of inseminations for any cow was three.

Plasma NEFA, glucose, GH, IGF1, and insulin concentrations

Data presented in this section are for day 0–60 *postpartum* unless stated otherwise. Plasma NEFA concentrations were affected by treatment ($P < 0.001$), day ($P < 0.001$), and treatment by day ($P < 0.001$; Fig. 2A). Lactating cows had greater plasma NEFA concentrations than nonlactating cows (436 ± 31 vs

189 ± 30 $\mu\text{Eq/l}$ respectively). Plasma NEFA declined over time in both treatments so that NEFA for lactating and nonlactating cows converged toward the end of the sampling period (after day 40). The effects of status (pregnant after first insemination or not pregnant after first insemination), status by treatment, status by day, and status by treatment by day were not significant ($P > 0.10$).

There was an effect of treatment on plasma glucose ($P < 0.001$; Fig. 2B). Lactating cows had lesser plasma glucose concentrations compared with nonlactating cows (65.7 ± 0.9 vs 77.4 ± 0.9 mg/dl for lactating and nonlactating cows). There was also a status by day *postpartum* interaction for plasma glucose ($P < 0.01$). On several days, particularly during the first month after calving, plasma glucose concentrations were greater in those cows that subsequently became pregnant at first insemination compared with those cows that were not pregnant after first insemination. When data were analyzed as two separate periods (day 0–30 or day 31–60), plasma glucose concentrations were greater for first insemination pregnant (status=1) than first insemination not pregnant cows (status=0) during period 1 (day 0–30; 72.9 ± 0.9 vs 68.8 ± 1.2 mg/dl for statuses 1 and 0 respectively; $P < 0.01$), but were similar for statuses 1 and 0 during period 2 (day 31–60; 72.4 ± 0.8 and 72.0 ± 1.1 mg/dl for statuses 1 and 0 respectively; $P > 0.10$).

There was an effect of treatment ($P < 0.01$), days *postpartum* ($P < 0.001$), and treatment by days *postpartum* ($P < 0.01$) on plasma GH concentrations. Lactating cows had greater plasma GH concentrations than nonlactating cows ($P < 0.002$; 14.9 ± 1.7 and 6.8 ± 1.7 ng/ml for lactating and nonlactating cows respectively; Fig. 2C). In both lactating and nonlactating cows, GH concentrations decreased with increasing days (12.9 ± 2.2 ng/ml on day 0– 6.7 ± 2.8 ng/ml on day 60). Plasma GH concentrations for cows that became pregnant after first insemination (status=1) were similar to plasma GH concentrations for cows that were not pregnant after first insemination (status=0). The effects of status by treatment, status by day, and status by treatment by day were not significant ($P > 0.10$).

There was an effect of treatment ($P < 0.001$), days *postpartum* ($P < 0.001$), and treatment by days *postpartum* ($P < 0.001$) for plasma IGF1 (Fig. 2D). Lactating cows had lesser plasma IGF1 concentrations than did nonlactating cows (115.0 ± 6.9 and 181.7 ± 6.9 ng/ml for lactating and nonlactating cows respectively). Regardless of treatment, plasma IGF1 concentrations increased with increasing days *postpartum* but this increase was more-rapid in nonlactating cows. There was an effect of status ($P < 0.017$) on plasma IGF1 concentrations because, regardless of treatment, cows that became pregnant at first insemination (status=1) had greater IGF1 concentrations than cows that did not become pregnant after first insemination (status=0) (lactating cows, 125.2 ± 7.7 and 104.7 ± 11.6 ng/ml for

Table 1 Least square means $\pm$ S.E.M. for measures of *postpartum* reproduction and fertility for primiparous *Bos taurus* Holstein cows that were either lactating or nonlactating (dried off immediately after calving).

Item	Treatment		$P <^a$
	Lactating	Nonlactating	
n	23	20	
Days to first ovulation (day) ^b	28.2 ± 2.8	18.3 ± 2.8	0.018
Days to first insemination (day)	60.2 ± 0.8	60.8 ± 0.9	NS
First insemination conception rate (%) ^c	69.6 (16/23)	55.0 (11/20)	NS
Second insemination conception rate (%) ^c	66.7 (4/6)	44.4 (4/9)	NS
Inseminations per conception	1.36 ± 0.17	1.68 ± 0.18	NS
Calving to conception (day)	69.2 ± 5.0	79.6 ± 5.4	NS
Pregnancy rate (%) ^d	100	95	NS
Number of corpora lutea at slaughter	1.2 ± 0.1	1.2 ± 0.1	NS
Cows with twin pregnancies (%)	8.7	15.8	NS

^a P value for the effect of treatment; NS, not significant, $P > 0.10$.

^bDefined as the sample day before the first sustained increase plasma progesterone above 0.5 ng/ml. ^cHundred times the number of pregnant cows divided by the number of inseminated cows. ^dHundred times the number of pregnant cows divided by the total number of lactating or nonlactating cows.

