BIOINFORMATICS ANALYSIS OF LIVER AND ADIPOSE TISSUE MICROARRAY DATA FROM PERIPARTURIENT DAIRY CATTLE AND DEVELOPMENT OF A WEB-BASED RUMINANT SPECIFIC MICROARRAY DATABASE

BY

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THESIS

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ABSTRACT

To-date bovine liver and adipose tissues have been investigated through several molecular techniques. However, for the most part molecular work in these tissues has been conducted using a reductionist approach discussing the individual genes, proteins or pathways. This approach does not necessarily evaluate the overall biological effect of a particular condition (e.g. treatment, physiological state) on a pathway. In some instances these tissues have been investigated at the transcriptomic and proteomic levels using several bioinformatics techniques such as gene enrichment analysis or over-represented approach (ORA).The ORA analysis is widely-used in scientific community for functional analysis of high-throughput data such as microarray or RNA-sequencing. It is performed to associate gene sets with their relevant biological pathways or functions using statistical methodologies. However, this approach has several limitations including inability to compare time series experiments or multiple treatments within the same experiment. In order to overcome these limitations, the Dynamic Impact Approach (DIA) has been developed by our group. This approach takes the concept of dynamism into consideration using proportion of differentially expressed genes compared to the genes present in array, statistically significant p-values and fold change values. This approach provides a holistic view of dynamic changes along time series experiments by means of impact and flux. The impact and flux take into account the overall biological perturbation and its direction (e.g., either induced or inhibited) to interpret the biological functions. For our time series microarray datasets, we employed both the DIA and enrichment analysis approaches together to infer the biological meanings. The current studies are mainly focused on the systematic evaluation of the tissues during the periparturient period, i.e. the last three weeks through the first three weeks around parturition. The results indicated a significant amount of biological adaptation in the liver

and adipose tissue during pregnancy into early lactation. The large amount of data generation and curation needs a way to be systematically stored, retrieved and analyzed using bioinformatics techniques. For this purpose, we have laid the foundation of a ruminant specific microarray database (Ruminant Physiome) for the data mining and representation. The objectives of this research were, 1) to evaluate the metabolic responses in the liver of dairy cows that were overfed or restricted-fed dietary energy prepartum, 2) to evaluate the metabolic changes and physiological adaptations in the bovine adipose tissue occurring during pregnancy into early lactation, and 3) to develop a ruminant specific ‘omics’ website to provide public access to the expression profiling datasets along with their functional results.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BER	Base Excision Repair
BP	Biological Processes
CC	Cellular Components
CP	Cellular Processes
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DEG	Differentially Expressed Genes
DIA	Dynamic impact approach
DIM	Days in milk
ECM	Extra cellular matrix
EIP	Environmental information Processing
FA	Fatty acid
FC	Fold Change
FDR	False discovery rate
GEO	Gene Expression Omnibus
GIP	Genetic Information Processing

GO	Gene Ontology
KEGG	Kyoto encyclopedia of genes and genomes
MAPKKK	Mitogen-activated protein kinase kinase kinase
MF	Molecular Function
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty acid
OF	Overfed
ORA	Over Represented Approach
OS	Organismal System
PPAR	Peroxisome proliferator-activated receptor
PPP	Pentose Phosphate Pathway
RE	Restricted Energy
SSH	Secure Shell
URL	Uniform Resource Locator

CHAPTER 1: LITERATURE REVIEW

Applications of Top-Down and Bottom-up Systems Approaches in Ruminant Physiology and Metabolism[1]

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1.1. INTRODUCTION

Bioinformatics and systems biology are the key areas to handle and analyze the enormous amount of biological data that are being generated through high throughput 'omics' technologies (Bionaz and Loor, 2012). Bioinformatics provides a platform to store, handle, retrieve and analyze the biological data (Claverie and Notredame, 2011) while systems biology deals at systems level information of living organisms by means of experimental and computational approaches using bioinformatics techniques (Shahzad and Loor, 2012). Both work in conjunction with each other to unravel complex biological systems (Van Regenmortel, 2004).

Systems biology is an interdisciplinary field that concentrates on experimental and computational biology. At the core of this approach, which is not novel, is the concept of dealing with a system as a whole rather than its constitutive parts. Advancements in computational biology, genome sequencing, and high-throughput technologies in the last decade have increased the awareness of the scientific community for approaching biological systems in an integrative fashion, i.e., allow access to the functional capabilities of an individual organism en masse. However, the notion of dealing with a system as a whole was proposed several decades earlier. For instance, in 1934 the Austrian biologist Ludwig von Bertalanffy proposed the application of the "general systems theory" (GST) in biology, cybernetics (structural study of regulatory systems) and other areas (Weckowicz, 1989). In the mid-20th century, the geneticist and biochemist Henrik Kacser focused on the use of systematic approaches instead of analyzing separate components of a metabolic system (Cornish-Bowden, 2005). Mihajlo Mesarovic (1968), a mathematician and engineer at Case Western Reserve University, also emphasized the need for systematic applications in biology (McNamara, 2010b).

