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Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis

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Walsh SW, Mehta JP, McGettigan PA, Browne JA, Forde N, Alibrahim RM, Mulligan FJ, Loftus B, Crowe MA, Matthews D, Diskin M, Mihm M, Evans AC. Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis. *Physiol Genomics* 44: 504–517, 2012. First published March 13, 2012; doi:10.1152/physiolgenomics.00178.2011.—Cellular mechanisms that contribute to low estradiol concentrations produced by the preovulatory ovarian follicle in cattle with a compromised metabolic status are largely unknown. To gain insight into the main metabolic mechanisms affecting preovulatory follicle function, two different animal models were used. *Experiment 1* compared Holstein-Friesian nonlactating heifers ($n = 17$) and lactating cows ($n = 16$) at three stages of preovulatory follicle development: 1) newly selected dominant follicle in the luteal phase (Selection), 2) follicular phase before the LH surge (Differentiation), and 3) preovulatory phase after the LH surge (Luteinization). *Experiment 2* compared newly selected dominant follicles in the luteal phase in beef heifers fed a diet of 1.2 times maintenance (M, $n = 8$) or 0.4 M ($n = 11$). Lactating cows and 0.4 M beef heifers had higher concentrations of β -hydroxybutyrate, and lower concentrations of glucose, insulin, and IGF-I compared with dairy heifers and 1.2 M beef heifers, respectively. In lactating cows this altered metabolic environment was associated with reduced dominant follicle estradiol and progesterone synthesis during Differentiation and Luteinization, respectively, and in 0.4 M beef heifers with reduced dominant follicle estradiol synthesis. Using a combination of RNA sequencing, Ingenuity Pathway Analysis, and qRT-PCR validation, we identified several important molecular markers involved in steroid biosynthesis, such as the expression of steroidogenic acute regulatory protein (*STAR*) within developing dominant follicles, to be downregulated by the catabolic state. Based on this, we propose that the adverse metabolic environment caused by lactation or nutritional restriction decreases preovulatory follicle function mainly by affecting cholesterol transport into the mitochondria to initiate steroidogenesis.

ovary; estradiol; theca cells; granulosa cells; RNA sequencing

THE METABOLIC ENVIRONMENT of females can be affected by both chronic and acute stressors at critical time points of the reproductive cycle and can be deleterious to reproductive function (7, 17). For instance, in beef animals acute dietary restriction

reduced systemic concentrations of metabolic hormones (insulin, IGF-I) and resulted in increased number of nonovulatory follicles; in addition, follicles that did ovulate had reduced growth rate, maximum diameter, and estradiol secretion (8, 9, 52, 53). This situation is similar to the observed metabolic adaptations to lactation in the early postpartum period in the lactating dairy cow, when they exhibit similar metabolic profiles and have reduced preovulatory follicle function i.e., reduced systemic estradiol despite increased ovulatory follicle diameter (68). Dietary manipulation to overcome these metabolic stressors by nutritionally increasing circulating insulin shortened the interval from calving to first ovulation (32) and increased the number of small follicles (<5 mm diameter) and diameter of the ovulatory follicle. Circulating estradiol concentration is a key component of the physiological cascade involved in stimulating estrous behavior and inducing the gonadotropin surge (27). Recent studies have reported lower serum estradiol concentrations and reduced length and intensity of estrus, as well as reduced subsequent luteal phase progesterone concentrations in lactating cows compared with dairy heifers (68, 69, 84). It is proposed that this is caused by the altered metabolic environment associated with high milk production.

Estradiol synthesis occurs sequentially in the ovarian follicle through a well-established “two-cell, two-gonadotropin” model (20, 28, 37). In response to LH, androgens are synthesized by theca cells and transported across the basement membrane to the granulosa cells where they are aromatized to estrogens under the influence of FSH. In the follicular phase increased LH pulse frequency and amplitude lead to enhanced estradiol production stimulating estrous behavior and inducing the preovulatory LH surge (27). The LH surge triggers a switch from estradiol to progesterone synthesis by triggering the transformation of granulosa and theca cells into large and small luteal cells, respectively (20). Early formation of a functional corpus luteum (CL) capable of secreting high progesterone postconception has been associated with an advancement of conceptus elongation (13, 70) and higher pregnancy rates in cattle (39, 77). As there is an association between preovulatory follicle estradiol production and subsequent postovulatory progesterone concentrations, lactating dairy cows with low circulating estradiol in the periestrus period may have subsequently lower

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progesterone concentrations leading to higher rates of embryo mortality (60, 74).

Here, we used two different animal models to test the hypothesis that the metabolic status of the animal affects the molecular mechanisms that regulate preovulatory follicle function at key developmental stages. Therefore, the objective of this study was to identify follicular cell target genes that are susceptible to metabolic influences. First, RNA sequencing (RNA-Seq) analysis and real-time quantitative reverse transcription PCR (qRT-PCR) were used to determine the effects of metabolic/lactational environment on differential expression of genes in theca and granulosa cells at three distinct stages of preovulatory follicle development between lactating dairy cows and nulliparous heifers. Second, to confirm that the expression of these genes is altered by the metabolic environment (rather than lactation per se) we examined ovarian follicles in an animal model of dietary restriction affecting the metabolism of nonlactating beef heifers.

MATERIALS AND METHODS

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the cruelty to animals act (Ireland 1897) and European Community Directive 86/609/EC. In addition, this experiment was approved by the Animal Research Ethics Committee of University College Dublin.

Experiment 1: Identification of Target Genes Susceptible to Metabolic/Lactational Influences

The aim of this experiment was to determine the effects of metabolic/lactational environment on differential expression of genes in theca and granulosa cells at three distinct stages of preovulatory follicle development between lactating dairy cows and nulliparous heifers.

Animal model. The estrous cycles of 16 Holstein-Friesian lactating cows (lactation number 3.63 ± 0.48 , days in milking postpartum 81 ± 2.4 ; mean $\pm$ SE) and 17 nulliparous Holstein-Friesian dairy heifers ($1.6 \text{ yr} \pm 0.06$; kept on the same farm) were presynchronized by insertion of a controlled intravaginal drug releasing device (CIDR) (containing 1.38 g of progesterone, CIDR; Pfizer Pharma, Karlsruhe, Germany) and a 2 ml intramuscular injection of a prostaglandin $F_{2\alpha}$ analog (PG, Estrumate; Chanelle, Loughrea, Co. Galway, Ireland, equivalent to 0.5 mg cloprostenol). Subsequent to detection of estrus, animals were treated with a used CIDR on *day 8* (*day 0* = observed estrus), and the development of the second dominant follicle in the cycle was monitored daily by transrectal ultrasonography (Aloka SSD-900 linear array transrectal probe, 7.5-MHz transducer, BCF Ireland) until the day of slaughter for recovery of ovaries at three developmental stages, selection, follicular phase differentiation, and onset of luteinization. The second dominant follicle was chosen for molecular analyses based on several studies using beef heifers, where in a similar model selection occurred on *day 13*, and follicles achieve optimal function and fertility following luteolysis, highly synchronized follicular phase differentiation and ovulation (2, 55, 58).