Figure 2 Least squares means and S.E.M. (bar) for plasma concentrations of (A) NEFA, (B) glucose, (C) GH, (D) IGF1, and (E) insulin during the first 60 days *postpartum* for *Bos taurus* primiparous Holstein cows that were either lactating (Lact) or nonlactating (Nonlact; dried off immediately after calving) and were either pregnant or not pregnant at first insemination. Symbols in panel A apply to all graphs.

statuses 1 and 0 cows respectively; nonlactating cows, 195.9 ± 9.2 and 167.4 ± 10.2 ng/ml for statuses 1 and 0 cows respectively). Data were subsequently analyzed for plasma IGF1 concentrations during periods 1 and 2 (0–30 and 31–60 days *postpartum* respectively). Cows that became pregnant after first insemination (status=1) had greater plasma IGF1 concentrations during period 1 than cows that failed to become pregnant after first insemination (status 1, 152.8 ± 7.0 ng/ml and status 0, 121.6 ± 9.1 ng/ml; $P < 0.01$). During period 2 (31–60 days *postpartum*), IGF1 concentrations only tended to differ for statuses 1 and 0 cows (167.8 ± 6.0 and 149.8 ± 7.7 ng/ml respectively; $P < 0.08$).

There was an effect of lactation on plasma insulin ($P < 0.001$; Fig. 2E). Lactating cows had lesser plasma insulin than nonlactating cows (6.0 ± 0.5 and

11.7 ± 0.5 ng/ml for lactating and nonlactating cows respectively). Neither the interactions of main effects with treatment nor interactions of main effects with status were significant for plasma insulin ($P > 0.10$).

Plasma progesterone and estradiol concentrations

Eleven lactating cows and 18 nonlactating cows had a luteal phase (basal plasma progesterone followed by a sustained elevation in progesterone) after the first $\text{PGF}_{2\alpha}$ injection (~ 25 days *postpartum*; luteal phase 1; Fig. 3). The reasons for excluding cows from the analysis of luteal phase 1 were that they were either anovular (six lactating and two nonlactating), they failed to regress the CL after the $\text{PGF}_{2\alpha}$ injection (five lactating and 0 nonlactating; progesterone > 1 ng/ml at 2 days after

Figure 3 Least squares means and S.E.M. (bar) for plasma concentrations of progesterone for *Bos taurus* primiparous Holstein cows that were either lactating or nonlactating (dried off immediately after calving) and were either pregnant or not pregnant at first insemination. Means are for the main effects of treatment (lactating or nonlactating) and status (pregnant or not pregnant at first insemination) during cycle 1 (luteal phase after first PGF_{2α} injection), cycle 2 (luteal phase after second PGF_{2α} injection), and cycle 3 (luteal phase after third PGF_{2α} injection when cows were inseminated).

PGF_{2α}) or they failed to ovulate after the PGF_{2α} injection (one lactating and 0 nonlactating; <1 ng/ml at 14 days after PGF_{2α}). As expected, there was an effect of day ($P<0.001$) on plasma progesterone concentrations during luteal phase 1. There was no effect of lactation, status, or their interactions on plasma progesterone during luteal phase 1.

Fifteen lactating cows and 15 nonlactating cows had a luteal phase after the second PGF_{2α} injection (~39 days postpartum; Fig. 3; luteal phase 2). The reasons for excluding cows from the analysis were that they were either anovular (five lactating and one nonlactating), they failed to regress the CL after the PGF_{2α} injection (three lactating and two nonlactating) or they failed to ovulate after the PGF_{2α} injection (0 lactating and two nonlactating). There was an effect of day ($P<0.001$) on plasma progesterone concentrations during luteal phase 2. There was no effect of lactation, status, or their interactions on plasma progesterone during luteal phase 2.

Twenty-two lactating and 18 nonlactating cows had a luteal phase after the third PGF_{2α} injection (the PGF_{2α} that immediately preceded timed artificial insemination (AI); luteal phase 3)). The reasons for excluding cows from the analysis were that they were either anovular at PGF_{2α} (one nonlactating), they failed to ovulate after the PGF_{2α} injection (one nonlactating), or they were inseminated before the PGF_{2α} injection (one lactating). Plasma progesterone concentrations were similar for lactating and nonlactating cows before and after the third PGF_{2α} injection (Fig. 3). As expected, there were

effects of status, day, and status by day ($P<0.001$). Cows that did not conceive at first insemination (status=0) underwent luteal regression toward the end of the sampling period, whereas pregnant cows (status=1) had a sustained elevations in progesterone. If only data from the mid-luteal phase (day 7–16) were included, then there was a tendency for an effect of status ($P<0.10$) because cows that became pregnant had greater plasma progesterone than cows that did not become pregnant (5.3 ± 0.3 and 4.5 ± 0.4 ng/ml respectively).

Plasma estradiol concentrations were greater ($P<0.001$) at 0 and 48 h after the third injection of PGF_{2α} for cows that became pregnant at the time of first AI compared with those that did not become pregnant (Fig. 4A). There was also an effect of time because plasma estradiol increased from 4.0 ± 0.4 to 6.8 ± 0.4 pg/ml from 0 to 48 h respectively. There was no effect of treatment on plasma estradiol after the third PGF_{2α} injection (Fig. 4B).