The field of genomics and transcriptomics has already provided an enormous amount of biological information. Currently, there is a need to communicate biological knowledge systematically, e.g., linking the genome to the whole organism. Newly emerging bioinformatics techniques along with biological data generated from genomics and transcriptomics studies have already allowed biologists to apply modern systems approaches to study interactions occurring inside living systems. The work of Palsson's group from the 1990's onward contributed to the development of genome-scale mathematical models to understand the biological interactions from simpler organisms (e.g., microbes) to humans. From 1999 onward, with the first genome-wide metabolic reconstruction of *Haemophilus influenza* (Edwards and Palsson, 1999), research in the field of modern systems biology has exploded. Several genome-wide and tissue-specific reconstruction projects across a broad range of species have been published, e.g., more than 50 in 2009 (Oberhardt et al., 2009) to more than 80 in 2011. It is likely that work in this area will continue to grow. Currently available genome-scale metabolic reconstructions ranging from bacteria, archaea, to multicellular eukaryotes are shown in Figure 1.1. (Retrieved from Systems Biology Research Group, University of California San Diego; on June 19, 2011 [http://systemsbiology.ucsd.edu/In_Silico_Organisms/Other_Organisms]).

Genome-scale metabolic network reconstructions of model organisms have been assembled in a BiGG (biochemically, genetically, and genomically structured) knowledgebase (Schellenberger et al., 2010) that aims to represent all known metabolic pathways of an organism. The BiGG knowledgebase works with the COBRA (constraint based reconstruction and analysis) toolbox (Schellenberger et al., 2011), while metabolic network reconstructions hosted by it are created using the steps described in details by Reed et al. (Reed et al., 2006), Feist et al. (Feist et al., 2009) and Thiele and Palsson (Thiele and Palsson, 2010). These

reconstructions have been assembled for more than 80 different organisms ranging from unicellular (e.g., bacteria (Edwards and Palsson, 1999; Feist et al., 2007) and yeast (Mo et al., 2009)), to multicellular organisms (e.g., mouse (Sigurdsson et al., 2010), Arabidopsis thaliana (Radrich et al., 2010), and humans (Duarte et al., 2007; Ma et al., 2007)).

The expanding suite of tools for applying modern systems biology requires bioinformatics expertise. Bioinformatics is generally defined as a field that relies on computational resources to analyze biological data (e.g., genome, transcriptome, metablome, or fluxome) on a large scale (Luscombe et al., 2001). It also encompasses the development of tools ranging from genome to proteome analyses including transcriptomics data (Butcher et al., 2004; Loor and Cohick, 2009). One of the goals of bioinformatics is to accelerate the interpretation of large amounts of 'omics' data (Loor and Cohick, 2009). For instance, Lemay et al. (2007) applied this technique on mouse mammary tissue microarray data that was generated during pregnancy, involution and lactation time points.

With the rapid development of bioinformatics analysis tools, there is a need to tailor some of those to help in the automation of ruminant genomics. From a ruminant animal perspective, one long-term goal of this process involves the development of mathematical and mechanistic models that would link the genome (e.g., bovine, caprine) to the whole organism (Sumner-Thomson et al., 2011). The pioneering work of Baldwin and his colleagues (Baldwin et al., 1987a; Baldwin et al., 1987b; Baldwin et al., 1987c) provided one of the first comprehensive mathematical models ('Molly') that attempted to link genotypic to phenotypic data (Baldwin, 1995). The model was aimed at determining the relationship between diet and animal performance (McNamara, 2010a). In essence, the goal was to develop "simple" models to understand the relationship between digestive processes and their effects on metabolic pathways

in liver, mammary, and adipose tissue of dairy cattle (*Bos taurus*). Upon successful completion of the cattle genome sequencing project (Elsik et al., 2009), the process of genome-wide and tissue-specific reconstructions in this species was accelerated with the application of both "top-down" and "bottom-up" approaches. An initial attempt to assemble genome-wide metabolic pathway information has already been performed by Seo and Lewin (2009). Further information about these metabolic pathways can be found using the online BioCyc and MetaCyc databases (Krieger et al., 2004; Caspi and Karp, 2007; Caspi et al., 2008).

The aim of this review is to provide a brief description of modern systems biology concepts and their applications in high-producing ruminants (i.e., dairy cattle). We succinctly describe the top-down and bottom-up approaches but mainly focus on the top-down approach for metabolic pathways reconstruction and analysis. The overall goal is to underscore the uniqueness of these approaches to provide a holistic view of complex biological interactions occurring in ruminants. We also discuss current methodologies that would help to accelerate metabolic reconstruction in ruminants as a means to enhance our biological and practical knowledge. In particular, we provide tissue-specific examples of ongoing efforts in the top-down reconstructions in the bovine. We believe that such knowledge will, in the long-term, help to improve efficiency of nutrient use in particular, and contribute in meeting the growing needs of high-quality food for human consumption.