Animals randomly assigned to the Selection group (cows $n = 5$, heifers $n = 6$) had their used CIDRs removed on the morning of *day 14* and were slaughtered. On the last ultrasound scan before slaughter all animals had a newly selected dominant follicle and a CL present. Selection of the dominant follicle was determined based on established morphological and hormonal criteria (29, 31, 56). Animals randomly assigned to the Differentiation and Luteinization groups received a luteolytic dose of PG on *day 13* and the CIDR was removed on the morning of *day 15* to accurately synchronize the follicular phase differentiation of selected dominant follicles. Animals assigned to the Differentiation group (cows $n = 7$, heifers $n = 5$) were then

slaughtered on *day 16* between 24 and 30 h after CIDR removal and before any animals displayed estrus indicating the onset of the gonadotrophin surge. Animals assigned to the Luteinization group (cows $n = 4$, heifers $n = 6$) received an injection of 5 ml GnRH im (Receptal; Intervet/Schering-Plough Animal Health, Bray, Co. Wicklow, Ireland) 30 h post-CIDR removal, which induces a synchronized gonadotrophin surge, and were slaughtered 18–22 h after the administration of GnRH (*day 17*) timed to occur before ovulation but after the onset of luteinization (44).

Experiment 2: Effect of Acute Dietary Restriction on the Expression of Genes Associated With Steroid Synthesis

The aim of this experiment was to confirm the findings of *experiment 1* using qRT-PCR specifically that it is the metabolic environment (rather than lactation per se), which influences ovarian steroid production by altering the expression of genes involved in cholesterol biosynthesis and utilization.

Animal model. The study used a validated beef heifer model of acute metabolic/nutritional stress (52) and was conducted using 19 Charolais crossbred heifers ($\sim 1.9 \text{ yr}$) exhibiting regular estrous cycles. Estrus was synchronized using an 8-day CIDR protocol as described for *experiment 1*. During the synchronization protocol and before commencement of dietary treatments, heifers were individually fed a grass silage and concentrate diet (fed 1:1 on an energy content basis) supplying the energy for 1.2 times maintenance (M), in which the energy requirement for maintenance was calculated as $8.3 + 0.091 \times \text{body weight in kilograms}$ [MJ metabolizable energy (ME)/kg dry matter (DM) per day] (52). The energy density of each feed, following feed analysis, was recorded as 9.9 MJ ME/kg DM for silage and 12.1 MJ ME/kg DM for concentrates. The crude protein of each feed was 137 g/kg DM for silage and 170 g/kg DM for concentrate. One day before CIDR removal, heifers were allocated randomly to a diet supplying 0.4 M ($n = 11$) or kept at 1.2 M ($n = 8$) for 18 days. Heifers continued to be individually fed using an electronic feeding system (Calan, Northwood, NH). Ovarian follicle development was monitored from the day of CIDR removal until day of slaughter using transrectal ultrasonography. All heifers showed estrus on *day 2* or *3* after CIDR removal (*day 0*), and on the 11th day after diet allocation (equivalent to *day 7* or *8* of the next cycle), heifers received a luteolytic dose of PG to induce luteolysis, estrus, and ovulation of the first-wave dominant follicle. Heifers were slaughtered on *day 17–19* after diet allocation [equivalent to *day 4–6* after the last estrus, and at the predicted time of selection of the first-wave dominant follicle (22, 56)] and the early dominant follicle from the first wave of the cycle was recovered. Steroidogenic and molecular function of this follicle has been characterized in detail previously (1, 22, 57, 78), which made it suitable for evaluating the impact of nutritional restriction on cellular processes.

Tissue Collection

On the morning of slaughter blood samples were collected from each animal. Blood collection tubes were obtained from BD Diagnostics (BD Vacutainer Systems; Preanalytical Solutions, Bellerive Industrial Estate, Plymouth, UK). In *experiment 1*, blood was collected into plain evacuated serum tubes for the analysis of nonesterified fatty acids (NEFA), β -hydroxybutyrate (BHB), cholesterol, and progesterone. For the analysis of insulin and IGF-I, blood was collected into evacuated tubes containing lithium heparin as an anticoagulant, while for the analysis of glucose, blood was collected into evacuated tubes containing potassium oxalate-sodium fluoride as a glycolytic inhibitor for glucose analysis. In *experiment 2*, blood was collected into evacuated tubes containing lithium heparin as an anticoagulant for the analysis of BHB, cholesterol, insulin, IGF-I, and glucose. Samples collected into plain evacuated serum tubes were refrigerated (4°C) for 12–24 h before centrifugation, while all other samples were processed within 2 h of collection and were subsequently stored at -20°C .

Following slaughter, each pair of ovaries from an individual animal were removed and placed in ice-cold phosphate-buffered saline (PBS; Sigma, Dublin, Ireland). Identification of dominant follicles was aided by ovarian diagrams recorded daily during ultrasonography in both experiments. Follicles were dissected from the stroma, the external diameter was measured using a calipers, and follicular fluid was aspirated from both the dominant and the largest subordinate follicle and snap-frozen in liquid nitrogen for subsequent analysis of estradiol and progesterone to estimate estrogen activity, a crucial determinant of dominant follicle differentiation (40, 78). Granulosa and theca interna layers were harvested according to published procedures (23, 24, 86). Briefly, each follicle was hemisected in PBS and quartered, and the theca-basement membrane-granulosa layers were peeled from the stroma with forceps. A glass scraper was used to separate granulosa cells from the basement membrane, followed by transfer of the PBS containing the dispersed granulosa cells to a 1.5 ml microcentrifuge tube, and centrifugation for 1 min at 5,000 rpm at room temperature. Pelleted granulosa cells were snap-frozen in liquid nitrogen. The theca-enriched layer was removed from PBS and slightly minced using a scalpel blade, transferred to a 1.5 ml microcentrifuge tube and snap-frozen in liquid nitrogen. All samples were stored at -80°C prior to further processing.

Radioimmunoassay and Biochemical Analyses

Progesterone and estradiol concentrations were measured in follicular fluid using validated radioimmunoassay (RIA) procedures as previously described and two commercial kits (Estradiol Maia, Adaltis Italia, Casalecchio di Reno, Italy; progesterone: Coat-a-Count, DPC, Diagnostic Products, Los Angeles, CA) (26, 86). Assay sensitivities were 0.2 pg/ml (estradiol) and 0.03 ng/ml (progesterone). Intra-assay coefficients of variation (CVs) ranged between 1.0 and 16.2% for low, medium, and high reference samples for both estradiol and progesterone assays. The dominant follicle was identified as being larger and having higher estradiol concentrations than other (subordinate) follicles (40, 78).

Total cholesterol in follicular fluid (*experiment 1*, 2), serum (*experiment 1*) and plasma (*experiment 2*) and BHB, NEFA, and glucose in serum and plasma (collected on the morning of slaughter) were analyzed using commercial biochemical assay kits (Olympus Diagnostics, Tokyo, Japan, and Randox Laboratories, Crumlin, Co. Antrim, Northern Ireland). All metabolite concentrations were quantified using an RX imola clinical biochemistry analyzer (Randox Laboratories). Intra-assay CVs ranged between 0.27 and 8.33% for low, medium, and high reference samples. Interassay CVs ranged between 0.37 and 13.87%. The assay sensitivity for cholesterol was 0.865 mmol/l, for BHB was 0.1 mmol/l, for NEFA was 0.072 mmol/l, and for glucose was 0.662 mmol/l. All measurements were carried out according to the manufacturers' instructions.

