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Running Title: CALVING SEASON AND THE LIVER TRANSCRIPTOME

The impact of calving in the summer on the hepatic transcriptome of Holstein cows during the peripartal period

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ABSTRACT

The liver is the main metabolic organ coordinating the adaptations that take place during the peripartal period of dairy cows. A successful transition into lactation, rather than management practices alone, depends on environmental factors such as temperature, season of parturition and photoperiod. Therefore, we analyzed the effect of calving season on the hepatic transcriptome of dairy cows during the transition period. A total of twelve Holstein dairy cows were assigned into two groups based on calving season (6 cows March-April, Spring, SP; and 6 cows June-July, Summer, SU). RNA was extracted from liver samples obtained at -30, 3 and 35 DIM via percutaneous biopsy, and hybridized to the Agilent 44K Bovine (V2) Gene Expression Microarray. qPCR on 22 target genes was performed to verify and expand the analyses. A total of 4,307 differentially expressed genes (DEG) were detected (FDR ≤ 0.05) in SU compared with SP. Furthermore, 73 unique DEG were detected in SU compared with SP cows after applying a fold-change threshold $\geq \pm 3$. For KEGG pathways analysis of DEG, we used the Dynamic Impact Approach, while Ingenuity Pathway Analysis software was used to analyze upstream transcription regulators and perform gene network analysis. Among metabolic pathways, energy metabolism from lipids, carbohydrates and amino acids was strongly impacted by calving in SU, with a reduced level of fatty acid synthesis, oxidation, re-esterification and synthesis of lipoproteins, leading to hepatic lipidosis. Glycan-synthesis was downregulated in SU cows probably as a mechanism to counteract the progression of this lipidosis. In contrast, calving in the SU resulted in upregulation of gluconeogenesis but also greater use of glucose as an energy source. Among non-metabolic pathways, the heat shock response was obviously activated in SU cows, but was also associated with inflammatory and intracellular stress response. Furthermore, data support a recent finding that cows experience ER stress around parturition. Transcription

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regulator analysis revealed how metabolic changes are related to important regulatory mechanisms, including epigenetic modification. The holistic analyses of the liver transcriptome response to calving in the summer at high environmental temperatures underscore how transition cows should be carefully managed during this period, as they experience alterations in liver energy metabolism and inflammatory state increasing susceptibility to health disorders in early postpartum.

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INTRODUCTION

During the transition period, several environmental factors including heat stress, length of photoperiod and nutritional management affect the health and production efficiency of dairy cows (do Amaral et al., 2009). The intensity of heat stress during both dry period and lactation can reduce voluntary DMI and negatively affect milk production (do Amaral et al., 2011). Despite the reduced DMI, body fat mobilization does not increase during heat stress, partly due to a reduction in lipolysis and alterations in insulin sensitivity (Baumgard and Rhoads, 2013). The immune system of heat-stressed cows also is compromised (Lacetera et al., 2006) due in part to alterations in gene expression including upregulation of pro-inflammatory cytokines (Tao et al., 2013). Another well-studied component of cellular responses to heat stress is the heat shock proteins, which appear to play a major role in eliciting immune responses under increased environmental stress in rodents (Campisi et al., 2003) and cows (Catalani et al., 2010).

Although there is some evidence from target-gene analysis that heat stress alters metabolic mRNA expression in liver (do Amaral et al., 2011), it is unknown if and to what extent other signaling pathways might be affected. For instance, it is well established that the plane of energy nutrition during the dry period can influence the hepatic transcriptome and alter tissue function (Loor et al., 2013; Shahzad et al., 2014). Because of the well-established decrease in DMI induced by heat stress, it is likely that cows calving in the summer (**SU**) compared with spring (**SP**) would experience more pronounced drops in DMI leading to consequent changes in the hepatic transcriptome. Thus, the main objective of this study was to evaluate the effect of calving in the summer than spring on hepatic molecular adaptations in peripartal cows. Furthermore, we sought to utilize bioinformatics approaches to uncover novel signaling pathways and gene networks that are affected.

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MATERIALS AND METHODS

Experimental Design and Liver Biopsies

Complete details of the experimental design have been published elsewhere (Basiricò et al., 2011). A total of twelve Holstein dairy cows (6 cows March-April, Spring, SP; and 6 cows June-July, Summer, SU) were used for transcriptomics. Those are a subset of 24 Holstein dairy cows, of which 12 calved in spring, and 12 calved in the summer. Mean temperature-humidity indices for SP (day/night: below 72) and SU (day: 79.5±2.9, night: 70.1±4.7) were recorded. At the time of calving, no clinical health problems were observed in cows and no cow received any treatment for metabolic problems (Basiricò et al., 2011). Liver tissue was harvested via percutaneous biopsy at -30, 3 and 35 d relative to parturition. The biopsies were performed under local anesthesia and tissue samples were first frozen in liquid nitrogen and then stored at -80 °C until real time PCR and microarray analysis.

RNA Extraction

The RNA was extracted from frozen liver tissues using QIAzol Lysis reagent (Qiagen, Chatsworth, CA, USA) and following the manufacturer's protocol. During the procedure, the homogenate was separated into aqueous and organic phases by centrifugation. RNA was precipitated from the aqueous phase by addition of isopropanol. The isolated RNA was resuspended in DNAse-free water and stored at -80 °C until qPCR and microarrays. The RNA quality evaluated via RNA integrity number (RIN) in the Agilent Bioanalyzer (Agilent) was greater than 6.5 for all samples.

Microarrays

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cRNA Synthesis, Labeling, and Purification. The microarray experiment was conducted using the 44K-Agilent bovine (V2) gene expression microarray chips (Agilent Technologies; cat# G2519F-023647). The methods used for labeling and hybridization were those outlined by Agilent Technologies. Briefly, a total of 200 ng of RNA per sample was used to generate first-strand cDNA, which was subsequently reverse-transcribed to cRNA using a low-input quick amp labeling kit (Agilent Technologies; Cat#. 5190-2306). The resulting cRNA was labeled with either Cy3 or Cy5 fluorescent dye according to the manufacturer's instructions. Purification of the labeled cRNA product was performed with RNeasy mini spin columns (Qiagen, Cat#. 74104), and subsequent eluted in 30 µL of DNAse-RNAse-free water. The eluted labeled cRNA was quantified in a NanoDrop ND-1000 (Thermo Scientific) to confirm the manufacturer's recommended criteria for yield and specific activity of at least 0.825 µg and ≥6.

Fragmentation and Slide Scanning. The labeled cRNA was fragmented and then hybridized to the microarray slide following the manufacturer's protocol. Briefly, 825 ng of Cy3 and Cy5 labeled cRNA sample were combined, mixed with 11 µL of 10X Blocking Agent (Agilent Technologies; Cat#. 5188-5281), 2.2 µL of 25X Fragmentation Buffer (Agilent Technologies; Cat#. 5185-5974), and nuclease-free water (to a final volume of 55 µL), and fragmented at 60°C for 30 s. The reaction was then stopped by adding 55 µL of 2X GEx Hybridization Buffer (Agilent Technologies; Cat#. 5190-0403) and the samples were loaded onto the slide. These were hybridized in a rotating hybridization oven at 65° for 17 h. The slides were washed according to the procedures recommended by the manufacturer and scanned using a GenePix 4000B scanner (Axon Instruments, Inc., CA, USA) and GenePix Pro v.6.1 software. Resulting spots where features were sub-standard were flagged as "bad", and excluded from subsequent analysis.