1.2. MODERN SYSTEMS BIOLOGY

Modern systems biology refers to the use of both mathematical and 'omics' approaches to expand the knowledge of biological functions (Van Dien and Schilling, 2006). In this context, one of the widely-accepted approaches for mathematical modeling is the use of constraint-based

modeling established by Price et al. (2004). Within this approach, constraints are applied under mathematical frameworks to mimic real-life biological activities (e.g., the interaction between reactants and products) *in silico*. These constraints implicitly define the solution space of a metabolite and its reactions with respect to other metabolites. The solution space is a mathematical term that can be defined using biological phenomena such as an allowed region in a biological network where reactants can be converted into one or more possible products (Price et al., 2004). During such conversions a steady-state flux distribution is required through all the reactions. These steady-state flux distributions are described in terms of extreme pathways whereas these extreme pathways are categorized into three main types that measure the flux distributions among the participating substrates, cofactors, and products during a series of reaction steps (Schilling et al., 2000; Price et al., 2002).

The detailed methodology of constraint-based modeling was developed into a computational tool called COBRA by Becker and his colleagues (2007). The COBRA toolbox is widely used in systems biology to reconstruct genome-scale mathematical models. This toolbox performs flux-balance analysis (FBA) that is used to define the metabolic behavior of substrates and their products within a solution space context (Covert and Palsson, 2002; Orth et al., 2010). Recently, this tool is further modified into a new version 2.0 by Schellenberger et al. (2011) to contain improved functions such as "network gap filling, ^{13}C analysis, metabolic engineering, omics-guided analysis, and visualization". This tool has facilitated efforts to integrate biological systems, effectively expanding from the reductionist methodologies.

The reconstructed mathematical models are used to simulate user-defined biological conditions *in silico*. For example, these models can be used in drug designing (Davidov et al., 2003), biofuel production (Rupprecht, 2009), or in numerous other related applications. An

important focus of systems biology has been to uncover new characteristics emanating from the network interactions, all of which should lead to a more holistic view of an organism (Loor and Cohick, 2009) and its useful applications for the benefits of humans. This emerging field also is dedicated to understanding the physiology of normal and abnormal (diseased) states from a cellular level to the whole organism (Butcher et al., 2004).

1.3. SYSTEMS BIOLOGY APPROACHES

The metabolic behavior of a cell can be approached in either a bottom-up or top-down directionality. The former encompasses the development of automated tools and implementation of mathematical models; whereas, the latter encompasses data processing from 'omics' levels to pathways and/or individual gene levels of an organism (Bruggeman and Westerhoff, 2007). Oltvai and Barabasi depicted these approaches in the form of a pyramid describing two different levels in terms of "organism specificity" and "universality". They emphasized that a cell can be approached from both bottom to top (universality) or from top to bottom (organism specificity) equally, i.e., from molecules to the scale-free networks or modules, or moving from a network scale-free and hierarchical nature to organism-specific modules (2002). In contrast, Kummel et al. (2006) combined these two sets of approaches with the second law of thermodynamics under the name of "network embedded thermodynamics (NET) analysis". NET analysis essentially combines these three ideas into a single approach to reveal functional behavior of the metabolic network interactions. This is indeed a novel approach to deal with biochemical properties in terms of physical laws of thermodynamics and aimed to help us improve our knowledge of cell physiology. There also are ongoing efforts for building automated tools that incorporate the steps of the bottom up approach to automatically create genome-scale models. One example is the

availability of a software called SEED which was initially validated with *Staphylococcus aureus* (DeJongh et al., 2007).

A) Bottom-up Approach:

The bottom-up approach is aimed at thoroughly crafting detailed models that can be simulated under different physiological conditions. This approach combines all organism-specific information into a complete genome-scale model to provide an integrative view of the biological interactions occurring inside living systems. It employs the methodology built on constraint-based modeling (Price et al., 2004), that allows to build genome-scale mathematical models using four main steps, which are i) draft reconstruction, ii) manual curation, iii) converting curated models into mathematical format, and then iv) validation of these models using literature reviews (bibliomics data), biochemical assays, and 'omics' data (Feist et al., 2009; Thiele and Palsson, 2010). These four steps are summarized below:

i) **Draft reconstruction**: Draft reconstruction encompasses data collection from different online resources such as genomics, biochemical, metabolic, and/or organism-specific databases. The data are extracted through bioinformatics software tools e.g., pathway tools (Karp et al., 2002) and metaSHARK (Pinney et al., 2005). In the case of ruminant draft reconstruction projects, freely accessible genomics databases include NCBI (Cates, 2006), EntrezGene (Maglott et al., 2005), UCSC Genome Browser (Kent et al., 2002), UniPort (2012) and BGD (Bovine Genome Database) (Childers et al., 2011); biochemical databases include KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa and Goto, 2000), BRENDA (BRaunschweig ENzyme DAtabase) (Schomburg et al., 2004; Chang et al., 2009), PubChem identifier (Austin et al., 2004), CAS (Chemical Abstracts Service) (Buntrock, 2001), CheBI (Chemical Entities of Biological Interest)

(Degtyarenko et al., 2008), and Transport DB (Ren et al., 2007); and among the metabolic- and organism-specific reconstruction databases are (but not limited to) Reactome (Matthews et al., 2009), BioCyc and MetaCYC (Krieger et al., 2004; Caspi and Karp, 2007; Caspi et al., 2008). Draft reconstruction is an automated process; hence, there are equally likely chances of incorporating incorrect information of metabolites or failing to include key metabolites or their reaction information (Thiele and Palsson, 2010). To avoid this misrepresentation, further manual curation is required, which is briefly described in the following step.