IGF-I concentrations in follicular fluid and plasma were determined using an RIA following acid-ethanol extraction as previously described (6) with recombinant IGF-I (Upstate, Millipore, Temecula, CA) as tracer and standard, and anti-human IGF-I (NHPP-NIDDK AFP 4892898; National Hormone and Peptide Program, Torrance, CA; dilution 1:750,000) as the primary antibody. The sensitivity of the IGF-I assay was 6 ng/ml. Intra-assay CVs ranged between 13.4 and 18.9% for low, medium, and high reference samples. Interassay CVs ranged between 10.1 and 12.2%, respectively. In *experiment 1*, plasma insulin concentrations were determined by using a solid-phase fluorimmunoassay (AutoDELFLIA, PerkinElmer Life and Analytical Science, Turku, Finland) in a single assay. The intra-assay CV was 3.2% for samples containing a mean concentration of 13 $\mu\text{IU/ml}$. Sensitivity of this assay was 0.1 $\mu\text{IU/ml}$. In *experiment 2*, plasma insulin concentrations were measured by direct RIA using a Coat-A-Count kit (Siemens Healthcare Diagnostics). The intra-assay and interassay CVs were 6.6 and 6.6% for samples containing a mean

concentration of 26 $\mu\text{IU/ml}$. The sensitivity of the assay was 1.2 $\mu\text{IU/ml}$.

RNA Extraction and Purification

Total RNA was extracted from theca and granulosa cells using TRIzol reagent (Molecular Research Center) according to the manufacturer's instructions and as previously described (23) with the following modifications: homogenization protocol, TRIzol extraction with BCP and isopropanol. RNA purification involved on-column DNase digestion to eliminate genomic DNA contamination and cleanup columns to remove free nucleotides, salts, and proteins using the Qiagen RNeasy mini kit (Qiagen, Crawley, UK) according to the supplier's recommendations. Total RNA concentration was quantified using a Nanodrop spectrometry at 260 nm (Thermo Fisher Scientific, Waltham, MA). Total RNA quality was confirmed using the RNA integrity number (RIN) generated by Agilent Bioanalyzer 2100 using an RNA 6000 Nano LabChip kit (Agilent Technologies Santa Clara, CA). All theca and granulosa samples had an RIN value of ≥ 7.5 and were processed further for RNA-Seq.

RNA-Seq Sample Processing, Library Preparation, and Cluster Generation

Analysis of theca and granulosa cell samples was performed using the Illumina GA2 sequencer. All samples were prepared for sequencing according to the standard Illumina protocol for sequencing cDNA samples (<http://www.illumina.com>). Briefly, 5 μg of total RNA from individual follicles was diluted to a volume of 50 μl and incubated at 65°C for 5 min to disrupt the secondary structures. Dynal oligo(dT) beads were washed and resuspended in 50 μl of binding buffer following which total RNA was added and rotated at room temperature for 5 min. The supernatant was removed, and the mRNA fraction was eluted in 10 mM Tris-HCl by incubation at 80°C for 2 min and subsequently at 65°C for 5 min. Next, the mRNA was fragmented by adding 1 μl of fragmentation buffer (Ambion, Carlsbad, CA) to 9 μl of mRNA and incubated at 70°C for 5 min. After that, 1 μl of stop buffer (Ambion) was added, and the sample was immediately placed on ice. Elution of the fragmented mRNA involved the addition of 1 μl 3 M NaOAc (pH 5.2), 2 μl glycogen, and 30 μl 100% EtOH to the sample, incubation at -80°C for 30 min, centrifugation at 4°C for 25 min at 14,000 rpm, washing of the mRNA pellet with 70% EtOH, and then resuspension in RNase DNase-free H_2O . First-strand cDNA synthesis was carried out by adding random hexamers (3 $\mu\text{g}/\mu\text{l}$) and Superscript II (Invitrogen) to fragmented mRNA and incubating sample in a thermocycler (G-Storm, Surrey, UK) at 25°C for 10 min, 42°C for 50 min, 70°C for 15 min and placed on ice. This was followed by second-strand cDNA synthesis using RNaseH (2 U/ μl) and DNA polymerase I (10 U/ μl) and incubating the sample at 16°C in a thermocycler for 2.5 h. Purification of the cDNA was carried out using the QIAquick PCR spin column according to the supplier's recommendations (Qiagen).

Further processing of the cDNA fragments involved end repair [addition of T4 DNA polymerase (3 U/ μl), *Escherichia coli* DNA polymerase I Klenow fragment (5 U/ μl), and T4 PNK (10 U/ μl) to sample and incubation at 20°C for 30 min], addition of a single "A" to the 3' end of the fragmented DNA [addition of Klenow 3'-5' exo-enzyme (5 U/ μl) and incubation at 37°C for 30 min] and ligation of indexed adapters [addition of T4 DNA ligase (2 U/ μl) and incubation at room temperature for 15 min]. Libraries were size-selected (excision at 200 bp) on 2% agarose gels and isolated from the agarose gels using the QIAquick gel extraction kit. Finally, PCR enrichment of purified adapter ligated DNA was performed by adding Phusion polymerase (New England Biolabs, Ipswich, MA) and PCR primers (Illumina, San Diego, CA) to samples and incubation with the following cycle conditions: 98°C for 30 s, 15 cycles of 98°C for 10 s, 65°C for 30 s, 72°C for 30 s, and 72°C for 5 min. Purified libraries were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA).

and a Quant-iT double-stranded DNA High-Sensitivity Assay Kit (Invitrogen). Clustering and sequencing of the products were carried out as per supplier's recommendations - v2 of the Illumina chemistry kits (Illumina) and 36 cycles of sequencing was performed on each flow cell using the Illumina GA2 sequencer.

Alignment of Reads

The RNA-Seq data were processed through the standard software pipeline for the Genome Analyzer (http://bioinfo.cgrb.oregonstate.edu/docs/solexa/SCS2_01_IPAR1_01_Release_Notes.pdf). The sequenced reads were aligned against the BosTau4 assembly of the bovine genome (downloaded from the UCSC genome browser; 64) using the ELAND alignment software from Illumina. A splice file was generated using the bovine exon information from Ensembl version 54 (38). The CASAVA module v1.0 from Illumina was then used to determine which reads mapped to the Ensembl annotated exons and splice junctions. Several papers have shown that RNA-Seq libraries can be subject to certain types of bias such as transcript length (12, 59) and GC bias (19). A key diagnostic plot for determining the presence and extent of such bias is a histogram or density plot of the sorted counts for each transcript in each library. Biased libraries show different/nonoverlapping profiles. The density plots for the 73 libraries profiled in this experiment show very consistent profiles, and so it was determined that further normalization of the data was not required for this dataset. The raw counts for each transcript as well as tags per million (TPM) and reads per kilobase of exonic sequence per million values were calculated and loaded into R. Transcripts for which no tags mapped in any sample were filtered out. In addition, transcripts where <5 samples had a TPM of 2 or greater or where the transcript had a CV ($100 \times \text{standard deviation/mean}$) of >300 were also removed.

