Conceptus development and transcriptome at preimplantation stages in lactating dairy cows of distinct genetic groups and estrous cyclic statuses

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ABSTRACT

The objectives were to compare development and transcriptome of preimplantation conceptuses 15 d after synchronized ovulation and artificial insemination (AI) according to the genetic background of the cow and estrous cyclicity at the initiation of the synchronization program. On d 39 ± 3 postpartum, Holstein cows that were anovular (HA; $n = 10$), Holstein cows that were estrous cyclic (HC; $n = 25$), and Jersey/Holstein crossbred cows that were estrous cyclic (CC; $n = 25$) were randomly selected in a grazing herd and subjected to the Ovsynch protocol. All cows were inseminated on d 49 ± 3 postpartum, which was considered study d 0. Blood was sampled and analyzed for concentrations of progesterone, estradiol, insulin, and insulin-like growth factor 1 (IGF-1) on study d -10 , -3 , -1 , 7, and 15 relative to AI. On study d 15, uteri were flushed and recovered fluid had IFN- τ concentrations measured and subjected to metabolomic analysis. Morphology of the recovered conceptuses was evaluated, and mRNA was extracted and subjected to transcriptome microarray analysis. Compared with HC, CC presented greater concentrations of progesterone and estradiol in plasma, with corpora lutea and preovulatory follicles of similar size. Conceptuses from CC were larger, tended to secrete greater amounts of IFN- τ , and had greater transcript expression of peroxisome proliferator-activated receptor gamma (PPAR γ), an important transcription factor that coordinates lipid metabolism and elongation at preimplantation development. In addition, pregnant

CC had greater concentrations of anandamide in the uterine flush, which might be important for elongation of the conceptus and early implantation. Conceptuses from HA were also longer and secreted greater amounts of IFN- τ than conceptuses from HC, likely because of the distinct progesterone profiles before and after AI. Nonetheless, anovular cows had reduced concentrations of IGF-1 in plasma, and their conceptuses presented remarkable transcriptomic differences. Some of the altered transcripts suggest that conceptus cells from anovular cows might be under greater cellular stress and presented markers suggesting increased apoptosis and autophagy, which could lead to increased mortality after d 15 of development. Estrous cyclicity had more impact on transcriptome of bovine conceptus than genetic background, and the developmental changes observed during the preimplantation period might be linked to differences in fertility among groups.

Key words: conceptus development, crossbreeding, dairy cow, estrous cyclicity

INTRODUCTION

Anovulation is a normal and temporary physiological condition of most mammals including dairy cows during early postpartum. It is characterized by lack of regular estrous cycles and ovulation, although follicle growth is still present (Wiltbank et al., 2002). Time for resumption of estrous cyclicity postpartum varies among cows and is directly associated with nutritional balance in the first weeks of lactation (Butler, 2003). As consequence, 18 to 43% of dairy cows remain anovular at the end of the voluntary waiting period, constituting an important problem in achieving adequate reproductive performance in dairy herds (Rhodes et al., 2003; Santos et al., 2009).

Adoption of timed AI programs maximizes submission to AI and lessens the problem of anovular cows reducing reproductive performance. Nevertheless, pregnancy per AI (P/AI) of anovular cows after synchro-

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nized estrus or ovulation is reduced compared with that of estrous cyclic herd mates (McDougall et al., 2005; Santos et al., 2009). Thus, anovulation impairs pregnancy rate and reproductive performance even when timed AI programs are implemented. It has been shown that reduced fertility results from impaired capability to establish or maintain pregnancy (Bisinotto et al., 2010). Nonetheless, timing and biology of the events leading to impaired zygote development are unknown.

Genetics is an important component influencing several traits related to production, health, and reproduction in dairy cows (Egger-Danner et al., 2015). One strategy that has been used in commercial herds is crossbreeding of complementary pure breeds not only to obtain a desired phenotype, but also to reduce inbreeding and maximize heterosis (Weigel and Barlass, 2003). The crossbreeding between Holstein and Jersey cattle, for instance, is common and aims to combine the high milk volume yield of Holsteins with the high solids content in milk of Jerseys. In addition, improvements in reproduction have been reported with this crossbreeding strategy (Heins et al., 2008; Ribeiro et al., 2011), and might be a result of the heterosis generated by the combination of the 2 distinct genetic backgrounds. Nonetheless, the effect of crossbreeding or heterosis on early developmental biology remains largely unknown.

We hypothesized that both anovulation at the onset of synchronization of ovulation protocol and crossbreeding affect conceptus development in dairy cows and would reflect in differences in fertility. The first study aimed to evaluate fertility data to further document the effect of estrous cyclicity status at the beginning of the synchronization program and genetic background of the cow on reproductive performance at first AI postpartum. The second study aimed to compare preimplantation conceptus development to the initial elongation stages and their transcriptome in lactating cows with distinct combinations of genetic background and estrous cyclicity to elucidate some of the biology involved in their fertility differences.

MATERIALS AND METHODS

All procedures involving cows were approved by the animal care and use committee of the University of Florida (Gainesville).

Study 1: Fertility Data

This study followed a prospective cohort design. Data comprising breed of cow, estrous cyclicity at the initiation of the synchronization program, and P/AI at the first AI postpartum were collected in 3 seasonally calving farms. Data were collected in 2 breeding seasons on

farms A and B, and in 1 breeding season on farm C. Dairies were located in north central Florida and used similar cow genetics and management practices. The herds were composed of Holstein (H), Jersey (J), and Holstein/Jersey crossbreds, mostly F₁ (50H:50J) and F₂ (25H:75J or 75H:25J) generations of crossbreeding between Holstein and Jersey genetics. A total of 3,196 lactating cows (737 Holsteins, 1,825 Holstein/Jersey crossbreds, and 634 Jerseys) were enrolled in the study. Primiparous cows represented 31% and multiparous cows represented 69% of the study animals. The mean ($\pm$ SD) lactation number was 2.2 ± 1.1 . The different genetic groups were represented in all herds and breeding seasons, managed in a pasture-based system, and supplemented with concentrates (Ribeiro et al., 2011). Cows were milked twice a day and the average milk production per cow across the different herds and breeds of the cows was approximately 6,000 kg/lactation.

Cows in all herds were enrolled in a preplanned breeding season and received timed AI on the first day of breeding (study d 0). For that, the estrous cycle was presynchronized and then cows were subjected to synchronization of ovulation programs. The presynchronization consisted of 2 injections of PGF_{2 α} (Lutalyse sterile solution; 5 mg/mL of dinoprost tromethamine, Zoetis, Florham Park, NJ) administered 14 d apart. The synchronization protocol was initiated 10 to 12 d after the second PGF_{2 α} of the presynchronization and consisted of GnRH (Cystorelin; 50 μ g/mL of gonadorelin diacetate tetrahydrate, Merial Ltd., Iselin, NJ) on d -8, PGF_{2 α} on d -3 and -2, and GnRH + AI on d 0. Cows were artificially inseminated using sires with North American genetics, 20 Jerseys, 9 Holsteins, and 4 Swedish Red sires. On d -3, tailheads were painted using oil-based tail paint for detection of estrus based on rubbed off tails on the day of AI. On the day of the first GnRH injection of the timed AI program, blood was sampled and analyzed for concentration of progesterone. Cows with progesterone concentration ≥ 1.0 ng/mL were considered estrous cyclic and those with progesterone concentration < 1.0 ng/mL were considered anovular. Mean and median DIM at first AI were 117 and 102, respectively.

Pregnancy was diagnosed in all cows on d 30 via ultrasonography of the uterus and its contents, and was characterized by visualization of an embryo with heartbeat. Cows diagnosed as pregnant on d 30 were reexamined by transrectal palpation 35 d later. Pregnancy per AI was calculated as the number of pregnant cows on d 30 and 65 after an insemination divided by the total number of cows inseminated. Pregnancy loss was calculated as the number of cows that lost pregnancy between gestation d 30 and 65 divided by the number of pregnant cows on d 30.

Study 2: Preimplantation Conceptus Development

Cows and Management. This study was conducted in a seasonally calving grazing dairy located in north central Florida with the same genetics and general management described above for study 1. The farm milked approximately 1,200 cows twice daily and the rolling herd average for milk yield during the study period was 6,500 kg/cow.

Study Design. This study followed a prospective cohort study design. Four weekly cohorts of purebred Holstein and crossbred Holstein/Jersey cows had their ovaries examined by ultrasonography (Easy Scan, BCF Technology, Livingston, UK) using a 7.5-MHz linear transducer on d 29 and 39 ± 3 postpartum. Presence of a corpus luteum (CL) in at least 1 of the 2 ultrasonographic examinations characterized an estrous cyclic cow, whereas lack of CL in both examinations characterized an anovular cow. Three groups were then formed (Figure 1): Holstein anovular cows (HA), Holstein cyclic cows (HC), and crossbred cyclic cows (CC). Sixty lactating cows were used in the study (HA = 10; HC = 25; CC = 25). Cows were enrolled in 4 consecutive weeks, and random selection of cows presenting the predefined conditions was used.