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Primer Design and Real-Time Quantitative PCR (qPCR)

Protocols for primer design, primer testing, selection of internal control genes (**ICG**) for normalization, and other protocols for the qPCR analysis were previously described (Graugnard et al., 2013). Briefly, genes selected as suitable ICG based on geNorm analysis included *UXT*, *GAPDH*, and *RPS9*. The geometric mean of these genes was used to normalize gene expression data. The mRNA was used to measure the expression of 22 target genes (Table 1) associated with the heat shock response (*HSP70A1A*, *HSTF1*), fatty acid oxidation (*CPT1A*, *PPARA*, *ACOX1*), hepatokines (*FGF21*, *ANGPTL4*), esterification and VLDL assembly (*MTTP*, *APOB*, *DGAT1*, *SREBF2*), glucose metabolism (*PC*, *PCK1*, *PDK4*) inflammatory mediators (*TNF*, *GPX1*, *SOD1*, *SOD2*), acute phase protein (*SAA3*, *HP*, *HAMP*) and endoplasmic reticulum stress (*EIF2AK3*). Primer sequences have been reported by Graugnard et al. (2013) and Khan et al. (2014).

Statistical Analysis

Statistical analysis of microarrays data was performed using SAS (SAS, SAS Inst. Inc., Cary, NC). Data from a total of 18 microarrays were adjusted for dye and array effects (Lowess Normalization and array centering). A MIXED model with repeated measures was then fitted to the normalized $\log_2$-tranformed adjusted ratios using Proc MIXED. The model included the fixed effects of time (-30, 3 and 35 d), season (SP and SU) and interaction of time × season. Cow was considered as uncorrelated random effect. The raw *P*-values were adjusted for the number of genes tested using Benjamini and Hochberg's false discovery rate (**FDR**) (Benjamini and Hochberg, 1995) to account for multiple comparisons. Differences in transcript profiles were

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136 considered significant at an FDR-adjusted $P \leq 0.05$. The focus of this manuscript is on the
137 overall differences in liver transcriptome in response to the main effect of group, i.e. SU vs. SP.

138 The qPCR data were normalized with the geometric mean of the ICG, and log-2
139 transformed prior to statistical analysis. A repeated measures model was fitted to gene expression
140 data using Proc MIXED in SAS. The model included the fixed effects of time (-30, 3 and 35 d),
141 season (SP and SU) and interaction of time × season. Cow was considered as uncorrelated
142 random effect. An autoregressive covariate structure was used. All means were compared using
143 the PDIFF statement of SAS, and differences were considered statistically significant at $P <$
144 0.05.

145 ***Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways Analysis***

146 For KEGG pathways analysis of DEG in the comparison of SU vs. SP, the Dynamic
147 Impact Approach (**DIA**) was used. The DIA calculates the overall impact (relevance of a given
148 pathway) and flux (direction of impact), thus, allowing evaluation of transcriptome profiles in a
149 more holistic fashion. The detailed methodology of DIA is described elsewhere (Bionaz et al.,
150 2012). Briefly, the whole data set with Entrez gene ID, FDR ≤ 0.05, fold-change (**FC**) and *P*-
151 value ≤ 0.05 was uploaded to DIA. For the analyses a minimum of 30% annotated genes on the
152 microarray vs. the whole genome (Bionaz et al., 2012) was selected. The results of these
153 analyses are reported in figures and tables.

154 ***Transcription Regulators and Gene Network Analysis***

155 Ingenuity pathway analysis (**IPA**) software (http:/www.ingenuity.com) was used to
156 analyze the upstream transcription regulators and their connections with other downstream genes
157 that were differentially expressed. For this purpose a list of differentially expressed genes (**DEG**)

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158 along with FC ≥ ± 2 and *P*-values (≤ 0.05) was uploaded into IPA. The results were downloaded
159 and saved for further analysis described below.

160 RESULTS AND DISCUSSION

161 The impacts of heat stress and its adverse consequences on DMI, health, milk production
162 and overall productivity have been widely investigated (St-Pierre et al., 2003, Bernabucci et al.,
163 2010; Liang et al., 2013). Several recent studies have reported target-gene expression responses
164 associated with heat stress in mammary, immune cells, and liver (do Amaral et al., 2009; do
165 Amaral et al., 2011; Tao et al., 2013). Thus, in this study we have combined target gene
166 expression, whole-transcriptome microarrays and extensive bioinformatics analyses to study the
167 hepatic response of cows calving in the summer compared with spring.

168 When comparing SU vs. SP, out of ~44,000 probes on the microarray a total of 4,307
169 (9.79 %) DEG (i.e. 3,820 annotated with Entrez gene ID) were detected at an FDR ≤ 0.05. After
170 application of a fold-change threshold, the overall number of DEG was considerably reduced and
171 only 538 were among those upregulated. It also can be discerned that the number of upregulated
172 genes was higher in both analyses with and without FC threshold. For a clearer picture of DEG
173 during the SU vs. SP, we further applied a more stringent criterion of FC threshold at ≥ ± 3 and
174 obtained a total of 73 unique genes that were differentially expressed. The results are reported in
175 Table 2 and 3. Table 2 contains the genes with positive FC values and Table 3 the genes with
176 negative FC values.

177 *Expression Patterns of DEG*

178 The large number of DEG (3,820) underscores the marked effect of calving season on the
179 transcriptome. A total of 2,168 DEG were upregulated and 1,652 downregulated ($P < 0.05$) when

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cows calved during the SU vs. SP. Even after applying a FC criterion of ≥ ± 2 and ± 3 a similar trend was observed, e.g. a total of 531 DEG had a ≥ ± 2 response of which 342 were upregulated and 189 downregulated. These highly-affected genes encompass a vast variety of metabolic and non-metabolic physiological adaptations due to calving season. A similar study dealing with stress response in bovine mammary epithelial cells highlighted the role of several DEG we uncovered (Collier et al., 2006). For example, a fair amount of upregulated DEG in the present study belong to the stress response and repair mechanism, while downregulated DEG belong to metabolism and cell cycle. Bioinformatics analyses in the current study allowed for a deeper evaluation encompassing a broader number of biological/molecular pathways (Figure 1).

Summary of the KEGG Pathways

Among the KEGG categories and sub-categories that were enriched with DEG (Figure 2), we focused on the top five. Among these, 'Metabolism', 'Genetic Information Processing', and 'Cellular Processes' were overall activated (Figure 1), while 'Environmental Information Processing' and 'Organismal Systems' were moderately activated. The discussion of transcription profiles will focus on the top 20 most-impacted metabolic and 20 most-impacted non-metabolic pathways (Figure 2 and 4).