ANOVA

ANOVA was used for each tissue separately for differential transcript analysis. Using the limma and puma libraries in R we conducted a two-way ANOVA (cow vs. heifer, stage of dominant follicle development). A key assumption for ANOVA is homogeneity of variance among the different classes. To control for multiple testing the ANOVA results were corrected using the Benjamini-Hochberg adjustment using a q value of 0.05. The Levene test was used to determine if heterogeneity of variance existed for any transcript in the ANOVA. If the variance differed significantly between classes then separate student's t -tests for each of the contrasts of interest were used to determine differences. Thus, the total number of significant genes for each contrast (cow vs. heifer or stage of follicle development) reported at each time point represents the sum of the transcripts identified from the ANOVA or the t -test analyses. Between-group analysis (BGA) plots were generated both as a quality control (to determine how well biological replicates clustered and as a means to identify any outliers) and as a dimensional reduction technique to enable visualization of the broad trends in the data. The BGA plot was generated independently for each tissue using the MADE4 library in Bioconductor using all of the transcripts that passed the initial quality filtering (18). The data discussed in this publication are MIAME compliant (11), have been deposited in the National Center for Biotechnology Information's (NCBI's) Gene Expression Omnibus (GEO) database (4), and are accessible through GEO Series accession number GSE34317 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34317>).

Ingenuity Pathway Analysis

In the current study, differentially expressed genes (DEGs) that increased or decreased during the transition from Selection to Differentiation and Differentiation to Luteinization stages in theca and granulosa cells were analyzed through the use of Ingenuity Pathways Analysis (IPA; Ingenuity Systems, <http://www.ingenuity.com>) for

identification of canonical pathways. Transcripts that increased or decreased within each comparison (listed in IPA as "molecules") were included in the analysis; however, no p value or log expression fold-change value cut-off was applied prior to IPA, as the uploaded gene list had previously been filtered to fit stringent false discovery rate criteria. Where multiple transcripts variants existed for a single gene, the average log expression fold change was used. The significance of the association between our data set and the large library of known canonical pathways in the IPA database was measured in two ways. First, Fisher's exact test was used to calculate a P value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. Second, a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway was determined.

qRT-PCR

To validate the findings from the RNA-Seq procedure, markers of follicle development 3-beta-hydroxysteroid dehydrogenase (*3HSD*), cytochrome P450 aromatase (*CYP19A1*), follicle stimulating hormone (*FSHR*), and luteinizing hormone receptor (*LHCGR*) were selected for qRT-PCR. Briefly, complementary DNA was synthesized from 1 μ g of total RNA (previously extracted for RNA-Seq study) using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems). All primers were designed using PrimerBLAST (NCBI; <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and manufactured by Eurofins MWG (Ebersberg, Germany) (Table 1). Each reaction was carried out in duplicate and consisted of 5 μ l of cDNA (equivalent to 3.0 ng of RNA), 300 nM primer concentration, and 10 μ l of SYBR Green Mastermix (Applied Biosystems), with a final reaction volume to 20 μ l with RNase/DNase-free water. Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. A dissociation curve was included to ensure specificity of amplification. Following confirmation that the expression of *CYP19A1*, *LHCGR*, and *TSPO* agreed with results from RNA-Seq procedure, a comprehensive analysis of the DEGs was performed.

Analysis of the most appropriate qRT-PCR normalization for theca and granulosa cells was performed using the geNorm application in the Biogazelle qBase^{plus} software (<http://www.qbaseplus.com>; Biogazelle, Ghent, Belgium) (35). In *experiment 1*, the optimal normalization factor was calculated as the geometric mean of *DDX39B* and *UBIQ* for theca cells, while the geometric mean of *DAD1* and *UBIQ* was recommended for granulosa cells. For samples in *experiment 2*, the optimal normalization factor was calculated as the geometric mean of *DDX39B* and *ACTB* for theca cells, while the geometric mean of *DAD1* and *UBIQ* was recommended for granulosa cells. All expression data for genes of interest were calibrated normalized, and the expression values for each gene were determined in arbitrary units.

Statistical Analysis of Physiological and qRT-PCR Measurements

Serum, plasma, and follicular fluid samples and qRT-PCR results were analyzed using Statistical Analysis Systems (SAS Institute, Cary, NC). Normality and homogeneity of variance of the data were determined using histograms, qqplots, and the UNIVARIATE procedure in SAS. When heterogeneity of variance was detected, the data were transformed by raising the variable to the power of lambda as determined by Box-Cox transformation analysis using the TRANSREG procedure in SAS. Hypothesis tests (P values) were performed where appropriate on transformed and nontransformed data. Least squares means and standard errors reflect analysis of the nontransformed data.

In *experiment 1*, the effect of stage of follicle development, animal status (cow/heifer), and their interaction on serum analytes, follicular fluid, and relative gene expression were determined using mixed

Table 1. Accession number, gene symbol, and name used to generate forward and reverse primer sequences for qRT-PCR analysis

Accession Number	Gene	Gene Name	Forward Primer	Reverse Primer
NM_001076363	<i>UBIQ</i>	Bos taurus ubiquitin a-52 residue ribosomal protein fusion product 1, mRNA	CGTCTGCCAGGCGGCATCAT	GTGCAGGCGGGCGTAACACT
NM_001033629.1	<i>DDX39B</i>	Bos taurus dead (asp-glu-ala-asp) box polypeptide 39b (ddx39b), mRNA	TGCCTTGCTCAGCTCTTCTGTCAGA	CCGCCATAACTGAGCCGGCA
NM_001034761.1	<i>DAD1</i>	Bos taurus defender against cell death 1 (dad1), mRNA	AGCTTCATCCTAGCGGTTTGTCTG	CGCTCTGGGAGATGCCTTGG
NM_001105613	<i>HMGR</i>	3-hydroxy-3-methylglutaryl-coenzyme A reductase	TGGGTATTGCTGGACTCTTCA	AGCAGGAAAAAGGGCAAAG
NP_001091530.1	<i>SQLE</i>	Squalene epoxidase	TGAAACTCGAGTGCTTGTGG	AGGAATGGTCCTTTCAGGTG
NP_001092333.1	<i>SC4MOL</i>	sterol-c4-methyl oxidase-like	CTGGGTGACTGTTCTGTTGA	GCCGAGAACCAGCATAGAAA
NP_776614.1	<i>STAR</i>	steroidogenic acute regulatory protein	CCTCAAGGACCAAACTCACC	GTGATTGGCAAAATCCACCT
XP_874020.1	<i>LDLR</i>	low-density lipoprotein receptor fragment (ldl receptor)	CAAATGCGAGTGTGAAGAGG	GTGGCGGTGGTAAAGAAGA
NM_174343	<i>HSD3B1</i>	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	CGCGAGAGACCATCATGAAC	CTATGGTGTGGTGTGGATAAAG
NM_174305	<i>CYP19A</i>	cytochrome p450, family 19, subfamily a, polypeptide 1	CTTGAAGAATGCATAGACTCG	CCAAATGGCTGAAAGTACCTGT
NM_174381.1	<i>LHCGR</i>	luteinizing hormone/choriogonadotropin receptor	GAAAGCACAGCAAGGAGACC	TGGAGTGTCTGGGTAAGCA
NP_776486.1	<i>FSHR</i>	follicle stimulating hormone receptor	CAGGATTTGGAGACCTGGAG	ATGTAGTTTGGGCAGGTTGG
NP_776519.2	<i>INHHA</i>	inhibin, alpha	AGATGTCTCCAGGCCATC	GGCTGGAACACATACGTGAA