ii) **Manual Curation:** This step is human-intensive and dependent on the actual organism-specific genome, metabolome, or fluxome information. Software-assisted (e.g., pathway tools) draft construction steps help to add missing data or to remove unnecessary information. To validate the constructed draft, textbooks, scientific articles, literature reviews, biochemical assays (i.e., validation), and organism-specific databases are used (2009; Thiele and Palsson, 2010). For ruminant-specific reconstructions, knowledge of metabolic pathway conservation relative to other mammals (e.g., mouse, human) is also useful. For example, evolutionary divergence of metabolic pathways can be helpful to uncover similarities and differences between the organism of interest (e.g., bovine) and known organisms (e.g., human) to build a common evolutionary relationship. This illustration can be exemplified using the creation of fish metabolic network (MetaFishNet) (Olah et al., 2010). This metabolic network is built upon homology-based searches using relationships from diverse species.

iii) **Conversion to Mathematical Models:** Following the completion of a curated draft, it is transformed into a mathematical language to perform simulations. For this purpose, mathematical software tools such as Matlab (Mathwork, Natwick, MA, USA) embedded COBRA toolbox (Becker et al., 2007), SBML (systems biology markup language) software (Hucka et al., 2003), and linear programming (LP) or quadratic programing (QP) solver can be used. During this step, balanced stoichiometric matrices are constructed, biomass objective functions (Feist and Palsson, 2010) are defined, FBA (Orth et al., 2010) is performed, and then flux variability analysis (FVA) is conducted to verify the robustness of the model (Gudmundsson and Thiele, 2010).

iv) **Network Validation:** The fourth and final step involves the iterative refinement of the model using different gap-filling algorithms. The model is checked for inconsistencies using defined objective functions. If a reconstructed model is not consistent with the expected results, then the draft is rechecked from step 2 and necessary changes are made. Due to the missing metabolic knowledge in some species, such as gaps (a missing reaction that consumes or produces a metabolite) and orphan reactions (reactions with incomplete or absent information about genes or enzymes), this approach faces some real challenges (Thiele and Palsson, 2010). These gaps and orphan reactions can be treated by implementing several gap-filling algorithms described by Orth and Palsson (Orth and Palsson, 2010). However, in version 2.0 of COBRA toolbox, gap-filling properties are also included. Following these metabolic network reconstructions, condition-specific models can be derived from a single reconstruction (Palsson, 2009). Figure 1.2 represents the summary of these four steps.

B) Top-down Approach:

The top-down approach originates from experimental data and information is spanned to reconstruct metabolic models. It can help to unravel biological behavior and underlying interactions using 'omics' data, which can be obtained via standard top-down methodologies such as DNA microarrays (Schena et al., 1995), RNA-Seq (Wang et al., 2009b), or other genome-enabled technologies. According to Van Dien and Schilling (2006), the flow of information in the top-down approach occurs from the transcriptome and proteome to flux-balanced metabolic pathways. This approach covers the whole genome; thus, it is considered as a "potentially complete" approach in that it deals with all the genome-wide transcriptomic information (Westerhoff and Palsson, 2004; Bruggeman and Westerhoff, 2007). From our perspective, the top-down approach can be explicitly divided into the following five stages. We have presented these stages using the existing DNA microarray case studies (Figure 1.3):

Stage 1: Sample Collection and Laboratory Experiments

Experiments are designed such that animals are allowed sufficient amounts of time for specific treatments or stimuli to have their effects on selected physiological parameters (e.g., milk production, growth, or fat deposition). More comprehensive studies involve repeated sampling of the same animal over extensive periods of time (e.g. the lactation cycle in dairy cattle or the neonatal period in calves). At the end of a suitable treatment period, tissue samples are collected (e.g., via biopsy or at slaughter) from control and treated animals. Some experiments may not necessarily deal with a treatment per se, but may involve evaluation of ontogenic changes of the transcriptome, proteome, metabolome, or fluxome (e.g. during the lactation cycle). After sample collection, RNA is extracted for subsequent analyses. The RNA extraction protocols may vary, but for most experiments, these involve reagents containing

phenol and are based on a classical method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The purification steps involve the use of commercial columns, while extra impurities including residual DNA (if acid phenol-chloroform is not used during extraction) are removed using a commercial DNase I enzyme. The extracted RNA is then reverse-transcribed to cDNA or cRNA and subsequently used for hybridization to DNA, oligonucleotide, or other types of expression microarrays.

Stage 2: Microarray Platform

DNA microarrays are widely used to determine the expression level of mRNA in specific cell or tissue types. Custom microarray platforms or commercially available platforms, such as Affymetrix (Butcher et al., 2004), Agilent (Westerhoff and Palsson, 2004), and Amersham BioSciences (Lemay et al., 2007) are generally used. Each microarray slide contains a fixed number of spots, and each spot represents a particular gene. The experiment is performed according to standard protocols mainly involving cDNA synthesis via reverse transcriptase polymerase chain reaction (RT-PCR) from extracted RNA, labeling with fluorescent dyes (e.g., Cy3 and Cy5), hybridization to the arrays, washing, and then scanning of these arrays using confocal laser scanners (Loor et al., 2005; Loor et al., 2006; Quackenbush, 2006; Michiels et al., 2007). After scanning array images, data are readily available for normalization and statistical analysis.