Pregnancy-associated glycoproteins

Plasma pregnancy-associated glycoprotein (PAG) concentrations were analyzed after insemination for cows that were known to be pregnant after first, second, or third insemination (Fig. 5). Three periods were analyzed separately (day 15–27; pregnant cows slaughtered on day 28, 35, or 42, all cows; day 29–34, pregnant cows slaughtered on day 35 or 42; and day 36–41, pregnant cows slaughtered on day 42). There was no

Figure 4 Least squares means and S.E.M. for plasma concentrations of estradiol at the time of the third PGF_{2α} injection (0 h; 3 days before first insemination) and 48 h later for *Bos taurus* primiparous Holstein cows that were either (A) pregnant or not pregnant at first insemination, or were either (B) lactating or nonlactating (dried off immediately after calving). Means are for the main effects of status (pregnant or not pregnant at first insemination) and treatment (lactating or not lactating).

effect of treatment or treatment by day interaction for PAG concentrations during any of the intervals tested. There was an effect of status for plasma PAG on day 15–27 ($P < 0.05$) and day 29–34 ($P < 0.09$) because cows that were pregnant after first insemination had greater plasma PAG concentrations when compared with cows that were pregnant after second or third insemination. Status was not significant for PAG concentrations from day 36 to 41 ($P > 0.10$).

Development of the CL, fetus, and placenta

There was an effect of day on CL weight ($P < 0.064$; Table 2) because the average weight of the CL increased from day 28 (6.2 ± 0.6 g) to day 42 (8.4 ± 0.6 g). There was no effect of treatment or status on CL weight or the number of CL on the ovary (1.2 ± 0.1 ; Table 1).

There was an effect of treatment ($P < 0.05$; Table 2), status ($P < 0.01$; Table 3), and day ($P < 0.001$) for fetal weight. The interactions of the main effects were not significant. The fetus grew larger from day 28 to 42, but that increase was greater in nonlactating compared with lactating cows (Table 2). There was also an effect of status because cows that conceived at first AI had fetuses of greater weight when compared with cows that conceived at second or third AI (Table 3). Fetal length was affected by day ($P < 0.001$) but not by the additional effects in the model. There was an effect of treatment ($P < 0.01$), day ($P < 0.001$), and a tendency for an effect of treatment by day ($P < 0.056$) on placental weight. After day 28, the placenta was heavier in nonlactating cows compared with the lactating cows. The effect of status was not significant for placental weight. Placental fluid volume was affected by treatment ($P < 0.024$; Table 2), day ($P < 0.001$; Table 2), and status ($P < 0.014$; Table 3). The placental fluid volume increased, as expected, from day 28 to 42 and nonlactating cows had greater placental volume than lactating cows. Cows that

conceived at first AI had greater placental volume than cows that conceived at second or third AI (effect of status; Table 3).

Twin pregnancies were found in two lactating and three nonlactating cows. Individual fetal weights for the twin pregnancies were: lactating day 28 pregnancy, 0.03 and 0.07 g; lactating day 35 pregnancy, 0.14 and 0.67 g; nonlactating day 35 pregnancy, 0.19 and 1.19 g; nonlactating day 35 pregnancy, 0.81 and 1.72 g; and nonlactating day 42 pregnancy, 1.51 and 1.87 g.

Discussion

The study described herein is one of the first of its kind to specifically address the direct effects of lactation on pregnancy development between day 28 and 42 in dairy cows. The first major finding from this study was that lactation led to slower pregnancy development (smaller fetuses and placenta and less placental fluid at a given day of pregnancy). The development of a placenta is an obvious requirement for the establishment of pregnancy but whether or not slower pregnancy development predisposes a lactating cow to embryonic loss is a question that was not specifically addressed in this trial. Pregnancy loss declines greatly after 60 days of pregnancy perhaps because the placentomes are fully formed and tenuous contacts between the uterus and placenta no longer exist (Santos *et al.* 2004, Diskin & Morris 2008). Before day 60, the trophoblast is loosely attached or is not attached to the uterus via the placentome (King *et al.* 1979, 1980). A slower developing fetus and placenta may be at risk because the fetal/placental unit spends more time in this loose attachment phase (after maternal recognition of pregnancy). This argument assumes, of course, that the smaller fetal/placental unit is at an earlier developmental

Figure 5 Least squares means and S.E.M. for plasma concentrations of PAG from day 15 to 42 of pregnancy for *Bos taurus* primiparous Holstein cows that were either lactating or nonlactating (dried off immediately after calving) and were either pregnant at first insemination or were pregnant at second or third insemination.

Table 2 Least square means $\pm$ S.E.M. for weight of the corpus luteum, and for fetal and placental measurements for primiparous *Bos taurus* Holstein cows that were either lactating or nonlactating (dried off immediately after calving) and had tissues collected on either day 28, 35, or 42 of pregnancy.

