Level of nutrient intake affects mammary gland gene expression profiles in preweaned Holstein heifers¹

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ABSTRACT

Bovine mammary parenchyma (PAR) and fat pad (MFP) development are responsive to preweaning level of nutrient intake. We studied transcriptome alterations in PAR and MFP from Holstein heifer calves ($n = 6/\text{treatment}$) fed different nutrient intakes from birth to ca. 65 d age. Conventional nutrient intake received 441 g of dry matter (DM)/d of a control milk replacer (MR) [CON; 20% crude protein (CP), 20% fat, DM basis]. Calves in the accelerated nutrition groups received 951 g/d of high-protein/low-fat MR (HPLF; 28% CP, 20% fat, DM basis), 951 g/d of high-protein/high-fat MR (HPHF; 28% CP, 28% fat, DM basis), or 1,431 g/d of HPHF (HPHF+) MR. Out of 13,000 genes evaluated, over 1,500 differentially expressed genes (DEG) were affected (false discovery rate < 0.10) by level of nutrient intake in PAR or MFP. Feeding HPLF versus CON resulted in the most dramatic changes in gene expression, with 278 and 588 DEG having ≥ 1.5 -fold change in PAR and MFP. In PAR, the most-altered molecular functions were associated with metabolism of the cell (molecular transport and lipid metabolism) with most of the genes downregulated in HPLF versus CON. In MFP, DEG also were primarily associated with metabolism but changes also occurred in genes linked to cell morphology, cell-to-cell signaling, and immune response. Compared with CON, feeding HPHF or HPHF+ did not result in substantial additional effects on DEG beyond those observed with HPLF. The pentose phosphate, mitochondrial dysfunction, and ubiquinone biosynthesis pathways were among the

most enriched due to HPLF versus CON in PAR and were inhibited, whereas glycosphingolipid biosynthesis, arachidonic acid metabolism, and eicosanoid synthesis pathways were among the most enriched due to HPLF versus CON in MFP and were inhibited. These responses suggest that, in PAR, doubling nutrient intake from standard feeding rates inhibited energy metabolism and activity of oxidative pathways that partly serve to protect cells against oxidative stress. The MFP in those heifers appeared to decrease production of lipid-derived metabolites that may play roles in signaling pathways within the adipocyte. Overall, results indicated that prepubertal/preweaned mammary transcriptome is responsive to long-term enhanced nutrient supply to achieve greater growth rates before weaning. The biological significance of these results to future milk production remains to be elucidated.

Key words: mammary gland development, dairy calf, gene expression

INTRODUCTION

Heifer rearing typically has focused on achieving earlier puberty and earlier breeding to increase profitability on farms (Ettema and Santos, 2004). Overfeeding during the allometric phase of mammary growth (2 to 3 mo of age through puberty) can increase ADG and accelerate onset of puberty (Meyer et al., 2006a). An increase in nutrient intake during this phase may impair mammary development (Sejrsen et al., 1982) and decrease milk yield at first lactation (Radcliff et al., 2000), but decreased first-lactation milk yield is not always observed (Waldo et al., 1998). However, more recent research suggests that chronological age and not level of nutrient intake determines the rate of bovine mammary parenchyma (PAR) mass accumulation in the prepubertal heifer (Meyer et al., 2006a,b).

The preweaning period (birth through 2 to 3 mo of age) is the first developmental stage in which nutrition can stimulate greater rates of lean gain (Blome et al., 2003; Brown et al., 2005b), presumably without causing

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impaired mammary development (Brown et al., 2005a; Meyer et al., 2006a). An increase in udder size during this period appears to be more related to an increase in bovine mammary fat pad (MFP) and connective tissue than PAR tissue (Sinha and Tucker, 1969; Meyer et al., 2006a). However, recent studies showed that greater energy and protein intake during the preweaning period (i.e., enhancing nutrient intake) has the potential to increase both PAR mass (Brown et al., 2005a) as well as epithelial cell proliferation (Meyer et al., 2006b). This may translate into greater first-lactation milk yield in animals fed such diets. For instance, Moallem et al. (2010) showed that calves allowed two 30-min periods per day to drink either fresh whole milk to satiety [as opposed to milk replacer (MR)], averaged 10% more milk and 7% more ECM in their first lactation than heifers fed MR. Also, a recent study by Davis Rincker et al. (2011) showed that heifers fed a diet designed to achieve 0.68 kg of daily gain versus a conventional diet to attain 0.45 kg of daily gain before weaning and managed the same thereafter, had no differences in energy-corrected or age-uncorrected 305-d milk yield; however, a tendency existed to produce more age-uncorrected ECM in heifers fed the intensified diet.

Prepubertal level of nutrient intake has proven to be an important stimulus that can affect bovine mammary mRNA expression and potentially the rate of tissue development. Early studies mentioned above indicated that level of nutrient intake during the preweaning period could have long-lasting effects on productivity and profitability. Therefore, identifying molecular mechanisms that are susceptible to different levels of nutrient intake in PAR and MFP before weaning might help us more thoroughly understand mammary gland development and, in the future, achieve practical goals such as maximizing PAR mass and, consequently, milk production. The general hypothesis we sought to test was that enhanced level of nutrient intake affects MFP and PAR tissue gene expression. Specific objectives were to evaluate large-scale gene expression profiles in PAR and MFP tissues using bioinformatics analysis and to relate gene network expression to tissue development and function.