Synchronization of the Estrous Cycle and Ovulation. All cows received an injection of 25 mg of PGF_{2α} on d 29 ± 3 postpartum. Ten days later,

cows were enrolled in an Ovsynch protocol. For the 2 cyclic groups, only cows with a functional CL when the Ovsynch started were used. Cows received an injection of 100 µg of GnRH on d 39 ± 3 postpartum, an injection of 25 mg of PGF_{2α} on d 46 ± 3 postpartum, and a final injection of 100 µg of GnRH on d 48 ± 3 postpartum, 56 h after the PGF_{2α} injection. All cows were inseminated using a single Holstein sire on d 49 ± 3 postpartum.

Samples and Data Collection. Ovaries were evaluated by ultrasonography concurrent with blood sampling on d -10 (a.m.), -3 (a.m.), -1 (p.m.), 7 (a.m.), and 15 (a.m.) relative to AI. Follicle and CL diameters and ovulatory responses to hormonal treatments were evaluated using an ultrasound equipped with a 7.5-MHz linear-array transducer. Blood was sampled by puncture of the coccygeal vessels into evacuated tubes containing K₂ EDTA (Becton, Dickinson, Franklin Lakes, NJ). Samples were placed immediately in ice and centrifuged at $2,000 \times g$ for 15 min, and aliquots of plasma were frozen at -25°C for later analyses. All activities with cows were performed after the morning milking, except for those on d -1, which were performed after the evening milking.

On d 15, the uterine horn ipsilateral to the CL was transcervically flushed for conceptus collection. Epidural anesthesia was used and the vulva and vagina were thoroughly cleansed. Initially, 20 mL of PBS (0.01 M

Figure 1. Diagram of activities for study 2. Ultrasonographic examination of the ovaries was used to determine the estrous cyclic status. Ovarian follicle and corpus luteum (CL) dynamics were evaluated by ultrasound. Uteri were flushed on d 15 after AI for recovery of conceptuses and uterine fluid, and endometrial tissue was collected for biopsy. Blood samples were collected for evaluation of concentration of progesterone, estradiol, IGF-1, and insulin in plasma. BS = blood sample; US = ultrasonography of ovaries.

phosphate buffer and 0.154 M sodium chloride, pH 7.4) with 0.1% (vol/vol) of polyvinyl alcohol (**PBS+PVA**) solution was infused using a 60-mL plastic syringe (Becton, Dickinson) connected to a 20 to 24 French silicone-coated latex Foley catheter (Bard, Covington, GA). The fluid infused was recovered into the same syringe used for infusion and then examined in a Petri dish under stereoscope for location of conceptus tissue. Up to 5 additional flushings were performed if no conceptus tissue was recovered. The recovered conceptuses were washed in fresh drops of PBS+PVA, had their length measured, and were snap-frozen in liquid N₂ and later stored at -80°C until nucleic acid extraction. Conceptuses <5 mm in length were classified as ovoid, whereas those >5 mm long were classified as elongated. The uterine fluid of the first flushing was placed in conical tubes in ice and later centrifuged at $2,000 \times g$ at 4°C for 15 min. The supernatant was collected, homogenized, aliquoted, and stored at -80°C for later analyses of fluid composition.

Transcriptome Analysis of Conceptus Cells.

Nucleic acids, mRNA and genomic DNA from conceptus cells were extracted using the AllPrep DNA/RNA kit (Qiagen, Germantown, MD) according to manufacturer instructions. Quality and integrity of purified mRNA were evaluated using Nanodrop (Thermo Fisher Scientific, Waltham, MA) and Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA) analyses. A subsample of only elongated conceptuses from each group (6 HC, 6 HA, and 6 CC) with ratio of absorbance at 260 to 280 nm between 1.95 and 2.1, and with RNA integrity number >7.5 were selected randomly within each experimental group and subjected to transcriptome analysis using the GeneChip Bovine Genome Array (Affymetrix, Santa Clara, CA) at the Interdisciplinary Center for Biotechnology Research Gene Expression and Genotyping Core at University of Florida (Gainesville). All microarray sample preparation used the GeneChip 3' In Vitro Transcription Express kit (Affymetrix), and reactions were performed following the manufacturer's protocols. Briefly, cDNA was synthesized from 200 ng of total RNA and template for in vitro transcription during which a biotin-modified nucleotide was incorporated. The biotin-labeled amplified RNA was then purified and fragmented. Samples were hybridized with rotation at 45°C for 16 h to the Affymetrix GeneChip Bovine arrays. The arrays were washed and stained with the reagents supplied in GeneChip Hybridization Wash and Stain kit (Affymetrix) on an Affymetrix Fluidics Station 450, and scanned with a GeneChip 7G Scanner (Affymetrix).

Concentration of IFN- τ in the Uterine Flushing. Concentrations of IFN- τ in the uterine fluid

samples were determined based on their ability to prevent vesicular stomatitis virus (**VSV**)-induced lysis of Madin-Darby bovine kidney (**MDBK**) cells and using recombinant hIFN- α (100 IU/ μL ; EMD Biosciences Inc., Gibbstown, NJ) as standard (Rodina et al., 2009). Briefly, 100 μL of Dulbecco's modified Eagle medium (**DMEM**; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (**FBS**; HyClone, Logan, UT) were pipetted into each well of 96-well plates. Then, 50 μL of either standard or uterine fluid sample was added to the first well of each row and serial 1:3 dilutions were performed until the last well of the row. The standard was evaluated in every plate and each sample was assayed in duplicate. Fifty microliters of MDBK cell preparation was then added to each well and incubated at 37°C in 5% CO₂ for 24 h. Medium was then removed and cells were challenged with VSV by adding 50 μL of VSV solution and incubating at 37°C in 5% CO₂ for 1 h. The VSV solution was then removed and 100 μL of DMEM with 10% FBS was added for additional 20 h of incubation at 37°C in 5% CO₂. Medium was then removed and cells were fixed with 70% ethanol and stained with 0.5% gentian violet. Inhibition of VSV-induced lysis of MDBK cells was then assessed visually and compared with standard activity. Concentration was then calculated based on previously described activity of bovine IFN- τ (8.03×10^8 IU/mg; Rodina et al., 2009). The sensitivity of the assay was 1 pg/mL and the intra- and inter-assay coefficients of variation were, respectively, 1.0 and 1.7%.

Metabolomics of Uterine Fluid. Histotroph composition in pregnant cows was evaluated by metabolomics of uterine fluid. A total of 18 uterine fluid samples from pregnant cows (6 HA, 6 HC, and 6 CC) were subjected to liquid chromatography and mass spectrophotometry (**LC-MS**) in the Southeast Center for Integrated Metabolomics at the University of Florida (Gainesville). Uterine fluid samples used for metabolomics were from the same flushings that recovered conceptuses used for transcriptome of conceptus cells.

Analyses were performed on a Thermo Q-Exactive High Resolution Mass Spectrometer coupled with a Dionex UHPLC and autosampler (Thermo Fisher Scientific Inc., Waltham, MA). The instrument runs both positive and negative ion modes with heated electrospray ionization. Mass resolution was 70,000 at m/z 200 with mass accuracy of <5 ppm in positive mode and <10 ppm in negative mode. Separation of metabolites was achieved on an Ace C18-PFP column (100×2.1 mm, 2 μm ; Advanced Chromatography Technologies, Aberdeen, UK) with 0.1% formic acid in water as mobile phase A and acetonitrile as mobile phase B with a column temperature of 25°C .

Sample preparation was performed by mixing 100 μL of each thawed uterine fluid sample with 20 μL of internal standard mix (creatine-D3, L-leucine-D10, L-tryptophan-D3, and caffeine-D3) and 800 μL of 8:1:1 acetonitrile:methanol:acetone solution. Samples were then vortexed for 10 min and cooled in a refrigerator for 30 min. The tubes were centrifuged at $20,000 \times g$ for 10 min to pellet the protein. The supernatant was then transferred to a new tube and dried with N_2 . The dried material was reconstituted by adding 100 μL of 0.1% formic acid in LC-MS grade water. Flow rate was set to 350 $\mu\text{L}/\text{min}$ with a total run time of 20 min. A total of 2 μL of each sample was injected for positive ion mode analysis and 4 μL was injected for negative ion mode analysis. The LC-MS data were processed using MZmine 2 software (Pluskal et al., 2010) for peak picking and alignment, and for generation of intensity tables for statistical analyses. An in-house-curated retention time metabolite library was first used to identify metabolites. We then used additional external databases such as the human metabolome database (www.hmdb.ca) to expand potential identifications.