Item	Day of pregnancy			$P <^a$		
	28	35	42	Trt	Day	$T \times D$
CL wt (g)				NS	0.064	NS
Lactating	6.0 $\pm$ 1.0	7.9 $\pm$ 0.8	8.6 $\pm$ 0.8			
Nonlactating	6.5 $\pm$ 0.8	7.1 $\pm$ 1.1	8.1 $\pm$ 0.9			
Fetal weight (g)				0.049	0.001	NS
Lactating	0.10 $\pm$ 0.13	0.61 $\pm$ 0.10	1.64 $\pm$ 0.10			
Nonlactating	0.12 $\pm$ 0.10	0.79 $\pm$ 0.13	2.02 $\pm$ 0.11			
Fetal length (mm)				NS	0.001	NS
Lactating	7.2 $\pm$ 1.4	14.7 $\pm$ 1.1	24.8 $\pm$ 1.0			
Nonlactating	8.0 $\pm$ 1.1	16.7 $\pm$ 1.4	26.3 $\pm$ 1.2			
Placental weight (g)				0.005	0.001	0.056
Lactating	1.1 $\pm$ 1.9	8.3 $\pm$ 1.6	16.8 $\pm$ 1.4			
Nonlactating	1.2 $\pm$ 1.6	12.7 $\pm$ 2.0	25.0 $\pm$ 1.5			
Placental fluid (ml)				0.024	0.001	NS
Lactating	8.1 $\pm$ 11.6	52.5 $\pm$ 9.4	121.8 $\pm$ 8.6			
Nonlactating	11.1 $\pm$ 9.1	78.7 $\pm$ 11.6	152.7 $\pm$ 9.7			

^a P value for the effect of treatment (Trt), day of pregnancy (day), and the interaction of treatment and day of pregnancy ($T \times D$). NS, not significant, $P > 0.10$.

stage and not simply smaller and at the same developmental stage with equivalent placentome development. We did not perform a detailed developmental analysis of the fetus or placenta itself. Our general impression was that lighter fetal/placental units in lactating cows were less developed (earlier stage), but this will need to be confirmed in subsequent experiments.

We observed considerable plasticity in terms of pregnancy development in *postpartum* cows. We assumed that the development of the fetus and placenta would be largely similar for a given day of pregnancy. This was clearly not the case (an example of this is depicted in Fig. 6). Cattle at a similar day of pregnancy may have vastly different fetal and placental development. What constitutes a 'normal' range in development in the *postpartum* cow between day 28 and 42 is largely unknown because few slaughter studies have been completed. Whether or not these differences in terms of fetal and placental development have a consequence

in terms of pregnancy loss are unknown. Presumably the slower developing pregnancy is at risk but this assumption will need to be tested. In addition to large variation in single pregnancies (between cattle), we also found that twin pregnancies found in the same cow could be comprised of two similar sized fetuses (e.g. 1.51 and 1.87 g at day 42 in Cow 6) or one large and one small fetus (e.g. 0.14 and 0.67 g at day 35 in Cow 10). Apparently two fetuses can develop at entirely different rates even when they reside within the same uterus. Again, the consequences of these differences in development are not known, i.e. does the smaller fetus eventually die or does it catch-up developmentally to the larger fetus at some later time?

The results of this trial were also remarkable in terms of the treatment differences that were not found. For example, regardless of treatment (lactating or nonlactating), there was very high fertility (conception rate) for the cows enrolled in this trial ($> 50\%$ conception rate for inseminations that occurred at ~ 60 days *postpartum*).

Table 3 Least square means $\pm$ S.E.M. for fetal and placental measurements for primiparous *Bos taurus* lactating and nonlactating Holstein cows that had tissues collected on either day 28, 35, or 42 of pregnancy and were pregnant from either the first or second/third insemination.

Item	Day of slaughter			$P <^a$		
	28	35	42	Status	Day	$S \times D$
Fetal weight (g)				0.003	0.001	NS
First insemination	0.14 $\pm$ 0.10	0.91 $\pm$ 0.08	2.05 $\pm$ 0.10			
Second or third insemination	0.08 $\pm$ 0.13	0.49 $\pm$ 0.16	1.61 $\pm$ 0.11			
Placental weight (g)				NS	0.001	NS
First insemination	1.49 $\pm$ 1.40	11.0 $\pm$ 1.29	21.5 $\pm$ 1.39			
Second or third insemination	0.73 $\pm$ 2.10	10.0 $\pm$ 2.28	20.2 $\pm$ 1.57			
Placental fluid (ml)				0.014	0.001	NS
First insemination	14.1 $\pm$ 8.6	75.9 $\pm$ 7.2	155.2 $\pm$ 8.6			
Second or third insemination	5.1 $\pm$ 12.1	55.3 $\pm$ 14.1	119.3 $\pm$ 9.7			

^a P value for the effect of status, day of pregnancy (day), and the interaction of status and day of pregnancy ($S \times D$). NS, not significant, $P > 0.10$.

Figure 6 Postpartum (A) plasma progesterone and (B) PAG for two *Bos taurus* primiparous Holstein cows whose fetuses (C) were collected on day 35 of pregnancy (Cow 35, fetus on the left, lactating pregnant at second insemination, fetal wt=0.1 g, placental wt=6.6 g; Cow 24, fetus on the right, not lactating and pregnant at first insemination, fetal wt=1.2 g, placental wt=17.8 g). First insemination occurred at 61 days postpartum. Cow 24 was CIDR-treated because she did not have a corpus luteum at the time of first GnRH of the synchronization program. Cow 35 was not pregnant at first AI but she was not observed in estrus between day 80 and 85 so she was retreated with PGF on day 93 and reinseminated.