MATERIALS AND METHODS

Animals and Sampling

All procedures were conducted under protocols approved by the Virginia Tech Institutional Animal Care and Use Committee. Specific details on feeding and management have been reported previously (Hill et al., 2008; Daniels et al., 2009) and a brief summary is presented in the Supplemental Material (available online

at <http://www.journalofdairyscience.org/>). Heifers ($n = 5-6$ per treatment) were euthanized at ca. 65 d age by phenobarbital injection (Euthasol; Virbac AH Inc., Fort Worth, TX; 10 mg/kg of BW) and exsanguinated. Internal organs and mammary glands were removed and their weights recorded (Hill et al., 2008). Mammary fat pad was collected from the fat adjacent to the body wall, whereas PAR was collected from the macroscopic central mass of the epithelial portion of the gland. Subsamples of PAR and MFP were snap-frozen in liquid N_2 , shipped overnight to the University of Illinois, and then stored in liquid N_2 until RNA extraction (details are given in the Supplemental Material; available online at <http://www.journalofdairyscience.org/>).

Microarrays

A bovine oligonucleotide microarray developed at the University of Illinois (Loor et al., 2007) with more than 13,000 bovine oligonucleotides (70-mers) was used to identify large-scale changes in gene expression. Details on the development, annotation, and use of this microarray have been reported previously (Loor et al., 2007). Methods for microarray hybridizations and scanning were as reported by Loor et al. (2007). Briefly, slides were hydrated, dried, and placed in a UV cross-linker (UV Stratalinker 1800; Stratagene, Agilent Technologies Inc., Santa Clara, CA) for approximately 5 min. Slides were washed with 0.2% SDS solution, rinsed with distilled deionized H_2O , and placed in warm prehybridization solution for 45 min at 42°C. The same amount of Cy3- or Cy5-labeled mammary cDNA and a reference standard RNA pool (made of different bovine tissues) were co-hybridized using a dye-swap design (i.e., 2 microarrays per tissue sample). Slides were incubated for 40 h at 45°C before scanning. Criteria for evaluation of slide quality included identification of number of spots with a minimum median signal intensity of 3 standard deviations above background, keeping slides with a minimum of 20,000 spots with minimum median signal intensity of 3 standard deviations above background in both Cy3 and Cy5 channels, and keeping slides with a minimum mean intensity of 400 relative fluorescent units in both Cy3 and Cy5 channels across the entire slide.

Data Analyses

Data from a total of 82 microarrays (38 PAR and 44 MFP; 41 samples from 19 animals contributing both PAR and MFP and 3 animals contributing only MFP) were normalized for dye and array effects (i.e., lowess normalization and array centering) and used for statistical analysis. All data were analyzed using the

Proc MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Fixed effects in the model were tissue, level of nutrient intake [441 g of DM/d of control MR (CON; 20% CP, 20% fat, DM basis), 951 g/d of high-protein/low-fat MR (HPLF; 28% CP, 20% fat, DM basis), 951 g/d of high-protein/high-fat MR (HPHF; 28% CP, 28% fat, DM basis), or 1,431 g/d of HPHF (HPHF+)], and dye, whereas random effects included calf and microarray. Separate analyses were conducted to evaluate treatment effects within PAR and within MFP. Results of tissue-specific differences in the transcriptome have already been published (Piantoni et al., 2010). Raw *P*-values were adjusted using the Benjamini and Hochberg false discovery rate (FDR; Reiner et al., 2003). Differences in relative expression between diets were considered significant at an FDR-adjusted 0.10 within PAR and within MFP, which was equivalent to unadjusted *P* < 0.01. Scheffé's adjustment of *P*-values between treatment was used to control the experiment-wise type I error. The cutoff for the Scheffé-adjusted *P*-value was 0.05 for treatment effect, unless otherwise specified. Data from real-time quantitative PCR (qPCR) were analyzed as described by Piantoni et al. (2008). The statistical model (SAS Institute Inc., Cary, NC) included the fixed effect of treatment and the random effect of heifer within treatment. The MIXED procedure of SAS was used.

Data Mining and Verification by qPCR

These details are presented in the Supplemental Material and results are in Supplemental Tables 14 and 15 (<http://www.journalofdairyscience.org/>). Briefly, microarray data were mined using the gene-enrichment approach [also known as the overrepresented approach (ORA); Huang et al., 2009] via the web-based software Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc., Redwood City, CA). The ORA is based on the assumption that in order for pathways, functions, or terms in a given gene list to be biologically relevant, they have to be overrepresented [i.e., must be present in the gene list at a greater proportion compared with the background or a list of genes selected randomly among the ones available (i.e., annotated microarray platform)]. As in previous studies with bovine mammary glands, using a similar FDR cutoff for overall treatment effects (i.e., FDR < 0.10; Li and Capuco, 2008), only those right-tailed Fisher exact test *P*-values < 0.05 were used to determine enrichment (e.g., Loor et al., 2007; Swanson et al., 2009). Using an FDR adjustment of those *P*-values did not lead to significantly enriched functions/pathways, which is biologically unlikely (Loor et al., 2011). Rather, such response underscored one important limitation of applying the ORA ap-

proach during bioinformatics [Huang et al., 2009; e.g., when the list of differentially expressed genes (DEG) is large, the likelihood of finding significantly enriched terms is lowered if FDR adjustments are applied]. For a thorough discussion of the ORA approach and its limitations the reader can evaluate Huang et al. (2009) and Loor et al. (2011). We attempted to provide biological interpretation of the ORA results by using the "effect on function" feature in IPA. Further details on this evaluation approach can be found in the Supplemental Material (<http://www.journalofdairyscience.org/>).