Hormone Assays. The concentration of progesterone in plasma was evaluated on samples collected on d -10, -3, -1, 7, and 15 relative to AI; estradiol was evaluated on samples from d -3 and -1; and IGF-1 and insulin were evaluated on samples from d 7 and 15. Concentrations of progesterone were determined by RIA using a commercial kit (Coat-a-Count; Siemens Healthcare Diagnostics, Los Angeles, CA). Only cows with low progesterone concentration ($<1 \text{ ng/mL}$) on d -1 and high progesterone concentration ($\geq 1 \text{ ng/mL}$) on d 7 and 15 remained in the final data set of the study. Concentrations of estradiol were evaluated using a commercial kit (Estradiol Double Antibody, Siemens Healthcare Diagnostics) and adjustments described by Ribeiro et al. (2012). Concentrations of total IGF-1 were determined by a commercial ELISA kit (Quantikine ELISA Human IGF-1 Immunoassay, R&D Systems Inc., Minneapolis, MN) designed for human IGF-1 but with 100% cross-reactivity with bovine IGF-1. Concentrations of insulin were determined using a commercial bovine ELISA kit (Mercodia Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden). At least 2 control samples of known hormone concentrations were included multiple times in all assays.

BCS and Diseases Postpartum. Cows were scored for body condition on a 1 to 5 scale with increments of 0.25 units (1 = emaciated, 5 = obese; Ferguson et al., 1994) at calving and 35 d postpartum. From calving until the end of the study, cows were monitored for the diagnosis of clinical diseases, including metritis, mastitis, and digestive, respiratory, and locomotor problems.

Statistical Analyses

Except for the microarray data, statistical analyses were performed using the GLIMMIX procedure of SAS (version 9.3; SAS/STAT, SAS Institute Inc., Cary, NC) and fitting adequate data distribution. Continuous variables were analyzed by ANOVA, and binary variables were analyzed by logistic regression. For study 1, the statistical models included the effect of breed, estrous cyclicity, the interaction between breed and estrous cyclicity, farm, calendar year, parity, DIM at AI, and BCS at AI. For study 2, the statistical models included the effect of group as HC, HA, and CC. For repeated measures, models included the effect of time, the interaction between group and time, and the random effect of cow nested within group. The covariance structure that resulted in the smallest Bayesian information criterion was selected. Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.15$ were considered a tendency.

Microarray data were analyzed using Bioconductor packages (www.bioconductor.org) in R version 3.1.1 (The R Foundation for Statistical Computing Platform; R Core Team, 2014; <https://www.r-project.org/foundation/>). Data were preprocessed using Gene Chip Robust Multi-Array function (Wu et al., 2004). The Limma package (Smyth, 2005) was then used to fit a linear model and to adjust variances by empirical Bayes adjustment according to Smyth (2004). Moderate *t*-test was performed for pairwise comparisons among breed-cyclicity groups and probability values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate (Benjamini and Hochberg, 1995). Adjustment for multiple group comparisons was performed by using the Global method within the *decideTests* function in Limma. Differently expressed genes were considered when adjusted $P < 0.05$ and fold change ≥ 1.5 . Networks, functional and causal analyses were performed using Qiagen's Ingenuity Pathway Analysis software (IPA, Qiagen, Redwood City, CA; www.qiagen.com/ingenuity). Emphasis was given to canonical pathways, predicted upstream regulators and predicted downstream effects with a z-score (**ZS**) $< \text{or} > 2.0$ and overlap *P*-value (**OP**) < 0.05 . Information regarding algorithms and statistical methods used in causal analysis of IPA were described by Krämer et al. (2014). The final lists of differently expressed transcripts among breed-cyclicity groups were compared with the list of transcripts that have expression modified during elongation of the bovine conceptus reported by Ribeiro et al. (2016). Because the present study used only elongated conceptuses for transcriptome analysis, the results were compared with the list of transcripts

for which expression changed during the tubular to filamentous transition.

Statistical analyses of metabolomics data were performed using the web server MetaboAnalyst 2.0 (www.metaboanalyst.ca; Xia et al., 2012). Analyses for positive and negative ion modes were run separately using the same approach. A table matrix of m/z peak intensities with samples in columns and features in rows were generated for each ion mode and imported to MetaboAnalyst 2.0. Features with 50% or more missing data were excluded from analyses. The remaining missing values were replaced by half of the minimum value in the original data. Preanalysis filtering was then performed based on the interquartile range to remove variables that are unlikely to be of use when modeling the data. In total, 25% of the variables were removed at this step considering the default approach used for data sets with 500 to 1,000 variables. Data were then normalized by the sum method, log-transformed, and scaled by the auto-scaling method. Statistical significances among groups (HC vs. HA, HC vs. CC, and CC vs. HA) were determined by ANOVA with Fisher's least significant difference post hoc adjustment. Orthogonal contrast was used to further explore the effect of anovulation (HC + CC vs. HA). Significance was declared when $P \leq 0.05$.

RESULTS

Study 1: Fertility Data

The prevalence of estrous cyclic cows in the study population was 82.9% (2,650/3,196), and it was affected ($P < 0.01$) by breed of the cow (Figure 2A). A greater proportion of crossbred cows were estrous cyclic compared with Holstein and Jersey (Figure 2A). In addition, the proportion of estrous cyclic cows tended ($P < 0.09$) to be greater for Jersey compared with Holstein.

The proportion of cows pregnant on d 30 after AI was influenced ($P < 0.01$) by estrous cyclicity at the initiation of synchronization program and tended ($P = 0.08$) to be influenced by breed of the cow (Figure 2B). Compared with estrous cyclic cows, anovular cows had a smaller ($P < 0.01$) P/AI on d 30 (33.9 vs. 56.3%; adjusted odds ratio = 0.40; 95% CI = 0.32 to 0.49). Holstein cows had reduced ($P = 0.03$) P/AI on d 30 compared with crossbred cows (41.5 vs. 48.2%; adjusted odds ratio = 0.76; 95% CI = 0.60 to 0.97), but neither differed from Jersey cows (44.9%). Within estrous cyclic cows, crossbred cows had greater ($P < 0.04$) P/AI on d 30 compared with Holsteins and Jerseys (61.7 vs. 50.6 vs. 56.4%, respectively; Figure 2B). No differences among genetic groups were observed within anovular cows, and the P/AI averaged 33.9%. The effects of

breed of the cow and estrous cyclicity at the initiation of the synchronization program on P/AI on d 30 were the same for P/AI on d 65. Estrous expression at AI was associated ($P < 0.01$) with P/AI (Figure 2C), and an interaction ($P < 0.01$) between estrous cyclicity and detection of estrus at AI was observed. The reduction in P/AI in anovular cows was only observed in those not detected in estrus at AI. Moreover, anovular cows that did not express estrus tended ($P = 0.06$) to have greater incidence of pregnancy loss between 30 and 65 d of gestation than cyclic cows or anovular cows that displayed estrus (Figure 2C).

Study 2: Preimplantation Conceptus Development

Reproductive Outcomes. As expected, concentrations of progesterone in plasma on the days of injections of the first GnRH and PGF_{2α} of the Ovsynch were lower ($P < 0.01$) for HA compared with HC and CC (Table 1). Although no difference in concentration of progesterone in plasma was observed between HC and CC on the day of the first GnRH, CC had greater ($P = 0.02$) concentrations of progesterone on the day of PGF_{2α}. An interaction ($P < 0.01$) between group and day of sampling was also observed (Table 1).

Diameter of the dominant follicle on the days of the PGF_{2α} and second GnRH of the Ovsynch protocol was greater ($P < 0.02$) for HA compared with HC or CC, and no differences were observed between the latter 2 groups (Table 1). There was an interaction ($P < 0.01$) between group and day of sampling for concentrations of estradiol in plasma. On the day of PGF_{2α}, HA had greater ($P < 0.02$) concentrations of estradiol than HC and CC; however, on the day of second GnRH, CC had greater ($P < 0.01$) concentrations of estradiol than HC and HA. Expression of estrus at AI was similar among the 3 groups and averaged 25.0% (Table 1).

Progesterone concentration in plasma on the day of the second GnRH of the Ovsynch protocol was similar among the 3 groups. Nonetheless, the concentrations of progesterone on d 7 and 15 after AI were smaller ($P < 0.05$) for HC compared with the other 2 groups (Table 1). Holstein anovular cows had larger ($P < 0.05$) luteal tissue volume on d 7 and 15 than HC cows. We detected no difference in luteal tissue volume between HC and CC.

Pregnancy per AI on d 15 based on detection of IFN- τ on uterine fluid was not affected by group, although CC approached a tendency ($P = 0.12$) to have greater P/AI compared with HC. Conceptuses tended ($P = 0.08$) to be longer for HA and CC groups compared with HC (Table 1). Conceptus length was smaller ($P = 0.01$) for HC compared with HA or CC, in part because HA and CC had only elongated conceptuses, whereas HC

had 27.3% ovoid conceptuses. Elongated conceptuses selected randomly for the microarray analysis, however, maintained similar differences in conceptus size as observed in the experimental population (Table 1). The concentration of IFN- τ in the uterine fluid was greater

($P < 0.01$) for HA compared with HC and tended ($P = 0.08$) to be greater for CC compared with HC. No differences were observed between HA and CC.