Metabolic Pathways

Lipid Metabolism. Microarrays along with qPCR results indicate that heat stress during SU led to modification of several lipid-related pathways. Among the most impacted pathways, the 'biosynthesis of fatty acids' and 'biosynthesis of unsaturated fatty acids' were inhibited in SU compared with SP cows (Figure 2). Use of qPCR also revealed several lipid-synthesis-related genes e.g., *SCD*, *HMGCS1*, *FADS2*, which were downregulated in SU. In ruminant animals, the

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liver is not a lipogenic organ per se but is the major organ which uptakes NEFA from the blood stream, and their removal from the circulation increases at higher concentrations (Bell, 1980). Thus, the observed downregulation was probably driven by a negative feedback mechanism due to the high level of circulating NEFA (Basiricò et al., 2011) in cows calving in SU.

Despite the higher lipomobilization, SU compared with SP cows also had downregulation of expression of genes associated with fatty acid oxidation (*CPT1A*, *PPARA*, and *ACOX1*), esterification and VLDL secretion (*APOB*, *DGAT1*, and *SREBF2*) (Figure 4). Thus, the net result of calving during the hot season appears to be an inability of the liver to oxidize the greater load of NEFA, hence, likely rendering cows more susceptible of liver lipidosis. In fact, SU cows had a greater degree of liver triacylglycerol concentration pre- and early post-partum, with a lower concentration of plasma cholesterol (a lipoprotein marker) (Basiricò et al., 2011). Overall, these changes indicate that during SU there is a greater risk for a severe alteration of metabolic status already 4–5 wk before calving independently of the adiposity status at calving (Murondoti et al, 2004).

The peroxisome proliferator-activated receptor alpha (*PPARA*) is involved in a variety of cellular functions in the liver of various species (Rakhshandehroo et al., 2010) including ruminants (Bionaz et al., 2013). Thus, at least in monogastrics, PPARA along with its target genes *CPT1A*, *HMGCS1* and *ACOX1* play an important role in lipid metabolism. It has been suggested that its function postpartum is increased in transition dairy cows to overcome the negative energy balance (**NEB**) status (Loor et al., 2006; van Dorland et al., 2009). However, its inhibition during SU compared with SP agrees with qPCR data and liver histology (Basiricò et al., 2011) indicating a lower rate of fatty acid oxidation and metabolism in heat-stressed cows.

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224 'Synthesis and degradation of ketone bodies' was also overall impacted, but a trend of
225 flux towards the positive or negative direction was not evident (Figure 2). This likely indicates
226 an equal response of ketone body synthesis and degradation in both SU and SP. The metabolism
227 of ketone bodies is altered during the transition into lactation (Loor et al., 2007, Osorio et al.,
228 2013) independently of calving season. Most probably the SP cows experienced the expected
229 physiological increase of ketone body synthesis after calving to overcome the NEB, while SU
230 cows had a reduction of this pathway due to the lower oxidation that led to accumulation of fatty
231 acids in liver (Basiricò et al., 2011). In view of the expected adaptations of lipid metabolism
232 pathways after calving, it was surprising to detect an overall induction of 'Fatty acid elongation
233 in mitochondria' and 'Fatty acid metabolism' pathways in SU compared with SP (Figure 2).
234 Further studies are needed to investigate the role of these pathways in liver during the transition
235 period.

236 ***Carbohydrate Metabolism.*** Among the most-impacted metabolic pathways, the
237 'metabolism of propanoate and butanoate' (Figure 2) was induced in SU compared with SP cows
238 indicating a greater degree of glucose metabolism, e.g. higher production and utilization. In fact,
239 these results suggest the activation of gluconeogenesis from its precursors (e.g., lactate, glycerol,
240 and alanine) (Aschenbach et al., 2010). Using qPCR, it was confirmed that the expression of
241 pyruvate carboxylate (*PC*) was greater ($P < 0.05$) in SU compared with SP cows (Figure 4),
242 supporting the notion of an increase in glucose synthesis from compounds other than propionate.
243 It was noteworthy that the expression of phosphoenolpyruvate carboxykinase 1 (*PCK1*) was
244 lower ($P < 0.05$) in the SU compared with SP cows, and that of pyruvate dehydrogenase
245 lipoamide kinase isozyme 4 (*PDK4*) did not differ ($P > 0.05$) between groups. These responses
246 could be related with the lower DMI, i.e. less ruminal propionate available, induced by heat

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247 stress and potentially greater insulin sensitivity within liver. Such response could have amplified
248 the effect of insulin on the hepatocytes, leading to a reduction in oxidation of fatty acids
249 (discussed above) and also allowing for greater TCA cycle flux.

250 Despite the apparent activation of gluconeogenesis in SU cows, the microarray and qPCR
251 results also are suggestive that those cows became more dependent on glucose for energy
252 production (via oxidation), and consequently reduced the amount of glucose available to the
253 mammary gland (Baumgard et al., 2007). This hypothesis agrees with the lower blood glucose
254 concentration postpartum detected in SU compared with SP cows (Basiricò et al., 2011).

255 ***Amino Acid Metabolism***. Heat stress seems to have affected the overall metabolism of
256 amino acids in SU cows (Figure 2). The biosynthesis and degradation of several amino acids was
257 activated likely to increase glucose synthesis. In this regard, such response partly explains the
258 activation of 'biosynthesis of phenylalanine, tyrosine and tryptophan'. Furthermore, the
259 'degradation of valine, leucine, isoleucine and lysine' was enhanced. The metabolism of other
260 amino acids, and amino acid-related molecules, was also increased including glutathione,
261 selenoamino acid and beta-alanine. Among these, glutathione plays an important role in
262 antioxidant related activities and functions as a regulatory element in disulfide bonds of proteins
263 (Mari et al., 2009). Increased glutathione and antioxidant enzyme synthesis in heat-stressed
264 transition dairy cows has been proposed as an adaptive mechanism to help alleviate oxidative
265 stress (Bernabucci et al., 2002).

266 The 'beta-alanine metabolism' pathway is linked with other metabolic pathways such as
267 pyrimidine, propanoate, and fatty acid biosynthesis. The ultimate goal of these pathways is to
268 provide nitrogen from skeletal muscle to liver for metabolism, e.g. energy synthesis (Timperio et
269 al., 2009). Skeletal muscle catabolism is another recognized mechanism that dairy cows use to

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provide amino acid carbon to the liver as energy source during periods of heat stress (Yunianto et al., 1997, Wheelock et al., 2010).

Glycan Biosynthesis and Metabolism. The 'biosynthesis and metabolism of glycan' was also decreased in SU compared with SP cows (Figure 2). Among the top 20 metabolic pathways, all the three glycan-related pathways ('globo series', 'ganglio series' and 'lacto and neolacto series') were inhibited. Glycan plays an important role in a wide range of cellular functions such as protein folding, cell-cell interactions, cellular differentiation, glycosylation and lysosomal activities (Helenius and Aebi, 2001, Hussain et al., 2012).

Data from mice in which glycan synthesis was suppressed suggest that glycans in hepatocytes are not essential for functions such as sterol, glucose, or lipoprotein metabolism (Jennemann et al., 2010). However, high-fat diet-fed mice treated with an inhibitor of glycan synthesis had a decrease in accumulation of triacylglycerol along with higher concentrations of biomarkers of liver pathology (Zhao et al., 2009). Treatment also reduced the expression of several genes associated with hepatic steatosis, including those involved in lipogenesis, gluconeogenesis, and inflammation. In addition, inhibiting glycan synthesis both prevented the development of steatosis and partially reversed preexisting steatosis (Zhao et al., 2009). Similar effects were reported in another study involving inhibition of glycan synthesis (Bijl et al., 2009). Thus, we hypothesize that the downregulation of the glycan-related pathways in SU cows could be a mechanism to counteract the progression of lipidosis due to the accumulation of lipid in the liver as a consequence of the downregulation of expression of genes involved in fatty acid oxidation and mobilization. Further bovine specific research is required to verify this hypothesis.