Stage 3: Statistical Analysis

Before employing the standard statistical analysis, data are preprocessed by using one of several available normalization techniques to remove systematic bias while preserving the variation in gene expression occurring due to biologically relevant or treatment-related changes

in transcription. Data are usually normalized by log-transformations (e.g., log base 2). Following log-transformations, fold-change values can be calculated relative to a control sample or to some reference time point. Subsequently, statistical tests (e.g., paired student t-test (Steel and Torrie, 1960)) can be applied using statistical software such as SAS (Statistical Analysis System (2000)) or R (Statistical Computing Language (Ihaka and Gentleman, 1993)). The statistical probability values (*p*-values) to determine differentially expressed genes (DEG) are obtained and adjusted for multiple comparisons using correction methods such as Bonferroni (Hochberg, 1988) or Benjamini and Hochberg's false discovery rate (FDR) (Benjamini and Hochberg, 1995; Reiner et al., 2003).

Stage 4: Implementation of Bioinformatics

Microarray (genes/oligonucleotides) inserts/spots are annotated using different databases such as NCBI (Cates, 2006), DAVID (Huang et al., 2009b), or bioDBnet (Mudunuri et al., 2009). Annotation helps discern the DEG affected by a particular stimulus or stimuli (e.g. dietary treatments, drug effects, or biological or developmental time points). Typically the FDR probability value cutoff criterion less than 1% ($p \leq 0.01$) or 5% ($p \leq 0.05$) is used to determine DEG. After selecting the list of DEG, bioinformatics software tools are applied to determine the functional significance of affected genes. There are several software packages for microarray data analyses and interpretation ranging from commercial (e.g., MAS 5.0 from Affymetrix platform; Ingenuity Pathway Analysis®) to open-source software (e.g., R bioconductor). According to a survey conducted by Huang and colleagues in 2009 (2009a) there are approximately 68 bioinformatics enrichment-analysis tools, which are available for curating DEG lists. Among these tools, the DAVID bioinformatics resource is a popular and user-friendly tool to extract biological information from large gene or protein lists (Huang et al., 2009b). This

resource has multiple applications including annotation of large gene lists, function prediction, and function categorization within “chromosomes”, “KEGG pathways” “biological processes”, “cellular components” and “molecular functions”.

To further analyze the biological interactions or pathways, DEG lists can be mined with software tools as implemented in several research projects such as GeneSpring GX (Seo and Lewin, 2009) used by (Loor et al., 2005; Loor et al., 2006), Ingenuity Pathway Analysis® (Loor et al., 2007), used by Loor et al. (2007), and Genesis (Sturn et al., 2002) used by Graugnard et al. (2009). Our research group also has recently developed a novel approach termed the Dynamic Impact Approach (DIA) (Bionaz et al., 2012a; Bionaz et al., 2012b) for functional analysis of expression profiling data. The KEGG database (Kanehisa et al., 2008) is used to visualize the DEG by uploading the list of gene IDs and their respective fold-change values to the KEGG array tool. Ultimately, the goal of these tools is to provide a visualization of the genes and their interactions (de la Fuente et al., 2002), protein-protein interaction networks (Bork et al., 2004), or more recently, the dynamic evaluation of changes in metabolic pathways evaluated in terms of overall impact or flux (Loor et al., 2011b). Table 1 provides a list of most commonly used tools for the systematic study of ruminant expression profiling data.

Stage 5: Data Interpretation and Knowledge Discovery

Following the bioinformatics analyses, the resulting pathway and network data are evaluated by using available scientific articles and organism-specific databases. Heat maps also can be generated from the expression profiling results obtained through DNA microarrays, RNA-Seq or other high-throughput technologies to provide a compact view of the ‘omics’ data (Rajaram and Oono, 2010). These heat maps of DEG provide results in the form of gene clusters,

which could represent an evolutionary relationship among closely and distantly related genes in the genome (Eisen et al., 1998). Despite the multitude of tools available, there is still a need to develop bioinformatics resources that provide more biologically relevant meaning to the ruminant data. Our group developed the DIA particularly for dealing with the functional analysis of time-course experiments. The approach takes into account the magnitude and significance of change in DEG (Bionaz et al., 2012b). Figure 1.3 summarizes the above five stages of the proposed top-down systems biology approach in ruminants.

As the top-down approach deals with the whole genome, it is considered as a potentially complete approach (Bruggeman and Westerhoff, 2007). There also are certain limitations (Loor et al., 2011a) in this approach; however, the major advantage of this approach is that it provides a more precise view of the fate of metabolites. Hence, it can help us to understand the molecular behavior (e.g., metabolism, signaling, transport) of genes or proteins under certain environmental or dietary conditions and physiological states, such as parturition (stressed condition), and negative energy balance in the post-partum period (Wathes et al., 2009).