Incidence of clinical diseases was similar among groups (Table 2). Body condition score at calving and

Figure 2. Reproductive outcomes from study 1: fertility data compilation. Data comprise information from 3,196 lactating cows (737 Holstein, 1,825 Holstein/Jersey crossbred, and 634 Jersey cows) subjected to synchronization of the estrous cycle and timed AI. (A) Proportion of estrous cyclic cows at the initiation of synchronization program according to the breed of the cow; (B) proportion of pregnant cows on d 30 after the first AI postpartum according to breed of the cows and estrous cyclic status at the initiation of the synchronization program; (C) proportion of pregnant cows on d 30 and 65 after first AI postpartum according to estrous cyclic status at the initiation of the synchronization program and expression of estrus at AI. Different letters above bars within a series represent statistical differences: $^{a,b,c}P \leq 0.05$; $^{A,B}P = 0.06$. Graphic bars represent the adjusted means and the error bars represent the SEM.

on d 39 postpartum, however, was smaller ($P < 0.01$) for HA compared with HC and CC, and the latter 2 groups did not differ (Table 2). Concentration of IGF-1 in plasma was smaller ($P < 0.01$) for HA compared with HC, but neither group differed from CC (Table 2). On the other hand, the concentration of insulin in plasma was lesser ($P < 0.01$) for CC compared with HC, but neither group differed from HA (Table 2).

Transcriptome of Conceptus Cells. The comparison of HA with HC resulted in 500 transcripts differently expressed (Table 3; Supplementary Table S1A and Supplementary Figure S1A; <http://dx.doi.org/10.3168/jds.2015-10315>). Of those, 262 were upregulated and 238 were downregulated in HA. Moreover, 87 out of 500 transcripts were related to the transition from tubular to filamentous conceptus (Ribeiro et al., 2016). Of those 87 transcripts, 60 were upregulated and 27 were downregulated in HA compared with HC.

The main molecular and cellular functions associated with the differently expressed genes were molecular transport (35 transcripts; $OP < 0.04$), lipid metabolism (24 transcripts; $OP < 0.04$), and small molecule biochemistry (59 transcripts; $OP < 0.04$). The main physiological system development and function were organismal development (46 transcripts; $OP < 0.04$), skeletal and muscular system development and function (22 transcripts; $OP < 0.04$), and embryonic development (43 transcripts; $OP < 0.04$). The main diseases and disorders were connective tissue disorders (16 transcripts; $OP < 0.04$), organismal injury and abnormalities (249 transcripts; $OP < 0.04$), and cancer (248 transcripts; $OP < 0.04$).

Among these categories, specific functional annotations with predicted increased activity included invasion of cancer cell lines ($OP = 2.3E-02$, $ZS = 2.2$), β -oxidation of lipid ($OP = 1.4E-02$, $ZS = 2.2$), and

Table 1. Ovarian dynamics, concentration of steroid hormones, pregnancy per AI, conceptus length, and concentration of IFN- τ in the uterine flush fluid in lactating cows with synchronized ovulation and uteruses flushed on d 15 after AI

Item	Group ¹			P-value
	HA	HC	CC	
Progesterone prior luteolysis,* ng/mL				<0.01
at first GnRH of Ovsynch	0.09 $\pm$ 0.73 ^b	3.72 $\pm$ 0.45 ^a	3.86 $\pm$ 0.46 ^a	
at PGF _{2α} of Ovsynch	2.23 $\pm$ 0.73 ^c	7.99 $\pm$ 0.43 ^b	9.42 $\pm$ 0.44 ^a	
Diameter of dominant follicle, mm				<0.01
at PGF _{2α} of Ovsynch	16.3 $\pm$ 0.99 ^a	13.2 $\pm$ 0.62 ^b	13.2 $\pm$ 0.62 ^b	
at second GnRH of Ovsynch	23.5 $\pm$ 0.99 ^a	20.7 $\pm$ 0.62 ^b	19.1 $\pm$ 0.62 ^b	
Estradiol concentration,* pg/mL				0.11
at PGF _{2α} of Ovsynch	3.08 $\pm$ 0.75 ^a	0.77 $\pm$ 0.47 ^b	0.90 $\pm$ 0.47 ^b	
at second GnRH of Ovsynch	4.83 $\pm$ 0.71 ^b	5.39 $\pm$ 0.45 ^b	7.11 $\pm$ 0.45 ^a	
Expression of estrus at AI, % (no./no.)	40.0 (4/10)	20.0 (5/25)	24.0 (6/25)	0.48
Progesterone after luteolysis, ng/mL				0.02
at second GnRH of Ovsynch	0.12 $\pm$ 0.39	0.28 $\pm$ 0.25	0.25 $\pm$ 0.25	
at d 7 after AI	4.08 $\pm$ 0.39 ^a	3.03 $\pm$ 0.25 ^b	4.02 $\pm$ 0.25 ^a	
at d 15 after AI	7.61 $\pm$ 0.39 ^a	6.67 $\pm$ 0.25 ^b	7.73 $\pm$ 0.25 ^a	
Luteal tissue volume, cm ³				0.02
at d 7 after AI	12.6 $\pm$ 1.26 ^a	7.7 $\pm$ 0.80 ^b	9.4 $\pm$ 0.80 ^b	
at d 15 after AI	12.4 $\pm$ 1.26 ^a	9.4 $\pm$ 0.80 ^b	11.0 $\pm$ 0.80 ^{ab}	
Pregnancy based on IFN- τ , ² % (no./no.)	70.0 (7/10)	64.0 (16/25)	84.0 (21/25)	0.28
Ovoid conceptus, ³ % (no./no.)	0.0 (0/0) ^b	27.3 (3/16) ^a	0.0 (0/0) ^b	0.05
Conceptus length, mm				
All recovered intact	47.8 $\pm$ 8.75 ^a	9.4 $\pm$ 5.83 ^b	25.3 $\pm$ 8.75 ^a	0.01
Used for microarray analysis	58.0 $\pm$ 9.12 ^a	15.0 $\pm$ 10.53 ^b	25.3 $\pm$ 9.12 ^a	0.04
IFN- τ , ⁴ pg/mL (95% CI)	2,830 ^a (762–10,511)	149 ^{b,B} (63–356)	430 ^{b,A} (202–917)	<0.01

^{a-c}Values with different lowercase superscripts in the same row differ ($P \leq 0.05$).

^{A,B}Values with different uppercase superscripts in the same row differ ($P \leq 0.10$).

¹HA = Holstein anovular; HC = Holstein cyclic; CC = crossbred cyclic. Estrous cyclicity was determined based on the presence of a corpus luteum in 1 of 2 ultrasound examinations of the ovaries 10 d apart, with the second examination on the day of the first GnRH of the Ovsynch protocol.

²Cows with detectable amounts of IFN- τ in the antiviral assay were considered pregnant. Data presented for continuous variables are LSM $\pm$ SEM, except for the concentration of IFN- τ , from which the 95% CI back-transformation from the logarithm distribution are reported.

³Conceptuses with length <5 mm.

⁴Concentration of IFN- τ in uterine flushing of pregnant cows. Cows with no detectable IFN- τ were classified as nonpregnant and removed from this analysis.

*Interaction ($P \leq 0.05$) between group and day.

Table 2. Incidence of clinical diseases postpartum, BCS postpartum, and plasma concentrations of IGF-1 and insulin in lactating dairy cows subjected to synchronized ovulation and AI on d 49 ± 3 postpartum¹

Item	Group ²			P-value
	HA	HC	CC	
Clinical diseases postpartum, % (no./no.)	20.0 (2/10)	16.0 (4/25)	20.0 (5/25)	0.93
BCS, 1–5 scale				<0.01
at calving	2.85 ± 0.07 ^b	3.08 ± 0.05 ^a	3.16 ± 0.05 ^a	
at d 39 ± 3 postpartum	2.73 ± 0.07 ^b	2.93 ± 0.05 ^a	2.97 ± 0.05 ^a	
Metabolic hormones, ³ ng/mL				
IGF-1	41.3 ± 3.81 ^b	54.3 ± 2.41 ^a	49.6 ± 2.41 ^{ab}	0.02
Insulin	0.35 ± 0.05 ^{ab}	0.41 ± 0.03 ^a	0.27 ± 0.03 ^b	<0.01

^{a,b}Values with different superscripts in the same row differ ($P < 0.05$).

¹Data presented for BCS and metabolic hormone concentrations in plasma are LSM ± SEM.

²HA = Holstein anovular; HC = Holstein cyclic; CC = crossbred cyclic. Estrous cyclicity was determined based on the presence of a corpus luteum in 1 of 2 ultrasound examinations of the ovaries 10 d apart, with the second examination on the day of the first GnRH of the Ovsynch protocol.

³There were no interactions between group and day of blood sampling for IGF-1 and insulin.

apoptosis of neuroglia ($OP = 3.1E-02$, $ZS = 2.0$). No specific function was significantly predicted ($ZS < -2.0$) with decreased function. Potential upstream regulators of the differently expressed genes included prolactin ($OP = 3.5E-02$, $ZS = 2.4$), peroxisome proliferator-activated receptor gamma (PPARG; $OP = 3.6E-03$, $ZS = 2.3$), kruppel-like factor 15 (KLF15; $OP = 3.5E-03$, $ZS = 2.2$), small ubiquitin-like modifier 2 ($OP = 3.2E-03$, $ZS = 2.0$), and small ubiquitin-like modifier 3 ($OP = 3.2E-03$, $ZS = 2.0$) with predicted increased activity; enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase (EHHADH; $OP = 6.7E-06$, $ZS = -2.2$) and 17 β -hydroxysteroid dehydrogenase 4 (HSD17B4; $OP = 4.8E-05$, $ZS = -2.2$) with predicted decreased activity.