Selection of genes for qPCR was based on level of fold change in the microarray data set as well as on their putative biological roles in PAR and MFP (e.g., *FOS*, *PPARG*, *LEPR*; Piantoni et al., 2008). For qPCR verification of microarray results, oligonucleotide sequences of those targets selected for verification were first analyzed via BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for homology with the respective target genes. In MFP, we verified the expression pattern (i.e., significance or direction of the response, or both) of 23 out of 32 genes (~72%) and in PAR 26 out of 31 genes (~84%). In MFP, we observed opposite responses by qPCR compared with microarray for *A2M*, *ACTB*, *CPT1C*, *CSN3*, *LPL*, *PRLR*, *S100A8*, *S100G*, and *TP53*, whereas in PAR, we observed opposite responses for *CSN3*, *DGAT2*, *LEP*, *RXRG*, and *TP53*. When verification was evaluated via correlation (e.g., Li and Capuco, 2008) using data for all genes examined, we observed the correlation coefficient (*r*) = 0.71 in PAR and *r* = 0.40 in MFP. The correlations without including genes with opposite responses were *r* = 0.94 in PAR and *r* = 0.87 in MFP. The degree of verification we obtained was similar to or greater than previous work in bovine mammary epithelial cells (~55–88%; Stiening et al., 2008), bovine embryos (~76%; Smith et al., 2009), or bovine liver (60%; Connor et al., 2010). Comparisons across studies, however, must be done with caution because of inherent differences in qPCR methodology, suitability of internal control genes used, and approach to establish congruency between microarrays and qPCR (i.e., correlation vs. actual statistical analysis).

RESULTS AND DISCUSSION

In the present study, MR were formulated to support increased daily gains either through varying the level of protein and fat or the rate of intake (Hill et al., 2008). In this context, the most relevant comparisons from an industry and biological standpoint were those between HPLF and CON and between HPHF and CON. Both HPLF and HPHF were fed at twice the level of CON, which more closely reflects current industry practices. The weight of MFP and PAR averaged 131.3 and 8.8

g, respectively, in calves fed HPLF and 84.1 and 5.9 g, respectively, in calves fed CON (Daniels et al., 2009). In contrast, the weight of MFP and PAR averaged 192.2 and 10.1 g, respectively, in calves fed HPHF, and 324.8 g and 84.1 g, respectively, in calves fed HPHF+ (Daniels et al., 2009). Differences in PAR tissue weight due to treatments were not statistically significant; however, enhanced nutrient intake resulted in greater MFP weight relative to CON (Daniels et al., 2009).

Our data, for the most part, confirmed previous studies evaluating the effects of level of nutrient intake on calf performance and mammary gland composition (e.g., Blome et al. 2003; Brown et al., 2005a,b). Previous data showed that feeding higher levels of nutrients enhances growth rate and is often accompanied by greater deposition of structural and lean tissue without additional fat deposition (Blome et al., 2003; Brown et al., 2005b). Extraparenchymal fat and total PAR increased when a high level of nutrient intake was fed from birth, suggesting that an increase in level of nutrient intake also affects rate of mammary development (Brown et al., 2005a). Meyer et al. (2006b) also suggested that a high level of nutrient intake from birth, and specifically at BW <100 kg, might increase PAR cell proliferation in the prepubertal mammary gland. Therefore, mammary tissue of young animals (BW <100 kg) may have an inherently greater capacity for enhanced rates of cell proliferation. First-lactation milk production from heifers that had been fed different MR or whole milk as calves also has been evaluated (Terré et al., 2009; Moallem et al. 2010), with results suggesting that higher intake of nutrients has the potential to result in greater milk production.

Growth performance and mammary development results from calves in the present study were discussed by Daniels et al. (2009), and seemed to confirm the notion that an increase in mammary size during the preweaning period is more related to enlargement of MFP than PAR tissue (Sinha and Tucker, 1969). This was supported by the fact that, in PAR, no differences were observed between HPHF and HPHF+ for total tissue weight (average 12.3 g), total lipid content (2 g), total DNA content (27.3 g), total protein content (0.48 g), cell proliferation rate, and amount of telomerase (0.5 amol/mg of mammary protein; Daniels et al., 2009). Nevertheless, the experiment had few animals per level of nutrient formulation, which makes it difficult to totally discard the possibility of an effect of higher nutrient intake on PAR tissue at this early stage of development. Despite the lack of effect on PAR, the opposite was the case for MFP (e.g., feeding HPHF and HPHF+ vs. HPLF increased total lipid by ~68 and 100 g; Daniels et al., 2009). In fact, the relative amount of lipid in MFP as a proportion of total MFP

mass was approximately 50% with HPLF but increased to approximately 69% and approximately 72% when HPHF and HPHF+ were fed. Those relationships seem to agree with data from Capuco et al. (1995), showing that the proportion of adipocytes relative to epithelium was markedly greater in prepubertal calves fed a high-energy diet designed to increase the rate of gain.

Despite the marked differences in MFP tissue weights observed when feeding HPLF compared with HPHF or HPHF+, the former resulted in the most changes in gene expression profiles relative to CON. In fact, no obvious changes were observed at the level of gene expression between the HPHF and HPHF+ diets compared with CON that could explain the increases in mass of MFP observed. Therefore, because of the practical relevance of feeding HPLF, the discussion of transcriptomics changes for both PAR and MFP is focused primarily on the comparison of HPLF with CON.