1.4. THE ROLE OF SYSTEMS BIOLOGY IN RUMINANT METABOLISM AND PHYSIOLOGY

Within the context of nutrient usage as it relates to physiology, ruminant systems biology focuses on the systematic study of complex biological interactions occurring in different tissues that are directly (mammary) or indirectly (liver, muscle, adipose tissue) involved in coordinating physiological adaptations, and particularly susceptible to nutritional management. Recent advances in bioinformatics and systems biology techniques have accelerated the genome-wide and tissue-specific reconstruction to enhance our knowledge at the systems level. Domestic cattle

(*Bos taurus*) are likely the most-extensively studied ruminant species. Here we present examples of tissue-specific metabolic network reconstructions from human and bovine species. The analysis of tissue-specific pathways and their functional behavior is an integral part of systems biology. This concept as it relates to ruminants has been discussed recently (Loor et al., 2011a) using liver, mammary gland and adipose tissue as an illustration.

A putative cattle genome-wide metabolic pathway assembly was conducted by Seo and Lewin (2009) using a bottom-up approach. They essentially applied the comparative analysis approach for the reconstruction process, and observed that between cattle and human metabolic pathways, there was ca. 35% similarity at the enzyme level and 54% similarity at the functional, level with the exception of some differences in individual enzymes and alternative reactions. They also observed that the most-conserved pathways include "energy and nucleotide/nucleoside metabolism," which are considered to be present in evolutionarily ancient pathways (Caetano-Anolles et al., 2007).

Genomic approaches may also help to identify previously unrecognized complex biological mechanisms that are unique to ruminants; hence, improving our opportunities for enhancing livestock productivity. Due to the high cost, few nutritional studies with ruminant species have been performed (Loor et al., 2011a); whereas, more extensive work in this area as it relates to livestock and agriculturally-important species has been conducted using chickens (2007). The high-throughput transcriptomics work conducted to date has greatly expanded our understanding of fundamental molecular mechanisms in ruminants (Lippolis and Reinhardt, 2008; Loor, 2010a). By analyzing the physiological conditions at critical levels in a ruminant species such as dairy cattle (e.g. lactation, dry period, parturition), in the future we might be able to increase the productive efficiency by optimizing management at the farm level. We and others

(McNamara, 2011) believe that this can be achieved by obtaining fundamental knowledge of genotypic to phenotypic transitions at the systems level using top-down approaches. Despite the progressive implementation of bioinformatics and systems biology tools in human and microbial species, their applications in livestock species are still in its infancy stages.

DNA microarray and other high-throughput sequencing techniques such as RNA-Seq, are used to measure the expression of the entire transcriptome of an organism in a single or series of experiments. These can detect not only mRNA from highly expressed genes but also from less abundant genes (Loor et al., 2005; Loor et al., 2006; Lippolis and Reinhardt, 2008). In fact, RNA-Seq has several advantages over DNA microarrays including the detection of single nucleotide polymorphisms (SNP), alternative splice variants, and RNA editing (Baldwin et al., 2012). These approaches have the ability to unravel genomic information at systems level in contrast to the reductionist paradigm. The resulting data can be used to create networks of genes and/or proteins or to incorporate molecular control points into mechanistic models (McNamara, 2011) leading to enhanced knowledge of network biology (Barabasi and Oltvai, 2004) and overall information at a functional level.

1.5. TISSUE-SPECIFIC APPLICATIONS

As indicated above, the genome-scale reconstruction provides a holistic view of an organism; whereas the tissue-specific reconstruction provides a view of metabolic pathways in a tissue-specific manner. Clearly, each tissue has a unique set of metabolic objectives, some of which differ markedly between tissues. Differential expression of genes and proteins in a tissue specific manner plays an important role in determining metabolic fates (Shlomi et al., 2008).

Human tissue-specific applications using the systems-biology have been developed by Gille et al. (2010), Jerby et al. (2010), and Shlomi et al. (2008). For instance, Gille and colleagues (2010) reconstructed the human liver using bottom-up constraint-based modeling, which led to development of HepatoNet1. This model has the capability of recreating liver-specific functions, such as cholesterol biosynthesis, bile formation, and ammonia detoxification under optimal conditions. These authors performed FBA on 442 metabolic objective functions to test the liver-specific stoichiometric model as a way to examine hepatic cell behavior. This tissue-specific reconstruction project provided a complete mathematical approach to assess biological functions. The model also allows for evaluating effects of minimal nutritional requirements on pathway behavior. Recently, a tissue-specific metabolic scale-free network using systems biology approaches has also been reconstructed for bovine mammary gland tissue (Wang et al., 2012).

The biological intricacy of livestock inexorably requires the systematic study of tissue-specific interactions. The above mentioned approaches are equally applicable to the study of tissue-specific transcriptomes. Liver, mammary, and adipose tissue-specific microarray studies have been conducted by our group and others (Table 2) in the last few years to evaluate the effects of nutrition and physiological state on the transcriptome. This technology allows us to examine the temporal expression of known components of metabolic networks, which is an appropriate means for addressing the issue of transcriptional regulation. This transcriptional regulation is related to tissue-specific metabolism as a response to growth and/or nutritional management in ruminants (Loor, 2010b). To date, more than 46 transcriptome expression profiling research articles using high-throughput genomics techniques on different bovine tissues have been published. Table 2 contains information from published articles between 2003 and

2012. The following liver and adipose tissue examples are two particular applications of tissue-specific, top-down reconstructions in cattle (*Bos taurus*).