The most significant canonical pathways included urea cycle ($OP = 5.9E-04$; transcripts: *ARG2*, *ASL*, *ASS1*), myo-inositol biosynthesis ($OP = 2.9E-03$; transcripts: *IMPA1*, *IMPA2*), and autophagy ($OP = 4.6E-03$; transcripts: *ATG4A*, *ATG4D*, *LAMP1*, *MAP1LC3B*, *VPS18*). The top 10 upregulated genes based

on fold change and having HC as reference group were *CD36*, *CDKN1C*, *C15H11orf96*, *ASS1*, *IGFBP7*, *PAG2*, *GALM*, *PPARG*, *PRUNE2*, and *EVL*, listed in decreasing order of absolute value in fold change. The top 10 downregulated genes based on fold change and having HC as reference were *AQP3*, *SLC38A11*, *FOLR1*, *PDGFRA*, *RNPC3*, *JAM2*, *TTC14*, *PPIF*, *INPP4B*, and *DIS3*, listed in decreasing order of absolute value in fold change.

Comparison of CC against HC resulted in only 42 transcripts differently expressed (Table 3; Supplementary Table S1B and Supplementary Figure S1A; <http://dx.doi.org/10.3168/jds.2015-10315>). From those transcripts, 10 were upregulated and 32 were downregulated in CC. Moreover, 4 out of 42 transcripts were related to the transition from tubular to filamentous conceptus reported by Ribeiro et al. (2016). From those 4 transcripts, 1 that had expression increased during elongation (*PPARG*) was upregulated in CC compared with HC. The other 3 whose expression decreased dur-

Table 3. Number of differently expressed transcripts in elongated conceptus cells

Item	Group ¹		
	HA	HC	CC
Number of elongated conceptus used	6	6	6
Number of differently expressed transcripts			
Upregulated	262	Reference	10
Downregulated	238	Reference	32
Total	500	Reference	42
Related to tubular/filamentous transition ²	87	Reference	4
Unrelated to tubular/filamentous transition ²	413	Reference	38

¹HA = Holstein anovular; HC = Holstein cyclic; CC = crossbred cyclic. Estrous cyclicity was determined based on the presence of a corpus luteum in 1 of 2 ultrasound examinations of the ovaries 10 d apart, with the second examination on the day of the first GnRH of the Ovsynch protocol.

²Contrasted with results published by Ribeiro et al. (2016), in which the transcriptome of tubular and filamentous conceptuses was compared.

ing elongation (*AOX1*, *F2R*, and *IL20RB*) were downregulated in CC compared with HC.

The few transcripts differentially expressed between CC and HC were related to the following molecular and cellular functions: cellular movement (5 transcripts; $OP < 0.05$), cell cycle (2 transcripts; $OP < 0.05$), and cell death and survival (4 transcripts; $OP < 0.05$). The only potential upstream regulator with predicted activity was LPS ($OP = 8.3E-03$, $ZS = -2.0$) and its activity was predicted to be reduced. The most significant canonical pathways involved with those genes were reelin signaling in neurons ($OP = 6.2E-03$; transcripts: *ARHGEF3*, *NDEL1*), guanosine nucleotides degradation III ($OP = 1.2E-02$; transcript: *AOX1*), and urate biosynthesis/inosine5'-phosphate degradation ($OP = 1.5E-02$; transcript: *AOX1*). The top 10 downregulated genes based on fold change and having HC as reference were *RNPC3*, *SLC13A2*, *SLC39A8*, *NR3C2*, *AOX1*, *GPRC5C*, *IL20RB*, *ARHGEF3*, *F2R*, and *PRR5L*, listed in decreasing order of absolute value in fold change. The top 8 and only annotated genes that were upregulated based on fold change and having HC as reference were *PPARG*, *BOLA-N*, *NDEL1*, *CCNY*, *RBM47*, *C1H8orf42*, *RPGRIP1*, and *KAT2B*, listed in decreasing order of absolute value in fold change.

The comparison of anovular (HA) against estrous cyclic (HC + CC) cows resulted in 333 transcripts differently expressed (Supplementary Table S1C; <http://dx.doi.org/10.3168/jds.2015-10315>). Of those, 192 were upregulated and 141 were downregulated in conceptuses from anovular cows. Moreover, 85 out of 333 transcripts were related to the transition from tubular to filamentous conceptus reported by Ribeiro et al. (2016). From those 85 transcripts, 61 whose expression increased during elongation were upregulated in HA compared with estrous cyclic cows. The other 24 whose expression decreased during elongation were downregulated in HA compared with estrous cyclic cows.

The main molecular and cellular functions associated with the differently expressed genes were cellular development (35 transcripts; $OP < 0.04$), energy production (10 transcripts; $OP < 0.04$), and lipid metabolism (27 transcripts; $OP < 0.04$). The main physiological system, development and function, included cardiovascular system development and function (24 transcripts; $OP < 0.03$), connective tissue development and function (24 transcripts; $OP < 0.03$), and organ morphology (31 transcripts; $OP < 0.03$). The main diseases and disorders were cancer (84 transcripts; $OP < 0.03$), organismal injury and abnormalities (96 transcripts; $OP < 0.03$), and renal and urological disease (46 transcripts; $OP < 0.03$). Among these categories, specific function annotations with predicted increased activity included invasion of cancer cell lines ($OP = 3.8E-03$,

$ZS = 2.2$), transport of lipid ($OP = 8.1E-03$, $ZS = 2.2$), and oxidation of lipid ($OP = 5.6E-03$, $ZS = 2.1$). Specific function annotations with predicted decreased activity included concentration of phospholipid ($OP = 1.9E-02$, $ZS = -2.0$) and hyperplasia of endocrine gland ($OP = 5.4E-03$, $ZS = -2.0$). Potential upstream regulators of the differently expressed genes included CCAAT/enhancer binding protein alpha (CEBPA; $OP = 5.9E-03$, $ZS = 2.5$), KLF15 ($OP = 6.2E-04$, $ZS = 2.1$), and PPARG ($OP = 8.1E-04$, $ZS = 2.1$) with predicted increased activity; mitogen-activated protein kinase 4 ($OP = 1.8E-02$, $ZS = -2.4$), insulin-induced gene 1 ($OP = 2.4E-02$, $ZS = -2.2$), EHHADH ($OP = 3.9E-05$, $ZS = -2.0$), and HSD17B4 ($OP = 1.7E-04$, $ZS = -2.0$) with predicted decreased activity.

The most significant canonical pathways included urea cycle ($OP = 1.9E-04$; transcripts: *ARG2*, *ASL*, *ASS1*), myo-inositol biosynthesis ($OP = 1.3E-03$; transcripts: *IMPA1*, *IMPA2*), and superpathway of citrulline metabolism ($OP = 1.9E-03$; transcripts: *ARG2*, *ASL*, *ASS1*). The top 10 upregulated genes based on fold change and having estrous cyclic as reference group were *CD36*, *CDKN1C*, *C15H11orf96*, *IGFBP7*, *ASS1*, *PAG2*, *PRUNE2*, *HES1*, *EFNA5*, and *CAND2*, listed in decreasing order of absolute value in fold change. The top 10 downregulated genes based on fold change and having estrous cyclic as reference were *AQP3*, *SLC38A11*, *FOLR1*, *PDGFRA*, *JAM2*, *RNPC3*, *INPP4B*, *TTC14*, *PPIF*, and *PHF17*, listed in decreasing order of absolute value in fold change.

Using the same list of differently expressed transcripts comparing anovular against estrous cyclic, we removed the 85 transcripts previously described to be related to conceptus elongation from tubular to filamentous transition (Ribeiro et al., 2016) and reran the functional analyses to search for differences in transcriptome and associated cell biology not directly related to elongation. This analysis comprised 248 transcripts differently expressed between anovular (HA) and estrous cyclic (HC + CC). From those transcripts, 131 were upregulated and 117 were downregulated in conceptuses from anovular cows.

The main molecular and cellular functions associated with this set of genes were cellular assembly and organization (29 transcripts; $OP < 0.04$), cellular function and maintenance (32 transcripts; $OP < 0.04$), and lipid metabolism (27 transcripts; $OP < 0.04$). Among these categories, specific function annotations with predicted increased activity included invasion of tumor cell lines ($OP = 2.8E-02$, $ZS = 2.3$), and invasion of prostate cancer cell lines ($OP = 1.1E-03$, $ZS = 2.2$). Specific function annotations with predicted decreased activity included glucose tolerance ($OP = 2.4E-02$, $ZS = -2.0$). Potential upstream regulators of the differently

expressed genes included choriogonadotropin ($OP = 3.9E-02$, $ZS = 2.6$) and CEBPA ($OP = 3.6E-02$, $ZS = 2.1$), both with predicted increased activity in HA compared with estrous cyclic cows.