Non-Metabolic Pathways

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Heat Shock and Endoplasmic Reticulum (ER) Stress Response. An increase in temperature can cause protein unfolding and aggregation, which can lead to a variety of cellular pathologies, such as defects of the cytoskeleton, fragmentation of the ER and Golgi apparatus, a decrease in number of mitochondria and lysosomes, and poisoning of RNA splicing (Richter et al., 2010). It is commonly believed that the deleterious accumulation of unfolded proteins is the signal starting cellular responses against heat stress, as many morphological and phenotypic effects of heat stress can be explained by the aggregation of proteins and an imbalance of protein homeostasis in general (Richter et al., 2010). The microarray data seem to confirm these mechanisms and highlight the expected activation of the heat shock response. In fact, different cellular pathways under 'folding sorting and degradation' (i.e. 'Mismatch repair', 'Homologous recombination', 'Base or Nucleotide excision repair') and 'replication and repair' were impacted by calving season with an overall positive flux and induction in liver of SU compared with SP cows.

The induction of sub-categories such as 'Protein processing in endoplasmic reticulum', 'Proteasome' and 'Ubiquitin mediated proteolysis' strongly support the idea that SU compared with SP cows experienced a greater degree of ER stress. This is also supported by the induction of pathways dealing with glycan synthesis (e.g. O-Mannosyl glycan and N-Glycan biosynthesis), because a potential role of N-Glycans in the liver is to handle the misfolded proteins in the ER during stress conditions. The ER stress and the unfolded protein response (**UPR**) are closely linked to oxidative stress (Malhotra and Kaufman, 2007). Dairy cows are known to undergo a period of oxidative stress during heat stress (Bernabucci et al., 2002). The simultaneous occurrence of these responses can initiate apoptotic cascades that are deleterious to liver function (Malhotra and Kaufman, 2007).

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Analysis by qPCR of two key heat shock proteins (HSPs) confirmed that cows in SU compared with SP were heat-stressed. The HSPs are molecular chaperones, which are induced by the heat shock response (Feder and Hofmann, 1999) as a mechanism to protect cells against physiological and environmental conditions including heat stress (Wang et al., 2003; Li et al., 2011). The HSP70 protein, encoded by the *HSP70A1A* gene, is one that can help protect cells against inflammation, oxidative stress, and heat stress (Morimoto and Santoro, 1998). It is also involved in the replication and repair mechanisms of protein synthesis under ER stress conditions (Wang et al., 2003). The higher expression of *HSP70A1A* post-partum (especially at 3 and 35 d) in SU compared with SP cows together with microarray results indicates the activation of cytoprotective mechanisms against stress and DNA damage. Thees mechanisms could also have been induced by the increased demand of energy for milk production (Gessner et al., 2014), and by inflammatory and immunological conditions typical of early lactation (Catalani et al., 2010).

Surprisingly, qPCR results revealed that the expression of heat shock transcription factor 1 (**HSF1**) was greater in SP compared with SU cows. HSF1 is at the center of the heat shock response, but also can be involved in several cellular regulatory mechanisms such as development, growth and protein synthesis (Trinklein et al., 2004). Furthermore, the expression of the ER stress sensor protein EIF2AK3 also was greater ($P < 0.01$) in SP compared with SU cows. EIF2AK3 acts as an ER stress sensor transcription factor, repressing global protein synthesis, while spliced-XBP1 is involved in regulation of chaperones and the UPR (Loor, 2010). Along with the DIA results indicating an impact and flux toward SU of pathways regarding protein processing and export, these data indicate the possibility that the protein synthesis mechanisms could have been increased by heat stress in SU compared with SP cows

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(Figure 3) to better cope with the stressful conditions. Further studies are necessary to assess the role of HSF1 and the ER stress in the transition period beside the heat stress response.

Immune System. The 'Antigen processing and presentation' was the most-impacted and induced KEGG pathway in SU compared with SP cows. Along with this pathway, 'Phagosome', 'Complement and coagulation cascade' and 'Cytosolic DNA-sensing pathway' (Figure 3) is indicative of a more pronounced inflammatory status in liver. The transition period is often characterized by a degree of inflammation (Trevisi et al., 2012) due to the effect of calving itself. However heat shock proteins that belong to Hsp70 family are capable of eliciting an immune response mainly as a result of heat stress to improve the host defense mechanisms (Asea et al., 2000, Campisi et al., 2003). During this response several inflammatory mediators can be activated. Microarray analysis and qPCR data revealed changes in expression of some of these mediators such as tumor necrosis factor alpha (*TNF*) and superoxide dismutase 1 (*SOD1*) (Figure 4), or interleukins (*IL36A*), major histocompatibility complex (*MHC Class I*), autophagy related 4B (*ATG4B*) and plasma glutathione peroxidase (*GPX3*) (Table 2). The greater expression of these genes indicates a higher inflammatory state during heat stress in SU cows.

In addition to regulatory elements of the immune system, the overall expression of genes encoding for acute phase proteins (**APP**), considered reliable biomarkers of inflammation, was higher in SU compared with SP cows (Table 2). Serum amyloid A 3 (*SAA3*) and haptoglobin (*HP*), two of the main APP, had greater ($P < 0.01$) expression in SU cows, together with hepcidin antimicrobial peptide (*HAMP)* ($P < 0.04$). SAA is immediately secreted from liver after an inflammatory response (Faty et al., 2012), i.e. it is a major APP which binds to toxins and helps clear lipoproteins out of the liver (Malle et al., 1993). Concentration of HP is also increased due to inflammation in the liver (Shahzad et al., 2014). Similarly, *HAMP* expression is

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361 increased shortly after acute inflammatory stimuli (Pigeon et al., 2011; Gaël et al., 2002),
362 supporting a role for hepcidin as a positive acute-phase response peptide in dairy cattle.

363 The inflammatory response has been studied in relation to several lipid- and
364 carbohydrate-related metabolic changes, and is known to elicit variation in blood concentrations
365 of cholesterol, NEFA, glucose and lactate, and increase the rate of lipolysis (Hardardottir et al.,
366 1994, Coghe et al., 2000, Kushibiki et al., 2000). Our results agree with these physiological
367 responses underscoring alterations in lipid metabolism, as also detected by blood analysis of the
368 cows used in this study (Basiricò et al., 2011) in which NEFA were greater in SU than SP cows.
369 Further the information of these genes along with their respective immune system related
370 pathways can be seen in the File S3 under non metabolic pathways.