Effects of Level of Nutrient Intake on PAR

Number of DEG. A total of 1,641 DEG had a significant overall effect of level of nutrient intake (FDR <0.10) in PAR (Table 1). When comparing the number of DEG from higher intake of nutrients with CON, feeding HPLF resulted in 598 DEG, constituting the largest effect (Table 1). Feeding HPHF+ resulted in the lowest number of DEG compared with CON. Overall, a total of 108 (33 upregulated, 75 downregulated) and 118 (29 upregulated, 89 downregulated) genes were simultaneously affected by higher nutrient intake compared with CON (Table 1; for details on the actual genes see Supplemental Tables 4–7, available in the online version of this paper). The number of DEG with an arbitrary 1.5-fold or greater expression when comparing HPLF with CON was relatively smaller (i.e., 278) than might have been expected, given the differences in nutrient intakes between both treatments. Nevertheless, this small difference on DEG is not surprising when we consider that PAR tissue weight in response to HPLF did not differ statistically from CON (Daniels et al., 2009). Besides, greater glucose concentration with HPLF, blood insulin, growth hormone, and IGF-1 were not statistically significant when comparing HPLF with CON (Daniels et al., 2008). Biologically, however, numerical differences in hormones and growth factors (e.g., greater IGF-1 with HPLF; Daniels et al., 2008) might have been associated with some of the transcriptomics responses observed.

Overall Effects of Feeding HPHF and HPHF+. Despite the much smaller numbers of DEG affected, and available for enrichment analysis, results from the bioinformatics analysis (Supplemental Table 14; <http://www.journalofdairyscience.org/>) revealed that genes

Table 1. Summary of statistical results in parenchyma and fat pad of heifer calves fed enhanced levels of nutrient intake (HPLF, HPHF, or HPHF+) compared with conventional level of nutrient intake (CON)¹

DEG (no.)	Tissue	Increased nutrient intake vs. CON	DEG affected simultaneously ²		Number of DEG ³					
			Up	Down	Expression ratio >1.0			Expression ratio >1.5		
					Up	Down	Total	Up	Down	Total
1,641	Parenchyma	HPLF			268	330	598	130	148	278
		HPHF			81	155	236	11	41	52
		HPHF+			74	122	196	17	17	34
		Total	33	75						
1,541	Fat pad	HPLF			433	612	1,045	240	348	588
		HPHF			56	178	234	28	54	82
		HPHF+			52	159	211	20	56	76
		Total	29	89						

¹HPLF = 951 g/d of high-protein/low-fat milk replacer (28% CP, 20% fat, DM basis); HPHF = 951 g/d of high-protein/high-fat milk replacer (28% CP, 28% fat, DM basis); HPHF+ = 1,431 g/d of HPHF; CON = 441 g of DM/d of control milk replacer (20% CP, 20% fat, DM basis). The complete list of differentially expressed genes (DEG) affected simultaneously in response to increased nutrient intake can be found in Supplemental Materials (<http://www.journalofdairyscience.org/>).

²Increased nutrient intake compared with CON.

³Upregulation and downregulation of DEG in parenchyma or fat pad tissue relative to the reference RNA sample. See Materials and Methods for additional details.

affected by either HPHF or HPHF+ compared with HPLF elicited the overall upregulation of pathways associated with oxidative stress and apoptosis, pentose phosphate pathway, and cellular growth and development, but at the expense of a downregulation of pathways that would (a priori) appear necessary for the cell to maintain homeostasis (e.g., glutathione metabolism) in the face of marked increase in nutrient availability. It was noteworthy that pathways associated with genome repair, phospholipid biosynthesis, glycolysis, and neuronal death were not affected by greater intake of nutrients. Thus, it appeared that PAR was able to tolerate greater nutrient availability but might be particularly sensitive to oxidative stress, which could be alleviated via supplementation of antioxidants (e.g., vitamin E and selenium) or cellular glutathione precursors (e.g., methionine).

Because we already established that PPAR γ and its lipogenic gene targets are more predominately expressed in MFP than PAR (Piantoni et al., 2010), the enlargement of MFP observed with HPHF and HPHF+ likely was driven by greater uptake of dietary long-chain FA rather than de novo synthesis. In fact, such response would partly explain the lack of enrichment in the bioinformatics analysis of genes associated with lipogenesis (see Supplemental Table 16; <http://www.journalofdairyscience.org/>). From that standpoint, it was quite remarkable that MFP from calves fed HPHF or HPHF+ compared with HPLF experienced an overall induction of glutathione metabolism and glycosphingolipid biosynthesis without changes in pathways associated with cell survival, repair, and removal of damaged proteins (Supplemental Table 17;

<http://www.journalofdairyscience.org/>). Further studies in this area of nutrition with a greater number of biological replicates seem warranted and would help to confirm the present findings based on relatively few DEG.