Two (one lactating and one nonlactating) of the 42 cows did experience embryonic loss as evidenced by a positive blood PAG test but fetal and placental tissue in the horn that appeared necrotic. Although fertility for the two treatments was the same, we did note the expected difference in interval to first ovulation with lactating cows ovulating later postpartum than nonlactating cows (Table 1). Although the interval to first ovulation was lengthened by lactation, there was no evidence for an effect of lactation on the weight of the CL (Table 2), blood progesterone concentrations (Fig. 3), or blood estradiol concentrations before insemination (Fig. 4B). Despite the lack of an observed effect of lactation, we did find that cows that were pregnant after first AI had greater blood estradiol concentrations preceding the first AI (Fig. 4A). This difference in estradiol implied greater functionality of the dominant follicle at the end of the synchronization program; an aspect previously related to fertility within timed AI programs (Perry *et al.* 2005). Progesterone after breeding was less during the mid-luteal phase in cows that did not become pregnant after the first AI (Fig. 3). Lesser progesterone in cows that failed to become pregnant after timed AI has been reported by other investigators as well (Wiltbank *et al.* 2010) and this may be related to the functionality of the

dominant follicle that we mentioned previously in this paragraph (lesser estradiol indicative of a less mature follicle during timed AI; Fig. 4A).

Our inability to detect progesterone and estradiol differences for lactating and nonlactating cows argues against the widely held theory that lactation causes a depression in blood steroid concentrations. Sangsritavong *et al.* (2002) found that lactating cows had greater metabolic clearance rate of progesterone than nonlactating cows. This difference was explained by greater feed intake in lactating cows. Sartori *et al.* (2004) showed that nulliparous heifers had greater steroid concentrations but smaller CL than lactating cows. Their conclusion was that poor reproduction in lactating cows was caused by lesser circulating steroid concentrations. The contrary results that we observed in terms of lactation effects on progesterone (Fig. 3) and estradiol (Fig. 4B) could be explained by the fact that the cows used in this trial were first parity and lower producing relative to multiparous cows. A similar study should be completed with multiparous cattle with greater milk production. The nonlactating cows also had greater BCS relative to the lactating cows and perhaps greater capacity to sequester steroids into body fat (thus affecting steroid clearance).

Plasma NEFA underwent a sustained period of elevated concentrations in lactating cows that did not achieve basal levels until ~40 days postpartum (Fig. 2A). In the nonlactating cow, NEFA concentrations were less but nonetheless elevated for ~15–20 days postpartum. Serum NEFA concentrations are correlated with follicular fluid NEFA concentrations (Leroy *et al.* 2005) and the elevated NEFA concentrations found in postpartum lactating dairy cows can negatively impact oocyte growth and quality (Leroy *et al.* 2008a). Nonetheless, we did not observe different NEFA profiles in cows that conceived or failed to conceive at first postpartum insemination. We did observe, however, an effect of first insemination status on plasma glucose concentrations. Regardless of whether cows were lactating or nonlactating, those that failed to conceive at first insemination had lesser plasma glucose during the first 30–40 days postpartum (Fig. 2B). After this time (>40 days postpartum), plasma glucose was largely the same for cows within the same treatment that conceived or failed to conceive at first insemination.

As a group, cows that failed to conceive at first insemination compared with those that conceived had lesser blood glucose concentrations (Fig. 2B) and lesser milk production (Fig. 1B). One possibility is that glucose availability was limiting the production of milk; a concept that was demonstrated when late lactation cows were treated with phlorizin to depress blood glucose concentrations (Bradford & Allen 2005, 2007). Perhaps depressed glucose availability not only affected the capacity of the cows to produce milk (presumably through availability of glucose for lactose synthesis) but

also in some manner had carry-over effects on their capacity to become pregnant after first insemination. This would most likely occur through the metabolic imprinting of reproductive tissues. The observation that greater milk production is associated with improved reproductive status has been made previously (Staples *et al.* 1990). The concept of metabolic imprinting of reproductive tissues has been presented as well (Britt 1992). The 'Britt hypothesis' states that early *postpartum* metabolism can exert carryover effects on the developing oocyte because the oocyte requires 60–80 days to fully develop within the preovulatory follicle. The oocyte that ovulates around the time of first insemination develops during the early *postpartum* period under adverse metabolic conditions. We would extend the Britt hypothesis to include the uterus and oviduct because these tissues are repaired through uterine involution that occurs primarily during the first 30 days after calving (Leslie 1983).