All primers were used at a final concentration of 300 nM.

model methodology (PROC MIXED) procedure in SAS. In *experiment 2*, the effect of treatment (1.2 M vs. 0.4 M) on plasma analytes, follicular fluid, and relative gene expression was determined using PROC GLM procedure in SAS. All gene expression data for genes of interest are presented as the means $\pm$ SE of the calibrated, normalized, relative expression values in arbitrary units. Pearson correlation coefficients among blood analytes and gene expression values were determined using PROC CORR of SAS. Correlations coefficients were classified as strong ($r > 0.6$), moderate (r between 0.4 and 0.6) or weak ($r < 0.4$), respectively.

RESULTS

Experiment 1

Metabolic profiles of lactating dairy cows and heifers. Analyte concentrations for lactating dairy cows and heifers collected on the morning of slaughter are shown in Table 2. Lactating dairy cows had higher NEFA ($P < 0.05$), BHB ($P < 0.001$), and total cholesterol ($P < 0.0001$) and lower glucose ($P < 0.0001$), insulin ($P < 0.01$), and IGF-I ($P < 0.0001$) concentrations than nulliparous heifers.

Table 2. Concentrations of analytes ($\pm$ SE) in serum collected on the day of tissue recovery in lactating dairy cows and heifers

	Cows	Heifers	P Value
Animals, <i>n</i>	16	17	
Glucose, mmol/l	3.42 $\pm$ 0.09	4.11 $\pm$ 0.09	<0.0001
NEFA, mmol/l	0.34 $\pm$ 0.08	0.19 $\pm$ 0.07	0.0473
BHB, mmol/l	0.67 $\pm$ 0.05	0.41 $\pm$ 0.05	0.0007
Insulin, μ IU/ml	6.08 $\pm$ 1.45	12.69 $\pm$ 1.24	0.0015
IGF-I, ng/ml	132.1 $\pm$ 25.42	466.5 $\pm$ 22.99	<0.0001
Cholesterol, mmol/l	5.59 $\pm$ 0.21	3.00 $\pm$ 0.19	<0.0001

Stage of follicle development (selection vs. differentiation vs. luteinization) was not different for any of the analytes therefore the overall differences between cows and heifers are presented.

Follicular fluid characteristics. The morphological and hormonal characteristics for dominant follicles across the three stages of follicle development confirmed that all follicles subsequently processed were at the expected stage of development (Fig. 1). Furthermore, stage of the estrous cycle was confirmed by measurement of circulating progesterone concentrations. All animals at Selection stage had elevated values of serum progesterone of >6 ng/ml (8.1 ± 0.51 ng/ml, mean $\pm$ SE), whereas concentrations were ≤ 0.22 ng/ml for all animals at Differentiation and Luteinization stages. Follicle diameter increased significantly from Selection (10.2 ± 0.41 mm) to Differentiation (16.9 ± 0.73 mm) ($P < 0.001$); however, there was no difference in diameter between Differentiation and Luteinization (18.0 ± 0.47 mm, Fig. 1A). Follicular fluid estradiol concentrations increased significantly from Selection to Differentiation ($P < 0.001$) and decreased from Differentiation to Luteinization ($P < 0.05$, Fig. 1B). Follicular fluid estradiol concentrations were overall higher in heifers than cows ($P < 0.05$). Furthermore, heifers had higher follicular fluid estradiol concentrations than cows at Differentiation ($P = 0.056$). Progesterone concentrations were not different in Selection vs. Differentiation follicles, but increased in Luteinization follicles ($P < 0.0001$, Fig. 1C). At this stage only heifers had higher follicular fluid progesterone concentrations ($P = 0.0036$). Follicular fluid cholesterol concentrations followed the same trend as follicular fluid estradiol concentrations, increasing from Selection to Differentiation ($P < 0.01$) and decreasing from Differentiation to Luteinization follicles ($P < 0.05$, Fig. 1D).

Temporal changes in gene expression of dominant follicles at distinct stages of the follicle wave. BGA using all transcripts that passed the initial quality filtering procedure (14,893 transcripts for theca and 14,482 transcripts for granulosa) was performed separately for theca cells and granulosa cells. All theca cell (Fig. 2A) and granulosa cell (Fig. 2B) samples were

Fig. 1. Mean ($\pm$ SE) diameter (A) measured after dissection and follicular fluid concentrations of estradiol (B), progesterone (C), and cholesterol (D) for the newly selected dominant follicle (Selection: cows $n = 5$, heifers $n = 6$), differentiated dominant follicle (Differentiation: cows $n = 7$, heifers $n = 5$), and luteinized dominant follicle (Luteinization: cows $n = 4$, heifers $n = 6$) for lactating cows (black bars) and heifers (white bars). Overall treatment effects are given in each panel for each follicular characteristic with significance set at $P < 0.05$. Significant differences ($P < 0.05$) among stages of follicle development are denoted by A–C, while significant differences ($P < 0.05$) between heifers and lactating cows within stage of follicle development are denoted by x and y. NS indicates $P > 0.05$.

discriminated into three distinct clusters corresponding to Selection, Differentiation, and Luteinization stages of the follicle wave. In theca cells, of a total of 14,893 transcripts the expression of 4,816 was altered between Selection and Differentiation, while the expression of 5,277 was altered between Differentiation and Luteinization. In granulosa cells, of the 14,482 transcripts, the expression of 2,331 was altered between Selection and Differentiation, while the expression of 7,374 was altered between Differentiation and Luteinization (Fig. 3). The largest number of differentially expressed transcripts between heifer and lactating cow theca cell samples was observed at Selection (2,603 transcripts different). This corresponds to the clear segregation observed between cow and heifer theca cell samples in the BGA plot (Fig. 2A). There were a total of 829 and 491 transcripts in theca cell samples different between cows and heifers at Differentiation and Luteinization, respectively. In granulosa cells, similar numbers of transcripts were different between cows and heifers at Selection (391 transcripts), Differentiation (226 transcripts), and Luteinization (257 transcripts).

qRT-PCR validation of RNA-Seq data. Four genes in granulosa cells were selected for validation to confirm that follicles were recovered at the correct stages of dominant follicle development. There was a significant stage effect for mRNA expression of the steroidogenic enzymes and gonadotrophin receptors ($P < 0.01$, Fig. 4). The expression of *3BHSD* and *LHCGR* increased ($P < 0.01$) from Selection to Differentiation. The expression of *CYP19A1*, *LHCGR*, and *FSHR* decreased ($P < 0.001$) from Differentiation to Luteinization. The expression of *3BHSD* was differentially expressed between cows and heifers ($P = 0.02$), with heifer follicles having higher mRNA expression in granulosa cells than cow follicles at Luteinization. There was a high degree of fidelity between qRT-PCR and RNA-Seq data.