371 ***Hepatokines and the Stress Response***. Hepatokines are mainly linked with energy
372 homeostasis and lipid metabolism, and due to the changes we observed in those pathways it is
373 not surprising that fibroblast growth factor 21 (*FGF21*) was among the upregulated DEG in SU
374 compared with SP cows (Table 2). FGF21 was further measured via qPCR together with another
375 hepatokine, angiopoietin-like 4 (*ANGPTL4*). Schoenberg et al. (2011) reported an increase of
376 plasma FGF21 after parturition in dairy cows, and positively related its concentration, coupled
377 with a greater hepatic mRNA expression, with NEB. FGF21 has been identified as an additional
378 hormonal factor involved in the regulation of metabolic adaptations during the transition period
379 (Schlegel et al., 2012). The calving effect on *FGF21* expression has been observed in other
380 studies concerning the transition period (Khan et al., 2014, Akbar et al., 2015).

381 Changes in FGF21 synthesis around parturition could be associated with the increased
382 concentration of growth hormone and glucagon typical of the postapartal period. In non-
383 ruminants both hormones have been recognized as regulators of *FGF21* hepatic expression either

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via direct activation or, indirectly, via the activation of PPARα by NEFA (Uebanso et al., 2011). Similar to FGF21, ANGPTL4, another hepatokine controlled by PPARα (Kersten et al., 2000), is involved in the regulation of energy and lipid metabolism (Hato et al., 2008). Because lipomobilization was greater in SU compared with SP cows (Basiricò et al., 2011) their greater expression in the SU cows underscores the fact that, as a consequence of the heat stress, SU cows underwent a greater NEB during the transition period.

These two hepatokines, rather than the metabolic adaptation itself, are linked to the stressful conditions of the transition period and the environmental stress of calving during the summer. For instance, the increase during the early postpartal period in dairy cows was linked with the ER stress phenomena, i.e. ER stress induces a greater hepatic expression of *FGF21* (Gessner et al., 2014). An intramammary LPS challenge with lipopolysaccharide in early-postpartum cows resulted in marked upregulation of *ANGPTL4* in liver tissue (Graugnard et al., 2013) confirming that it may function as an APR protein. As proposed by Akbar et al. (2014), our data agree with a possible role of circulating ANGPTL4 and FGF21 as APP reflecting metabolic stress. Their application as biomarkers during the peripartal period should undergo further investigation.

Transcription Regulators. To understand the transcription mechanisms occurring within liver during spring and summer, we studied the role of transcription regulators and their connections with other downstream genes. The IPA analysis using genes with FC = 2 resulted in a total of 29 transcription regulators identified. The upstream transcription regulator network is shown in Figure 5, and more detailed information is reported in Table 4. The network highlighted four transcription regulators (*HDAC5*, *IRF3*, *USF2*, and *XBP1*), all of which were

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406 upregulated in SU compared with SP cows. These could be considered central for the modulation
407 of the hepatic transcriptome around calving during heat stress.

408 The affected genes (Table 4) were mostly related to energy metabolism, with a large
409 portion being related to lipid anabolic and catabolic pathways (i.e., *ACADM*, *HADHA*, *CPT1B*
410 and *FASN*). Except *USF2*, all regulators were involved in the modulation of genes related to ER
411 stress, UPR and inflammation response (Table 4). XBP1 is well established as a central regulator
412 of the UPR (Lee et al., 2003). Several target genes related to cell division or apoptosis were
413 overall upregulated by the transcription regulators (Table 4). Recent data suggested the
414 involvement of HDAC5 in hepatic regeneration (Huang and Rudnick, 2014). As heat stress could
415 lead to cell damage, and many HSPs have antiapoptotic functions (Kregel, 2002), the fact that
416 this particular regulator was among the most-affected suggests that it can elicit a protective role
417 during the heat shock response. Overall, the IPA network analysis underscores the importance of
418 lipid metabolism together with the effect on hepatocyte health during the transition period in
419 heat-stressed cows.

420 As judged by number of target genes (Table 4), a noteworthy output generated from the
421 IPA analysis was the seemingly important role of *HDAC5* in coordinating hepatic response to
422 heat stress. The protein encoded by this gene belongs to a histone deacetylase (HDAC), a group
423 of enzymes that are part of the epigenetic mechanism of transcription regulation. HDACs can
424 cleave an acetyl group from a histone, resulting in a more compact DNA helix that prevents
425 access to genetic information by the cell transcription machinery (de Ruijeter et al., 2003). The
426 end result of deacetylation is downregulation or silencing of the expression of genes located in
427 the given DNA region. Data from Table 4 indicates that upregulation of HDCA5 was linked with
428 both downregulation and upregulation of target genes. The fact that most of its target genes were

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upregulated in SU cows leads us to speculate that some transcription inhibitor located in the acetylated histone area was silenced, hence, minimally affecting expression/upregulation of the target gene/s. It also is likely that the genes regulated by HDAC5 are subject to control from other transcription regulators, e.g. *SIRT7* is controlled by *MAGED1*, *NOCL2*, *SNW1*, and *SRSF2* (Table 4).

CONCLUSIONS

Management of the transition cow is already at the center of the modern dairy industry. The hepatic transcriptome of transition dairy cows is strongly affected by season of calving. Besides expanding our knowledge of molecular adaptations coordinating production responses (e.g. milk production, energy balance), results from this study underscore how environmental stress not only alters energy metabolism in liver, but also induces an inflammatory and intracellular stress response. When combined with the physiological inflammatory state characteristic of the peripartal period, the overall impact is a greater susceptibility to health disorders early postpartum. The holistic analyses of the liver transcriptome response to calving in the summer at high environmental temperatures supports recent findings that cows experience ER stress around parturition. Furthermore, novel mechanisms related to epigenetic regulation of DNA transcription appear to be involved in the response to extreme environmental conditions. Their functional relevance merits further study.

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452

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Table 1. Symbol and name of genes analyzed by qPCR.

Symbol	Gene name
ACOX1	acyl-Coenzyme A oxidase 1, palmitoyl
ANGPTL4	angiopoietin-like 4
APOB	apolipoprotein B
CPT1A	carnitine palmitoyltransferase 1A
DGAT1	diacylglycerol O-acyltransferase 1
EIF2AK3	eukaryotic translation initiation factor 2 alpha kinase 3
FGF21	fibroblast growth factor 21
GPX1	glutathione peroxidase 1
HAMP	hepcidin antimicrobial peptide
HP	haptoglobin
HSP70A1A	heat shock 70kDa protein 1A
HSTF1	heat shock transcription factor 1
MTTP	microsomal triglyceride transfer protein
PC	pyruvate carboxylase
PCK1	phosphoenolpyruvate carboxykinase 1
PDK4	pyruvate dehydrogenase kinase, isozyme 4
PPARA	peroxisome proliferator activated receptor alpha
SAA3	serum amyloid A 3
SOD1	superoxide dismutase 1, soluble
SOD2	superoxide dismutase 2, mitochondrial
SREBF2	sterol regulatory element binding transcription factor 2
TNF	tumor necrosis factor alpha

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Table 2. Differentially expressed genes with fold-change > 3 in liver of cows calving during the summer (SU) compared with spring (SP).