The possibility that glucose is a mediator of *postpartum* reproduction because it acts as a limiting substrate for both milk production and reproduction may be a new idea supported by these experimental findings. The lactating mammary gland is the primary glucose 'sink' in *postpartum* cows (Bell & Bauman 1997). Other tissues and cells function in the metabolic environment created by lactation. If the cow is deficient in terms of her blood glucose concentrations then she compromises her lactation. This state of inadequate blood glucose during lactation theoretically compromises the function of other tissues that depend on glucose. For example, glucose is an essential molecule for developing oocytes (Leroy *et al.* 2008b, Sutton-McDowall *et al.* 2010), for follicular granulosa and theca cells (Weber & La Barbera 1988), for the CL (Chase *et al.* 1992), and for the embryo (Sinclair *et al.* 2003) and fetus (Battaglia & Meschia 1978). Low blood glucose could potentially compromise a variety of essential processes in these cells and tissues.

Plasma GH concentrations were elevated in lactating cows (Fig. 2C) presumably because the somatotrophic axis remained uncoupled. The uncoupling of the somatotrophic axis at calving leads to a decrease in circulating concentrations of IGF1 that exists for at least 60 days *postpartum* (Fig. 2D). In the lactating cow, glucose is used for the production of lactose and also energy within the mammary gland. The demand for glucose during lactation leads to lesser glucose and lesser insulin concentrations during lactation (Fig. 2E). The lesser insulin theoretically prevents (or slows) the recoupling of the somatotrophic axis through its effects on GH receptor 1A (GHR1A; Butler *et al.* 2003, Lucy 2008). When cows were not milked, the somatotrophic axis recoupled within 10 days after calving (evidence by a plateau in plasma IGF1 within 10 days *postpartum*; Fig. 2D). In the absence of lactation, therefore, plasma IGF1 concentrations normalize rapidly in dairy cattle.

This rapid recoupling in nonlactating cows could perhaps be explained by the rapid normalization of insulin in *postpartum* cows that are not milked (Fig. 2E).

With respect to pregnancy status, plasma IGF1 was similar to plasma glucose because plasma IGF1 concentrations in cows that failed to conceive at first insemination were depressed, particularly during the first 30–40 days *postpartum*. As with glucose, the underlying physiology that dictates the relationship between IGF1 and reproductive function is incompletely understood. There are IGF receptors in the uterus and ovary and IGF1 is generally correlated with improved uterine/ovarian health through its actions as a growth factor in the reproductive tract (Velazquez *et al.* 2008). IGF1 has been shown to promote follicular growth and differentiation (Lucy 2000, Gong 2002, Leroy *et al.* 2008b). Additionally, low blood IGF1 concentrations are associated with a delay in the resumption of *postpartum* ovarian activity (Beam & Butler 1999), an observation that was made for lactating cows in this trial.

The second major finding from this work was that the development of the fetus was faster (greater fetal weight at a given day of gestation) and there was more fetal fluid in cows that conceived after first insemination when compared with cows that conceived after second or third insemination (Table 3). Differences in fetal development were apparently manifested as early as the third week after insemination as evidenced by greater PAG concentrations during the third and fourth weeks in cows that conceived after the first insemination (Fig. 5). We did observe different metabolic profiles for the respective classifications (pregnant or not pregnant to first insemination) but the observed differences only existed during the first 30 days *postpartum* and did not exist at the time of first insemination or immediately preceding slaughter. The observation made here imply that the early *postpartum* period (first 30 days after calving) imprints the reproductive tract to affect the likelihood of pregnancy at first insemination. The existing literature, of course, would support this concept in as much as numerous studies have reported risk factors for pregnancy at first insemination are manifested early *postpartum* (Dubuc *et al.* 2011). What is new here is that cows that become pregnant at a later insemination have a pregnancy that develops more slowly. Alleviating the origins of this compromised reproductive state could theoretically improve first insemination conception rate in dairy cows and perhaps improve pregnancy development in cows that fail to conceive at first insemination.

We measured PAG but only in cows that were known to be pregnant. The concentrations of PAG in the plasma were not affected by lactation but were affected by status (whether or not the pregnancy was derived from first insemination or second/third insemination). Cows that conceived at first AI had greater PAG than cows that conceived at second or third AI (Fig. 5) but this difference

only existed before day 35 of pregnancy. In addition to the results presented in Fig. 5 we found that larger fetuses produced greater amounts of PAG than the smaller fetuses (data not shown). When we examined the actual PAG concentrations in two day 35 fetuses with extremely different size we found numeric difference in PAG concentrations (Fig. 6). The PAG results (Fig. 5) supported the notion that cows that become pregnant at first insemination have larger pregnancies (Table 3). It may be possible to use plasma PAG concentrations to assess embryonic development; particularly during the first 35 days of pregnancy.

One limitation of this study was that the nonlactating cows had greater BW and BCS than the lactating cows. Cows were housed together in the same pen and fed the same diet. Keeping nonlactating cows in a separate pen and feeding a separate lower energy diet would have lowered BW and BCS but would have confounded pen and diet with treatment (lactating vs nonlactating). In this study, we opted to not to confound treatment and accepted the potential consequences that greater BW and BCS in nonlactating cows may have had on the results that we observed. There is an optimum BCS for reproduction in cattle that is centered at a BCS of 3.5 (Roche *et al.* 2009). Cows above and below this optimum have inferior reproduction. The nonlactating cows in this study had a BCS of 3.8 on week 12 (Fig. 1A). A caveat of this work, therefore, was the possibility that some of the effects that we observed were a consequence of the high BCS in nonlactating cows and not a consequence of the physiological state of lactation.