Pathway analysis. IPA software identified the top five canonical pathways containing genes that were temporally regu-

lated in theca and granulosa cells during follicle differentiation (Selection vs. Differentiation) and during luteinization (Differentiation vs. Luteinization) (Table 3). One of the top five canonical pathways linked with our data, the “biosynthesis of steroids” pathway was common to both theca and granulosa cells during follicle differentiation and in theca cells during luteinization. Further investigation of the biosynthesis of steroids pathway revealed that the DEGs clustered at significant points of the cholesterol biosynthesis pathway. In theca cells, 13 DEGs had an increase in expression from Selection to Differentiation (*NQO1*, *HMGCR*, *MVK*, *PMVK*, *MVD*, *IDII*, *FNTB*, *FDPS*, *FDFT1*, *SQLE*, *LSS*, *DHCR7*, *EBP*), and the same list had a decrease in expression from Differentiation to Luteinization. In granulosa cells, seven DEGs increased (*DHCR7*, *EBP*, *FDFT1*, *FDPS*, *HMGCR*, *PMVK*, *SQLE*), while one DEG (*GGPS1*) decreased between Selection and Differentiation while 13 DEGs (*COX10*, *DHCR7*, *EBP*, *FDFT1*, *FDPS*, *FNTB*, *HMGCR*, *IDII*, *LSS*, *MVD*, *MVK*, *PMVK*, *SQLE*) decreased in expression between Differentiation and Luteinization. The complete list of the IPA canonical pathways for all genes can be found in Supplemental Tables S1–S4.¹

Characterization of biosynthesis of steroids canonical pathway in experiment 1. Given that follicular fluid steroid concentrations were not only different between stages of follicle development, but also different between heifers and lactating cows we chose to focus this paper on the characterization of the differences in key components of the biosynthesis of steroids pathway to identify target genes within this pathway that may be susceptible to metabolic influences. Interestingly, in theca cells, lactating dairy cows had higher expression of five (*SQLE*, *SC4MOL*, *SC5DL*, *EPB*, *NQO1*) genes within the biosynthesis of steroids pathway than heifers at Differentiation. To confirm the temporal and animal status (cow/heifer) effects on such

¹ The online version of this article contains supplemental material.

Fig. 2. Between-group analysis (BGA) using correspondence analysis of the RNA-Seq data for theca (A) and granulosa (B) cells based on the overall transcriptional profile for the newly selected dominant follicle (SDF), differentiated dominant follicle (DDF), and luteinized dominant follicle (LDF) in heifers and lactating cows.

changes in gene expression qRT-PCR was used to validate two genes that RNA-Seq indicated were differentially expressed between cows and heifers at Differentiation (*SC4MOL* and *SQLE*) and also the first enzyme in the pathway (*HMGCR*) that was not different between cows and heifers (Fig. 5). Similar to the RNA-Seq results, the expression of *HMGCR*, *SC4MOL*, and *SQLE* increased from Selection to Differentiation and decreased

during transition from Differentiation to Luteinization ($P < 0.0001$), thus confirming the stage of ovarian follicle development effect. Theca cell expression of *SC4MOL* and *SQLE* mRNA determined using qRT-PCR was also higher in follicles from heifers than from lactating cows ($P < 0.01$). Since steroidogenic acute regulatory protein (*STAR*) is the rate-limiting step in steroidogenesis of theca cells (3), the expression of this enzyme was

Fig. 3. Numbers of differentially expressed transcripts (DEGs) in bovine theca and granulosa cells from dominant follicles collected from dairy heifers ($n = 17$) and lactating dairy cows ($n = 16$) at Selection (cows $n = 5$, heifers $n = 6$), Differentiation (cows $n = 7$, heifers $n = 5$), and Luteinization (cows $n = 4$, heifers $n = 6$) stages of preovulatory follicle development. Numbers within rectangles indicate the number of DEGs during differentiation (Selection vs. Differentiation) and luteinization (Differentiation vs. Luteinization) in theca and granulosa cells. Numbers within ovals indicate the number of DEGs between heifers and lactating cows within stage of follicle development in theca and granulosa cells. Arrows beside the numbers indicate the direction of change (up- vs. downregulated).

also validated using qRT-PCR. In support of the higher follicular fluid steroid concentrations, heifers had higher mRNA expression for this enzyme in dominant follicle theca cells than lactating dairy cows ($P = 0.0045$, Fig. 5).

Experiment 2

Metabolic profiles and follicular fluid characteristics of beef heifers offered 1.2 vs. 0.4 M. Plasma concentrations of glucose, insulin, and IGF-I were significantly higher in beef heifers fed 1.2 M than those offered 0.4 M (Table 4). Heifers fed 1.2 M also tended to have higher plasma cholesterol concentrations than those fed 0.4 M ($P = 0.084$). Heifers fed 0.4 M had significantly higher BHB concentrations.

There was no difference in follicle diameter (early dominant follicle from the first wave of the cycle) between heifers offered 1.2 M versus those offered 0.4 M. However, heifers offered 1.2 M had significantly higher follicular fluid concentrations of estradiol, IGF-I, and cholesterol than those offered

0.4 M. Follicular fluid progesterone concentrations did not differ between the groups.

Characterization of biosynthesis of steroids canonical pathway in experiment 2. In experiment 2, to determine if the relationship between *STAR* expression and estradiol production remained valid in another model of compromised metabolic state, qRT-PCR was performed on candidate genes in the cholesterol biosynthesis pathway (*HMGCR*, *SQLE*, *SC4MOL*, *STAR*) within theca cells identified as significantly different between lactating dairy cows and heifers in experiment 1. Follicles from heifers fed 1.2 M had higher mRNA expression for *SC4MOL* ($P < 0.05$), and *STAR* ($P < 0.01$) in theca cells and tended to have higher mRNA expression for *SQLE* ($P = 0.066$) compared with theca cells from heifers fed 0.4 M (Fig. 6). A schematic representation of a selection of genes involved in de novo cholesterol biosynthesis and steroidogenesis in the bovine preovulatory ovarian follicle is illustrated in Fig. 7.

Fig. 4. Relative expression values for *3BHSD* (A), *CYP19A1* (B), *FSHR* (C), and *LHCGR* (D) in bovine granulosa cells. Mean ($\pm$ SE) expression values are calibrated normalized relative expression (CNRQ) values in arbitrary units (AU) and are given for lactating dairy cows (solid black bars) and heifers (solid white bars) for the newly selected dominant follicle (Selection: cows $n = 5$, heifers $n = 6$), differentiated dominant follicle (Differentiation: cows $n = 7$, heifers $n = 5$), and luteinized preovulatory follicle (Luteinization: cows $n = 4$, heifers $n = 6$). Overall treatment effects are given in each panel for each gene with significance set at $P < 0.05$. Significant differences ($P < 0.05$) among stages of follicle development between heifers and lactating cows are denoted by x and y. NS indicates $P > 0.05$.