Symbol	Description	SU vs. SP
HP	haptoglobin	7.44
GPX3	glutathione peroxidase 3 (plasma)	6.85
IL36A	interleukin 36, alpha	6.52
MT1E	metallothionein 1E	5.03
ABHD1	abhydrolase domain containing 1	4.69
DEFB7	defensin beta 7	4.62
FAM47E	family with sequence similarity 47, member E	4.59
EPHX4	epoxide hydrolase 4	4.53
PRSS12	protease, serine, 12 (neurotrypsin, motopsin)	4.30
DEFB1	defensin, beta 1	4.15
TBATA	chromosome 28 open reading frame, human C10orf27	4.13
CA1	carbonic anhydrase I	4.08
MT2A	metallothionein 2A	4.00
TRAFD1	TRAF-type zinc finger domain containing 1	3.98
MT1A	metallothionein-1A	3.82
INMT	indolethylamine N-methyltransferase	3.79
DES	desmin	3.76
MIOX	myo-inositol oxygenase	3.67
PCP4	Purkinje cell protein 4	3.65
FOLR1	folate receptor 1 (adult)	3.64
LOC613867	trace amine-associated receptor 7a-like	3.63
TMC2	transmembrane channel-like 2	3.62
ATP6V1C2	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C2	3.57
HOPX	HOP homeobox	3.55
HPS4	Hermansky-Pudlak syndrome 4	3.51
FGF21	fibroblast growth factor 21	3.51
CATHL1	cathelicidin 1	3.50
JSP.1	MHC Class I JSP.1	3.43
SAA3	serum amyloid A 3	3.43
NTN4	netrin 4	3.40
ADCY7	adenylate cyclase 7	3.37
MUC15	mucin 15, cell surface associated	3.35
PPL	periplakin	3.35
ITGA11	integrin, alpha 11	3.32
C20H5orf49	chromosome 20 open reading frame, human C5orf49	3.31
TBC1D14	TBC1 domain family, member 14	3.30
ISM1	isthmin 1 homolog (zebrafish)	3.29
DYX1C1	dyslexia susceptibility 1 candidate 1	3.26
LOC517799	olfactory receptor, family 52, subfamily N, member 4-like	3.21
HM13	histocompatibility (minor) 13	3.19
MICAL2	microtubule associated monooxygenase, calponin and LIM domain containing 2	3.19
LBP	lipopolysaccharide binding protein	3.16
LOC519492	olfactory receptor, family 10, subfamily T, member 2-like	3.15
GPC3	glypican 3	3.15
NWD1	NACHT and WD repeat domain containing 1	3.07
PPP1CA	protein phosphatase 1, catalytic subunit, alpha isozyme	3.03

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ATG4B	autophagy related 4B, cysteine peptidase	3.02
LOC509354	nucleoside diphosphate kinase 6-like	3.01

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Table 3. Differentially expressed genes with fold change < 3 in liver of cows calving during the summer (SU) compared with spring (SP).

Symbol	Description	SU vs. SP
BOLA-DQB	major histocompatibility complex, class II, DQ beta	-6.77
CHAMP1	chromosome alignment maintaining phosphoprotein 1	-6.48
KIAA0895	KIAA0895 ortholog	-5.50
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	-5.09
LOC100850628	olfactory receptor 51L1-like	-5.06
PVRL1	poliovirus receptor-related 1 (herpesvirus entry mediator C)	-4.95
MBOAT2	membrane bound O-acyltransferase domain containing 2	-4.24
MYH7	myosin, heavy chain 7, cardiac muscle, beta	-4.10
ST8SIA1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	-3.91
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	-3.88
THRSP	thyroid hormone responsive	-3.79
KCTD20	potassium channel tetramerisation domain containing 20	-3.76
ANKS1B	ankyrin repeat and sterile alpha motif domain containing 1B	-3.66
KCNB2	potassium voltage-gated channel, Shab-related subfamily, member 2	-3.47
LRRC49	leucine rich repeat containing 49	-3.42
RPL4	ribosomal protein L4	-3.39
LOC100336905	uncharacterized LOC100336905	-3.19
SPAM1	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	-3.13
PCSK2	proprotein convertase subtilisin/kexin type 2	-3.11
HMGCS1	HMGCS1 protein-like	-3.11
ASH1L	ash1 (absent, small, or homeotic)-like (Drosophila)	-3.09
FADS2	fatty acid desaturase 2	-3.09
LOC613728	nuclear RNA export factor 3-like	-3.04
ACAA1	acetyl-CoA acyltransferase 1	-3.04
KCNJ13	potassium inwardly-rectifying channel, subfamily J, member 13	-3.04

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Table 4. Upstream differentially expressed transcription regulators and their target genes with fold change ± 3 in liver of cows calving during the summer (SU) compared with spring (SP).

Transcription regulator	Target gene and response in SU vs. SP
DNAJB6	↑*KRT8*, **PHKG2*, ↑*KRT18*
ELF3	↑*PRKCSH*, ↑*KRT18*
HDAC5	↑*TUBB*, ↑*ACAT1*, ↑*PKN1*, ↑*HADHA*, ↑*ACADM*, ↑*PPIA*, ↑*CPT1B*, ↑*TUBB4B*, ↑*EEF1G*, ↑*CKB*, ↑*PPP1CA*, ↓*MYH7*, ↓*RPL4*, ↓*ACLY*, ↓*SMC1A*, ↓*FASN*, ↓*RPL3*
ID1	↑*CTGF*, ↑*CDC20*, ↑*CDK4*
IRF3	↓*PPP2R3A*, ↓*IFNW1*, **IFNA16*, ↑*USP18*
MAGED1	↑*SIRT7*, ↑*BAG6*
NOC2L	↑*SIRT7*, ↑*PPP1CA*
PPP1R27	↑*PPP1CA*
SNW1	↑*SIRT7*, ↑*TUBB*, ↑*EEF1A1*, ↑*EEF1G*
SRSF2	↑*SNCA*, ↑*SIRT7*
USF2	↑*UCP2*, ↑*KARS*, ↑*S100A9*, ↓*FASN*, ↑*CPT1B*, ↑*CDK4*, ↓*THRSP*
XBP1	↓*FASN*, ↑*FKBP11*, ↑*HM13*