Top Biological Functions and Canonical Pathways. In PAR, significant molecular functions in the comparison of HPLF with CON were molecular transport (67 DEG), cellular development (34 DEG), lipid metabolism (42 DEG), carbohydrate metabolism (40 DEG), AA metabolism (11 DEG), cell morphology (27 molecules), and cellular growth and proliferation (12 DEG) (Supplemental Table 2; <http://www.journalofdairyscience.org/>). The most-enriched molecular functions were primarily related to cell metabolism (i.e., molecular transport and lipid, carbohydrate, and AA metabolism) and, in general, genes in these functions were downregulated. Interestingly, cellular development, cell morphology, and cellular growth and proliferation also included genes that were primarily downregulated despite the fact that calves fed HPLF had a numerical increase in PAR tissue weight (8.8 vs. 5.9 g; Daniels et al., 2009). In contrast, several genes associated with FA uptake (*CD36*) and energy metabolism (*UCP1*), as well as genes involved in cellular mitogenesis and response to inflammation (e.g., *F2R*) were upregulated >1.5 fold by feeding HPLF. Among metabolism-related functions, functional analysis revealed that overall quantity of lipid and carbohydrate, as well as production of lipid were overall inhibited with feeding HPLF compared with CON (Table 2). Such response might have been related to the fact that enhancing intake of nutrients with high-protein MR typically favors leaner tissue

Table 2. Functional evaluation of molecular and cellular functions that were enriched in differentially expressed genes (DEG) in parenchymal tissue from calves fed an enhanced level of nutrient intake as HPLF compared with the conventional level of nutrient intake (CON)¹

Function	Genes affected (no.)	Main effect on function ²
Molecular transport	67	↓Quantity of lipid
Cellular development	34	Lifespan of cells ³ ; differentiation of epidermal cells ³
Lipid metabolism	42	↓Quantity of lipid; ↓production of lipid
Carbohydrate metabolism	40	↓Quantity of carbohydrate
Cell morphology	27	↔Shape change of blood cells; ↓shape change of tumor cell lines
Immune and lymphatic system development and function	16	↑Quantity of neutrophils
Cell-to-cell signaling and interaction	52	Response of cells ³
Cell signaling	27	↔Tyrosine phosphorylation of protein; ↓release of Ca ²⁺
Nervous system development and function	40	↓Activation of neuroglia
Vitamin and mineral metabolism	16	↓Release of Ca ²⁺
Hematological system development and function	29	↔Aggregation of blood cells
Tissue development	27	↔Aggregation of blood cells
Post-translational modification	17	↔Tyrosine phosphorylation of protein

¹HPLF = 951 g/d of high-protein/low-fat milk replacer (28% CP, 20% fat, DM basis); CON = 441 g of DM/d of control milk replacer (20% CP, 20% fat, DM basis). Included are functions with at least 5 molecules affected.

²Arrows indicate stimulation (↑) of a particular effect on function or an inhibition (↓) of a particular effect on function according to criteria outlined in the Supplemental Materials (<http://www.journalofdairyscience.org/>). The horizontal arrows (↔) indicate that the effect on function was neither inhibited nor stimulated.

³Effects on functions that were considered to be affected (not stimulated or inhibited in a clear fashion) due to lack of information in the Ingenuity Pathway Analysis database (Ingenuity Systems Inc., Redwood City, CA).

mass deposition than standard protein MR (Blome et al., 2003), and that lipid content in PAR (mg/g of PAR) did not increase significantly in calves fed HPLF compared with CON (Daniels et al., 2009).

Among the genes uniquely affected by HPLF and with a relatively large fold change was *F2R* (verified by qPCR; Supplemental Table 10; <http://www.journalofdairyscience.org/>). This gene was associated with molecular transport, lipid metabolism, carbohydrate metabolism, as well as with cell morphology and cellular growth and proliferation (Supplemental Table 2). The overall evaluation of the likely functional significance of changes in *F2R* and other DEG (both up- and downregulated) in response to HPLF compared with CON indicated that PAR responded by downregulating energy metabolism pathways leading to a decrease in biological processes related to lipid synthesis and accumulation (Tables 3 and 4). The upregulation of *F2R* alone due to HPLF is not entirely surprising because in nonmammary epithelial cells, *F2R* encodes the thrombin receptor, which is required for activation of the phosphoinositide 3-kinase (PI3K)-Akt pathway. This signaling pathway is crucial for self-regeneration, active growth, and regression of nonmammary epithelial cells (Feutz et al., 2008). Thus, despite a likely involvement of this gene (and others among DEG in Supplemental Table 2; <http://www.journalofdairyscience.org/>) in cell growth, the overall changes revealed a broader set of adaptations that apparently resulted in a decrease in cellular metabolism when nutrient availability was enhanced by feeding HPLF.

Among cellular adaptations of potential importance was the inhibition of oxidative pathways (i.e., those that potentially prevent excessive buildup of reactive oxygen species and fall within the IPA categories of mitochondrial dysfunction and ubiquinone biosynthesis, which takes place in mitochondria). Derangements in the mitochondrial dysfunction pathway have been related with oxidative stress (Hashiguchi et al., 2004) and apoptosis (Eckert et al., 2003). It could be envisioned that enhanced nutrient delivery to mammary PAR cells when the feeding rate was doubled (i.e., due to HPLF) signaled adaptations to utilize those substrates for metabolism rather than maintaining flux through pathways, which, under basal conditions, provide most of the ATP required by cells (e.g., oxidative phosphorylation).