Table 3. Top five canonical pathways with the lowest *P* values from Fisher's exact test for pathways generated by IPA for theca and granulosa cells from heifers and lactating cows during follicle differentiation (Selection vs. Differentiation) and luteinization (Differentiation vs. Luteinization)

Comparison	Ingenuity Canonical Pathway	<i>P</i> Value (−log)	Ratio, %	Differentially Expressed Transcripts, <i>n</i>
<i>Theca</i>				
Selection vs. Differentiation	NRF2-mediated oxidative stress response	7.99	31	60 (↑ 46; ↓ 14)
	thrombin signaling	6.06	28	57 (↑ 26; ↓ 31)
	clathrin-mediated endocytosis signaling	5.90	29	50 (↑ 26; ↓ 24)
	biosynthesis of steroids	5.58	11	13 (↑ 13; ↓ -)
	ephrin receptor signaling	5.26	26	51 (↑ 20; ↓ 31)
Differentiation vs. Luteinization	mitochondrial dysfunction	9.06	30.9	54 (↑ 7; ↓ 47)
	oxidative phosphorylation	6.99	32.7	52 (↑ 1; ↓ 51)
	biosynthesis of steroids	5.05	10.7	13 (↑ -; ↓ 13)
	glycolysis/gluconeogenesis	4.76	24.6	33 (↑ 10; ↓ 23)
	ubiquinone biosynthesis	4.72	24.1	27 (↑ -; ↓ 27)
<i>Granulosa</i>				
Selection vs. Differentiation	EIF2 signaling	5.64	19.8	20 (↑ 6; ↓ 14)
	regulation of eIF4 and p70S6K signaling	5.15	16.7	22 (↑ 7; ↓ 15)
	biosynthesis of steroids	4.13	6.6	8 (↑ 7; ↓ 1)
	purine metabolism	3.86	9.5	37 (↑ 12; ↓ 25)
	polyamine regulation in colon cancer	3.12	24.1	7 (↑ 1; ↓ 6)
Differentiation vs. Luteinization	molecular mechanisms of cancer	11.50	36.3	137 (↑ 84; ↓ 53)
	ERK/MAPK signaling	10.30	41.2	84 (↑ 61; ↓ 23)
	breast cancer regulation by stathmin1	9.16	40.4	84 (↑ 61; ↓ 23)
	HGF signaling	8.73	48.6	51 (↑ 37; ↓ 14)
	glioma signaling	8.43	43.8	49 (↑ 32; ↓ 17)

Correlation coefficients for the association between follicular fluid steroid concentrations and metabolic traits of beef heifers with genes involved in cholesterol biosynthesis in bovine theca cells are presented in Table 5. Strong positive correlation coefficients were observed between follicular fluid estradiol concentrations with mRNA expression levels for *SQLE* and *STAR* and moderate correlation coefficients with mRNA expression levels for *HMGCR* and *SC4MOL*. Correlations between the mRNA expression levels for *STAR* and circulating IGF-I concentrations and follicular fluid cholesterol concentrations were moderately positive. Moreover, mRNA

expression levels for *STAR* tended to be negatively correlated with circulating BHB concentrations.

DISCUSSION

The main findings from this study are 1) significant temporal changes in the transcriptional profile of theca and granulosa samples from heifers and lactating dairy cows coincide with three stages of dominant follicle development, 2) genes within the biosynthesis of steroids pathway are important for follicle differentiation and luteinization in theca and granulosa cells,

Table 4. Least squares means for concentrations of analytes in plasma collected on the day of tissue recovery and diameter of the dominant follicle measured after dissection and follicular fluid concentrations of estradiol, progesterone, IGF-I, and cholesterol for the newly selected dominant follicle for control (1.2 M) and restricted (0.4 M) heifers

	Control	Restricted	SE Pooled	P Value
Animals, <i>n</i>	8	11		
Metabolic indicators				
Glucose, mmol/l	4.61	4.27	0.100	0.030
BHB, mmol/l	0.27	0.40	0.020	0.0004
Insulin, μ IU/l	5.40	3.27	0.637	0.035
IGF-I, ng/ml	345.33	207.76	30.05	0.006
Cholesterol, mmol/l	3.44	2.99	0.171	0.084
Follicle characteristics				
Diameter	11.3	10.5	0.62	0.378
Estradiol, ng/ml	7.92	3.55	1.14	0.006
Progesterone, ng/ml	3.28	5.07	1.67	0.681
*E2:P4 ratio	3.11	1.54	0.58	0.052
IGF-1, ng/ml	189.45	131.30	17.06	0.030
Cholesterol, mmol/l	1.12	0.83	0.06	0.007

*E2, estradiol; P4, progesterone.

3) the metabolic status of the animal alters the expression of genes in follicle cells between heifers and lactating dairy cows within each stage of follicle development, 4) we have shown for the first time that compromised metabolic environment lowers the expression of *STAR* and contributes to reduced capacity of the dominant follicle to produce estradiol. Previous studies have investigated differences between ovarian follicle development in lactating and nonlactating cattle at a specific stage of follicle development (68, 84); however, to our knowledge, this is the first study that has characterized the temporal changes in the transcriptomic profile of differentiated and luteinized theca and granulosa cells in heifers and lactating dairy cows.

The results from this study clearly illustrate the divergent metabolic profiles associated with lactation and acute dietary restriction as evidenced by the higher circulating BHB and lower circulating glucose, insulin, and IGF-I concentrations in lactating cows and in beef heifers on a low plane of nutrition. This is consistent with previous studies in lactating animals (25) and in animals on a lower plane of nutrition (21, 41, 53). Circulating metabolites are reflected in the follicular fluid of

dominant follicles (48, 50) and consequently may have a direct influence on granulosa cell function (80) and oocyte developmental competence (49). Despite the fact that metabolic environment did not influence follicle diameter, lactating cows and animals on a lower plane of nutrition had lower follicular fluid estradiol concentrations than heifers and animals on a high plane of nutrition. Furthermore, lactating dairy cows also had lower follicular fluid progesterone concentrations than heifers after the LH surge. This suggests that the *in vivo* metabolic environment can alter the production of steroids by the ovarian cells. Interestingly, IPA identified the biosynthesis of steroids pathway as a predominant pathway altered in both theca and granulosa cells during follicle differentiation and luteinization. Genes within this pathway encoded cholesterol biosynthesis enzymes, of which five had higher expression in theca cells of lactating dairy cows than heifers. This raises the question of whether cholesterol, the precursor of steroid hormones in all steroidogenic tissue (45), is limiting in lactating dairy cows compared with heifers.

To investigate if cholesterol availability was limiting to the biosynthesis of estradiol, we determined circulating and follicular fluid cholesterol concentrations in both animal models. In *experiment 1*, lactating dairy cows had higher circulating cholesterol concentrations but similar follicular fluid concentrations compared with heifers. In *experiment 2*, animals on a high plane of nutrition had similar circulating cholesterol concentrations but higher follicular fluid concentrations compared with animals on a lower plane of nutrition. Higher serum cholesterol concentrations were also observed in lactating compared with nonlactating animals (47). A possible explanation for higher circulating cholesterol in lactating cows than in heifers is that during lactation, cholesterol is required by the mammary gland for lactogenesis. A recent study reported higher expression of key enzymes in the cholesterol biosynthesis pathway in the liver of lactating dairy cows in the early postpartum period (81), and most likely this is reflected in higher circulating cholesterol that is observed following parturition (36, 81, 82). Furthermore, the mammary gland converts triglyceride-rich lipoproteins to cholesterol-rich lipoproteins, which are transported in the blood (5, 33, 62).