*Included in the IPA network but without any up or downstream regulation

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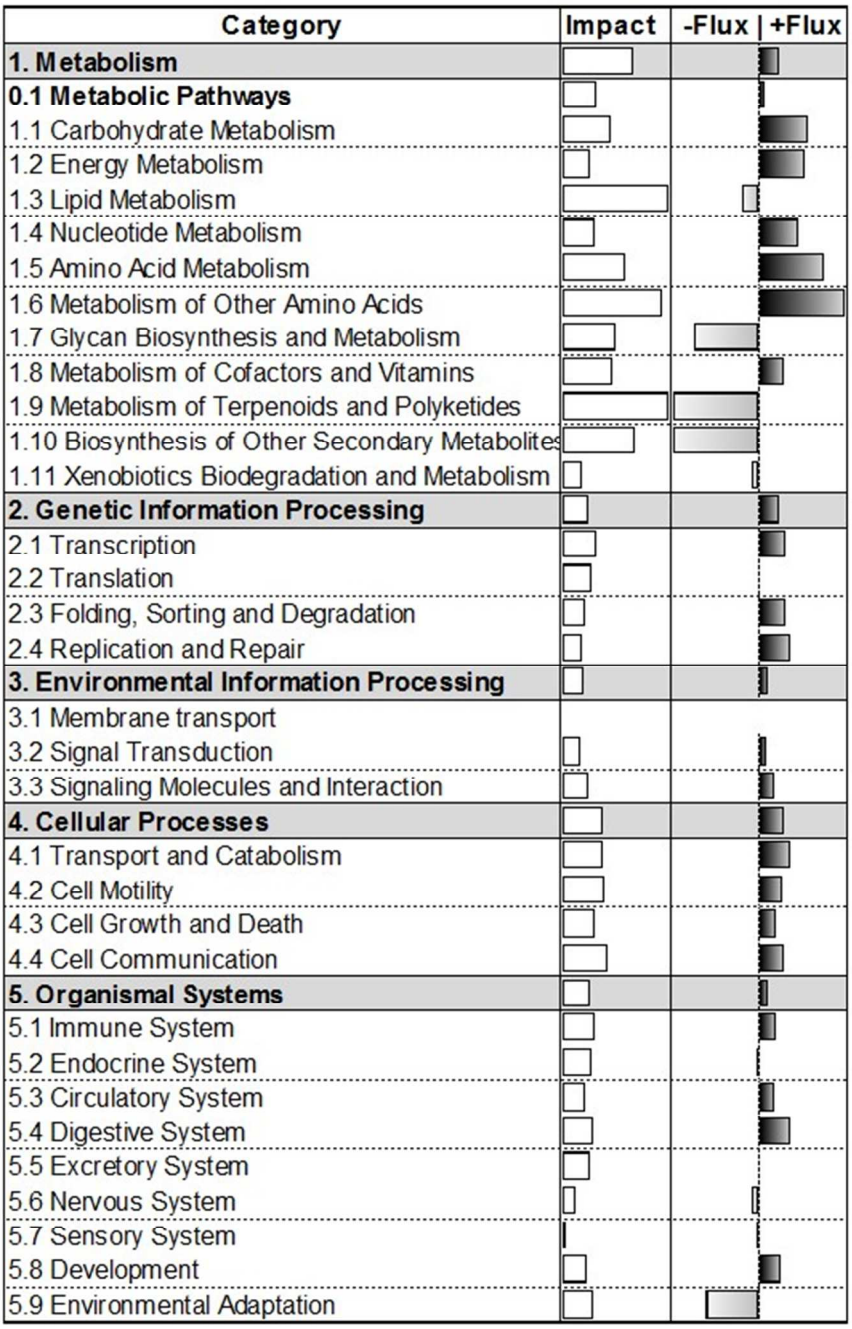

Figure 1.

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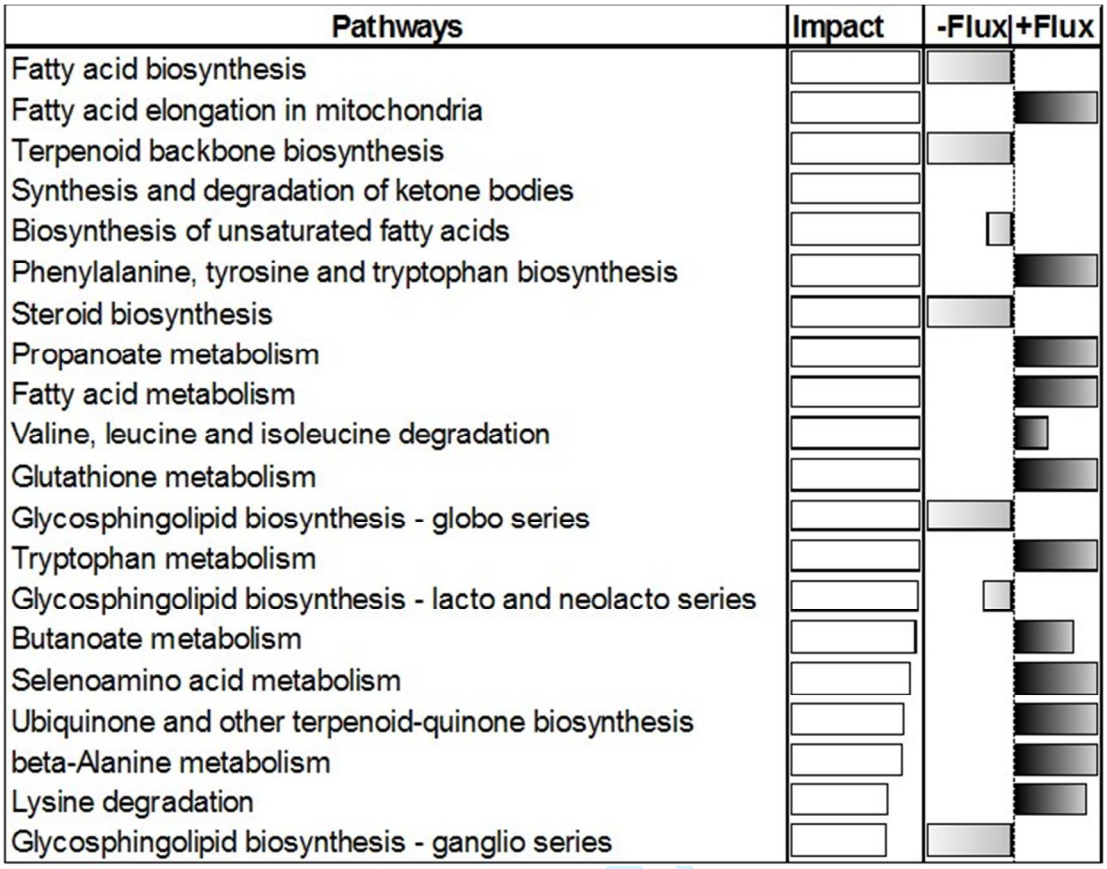

Figure 2.

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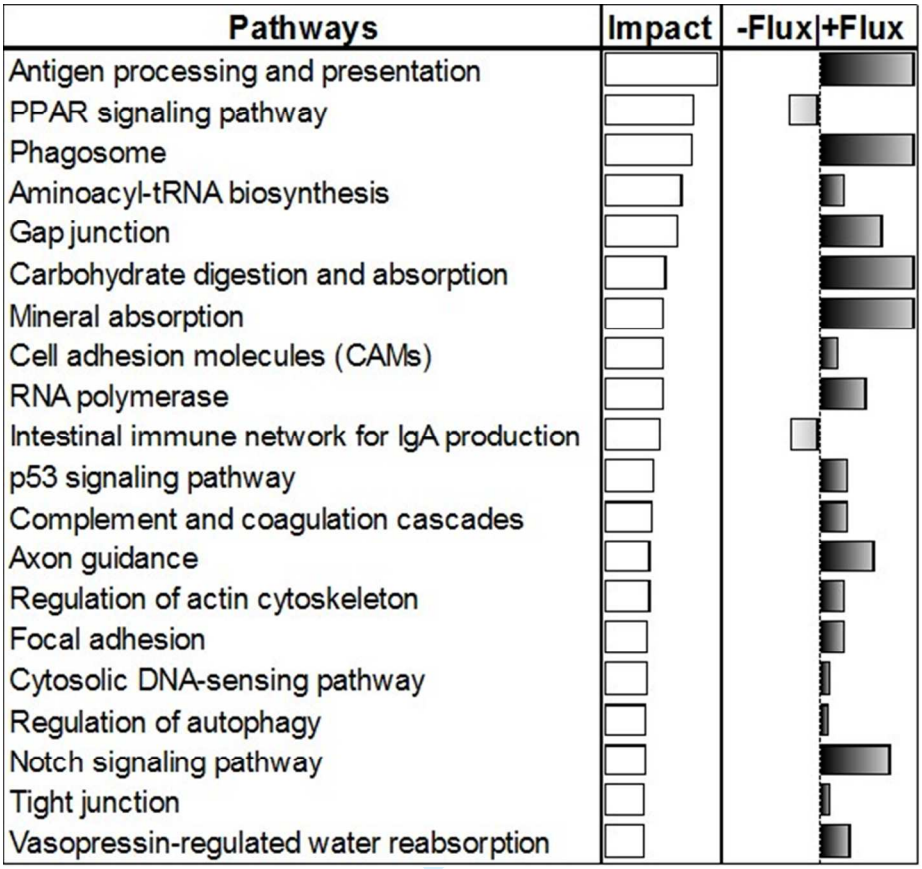