Another gene that was upregulated by HPLF compared with CON by qPCR was *CAMK2A* (Figure 1). In nonruminants, *CAMK2A* is ubiquitously expressed and is predominantly cytoplasmic (Hook and Means, 2001). Upon activation, one of the target genes of *CAMK2A* is *FOS*, which also was numerically upregulated by HPLF in PAR (Figure 1). The cyclic AMP (cAMP) response element-binding protein (*CREB1*), a ubiquitous transcription regulator that binds the response element of hundreds of target genes, is another substrate for activated *CAMK2A* (Montminy, 1997). The majority of nuclear CREB protein is bound to chromatin and ca. 50% becomes phosphorylated upon cAMP stimulus (Montminy, 1997). In fact, it is thought that CREB1 actually resides as a component of certain RNA poly-

Table 3. Most significant canonical pathways, number of genes affected, number of genes up or downregulated ($\uparrow/\downarrow$), effect related to end product (up- or downregulated or no change: $\uparrow$, $\downarrow$, or $\leftrightarrow$, respectively), function of the pathway, and genes affected in parenchyma tissue from calves fed an enhanced level of nutrient intake as HPLF compared with the conventional level of nutrient intake (CON)¹

Pathway	–Log (<i>P</i> -value)	No. of genes	$\uparrow/\downarrow$	Effect related to end product	Function (from IPA ²)	Genes
Pentose phosphate	2.35E+00	5	0/5	$\downarrow$	Substrates for glycolysis, gluconeogenesis, and purine, pyrimidine, and histidine metabolism	<i>GPI, ALDOB, G6PD, PFKM, BCKDHB</i>
Mitochondrial dysfunction	1.78E+00	10	1/9	$\downarrow$	β -Oxidation of FA; protection against oxidative stress	<i>SDHA, NDUFV1, NDUFB9, SOD2, PRDX5, NDUF6, CPT1B, NDUFB7, PSEN2, COX6A2</i>
PXR/RXR ³ activation	1.75E+00	6	0/6	$\downarrow$	Transport of xenobiotics and other organic compounds; phase II metabolism of xenobiotics (conjugation); drug-induced regulation of gluconeogenesis and lipid metabolism	<i>PRKACB, GSTA2, ABCC2, FOXO3, INSR, SLCO1B3</i>
One carbon pool by folate	1.74E+00	3	1/2	$\downarrow$		<i>FTCD, TYMS, MTHFR</i>
Androgen and estrogen metabolism	1.57E+00	4	1/3	$\downarrow$		<i>HSD3B1, AKR1D1, UGT1A6, HSD17B8</i>
Coagulation system	1.51E+00	4	1/3	$\downarrow$	Extrinsic and intrinsic pathways; fibrin degradation	<i>F10, F2R, FGB, F2</i>
Ubiquinone biosynthesis	1.31E+00	5	0/5	$\downarrow$		<i>NDUFV1, NDUFB9, NDUF6, NDUFB7, BCKDHB</i>

¹HPLF = 951 g/d of high-protein/low-fat milk replacer (28% CP, 20% fat, DM basis); CON = 441 g of DM/d of control milk replacer (20% CP, 20% fat, DM basis). Remaining comparisons can be found in the Supplemental Material (<http://www.journalofdairyscience.org/>).

²IPA = Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA).

³PXR/RXR = pregnane X receptor/retinoid X receptor.

Table 4. Functional evaluation of molecular and cellular functions that were enriched in differentially expressed genes (DEG) in fat pad tissue from calves fed an enhanced level of nutrient intake as HPLF compared with the conventional level of nutrient intake (CON)¹

Function	Genes affected (no.)	Main effect on function ²
Cell morphology	76	↔Outgrowth of neurites; ↔outgrowth of plasma membrane projections; size of cells ³
Cellular development	70	Developmental process of dermal cells ³ ; developmental process of epidermal cells ³ ; developmental process of red blood cells ³ ; developmental disorder of cells ³
Nervous system development and function	62	↔Outgrowth of neurites; ↓growth of neurites
Lipid metabolism	43	↔Hydrolysis of phospholipid; ↔hydrolysis of lipid
Molecular transport	74	↓Quantity of calcium
Cell-to-cell signaling and interaction	45	Adhesion of blood cells ³
Hematological system development and function	48	Cell movement of granulocytes ³ ; cell movement of myeloid cells ³ ; adhesion of leukocytes ³
Immune and lymphatic system development and function	45	Adhesion of leukocytes ³
Tissue development	38	Aggregation of blood cells ³
Post-translational modification	38	↔Activation of protein
Immune response	51	Inflammatory response ³

¹HPLF = 951 g/d of high-protein/low-fat milk replacer (28% CP, 20% fat, DM basis); CON = 441 g of DM/d of control milk replacer (20% CP, 20% fat, DM basis). Included are functions with at least 5 molecules affected.

²Arrows indicate stimulation (↑) of a particular effect on function or an inhibition (↓) of a particular effect on function according to criteria outlined in Supplemental Materials (<http://www.journalofdairyscience.org/>). The horizontal arrows (↔) indicate that the effect on function was neither inhibited nor stimulated.

³Effects on functions that were considered to be affected (not stimulated or inhibited in a clear fashion) due to lack of information in the Ingenuity Pathway Analysis database (Ingenuity Systems Inc., Redwood City, CA).

merase II complexes. In this regard, it was interesting that *POLR2H* mRNA was ca. 2-fold greater in response to HPLF than in CON (Supplemental Table 10; <http://www.journalofdairyscience.org/>). Although neither *CAMK2A* nor *POLR2H* were mapped to the top-enriched IPA molecular functions (Supplemental Table 2), it could be possible that external stimuli such as insulin, IGF-1, glucose, and additional molecules or nutrients themselves may have elicited changes in PAR gene expression through upregulation of *CAMK2A* and its putative downstream targets *FOS*, *CREB*, and *POLR2H*.