The most significant canonical pathways included myo-inositol biosynthesis ($OP = 8.2E-04$; transcripts: *IMPA1*, *IMPA2*), p53 signaling ($OP = 9.9E-04$; transcripts: *AKT2*, *CASP6*, *FAS*, *GSK3B*, *SERPINE2*, *SFN*), autophagy ($OP = 2.6E-03$; transcripts: *ATG4A*, *ATG4D*, *LAMP1*, *VPS18*), pentose phosphate pathway (nonoxidative branch) ($OP = 4.0E-03$; transcripts: *RPE*, *RPIA*), G β gamma signaling ($OP = 5.4E-03$; transcripts: *AKT2*, *CAV2*, *GNA15*, *GNAS*, *GNG10*), DNA damage-induced 14-3-3 signaling ($OP = 1.4E-02$; transcripts: *AKT2*, *GSK3B*, *SFN*, *TUBA4A*, *TUBB2B*), signaling by Rho family GTPases ($OP = 2.4E-02$; transcripts: *ARHGEF5*, *CDC42EP3*, *GNA15*, *GNAS*, *GNG10*, *PIP5K1A*, *SEPT8*), Wnt/ β -catenin signaling ($OP = 2.7E-02$; transcripts: *AKT2*, *GJA1*, *GSK3B*, *TGFBR3*, *WNT2B*). The top 10 upregulated genes based on fold change and having estrous cyclic as reference were *CD36*, *EFNA5*, *CAND2*, *KRT6A*, *TPM2*, *TMC6*, *HEYL*, *NR2F2*, *PODXL*, and *IRS1*, listed in decreasing order of absolute value in fold change. The top 10 downregulated genes based on fold change and having estrous cyclic as reference were *FOLR1*, *RNPC3*, *TTC14*, *PHF17*, *TUBA4A*, *NR3C2*, *MARCKS*, *CDC42EP3*, *TSPAN5*, and *QTRT1* listed in decreasing order of absolute value in fold change.

Metabolomics of Uterine Fluid. In the negative ion mode, 28 features in the uterine flushing were identified to have different abundances among the 3 groups (Table 4; Supplementary Figure S1B; <http://dx.doi.org/10.3168/jds.2015-10315>). Peak intensities from 12 features differed between HC and HA, of which 7 had increased and 5 decreased abundance in the HA group. Another 15 features differed between HC and CC, of which 8 had increased and 7 decreased abundance in the CC group. All features identified in negative ion mode are listed in Supplementary Table S2A (<http://dx.doi.org/10.3168/jds.2015-10315>) with the mean value of peak intensities and standard error of the mean for each experimental group.

In the positive ion mode, 24 features in the uterine flushing were identified to have different abundances among the 3 groups (Table 5; Supplementary Figure S1B; <http://dx.doi.org/10.3168/jds.2015-10315>). Peak intensities from 10 features differed between HC and HA and all were increased in the HA group. Another 10 features differed between HC and CC, of which 5 had increased and 5 decreased abundance in the CC group. All features identified in positive ion mode are listed in Supplementary Table S2B (<http://dx.doi.org/10.3168/jds.2015-10315>) with the mean value of peak intensities

and standard error of the mean for each experimental group.

DISCUSSION

The fertility data from the field study presented reinforce that Holstein/Jersey crossbred cows have greater P/AI compared with Holstein cows in grazing systems, as previously reported (Ribeiro et al., 2011). In agreement with data on P/AI, the study evaluating conceptus development showed advantages for CC compared with HC. Crossbred cows presented advanced conceptus development and there was a tendency for greater P/AI already on d 15. Contrary to HC cows that had some ovoid conceptuses, CC had only elongated conceptuses, which were longer and tended to secrete greater amounts of IFN- τ . Secretion of IFN- τ during conceptus elongation is pivotal for coordination of endometrium biology and maintenance of a functional CL secreting progesterone, a requirement for pregnancy maintenance (Thatcher et al., 1989). Crossbred cows had greater concentrations of ovarian steroids before and after AI. Estradiol concentrations increase in the periovulatory period, and they are critical for expression of estrus, ovulation, and endometrial function that affect pregnancy (Geary et al., 2013; Jinks et al., 2013; Madsen et al., 2015). Similarly, high concentrations of progesterone during growth of the preovulatory follicle benefit pregnancy in cattle (Bisinotto et al., 2010; Wiltbank et al., 2014). In addition, the increase in progesterone concentrations after AI stimulates conceptus elongation (Lonergan and Forde, 2014), which might explain the advanced conceptus development observed in CC compared with HC. Therefore, differences in concentrations of circulating ovarian steroids might be some of the determining factors for the differences in fertility between HC and CC.

The differences in concentration of steroid hormones observed between HC and CC were not associated with differences in size of the ovulatory follicle and CL, suggesting that these changes likely are not related to the number of secretory cells. Possible reasons for such differences might be a greater rate of production in crossbred than in Holstein cows; the smaller body size in crossbred cows, which results in less plasma volume; and the fact that crossbred cows produce less milk than Holsteins, which might reduce splanchnic blood flow and subsequent rate of hepatic catabolism of steroid hormones (Wiltbank et al., 2006). Regardless of the exact reason, the resulting greater concentration of progesterone before and after AI and the greater concentration of estradiol during proestrus in crossbred than Holstein cows likely have potential benefits for fertility. In addition to differences in concentration of steroids,

Table 4. Negative ion features in the metabolomic analysis of the uterine flushing that presented distinct abundance among pregnant cows from the 3 groups¹

Mass	Retention time (min)	Putative identification	Group ²			P-value
			HA	HC	CC	
94.9	0.9	—	8,607.0 ^b	115,384.1 ^a	98,928.9 ^a	0.003
247.0	1.0	—	150,612.2 ^a	121,086.3 ^a	27,982.8 ^b	0.005
130.1	2.4	5-Aminolevulinate	141,502.8 ^a	61,964.9 ^a	21,490.9 ^b	0.010
151.0	9.2	—	69,289.1 ^b	326,802.2 ^a	205,311.5 ^a	0.011
316.9	1.0	—	213,387.3 ^a	49,090.5 ^b	96,323.0 ^{ab}	0.011
333.1	3.1	<i>sn</i> -Glycero-3-phospho-1-inositol	169,076.0 ^a	185,362.8 ^a	47,237.3 ^b	0.013
456.7	0.9	—	225,836.5 ^b	234,659.7 ^b	1,051,616.6 ^a	0.015
161.8	1.2	—	1,463,467.7 ^b	2,430,838.1 ^{ab}	4,464,274.1 ^a	0.016
243.1	3.1	Uridine	25,409.0 ^b	16,707.5 ^b	123,954.9 ^a	0.016
111.0	1.1	—	56,169.2 ^a	158,995.7 ^a	11,222.5 ^b	0.016
334.7	1.0	Complex of 160.84 and 172.87 <i>m/z</i>	8,127.0 ^b	38,884.8 ^b	164,079.9 ^a	0.017
187.0	8.0	—	262,428.7 ^a	49,172.0 ^b	89,241.6 ^b	0.018
207.1	1.0	Dihydrolipoate	607,257.0 ^a	338,499.7 ^a	69,463.7 ^b	0.019
289.1	3.1	<i>cis</i> -3,4-Leucopelargonidin	21,638.9 ^b	14,776.1 ^b	131,112.0 ^a	0.020
253.9	1.0	—	129,385.6 ^a	3,019.1 ^b	3,959.4 ^b	0.021
195.1	1.0	D-Gluconic acid	17,871.3 ^b	52,231.6 ^a	73,713.9 ^a	0.021
644.6	0.9	Complex of 80.97 and 562.59 <i>m/z</i>	66,057.2 ^b	9,577.9 ^b	250,775.2 ^a	0.022
106.0	17.3	3-Fluoro-D-alanine	83,068.5 ^a	44,325.3 ^b	107,441.4 ^a	0.025
156.0	14.9	2-Aminomuconate	43,890.3 ^b	41,715.7 ^b	83,048.2 ^a	0.027
79.0	0.9	[M-H ₂ O+H] ⁺ -18.01 <i>m/z</i> adduct of 96.97 <i>m/z</i>	69,722.1 ^{ab}	14,395.7 ^b	148,117.1 ^a	0.034
161.0	2.5	4-Hydroxy-2-oxoglutarate	251,248.8 ^a	144,378.3 ^{ab}	106,127.5 ^b	0.036
92.9	0.9	—	87,282.1 ^b	299,916.7 ^a	87,568.3 ^b	0.038
294.0	1.8	—	395,369.9 ^a	182,760.0 ^{ab}	72,035.8 ^b	0.041
282.1	1.8	[M+CHOONa] 67.99 <i>m/z</i> adduct of 214.07 <i>m/z</i>	263,686.1 ^a	93,114.6 ^b	62,917.9 ^b	0.041
98.9	1.2	Potassium bicarbonate	61,303.1 ^a	4,155.1 ^b	30,233.0 ^{ab}	0.044
214.0	1.8	—	37,964.5 ^a	15,087.8 ^b	9,654.0 ^{ab}	0.046
209.8	1.2	—	11,070,786.0 ^{ab}	15,900,000.0 ^a	5,717,017.6 ^b	0.047
185.0	1.0	—	41,310.0 ^b	115,138.0 ^a	24,363.9 ^{ab}	0.048

^{a,b}Values with different superscripts differ ($P < 0.05$) according to Fisher's LSD in post hoc analysis from MetaboAnalyst 2.0 (<http://www.metaboanalyst.ca/>).