Liver:

In contrast to tissue-specific bottom-up reconstruction in human hepatocytes, the top-down approach as exemplified by the applications of DNA microarray data has been employed in studies of dairy cattle liver (13 of 46 papers published since 2003, Table 2). Similar to humans, bovine liver performs a wide range of tissue-specific functions, including cholesterol biosynthesis (Viturro et al., 2009), urea synthesis (Emmanuel, 1980; Lobley et al., 1995), gluconeogenesis (Aschenbach et al., 2010), oxidation of non-esterified fatty acids (NEFA), ketogenesis, or esterification of NEFA into triacylglycerol (TAG) (Drackley, 1999; Loor, 2010a). Despite the information generated by these studies, the scope of the bioinformatics analysis based on time-course experiments is quite limited due in part to the reliance on software tools built on the analytical features dealing with overrepresented approach (ORA) (Huang da et al., 2009). To overcome such limitations, particularly when dealing with time-course or multiple treatment transcriptome data, our group recently has developed and validated a novel DIA analysis (Bionaz et al., 2012a; Bionaz et al., 2012b), which outperforms over the ORA and produces biologically more meaningful interpretation of longitudinal transcriptome data.

We have recently applied DIA analysis to mine the hepatic transcriptome from late pregnancy through early lactation in cows receiving different levels of dietary energy prepartum. For this study, already available DNA microarray data were obtained from NCBI GEO (accession number GSE 3331) (Loor et al., 2005; Loor et al., 2006) and re-analyzed using the Proc MIXED model of SAS. The study was based on two dietary conditions i.e., overfed (OF) versus restricted energy (RE) intake. The tissue biopsies were harvested at days -65, -30, -14, +1,

+14, +28, and +49 relative to parturition. A Benjamini-Hochberg FDR correction resulted in a total of 4,111 DEG with a significant diet × time interaction (FDR <0.05). The bioinformatics analysis was carried out using the DIA methodology as described by Bionaz et al. (Bionaz et al., 2012b). This novel tool uses the information from the KEGG pathway database (http://www.genome.jp/kegg/pathway.html) and can help rank each pathway-based on higher or lower impacted values. In this particular experiment, DIA estimates the overall magnitude of physiological changes (impact) and direction (flux; activation, inhibition, or no change) over time and in response to a dietary treatment.

The Figure 1.4 contains a set of five highly-impacted pathways obtained from bovine liver data analysis. Among the top affected pathways by plane of nutrition, the five pathways include ubiquinone and other terpenoid-quinone biosynthesis, sulfur metabolism, arachidonic acid metabolism, complement and coagulation cascade and base excision repair. A preliminary interpretation of these results revealed unique responses of bovine liver during transition from pregnancy to lactation. For instance, ubiquinone (coenzyme Q) and other terpenoid-quinone biosynthesis are involved in oxidative phosphorylation as part of the cellular respiratory chain (Kawamukai, 2002), and during the transition into lactation a significant induction was observed in OF cows; while sulfur metabolism was inhibited.

From a biological standpoint, and because its anionic property, the observed adaptation in sulfur metabolism in OF cows might help the liver balance the cation-anion concentration (Tucker et al., 1991). Metabolism of sulfur also plays a role in the synthesis of sulfur-containing amino acids (Spears et al., 2011), and indirectly may play a role in lipid metabolism. The activation of arachidonic acid metabolism after parturition in OF cows, i.e. d 1 postpartum, could be related with the synthesis of signaling molecules that may play a role in the overall adaptation

of liver to the onset of lactation. Similarly, the inhibition of the complement and coagulation pathway before parturition coupled with its activation at 14 d postpartum in OF cows is an indication that they were more sensitive to mounting an inflammatory response (Carroll, 2004). The gradual activation of the base excision repair pathway between -14 d through 14 d around parturition in OF cows suggested a potentially greater degree of DNA damage because this pathway is central in repairing damaged DNA(Liu et al., 2007) and the control of cell proliferation (Reese, 2003). Overall, these results indicate that OF vs. RE prepartum elicited a stronger transcriptional response potentially leading to alterations in immune response, metabolism, and DNA damage. These findings are supported in part by the original studies conducted by Loor et al. (Loor et al., 2006).

Adipose tissue:

Relatively fewer transcriptome studies (6 published since 2003, Table 2) have been conducted on bovine adipose tissue (McNamara, 1994; Sumner and McNamara, 2007; Thering et al., 2009). Sumner et al. (Sumner and McNamara, 2007) performed transcriptome profiling of subcutaneous adipose tissue during the transition from pregnancy to lactation, and used the ORA approach to mine the DEG. In collaboration with the McNamara group, we used the KEGG-based DIA analysis to evaluate the impact of change in physiological states on biological pathways in bovine adipose tissue. The tissue biopsies were obtained on days -21, -7, +7, and +28 relative to parturition (Shahzad et al., 2011). The ANOVA with an FDR correction resulted in 1,802 DEG with a time effect (FDR < 0.10).