Effects of Higher Nutrient Intake on MFP Tissue

Number of DEG. More than 1,500 DEG had a significant overall effect (FDR <0.10) of higher nutrient intake in MFP. In the HPLF versus CON comparison, more DEG were present in MFP than PAR (Table 1). This likely reflects the fact that adipose tissue is more responsive to greater nutrient intake (Meyer et al., 2006a) and potentially more biologically active at this point of development than the PAR tissue. An increase in MFP weight corresponded with an increase in nutrient intake across all diets, with HPHF+ resulting in the highest MFP tissue weight (Daniels et al., 2009). Despite the substantial differences in MFP deposition between HPLF and HPHF+, the response in number of DEG was similar to that observed in PAR (i.e., feed-

ing HPLF resulted in the most changes compared with CON; Table 1).

Top Biological Functions and Canonical Pathways. In comparison with PAR, a substantially greater number of DEG belonged to several molecular functions that were affected in MFP in response to HPLF. Some of the most enriched and significant molecular and cellular functions affected by HPLF compared with CON were cell morphology (76 DEG), molecular transport (74 DEG), cellular development (70 DEG), immune response (51 DEG), cell-to-cell signaling and interaction (45 DEG), and lipid metabolism (43 DEG) (Supplemental Table 3; <http://www.journalofdairyscience.org/>). The large numbers of DEG associated with cell morphology, cellular development, cell-to-cell signaling, and immune response could be related to the fact that, at this stage in mammary development, the MFP is growing due to both hypertrophy and hyperplasia (Avram et al., 2007). The stable hydrolysis of triglycerides and phospholipids and the suggestion of reduced intracellular Ca²⁺ (Table 4) could be related to increased hypertrophy as well. In that regard, functional evaluation of enriched pathways underscored the fact that greater nutrient intake may have resulted in lower synthesis of secondary signaling molecules derived from phospholipids or arachidonic acid and eicosanoid metabolism, or both (Table 5). Thus, it could be possible that these lipid-derived signaling molecules are important for MFP enlargement via hyperplasia.

Figure 1. Expression pattern [fold change due to 951 g/d of high-protein/low-fat milk replacer (HPLF; 28% CP, 20% fat, DM basis) vs. 441 g of DM/d of control milk replacer (CON; 20% CP, 20% fat, DM basis)] of selected genes in parenchyma and fat pad. * $P < 0.05$; ** $P = 0.015$. A value of 1.0 or -1.0 = no change over CON and is denoted by a dashed line.

A signature gene that is associated with lipogenesis in nonruminants is *THRSP* [Thyroid hormone responsive (SPOT14 homolog, rat); Supplemental Table 11; <http://www.journalofdairyscience.org/>]. Thyroid hormone and insulin act synergistically to enhance lipogenic rates in white and brown adipocytes in rodents, a process driven at least in part via coordinated induction of *ACACA*, *FASN*, and *THRSP* mRNA expression (Freake and Moon, 2003). We did not detect differences due to HPLF on mRNA of *PPARG* (Figure 1), which is primarily expressed in MFP (Piantoni et al., 2010) and encodes a key nuclear receptor that drives lipogenesis (Desvergne et al., 2006). Thus, our results suggest that adipogenesis and lipid filling in MFP during the preweaning period was sensitive to enhanced nutrition and it seems, to some extent, that it is partly under control of *THRSP* through its effectors insulin, thyroid hormone, and likely glucose. Because insulin was nu-

merically lower and glucose significantly higher in blood from calves fed HPLF compared with CON (Daniels et al., 2008), these animals did not seem to have experienced insulin insensitivity, which is in agreement with an involvement of *THRSP* in MFP lipogenesis.

In MFP as in PAR, several DEG were classified across different molecular function categories, including *F2R*, *INSR*, *IL1B*, and *S100A8* (Table 5). Expression of *F2R* was upregulated by HPLF in MFP as it was in PAR, suggesting that signaling cascades transduced via this receptor are important in the development of both tissue compartments. The proinflammatory cytokine *IL1B* was downregulated due to HPLF, which partly agrees with recent data showing that a higher nutrient intake in heifer calves of slightly greater development than ours (i.e., ca. 100 kg vs. 86.7 kg of BW; Daniels et al., 2009) did not result in greater MFP mRNA abundance of *TNF* (Thorn et al., 2008). Thorn et al. (2006) concluded that, although expansion of the MFP occurred as heifers grew from 100 to 350 kg of BW, this was related to greater mRNA of inflammatory proteins (*TNF*, *IL-6*, plasminogen activator inhibitor-1, and transforming growth factor- β 1) and these were unlikely to depress PAR growth, based on their lack of effects on *in vitro* cell proliferation assays.

Despite those previous findings, our results suggest that other inflammatory-related proteins are responsive to higher intake of nutrients in MFP before weaning (e.g., *S100A8*, which was ca. 2-fold greater in MFP due to HPLF; Supplemental Table 11; <http://www.journalofdairyscience.org/>) and leads to formation of calprotectin, which plays a prominent role in the regulation of inflammatory processes and immune response, and serves as a marker for the influx of phagocytes during inflammation (Korndörfer et al., 2007). Because excessive fat deposition is often associated with a mild proinflammatory state in nonruminants (Boullu-Ciocca et al., 2008), it is possible that feeding HPLF elicited a similar effect.