¹Data presented are the LSM of peak intensities.

²HA = Holstein anovular; HC = Holstein cyclic; CC = crossbred cyclic. Estrous cyclicity was determined based on the presence of a corpus luteum in 1 of 2 ultrasound examinations of the ovaries 10 d apart, with the second examination on the day of the first GnRH of the Ovsynch protocol.

CC also had reduced concentration of insulin in plasma compared with HC, which might indicate a potential difference in their glycemic control. The BCS and the change in body condition in the first 39 DIM in cows of these 2 groups were similar, and IGF-1 did not change between HC and CC, thereby suggesting similar energy status. Less insulin under similar energy status might benefit oocyte quality in cows (Adamiak et al., 2005).

Analyses of the transcriptome of conceptus cells and metabolomics of uterine flush fluid resulted in differences between CC and HC that might be important for conceptus development and perhaps help explain the distinctive fertility in these 2 groups of cows. Conceptus cells from CC presented greater transcript expression of PPAR γ , which is a ligand-dependent transcriptional factor thought to be important in preimplantation conceptus elongation in cattle (Ribeiro et al., 2016). Comparison of conceptus cells at different stages of elongation showed a gradual increase in transcript expression of PPAR γ and several other genes with puta-

tive PPAR response element in their regulatory regions. Most of these genes are related to lipid metabolism, which seems to be important for conceptus elongation in cattle. In fact, lipids and lipid derivatives constitute an important component of uterine histotroph during the onset of conceptus elongation and might function as natural ligands for PPAR γ in conceptus cells (Ribeiro et al., 2016).

Cyclic crossbred cows had a greater abundance of anandamide in the uterine flush fluid than did HC. Anandamide is an endocannabinoid biosynthesized from membrane phosphatidylethanolamine (Wang and Ueda, 2009), and it has been shown to be more abundant in the histotroph of pregnant compared with nonpregnant cows (Ribeiro et al., 2016). In rodents and humans, anandamide has been implicated in important events in blastocyst activation, onset of implantation, synchrony of these 2 events, and risk of abortion (Wang et al., 2003; Wang and Dey, 2005; Rapino et al., 2014). Most of the effects of anandamide are mediated by

binding to membrane receptors, which do not seem to be expressed by conceptus cells at this stage. Nonetheless, anandamide has also been demonstrated to act through PPAR γ (O'Sullivan, 2007; Sun and Bennett, 2007), which again might be important for elongation of the conceptus. Moreover, the breakdown of anandamide generates arachidonic acid, which can be used for prostaglandin biosynthesis or incorporated into cell membranes.

Estrous cyclicity influenced P/AI of grazing dairy cows, regardless of the breed of the cow. Considering the difference in P/AI, it was initially predicted that HA would have poorly developed conceptuses. In contrast, d 15 conceptuses from HA were longer and secreted greater amounts of IFN- τ than conceptuses from HC. Nonetheless, the transcriptome of the conceptus cells had considerable differences and HA cows had reduced concentrations of IGF-1 in plasma compared with HC cows. A reasonable explanation for the difference in conceptus size between HC and HA is the distinct concentrations of progesterone in those 2 groups of cows. By design, HA had lesser concentrations of progesterone at the first GnRH of the synchronization

program because of the lack of CL, whereas HC cows had at least one CL in their ovaries. The difference in progesterone concentrations between HA and HC cows remained throughout the growth of the preovulatory follicle. Less progesterone allows greater secretion of gonadotropins and accelerated development of the dominant follicle, resulting in ovulation of a larger follicle and, therefore, a larger CL. The consequence of a larger CL is increased progesterone concentrations in the postovulatory period, which hastens conceptus elongation (Lonergan and Forde, 2014).

Although the conceptuses used for transcriptome analysis were all elongated and morphologically similar among the 3 groups, those from HA were still larger than those from the HC group, and it is possible that some of the differences between anovular and estrous cyclic cows were caused by the accelerated rate of conceptus development in HA compared with HC. In fact, 87 transcripts differently expressed between HA and HC were common to differences between tubular (5 to 19 mm) and filamentous (20 to 60 mm) conceptuses reported by Ribeiro et al. (2016). These transcripts included genes that are considered important

Table 5. Positive ion features in the metabolomic analysis of the uterine flushing that presented distinct abundance among pregnant cows from the 3 groups¹

Mass	Retention time (min)	Putative identification	Group ²			P-value
			HA	HC	CC	
103.0	1.1	—	285,846.2 ^a	14,252.3 ^b	7,518.7 ^b	0.005
229.1	1.1	Complex of 115.04 and 115.04 <i>m/z</i>	497,194.0 ^a	15,697.5 ^b	8,807.0 ^b	0.008
209.1	1.1	—	316,452.8 ^a	10,533.7 ^b	6,018.7 ^b	0.008
321.0	1.1	—	390,538.2 ^a	17,563.0 ^b	5,183.5 ^b	0.008
378.9	1.0	Complex of 196.87 and 183.087 <i>m/z</i>	557,003.5 ^a	450,829.5 ^a	151,960.7 ^b	0.011
188.1	2.1	6-Acetamido-3-oxohexanoate	143,082.4 ^a	74,322.7 ^a	15,412.7 ^b	0.011
256.2	14.7	—	899,595.3 ^a	1,250,959.9 ^a	306,883.4 ^b	0.014
354.3	14.2	Anandamide (20:1, n-9)	93,091.6 ^b	86,900.1 ^b	287,398.3 ^a	0.015
98.1	1.2	[M+Na-H] 21.98 <i>m/z</i> adduct of 76.08 <i>m/z</i>	97,739.2 ^a	170,254.2 ^a	43,898.4 ^b	0.018
238.0	1.1	—	527,587.9 ^a	18,706.6 ^b	10,683.8 ^b	0.018
203.0	2.8	—	125,551.6 ^a	48,383.3 ^b	32,962.4 ^b	0.019
223.2	13.4	(2E,6E)-Farnesol	616,741.8 ^a	358,250.9 ^b	776,187.2 ^a	0.021
232.1	1.6	N-Acetyl-L-2-amino-6-oxopimelate	39,784.1 ^b	45,359.2 ^b	213,605.0 ^a	0.021
169.1	1.0	[M+Na-H] 21.98 <i>m/z</i> adduct of 147.08 <i>m/z</i>	26,457.4 ^b	113,384.5 ^b	334,684.5 ^a	0.024
316.0	1.8	—	123,539.3 ^a	28,211.8 ^b	48,528.6 ^{ab}	0.024
125.5	1.4	—	261,585.6 ^a	153,772.9 ^{ab}	90,625.2 ^b	0.026
146.1	1.7	2R-aminoheptanoic acid	58,897.1 ^{ab}	36,596.9 ^b	142,013.0 ^a	0.026
161.1	10.0	Ethyl (R)-3-hydroxyhexanoate	261,292.9 ^a	8,388.9 ^b	69,357.0 ^{ab}	0.028
150.0	0.8	—	238,841.5 ^a	194,128.0 ^{ab}	39,240.1 ^b	0.041
170.0	1.6	L-Cysteate	276,470.2 ^{ab}	431,304.9 ^a	99,982.0 ^b	0.047
104.0	1.1	—	349,157.9 ^a	336,063.2 ^{ab}	8,473.0 ^b	0.047
300.0	1.9	—	189,414.9 ^a	63,874.8 ^{ab}	15,883.5 ^b	0.047
315.0	1.0	—	150,996.5 ^a	103,866.5 ^{ab}	33,572.1 ^b	0.048
174.6	10.7	—	9,143.6 ^b	242,713.3 ^{ab}	249,794.8 ^a	0.050

^{a,b}Values with different superscripts differ ($P < 0.05$) according to Fisher's LSD in post hoc analysis from MetaboAnalyst 2.0 (<http://www.metaboanalyst.ca/>).

¹Data presented are the LSM of peak intensities.

²HA = Holstein anovular; HC = Holstein cyclic; CC = crossbred cyclic. Estrous cyclicity was determined based on the presence of a corpus luteum in 1 of 2 ultrasound examinations of the ovaries 10 d apart, with the second examination on the day of the first GnRH of the Ovsynch protocol.

for conceptus elongation, such as *PPARG*, *SLC10A1*, *PEX11A*, *ACOX1*, *CRAT*, *AGPAT3*, *ASS1*, *ASL*, and *PAG2*, among others (Ribeiro et al., 2016), and they were all increased in HA compared with HC. Nonetheless, 413 other transcripts were differently expressed between the 2 groups of cows that were unrelated to the transition from tubular to filamentous stage. These differences might help explain the diverging fertility between anovular and estrous cyclic cows.