Cholesterol is a fundamental component of mammalian cell membranes and various homeostatic mechanisms are involved to control its synthesis, efflux, and influx in and out of the cell (66). Unlike most animals, in ruminants the primary site of cholesterol synthesis occurs in the small intestine and not the in liver (51, 72). The cholesterol biosynthesis pathway is initiated in the cytoplasm with the conversion of HMG-CoA to mevalonate by HMGCR, which is the rate-limiting step in *de novo* cholesterol biosynthesis (30). Results from our study reveal that there was no difference in expression of *HMGCR* between lactating cows and dairy heifers in theca cells. However, lactating cows had lower expression of five (*SQLE*, *SC4MOL*, *SC5DL*, *EPB*, *NQO1*) of the genes within this pathway than heifers. One possible explanation for the lower expression of these genes is that the theca cells of the lactating dairy cow, which are highly vascularized, are exposed to higher circulating cholesterol concentrations. Cholesterol-sensing transcription factors may detect the high cholesterol concentrations and inhibit the endogenous cholesterol production. Short-term nutrition did not have an effect on circulating cholesterol concentrations in *experiment 2*. Perhaps a longer period of dietary

Fig. 6. Relative expression values for *HMGCR*, *SC4MOL*, *SQLE*, and *STAR* in bovine theca cells. Mean ($\pm$ SE) expression values are CNRQ values in AU, given for heifers offered 1.2 maintenance (M) (black bars, *n* = 8) and 0.4 M (white bars, *n* = 11).

Fig. 7. A schematic model of a selection of genes involved in de novo cholesterol biosynthesis and steroidogenesis in the bovine preovulatory ovarian follicle (enzymes are depicted in rectangles). Uptake of extracellular cholesterol in the form of high-density lipoproteins (HDL) and low-density lipoproteins (LDL) from circulation is achieved through binding with their respective receptors (LDLR and SRB1) on the theca cell membrane, while intracellular cholesterol is generated through de novo synthesis from fatty acids. Free cellular cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane by StAR. This is a rate-limiting step in the formation of estradiol in the steroidogenic pathway involving enzymes (some of which are shown) in both the theca and granulosa cells. Activation and inhibition of the expression of StAR are regulated by trophic hormones, growth factors, various transcription factors, and multiple signaling pathways (see text for more details).

restriction would alter the cholesterol biosynthesis pathway. Regardless, we have shown that cholesterol availability is not rate limiting for steroidogenesis between lactating cows and nonlactating heifers and between animals on a high and low plane of nutrition. Therefore, this suggests that the control of cholesterol utilization may have a central role in the physiological differences observed in both animal models.

The rate-limiting step in steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane, where it is converted to pregnenolone by cytochrome P450 cholesterol side chain cleavage complex. *STAR*, a mitochondrial phosphoprotein, has been identified as the critical

cholesterol transport protein (14, 75) and has been extensively studied across numerous species (10, 83, 85). In bovine follicles, the expression of *STAR* is restricted to the theca interna (3, 73); however, it is acquired in granulosa cells following luteinization (3, 61). Results from *experiment 1* indicate that the expression of *STAR* is temporally regulated and this is consistent with observations in the rat ovary (67). There was a dramatic increase in expression during follicle differentiation (Selection vs. Differentiation) and a dramatic decrease in expression after the LH surge (Differentiation vs. Luteinization). Furthermore, lactating cows had lower expression of *STAR* in theca cells than heifers. Additionally, animals on a

Table 5. Correlations of genes involved in cholesterol biosynthesis in bovine theca cells with follicular fluid steroid concentrations and metabolic traits of beef heifers (n = 19)

	Metabolic Traits					Follicular Fluid Steroid Concentrations			
	Glucose	BHB	Insulin	IGF-I	Cholesterol	Estradiol	Progesterone	IGF-I	Cholesterol
HMGCR	-0.01	-0.08	0.06	0.38	-0.02	0.40*	0.25	0.21	0.14
SQLE	-0.09	-0.19	-0.07	0.34	-0.11	0.61**	0.26	0.04	0.11
SC4MOL	-0.003	-0.17	-0.03	0.35	0.00	0.58**	0.18	0.04	0.23
STAR	0.34	-0.39†	0.19	0.56*	0.20	0.75***	0.14	0.34	0.51*

Correlation coefficient in boldface are different from zero ($P < 0.10$). † $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

low plane of nutrition had lower expression of *STAR* in theca cells. Interestingly, the temporal expression of *STAR* coincided with follicular fluid estradiol concentrations. Previous studies have reported a physiological link between ovarian steroid concentrations and *STAR* protein synthesis (34, 43, 46, 61, 79); therefore, it is reasonable to suggest that differences in follicular fluid estradiol concentrations between cows and heifers and between animals on a high and low plane of nutrition can be explained by differences in *STAR* mRNA levels. This is further strengthened by the fact that *STAR* mRNA levels are tightly coupled to the active form of the protein (16).

The expression, activation, and inhibition of *STAR* are tightly regulated by the orchestrated involvement of trophic hormones, growth factors, as well as various transcription factors and multiple signaling pathways (54, 63, 76). In steroidogenic tissue, LH stimulates increased expression of *STAR* through cAMP-mediated signaling (15, 42, 75). In addition, insulin and IGF-I also stimulate increased expression of *STAR* mRNA (67, 71). In the present study, lactating dairy cows and animals on a low plane of nutrition had lower circulating insulin and IGF-I. Furthermore, lactating animals and animals on a low plane of nutrition have decreased LH pulse frequency and lower preovulatory LH surge than nonlactating animals and animals on an adequate plane of nutrition (9, 53, 65, 84). Thus, it is reasonable to conclude that in the present study exposure of the highly vascularized theca cells to the altered physiological environment resulted in lower *STAR* mRNA levels in lactating cows and animals on a low plane of nutrition.

In summary, we have shown that lactation and acute dietary restriction alter the metabolic environment in cattle and that follicular fluid estradiol concentrations were also lower in these animals. Despite the fact that the expression of cholesterol biosynthesis enzymes was lower in lactating cows than heifers, cholesterol availability for steroidogenesis was not rate limiting. However, we suggest here that steroid production is affected by the metabolic environment, not by an effect on steroidogenic enzymes but by an effect on *STAR* that is the true rate-limiting enzyme in steroidogenesis. Therefore, we propose that the adverse metabolic environment caused by lactation or nutritional restriction decreases preovulatory follicle function (and likely oocyte and subsequent CL quality) by affecting cholesterol transport into the steroidogenic pathway.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.W.W., J.A.B., R.A., F.M., and D.M. performed experiments; S.W.W. prepared figures; S.W.W. drafted manuscript; S.W.W., P.A.M., N.F., M.G.D., M.M., and A.C.O.E. edited and revised manuscript; J.P.M., P.A.M., J.A.B., and B.L. analyzed data; B.L. and A.C.O.E. interpreted results of experiments; M.A.C., M.G.D., M.M., and A.C.O.E. conception and design of research; A.C.O.E. approved final version of manuscript.

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