Figure 3.

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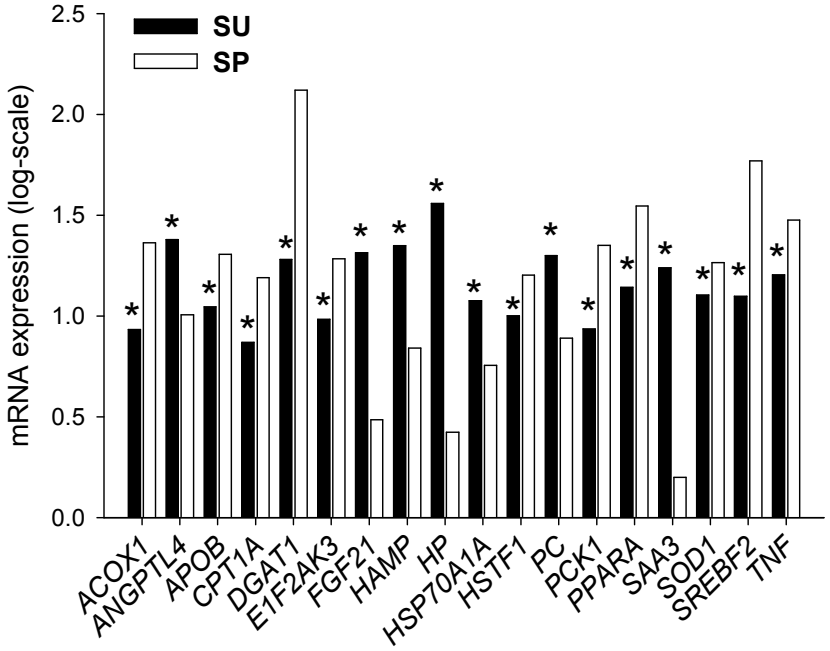

Figure 4.

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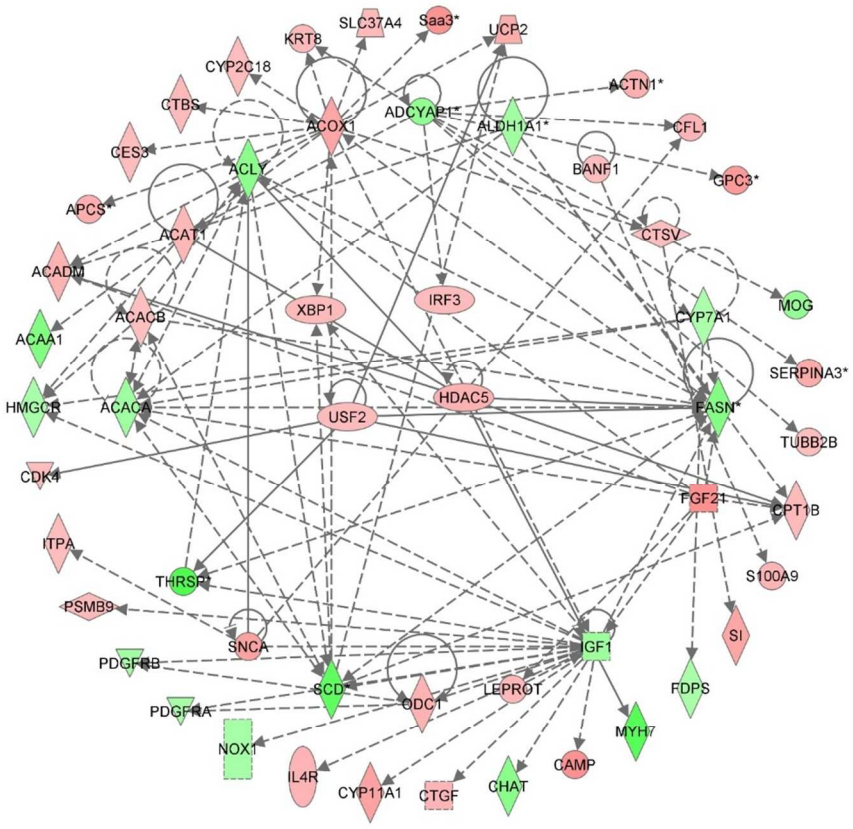

Figure 5.

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Figure legends

Figure 1. Summary of the KEGG categories and sub-categories resulting from DIA analysis in liver of cows calving during the summer (SU) compared with spring (SP). On the right hand side the two columns represent the impact and flux responses. The transparent bars represent the impact values (0 to 100), while the flux column represents negative (-) and positive (+) flux (-50 to +50) based on the direction of the impact. The negative flux is represented with grey bars, and positive flux is represented with black bars.

Figure 2. The top 20 most impacted metabolic KEGG pathways ranked by overall impact value in liver of cows calving during the summer (SU) compared with spring (SP). On the right hand side the two columns represent the impact and flux values. On the right hand side the two columns represent the impact and flux responses. The transparent bars represent the impact values (0 to 100), while the flux column represents negative (-) and positive (+) flux (-50 to +50) based on the direction of the impact. The negative flux is represented with grey bars, and positive flux is represented with black bars.

Figure 3. The top 20 most impacted non-metabolic KEGG pathways ranked by overall impact values in liver of cows calving during the summer (SU) compared with spring (SP). On the right hand side the two columns represent the impact and flux responses. The transparent bars represent the impact values (0 to 100), while the flux column represents negative (-) and positive (+) flux (-50 to +50) based on the direction of the impact. The negative flux is represented with grey bars, and positive flux is represented with black bars.

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Figure 4. mRNA expression by qPCR of 18 significantly-affected genes tested in liver of cows calving during the summer (SU) compared with spring (SP). *Differs in expression ($P < 0.05$) in SU compared with SP.

Figure 5. Ingenuity Pathway Analysis (IPA) upstream network analysis of differentially expressed genes (DEG) in liver of cows calving during the summer (SU) compared with spring (SP). Up-stream regulators are located at the center of the network while the downstream target genes are located at the periphery. The arrow represents the direction of the target molecule. The red color represents upregulation of genes in SU, and green color represents upregulation of genes in SP.

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