Based on the aforementioned findings, we could hypothesize that developmental problems in anovular cows occur before d 15 and the conceptuses that survived by d 15 would continue to develop comparably to conceptuses in estrous cyclic cows. In favor of this hypothesis is the phenotype of the conceptus and increased secretion of IFN- τ . On the contrary, one could suggest that although HA conceptuses were longer and secreted more IFN- τ , they might not necessarily be better, and perhaps the difference in transcriptome could indicate imbalances in cell biology that could result in greater incidence of pregnancy loss after d 15. In support of the latter, research has demonstrated increased pregnancy loss in anovular cows (Santos et al., 2004) and in those that develop the ovulatory follicle under low concentrations of progesterone (Bisinotto et al., 2010), and supplementing progesterone to anovular cows increases P/AI and reduces pregnancy loss (Bisinotto et al., 2015). The large number of differently expressed genes observed in HA compared with HC suggests metabolic differences that might be linked to the increased risk of pregnancy failure.

Insulin-like growth factor 1 is important for conceptus development in cattle, especially during elongation. It is an upstream regulator that coordinates changes in gene expression of conceptus cells during elongation, and it is associated positively with the amount of IFN- τ secreted by the conceptus and with conceptus survival in lactating dairy cows (Ribeiro et al., 2014; 2016). The main downstream effects of IGF-1 are stimulation of cell proliferation and survival, and signaling is mediated through phosphatidylinositol and v-akt murine thymoma viral oncogene (**AKT**; Siddle, 2011). Interestingly, conceptuses from HA had reduced expression of *IMPA1*, *IMPA2*, *PIP5K1A*, and *AKT2*, all of which are important for phosphatidylinositol/AKT signaling. Both IGF-1 and insulin receptors activate phosphatidylinositol signaling, which in turn activates AKT signaling that interacts with several other proteins to coordinate energy metabolism, cell survival, proliferation, and migration. One of the downstream targets of AKT is the mechanistic target of rapamycin (**mTOR**) signaling, which has been demonstrated to be needed for elongation of preimplantation ovine conceptuses

(Kim et al., 2010). Therefore, the reduced concentration of IGF-1 combined with reduced expression of key enzymes of the phosphatidylinositol/AKT signaling pathway might have negative consequences for cell survival and proliferation in conceptuses of HA cows.

The functional analysis of transcriptome data pointed to differences in canonical pathways related to apoptosis, 14-3-3 signaling, and autophagy. In fact, p53 signaling was predicted to be increased in HA conceptuses. Protein p53 has a central role in sensing cellular stress and controlling cellular processes such as cell cycle progression, autophagy, and apoptosis (Brady and Attardi, 2010). The prediction of increased activity of p53 in HA was linked to the increased expression of genes such as *CASP6*, *SFN*, *DAP*, *GSK3B*, *LAMP1*, and *ATG4A*, and the reduced expression of *AKT2*. Caspase 6 (*CASP6*) is an executioner caspase that functions as an endoprotease regulating apoptosis (McIlwain et al., 2013) and has been suggested to be important in early (Little et al. 2003) and late embryo development (Godefroy et al., 2013) in mice. The p53 protein interacts with the *CASP6* promoter, working as a transcription activator to increase expression and activity of *CASP6* (Yang et al., 2008; Ehrnhoefer et al., 2014). In fact, expression of *CASP6* reduces the threshold for apoptosis and is considered the most downstream component of the apoptosis pathway induced by p53 (MacLachlan and El-Deiry, 2002).

Another pro-apoptotic protein that had increased transcript expression in HA conceptuses is glycogen synthase kinase 3 β (*GSK3B*). When active, this protein inhibits a series of transcription factors associated with cell survival (Grimes and Jope, 2001). Interestingly, p53 is known to stimulate its activity, whereas AKT is known to inhibit its activity (Grimes and Jope, 2001; Watcharasit et al., 2002). In fact, part of the cell survival effects of AKT signaling is mediated through inhibition of *GSK3B*. Thus, not only was the transcript expression of *GSK3B* increased in HA but its activity was predicted to be increased considering the potential increase in activity of p53 and reduced expression of *AKT2* in HA conceptuses. Extensive apoptosis of conceptus cells is an event generally assumed to precede embryonic mortality and pregnancy loss (Toder et al., 2002). Therefore, upregulation of genes involved in apoptosis in HA conceptuses on d 15 might favor pregnancy loss later in development.

Autophagy was another canonical pathway predicted to be affected by estrous cyclicity and was linked to the differential expression of genes such as *ATG4A*, *MAP1LC3B*, *LAMP1*, *ATG4D*, and *VPS18*. The first 3 genes had increased expression and the latter 2 genes had decreased expression in HA compared with

HC. Both autophagy-related 4 homolog A (*ATG4A*) and microtubule-associated protein 1 light chain 3 β (*MAP1LC3B*) encode proteins involved in the biogenesis of autophagosomes (Reggiori, 2006). Moreover, lysosomal-associated membrane protein 1 (*LAMP1*) is a transmembrane glycoprotein located mostly in the lysosome membrane and responsible for regulating the fusion of lysosomes with autophagosomes, a process critical for the enzymatic degradation of cytoplasmic materials in the autophagosomes for recycling of nutrients (Saftig and Klumperman, 2009). Thus, the increased expression of these 3 genes suggests an increased rate of autophagy in HA conceptuses. Cells use autophagy during stress such as nutrient deprivation or to eliminate aberrant protein aggregates and malfunctioning organelles (Saftig and Klumperman, 2009). Nevertheless, autophagy can induce apoptosis in a process dependent on caspase activity (Scott et al., 2007). Interestingly, HA conceptuses had increased transcript expression of death-associated protein 1 (*DAP1*), another protein considered to be pro-apoptotic but whose activity was demonstrated to regulate the rate of autophagy by interacting with mTOR signaling in a “gas/brake” model (Koren et al., 2010) and to prevent overactivation of autophagy under nutrient deprivation or reduced mTOR signaling.

Energy metabolism is certainly critical for conceptus development, and anovular cows normally suffer from more severe negative nutrient balance postpartum compared with estrous cyclic cows that ultimately causes delayed resumption of estrous cyclicity (Butler, 2003). The impaired energy balance in anovular cows results in a legacy of events that perpetuate to the time of breeding and early embryo development, therefore potentially affecting early conceptus development and placentation. Nonetheless, it is important to point out that the low concentration of progesterone during ovulatory follicle development has been identified as a major cause of reduced fertility in anovular cows (Bisinotto et al., 2010; Wiltbank et al., 2014). Estrous cyclic cows induced to ovulate a follicle that develops under low concentrations of progesterone, mimicking the endocrine milieu observed in anovular cows, had a similar reduction in P/AI as that observed in anovular cows (Bisinotto et al., 2010). Moreover, progesterone supplementation during ovulatory follicle development was able to rescue P/AI in anovular or low progesterone cows (Bisinotto et al., 2013). It is noteworthy that only anovular cows that do not express estrus at AI presented reduced P/AI. This finding reinforces that events occurring before ovulation have remarkable effects in developmental biology during early pregnancy. Whether the reduced fertility in anovular compared

with estrous cyclic cows is mediated through the oocyte quality and its developmental competence or through carryover effects on the uterus and its capacity to support pregnancy survival or both is unknown and remains to be investigated.

CONCLUSIONS

Holstein/Jersey crossbred cows presented improved conceptus development on d 15 of development compared with Holstein cows. Conceptuses on d 15 from crossbred cows were longer, tended to secrete greater amounts of IFN- τ , and had greater expression of PPAR γ , an important transcription factor that coordinates lipid metabolism and elongation at preimplantation development. Moreover, crossbred cows had greater concentrations of anandamide in the uterine flush fluid, which might be important for activation of PPAR γ and for later coordination of implantation. Crossbred cows had greater concentrations of progesterone and estradiol in plasma with similar size CL and follicles, which suggest that these ovarian steroids during the periestrus period and early gestation are likely causative factors for improved fertility. In addition, differences in metabolic hormones observed in the current study might influence conceptus development. It is suggested that the differences in fertility observed between these 2 genetic groups are mediated by changes in metabolic and ovarian steroid hormones that influence conceptus development during the preimplantation period. Estrous cyclicity at the initiation of synchronization had a large effect on P/AI of grazing dairy cows, and this difference was observed in those that did not display signs of estrus at AI. Interestingly, d-15 conceptuses from anovular cows were longer and secreted greater amounts of IFN- τ than conceptuses from estrous cyclic cows, likely a consequence of the different concentrations of progesterone in these 2 groups of cows. Anovular cows had lesser concentrations of progesterone during ovulatory follicle development, resulting in a larger ovulatory follicle that, in turn, resulted in the formation of a larger CL and more progesterone that anticipated conceptus elongation compared with estrous cyclic cows. Nonetheless, anovular cows had reduced concentrations of IGF-1 in plasma and their conceptuses presented remarkable differences in the transcriptome. Some of the altered transcripts in conceptus cells from anovular cows indicate greater cellular stress and increased apoptosis and autophagy, which could influence subsequent survival of the pregnancy past d 15 of development. Nonetheless, further research is needed to understand the alterations in early developmental biology in anovular cows after induced ovulation.

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