In summary, lactation did not affect the establishment of pregnancy but instead negatively affected the weight of the placenta and fetus between day 28 and 42 in primiparous Holstein cows. The mechanisms through which this occurred may involve large differences in hormone (GH, IGF1, and insulin) and metabolite (glucose and NEFA) concentrations that existed between lactating and nonlactating cattle. Although lactation affected the interval to first ovulation, we did not observe an effect of lactation on plasma progesterone during three *postpartum* estrous cycles or an effect of lactation on plasma estradiol before insemination. One surprising result was that cows that were pregnant after the first insemination had larger conceptuses than cows that were pregnant after a second or third insemination. There was an association between pregnancy at first insemination and greater plasma glucose and IGF1 concentrations during the first 30 days *postpartum*. The implication is that hormonal and metabolic imprinting of the reproductive tract occurs during the early *postpartum* period. This imprinting can affect the likelihood of pregnancy at first insemination and fetal development for cows that become pregnant after a second or third insemination. Whether or not slower fetal development leads to embryonic loss is an important question that will need to be addressed in future studies.

Materials and Methods

Animals and sample collection

The project was approved by the University of Missouri-Columbia Animal Care and Use Committee. Cows were housed at the University of Missouri-Columbia Foremost Dairy Farm. Pregnant *Bos taurus* Holstein heifers ($n=43$) were randomly assigned to one to two treatment groups: lactating ($n=23$) and not lactating (nonlactating; $n=20$). After calving, the lactating group was milked twice daily. The nonlactating group was not milked after calving (i.e. dried off immediately, never milked). Both treatment groups were housed together in a two-row freestall barn. The freestalls were sand bedded and there was a solid concrete floor. A diet consisting of corn silage, alfalfa haylage, grass hay, concentrates, and minerals was provided with equal access via bunk feeding for both treatment groups.

Blood samples were collected from cows thrice weekly following parturition (Monday, Wednesday, and Friday mornings, ~2 h after feeding) and continued until cows were slaughtered for tissue collection. Blood was collected by venipuncture of the median caudal vein or artery into tubes containing 100 μ l of a 15% solution of EDTA (K_3) (Tyco Healthcare Group, Mansfield, MA, USA). Plasma was collected by centrifugation of whole blood (1500 g for 10 min) and was stored at -20°C in polypropylene tubes until analyzed for hormone and metabolite concentrations. Cows were weighed and scored for body condition on Monday mornings. BCS (1=thin to 5=obese) was the average score between two trained individuals that used a common scorecard.

Cows were synchronized with the a 'Presynch-Ovsynch' protocol (PGF_{2 α} (5 ml Lutalyse i.m., 25 mg dinoprost tromethamine; Pfizer Animal Health, New York, NY, USA), wait 14 days, PGF_{2 α} , wait 12 days, GnRH (2 ml Factrel i.m., 100 μ g gonadorelin hydrochloride; Pfizer Animal Health), wait 7 days, PGF_{2 α} , wait 56 h, GnRH, wait 16 h, AI) for first AI between 56 and 62 days *postpartum*. Ovarian morphology (CL and follicles ≥ 5 mm) was recorded weekly by using transrectal ultrasonography (Aloka 500-SSD equipped with 7.5 MHz transducer, Aloka, Tokyo, Japan) on Monday mornings. Cows that did not have a CL at the time of the first administration of GnRH were treated intravaginally with progesterone (CIDR device, Pfizer Animal Health) for 7 days (removed at the time of PGF_{2 α} injection; three lactating and two nonlactating cows were treated with CIDR). All cows were inseminated from a single ejaculate of a high fertility sire (Regancrest-RB Miles, 7HO7037; Select Sires, Plain City, OH, USA). Cows that did not conceive at first service were subsequently reinseminated 12 h after an observed return to estrus by using semen from the same sire and ejaculate used for first AI. Nonpregnant cows that were not observed in estrus were resynchronized by using an Ovsynch program for timed AI (GnRH injection, wait 7 days; PGF_{2 α} injection, wait 56 h; and GnRH injection, wait 16 h, AI).

Cows were diagnosed as pregnant by using transrectal ultrasonography and by using a rapid ELISA for PAGs (Green *et al.* 2009). Pregnant cows were slaughtered at 1 of 3 days of pregnancy (28 days (lactating, $n=6$; nonlactating, $n=6$; 105 ± 28 days *postpartum*), 35 days (lactating, $n=8$; nonlactating,

$n=6$; 108 ± 29 days *postpartum*), or 42 days (lactating, $n=8$; nonlactating, $n=6$; 114 ± 13 days *postpartum*). Three cows were not included in the analyses because they either did not have a viable pregnancy (dead embryo found) at the time of slaughter (lactating, $n=1$; nonlactating, $n=1$) or failed to become pregnant (nonlactating, $n=1$). The slaughter was done immediately after the morning milking. Cows were stunned by using a pneumatic captive bolt and then killed by exsanguination. The tract was collected and the weights of the gravid ovary, gravid CL, nongravid ovary, nongravid CL (if present), fetus, and placenta were measured. The volume of placental fluid and the crown–rump length of the fetus were also recorded.