Additional genes upregulated with HPLF in MFP with potentially important roles in regulation of gene transcription were *TAF9* (Supplemental Table 11; <http://www.journalofdairyscience.org/>) and *TTF1* (Figure 1). The former encodes a protein member of the TATA-binding protein that is used by all RNA polymerases during the process of gene transcription (Johnson et al., 2003), whereas *TTF1* encodes a transcription termination factor during RNA polymerase I transcription. Based on fold changes, both *TAF9* and *TTF1* responded to HPLF marginally. However, it is well established in nonruminants that changes in *TAF* expression are often lower than 2 fold in response to cellular signaling events, but the resulting changes in target gene expression are more dramatic (Johnson et

Table 5. Most significant canonical pathways, number of genes up- or downregulated ($\uparrow/\downarrow$), effect related to end product (up- or downregulated or no change: $\uparrow$, $\downarrow$, or $\leftrightarrow$, respectively), pathway function, and molecules affected in fat pad tissue from calves fed an enhanced level of nutrient intake as HPLF compared with the conventional level of nutrient intake (CON)¹

Pathway	–Log (<i>P</i> -value)	No. of genes	$\uparrow/\downarrow$	Effect related to end product	Function (from IPA ²)	Molecules
Glycosphingolipid biosynthesis—lactoseries	2.47E+00	4	1/3	$\downarrow$		ABO, ST3GAL1, FUT1, FUT3
Synaptic long-term depression	2.13E+00	15	8/7	$\downarrow$	Phosphorylation-dephosphorylation (PD) system; phosphorylation and internalization of parallel fiber AMPAR ³ ; endocytosis	PPP2R5C, MAPK1, GUCY2D, ADCY4, GNA11, GNAQ, PLA2G2A, GNA14, ITPR1, PRKG1, PPP2R1A, RRAS2, RNF111, PLCB3, PLCB1
Glycosphingolipid biosynthesis—neolactoseries	1.98E+00	5	0/5	$\downarrow$		ABO, FUT1, B3GNT1, FUT3, ST8SIA3
Arachidonic acid metabolism	1.92E+00	10	2/8	$\downarrow$		LTA4H, GPX3, ALOX15B, GGT5, CYP2E1, RNF111, EPHX2, ALOX12, PLA2G2A, CYP2S1
Chondroitin sulfate biosynthesis	1.69E+00	5	5/0	$\uparrow$		GAL3ST2, XYLT2, XYLT1, HS3ST5, SULT1B1
Eicosanoid signaling	1.39E+00	6	1/5	$\downarrow$	Inflammatory and allergic response; hyperalgesia, analgesia; cell proliferation, angiogenesis; among others	LTA4H, ALOX15B, RNF111, PTGER3, ALOX12, PLA2G2A
Fructose and mannose metabolism	1.34E+00	6	1/5	$\downarrow$		AKR1A1, PMM1, KHK, GALK1, FUK, PFKFB2
Pentose and glucuronate interconversions	1.33E+00	4	1/3	$\downarrow$		UCHL1, AKR1A1, UGT2A1, BCKDHB
GABA ⁴ receptor signaling	1.32E+00	5	0/5	$\downarrow$	Proteasomal degradation; activation of G proteins; inhibition of adenylate cyclase; inhibition of calcium and potassium channels	GABRA5, GABRB1, GABRE, GABRA1, GABRA2

¹HPLF = 951 g/d of high-protein/low-fat milk replacer (28% CP, 20% fat, DM basis); CON = 441 g of DM/d of control milk replacer (20% CP, 20% fat, DM basis). Remaining comparisons can be found in the Supplementary Material (<http://www.journalofdairyscience.org/>).

²IPA = Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA).

³AMPA = α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

⁴GABA = γ -aminobutyric acid.

al., 2003). A 25% increase in *TAF* mRNA in fibroblasts produced a 2-fold or greater increase in expression of 20 genes and a 2-fold repression of 30 genes (Johnson et al., 2003). Thus, the involvement of *TAF9* and *TTF1* in transcriptional adaptations in MFP due to greater nutrient intake are likely of biological relevance. The potential role of estrogen receptor signaling in MFP development preweaning is discussed in Supplemental Materials (<http://www.journalofdairyscience.org/>). The downstream targets of *TAF9* in bovine mammary tissue remain to be determined.

CONCLUSIONS

Considering the number of transcripts represented on the microarray platform and the marked differences in nutrient intake we studied, gene expression analysis at the end of the treatment period revealed fewer than expected DEG. However, it should be kept in mind that these transcriptomic adaptations represent endpoint responses to long-term higher nutrient intake (i.e., it is reasonable to suspect that temporal adaptations led to the stable expression patterns we uncovered (also see Daniels et al., 2009). Judging by the modest responses observed in tissue weights and blood plasma metabolites and hormones, the transcriptional responses observed due to enhancing nutrient intake by feeding HPLF appear relevant. Overall, enhancing nutrient intake beyond levels from what is commonly used in accelerated calf rearing programs (i.e., HPHF or HPHF+) during the first 2 mo of life did not affect substantially the mammary PAR and MFP transcriptome. Some of the transcriptomic changes might underlie the observed differences in tissue mass and development due to HPLF compared with CON [i.e., a numerical ~21% increase in MFP weight (g of MFP/100 kg of empty BW) and 18% increase in PAR weight (g of PAR/100 kg of empty BW); Daniels et al., 2009]. However, we did not find molecular signatures with the higher-fat MR that could explain differences observed in tissue mass. We conclude that enhancing nutrient intake to twice the intake of conventional MR might be sufficient to enhance mammary gland development through alterations in mRNA expression across several molecular functions and pathways. The biological significance of these results to future milk production remains to be elucidated.

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