Plasma hormone assays

Validated RIAs were used to determine the plasma concentrations of GH (Gorewit 1981), insulin (Kolath *et al.* 2006), and IGF1 (Rhoads *et al.* 2008) in the thrice weekly samples from calving up until the day of first AI (~ 60 days *postpartum*). Two RIAs for GH were completed with an intraassay CV of 8.5% and an interassay CV of 5.9%. Four RIAs for insulin were completed with an intraassay CV of 7.2% and an interassay CV of 11.2%. Two RIAs for IGF1 were completed with an intraassay CV of 6.9% and an interassay CV of 3.8%.

Plasma progesterone and estradiol concentrations were analyzed by validated RIA (Kirby *et al.* 1997). The plasma progesterone concentrations were measured in the thrice weekly samples from calving up until 28 days after first AI. Ten progesterone RIAs were completed with an interassay CV of 6.4% and the intraassay CV of 8.6%. Plasma estradiol concentrations were measured at 0 and 2 days relative to the final PGF_{2 α} injection of the synchronization program (i.e. -3 and -1 days before first timed AI). All estradiol determinations were done within a single assay with an intraassay CV of 9.4%.

PAG assay

Plasma PAG concentrations were measured in plasma samples of pregnant cows from day 15 until day of slaughter (day 28, 35, or 42). The antibodies and procedures for the ELISA were described by Green *et al.* (2005). The intraassay CV of was 7.0%.

Plasma metabolite assays

Plasma glucose concentrations were measured by using a commercially available colorimetric assay kit (Pointe Scientific, Inc., Canton, MI, USA) and by following the manufacturer's protocol. Plasma NEFA concentrations were measured by using the Wako NEFA-HR(2) kit (Wako Diagnostics, Richmond, VA, USA). The glucose and NEFA concentrations of the unknown samples run in duplicate were estimated from standard curves within the respective assays.

Statistical analyses

Cows were assigned to treatment at calving (lactating or nonlactating) and were subsequently assigned to first

insemination pregnancy 'status' based on whether they conceived or failed to conceive at first insemination (pregnant or not pregnant to first insemination; coded as 1 or 0 respectively for 'status' in the statistical model). Cows were sampled on repeated *postpartum* day for plasma hormone and metabolites (GH, IGF1, insulin, NEFA, and glucose). The statistical model used for these data include the effects of treatment, status, treatment by status, day, treatment by day, status by day, and treatment by status by day. The mixed models procedure (PROC MIXED) of SAS (SAS Inst., Cary, NC, USA) was used with day defined as the repeated variable and cow nested within treatment and status defined as a random effect.

Plasma progesterone concentrations were used to determine the interval to first ovulation (defined as the sample day before the first sustained increase plasma progesterone above 0.5 ng/ml). The interval to ovulation (day) was analyzed by using PROC GLM with a model that included treatment, status, and treatment by status. Plasma progesterone concentrations were analyzed by using repeated measures to determine effects of treatment and status during three *postpartum* periods. A cow was included in this analysis if she responded to an injection of PGF_{2 α} by having luteolysis and ovulation (indicated by a rise in progesterone within 1 week after PGF_{2 α}). Anovular cows were not included in these series of analyses. The first luteal phase (luteal phase 1) began 48 h after the first injection of PGF_{2 α} within the synchronization program (day 21–27 *postpartum*) and ended 14 days later when the second injection of PGF_{2 α} was administered (day 35–41 *postpartum*). The second phase (luteal phase 2) began 48 h after the second injection of PGF_{2 α} and ended 19 days later when the third injection of PGF_{2 α} was administered (day 50–64 *postpartum*). The third and final phase (luteal phase 3) included the period before luteolysis and began 2 days before the third and final injection of PGF_{2 α} and ended 28 days later. The progesterone data were analyzed for each phase by using PROC MIXED and a model that was identical to that used for the hormone and metabolite data described above (i.e. model included treatment, status, and day as well as interactions with cow nested within treatment and status defined as random and day defined as repeated). Plasma estradiol was only analyzed at two times (0 and 2 days after the third and final PGF_{2 α} ; immediately preceding timed AI) and a model identical to that used for progesterone was employed.

The concentrations of PAG after AI were only assessed in samples collected from cows that were pregnant (i.e. the sample analyzed may or may not have been from a cow following first insemination; second or third insemination samples were used if the cow became pregnant after second or third insemination respectively). The PAG data were analyzed by using PROC MIXED and a model that included treatment, status, and day as well as interactions. Cow nested within treatment and status was defined as random and day was defined as repeated.

Data collected at slaughter (size and weight of the embryo, placental weight, CL weight, etc.) were analyzed by using PROC GLM. The statistical model included the effects of treatment, status, day, and interactions. Data are reported as least square means $\pm$ S.E.M. A result was viewed as statistically significant if $P < 0.05$. A tendency for statistical significance was $0.05 < P < 0.10$. Comparisons with $P > 0.10$ were reported as not significant (NS).

Declaration of interest

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

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