The DIA approach revealed that the onset of lactation resulted in a gradual decrease in the utilization (metabolism) of glucose, lactate, and acetate to produce energy (e.g., most impacted pathways included metabolism of fatty acids, biotin, pyruvate, and TCA cycle)

(Shahzad et al., 2011). Furthermore fatty acid desaturation, elongation, and PPAR signaling were markedly inhibited during lactation. Among the significantly-affected, the complement and coagulation cascade pathway of the immune system also was induced. While implementing the DIA using the DAVID bioinformatics resources, it was observed that fatty acid biosynthesis, linoleic acid metabolism, biotin metabolism, and glycerolipid metabolism were markedly inhibited postpartum than prepartum; whereas, complement and coagulation cascades and riboflavin metabolism were among the only pathways with sustained induction postpartum relative to prepartum.

Overall, the preliminary evaluation of the combined results from both bioinformatics approaches indicated that the adipogenic capacity of adipose tissue is quite robust during late pregnancy while the innate immune response of the tissue is more predominant during early lactation. The latter may be a response of the tissue due to stressors such as cytokines/hepatokines, NEFA, and/or pathogens. Alternatively, it may represent a mechanism associated with tissue remodeling (Shahzad et al., 2011). The liver and adipose-specific applications provide evidence that systems biology approaches inevitably lead to a better understanding of the functional changes in an organism due to internal or external factors.

1.6. CONCLUDING REMARKS AND FUTURE CHALLENGES

The primary objective of this review was to provide a concise overview of the evolution of systems biology approaches and its potential applications in ruminants using transcriptomic data. To enhance our understanding of the complex biological behavior in ruminants, there is a need for integration of genome-enabled and computational techniques. Work during the previous

15 years on model organisms has clearly demonstrated the applicability of high-throughput technologies coupled with genome-scale models to elucidate systematic interactions (Feist and Palsson, 2008).

Bottom-up systems biology deals with the known stoichiometry of chemical reactions in biological systems by means of labor-intensive literature surveys and computational resources *in silico*. There is a substantial body of work on biochemical pathways and their regulation in the ruminant animal (Baldwin, 1995). That information will prove useful when applying the bottom-up approach within the systems framework. However, the bottom-up approach leaves some gaps in genome-scale models because of our incomplete knowledge in non-model organisms such as cattle. These gaps could be filled by using conserved evolutionary relationships among species. Top-down systems biology examines molecular interactions in complex biological systems through genome-wide 'omics' studies. As part of this approach we can uncover relationships among genes and proteins, but more importantly, among biological networks.

Both approaches are complimentary in the search for interrelationships between genotypes and phenotypes. With the availability of tissue-specific genome-scale models constructed from 'omics' data and already published research articles, our understanding of the impact of genomic background on an observed phenotype will be enhanced. Ultimately, these models will help to explain diverse molecular interactions among various networks, from the cellular level up to the organism level in an integrative manner (Kuepfer, 2010). It is also worth mentioning that both reductionist and integrative approaches can help describe the functional behavior of a cell (Barabasi and Oltvai, 2004).

Even though much progress has taken place in ‘omics’, bioinformatics, and systems biology, its specific applications in ruminants are still minor relative to model organisms (Loor et al., 2011a). To accelerate progress in ruminant systems biology, there is a need for automation to help handle the growing number of datasets originating from genome-enabled tools. The application of modern computational resources in ruminant biology can improve our understanding about molecular interactions *in silico*. Over the long term, the end result of this work could help to improve productive performance, and ultimately lead to more efficient ways of managing dairy cattle for production of milk and meat to meet the demands for highly nutritious food for humans worldwide.

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1.8. TABLES AND FIGURES

Table 1.1: List of bioinformatics software commonly used for data mining and analysis in ruminant research.

The reference column provides selected examples of published studies that have used these tools.

Sr. #	Name	Link	Reference
1.	DAVID	http://david.abcc.ncifcrf.gov/	(Loor et al., 2011b)
2.	GeneSpring	http://www.genomics.agilent.com/	(Loor et al., 2005; Loor et al., 2006)
3.	IPA	http://ingenuity.com/	(Loor et al., 2007; Walsh et al., 2012)
4.	Genesis	http://genome.tugraz.at/genesisclient/genesisclient_de	(Graugnard et al., 2009)
5.	KEGG	http://www.genome.jp/kegg/	(Loor et al., 2011b)
6.	DIA	Dynamic Impact Approach	(Bionaz et al., 2012a)
7.	MetaCore	http://www.genego.com/metacore.php	(Wickramasinghe et al., 2012)
8.	GOseq	http://www.bioconductor.org/packages/2.9/bioc/html/	(McCabe et al., 2012)

Table 1.2: Published bovine studies between 2003-2012 using high-throughput genomics technologies.

Title	Year	Tissue(s)	Technology used	Reference
"Bovine mammary gene expression profiling using a cDNA microarray enhanced for mammary-specific transcripts"	2003	Mammary	DNA Microarray	(Suchyta et al., 2003)
"Generation of a bovine oocyte cDNA library and microarray: resources for identification of genes important for follicular development and early embryogenesis"	2004	Fetal ovary	DNA Microarray	(Yao et al., 2004)
"Transcriptional profiling of skeletal muscle tissue from two breeds of cattle"	2004	Skeletal muscle	DNA Microarray	(Wang et al., 2005)
"Pregnancy-associated changes in genome-wide gene expression profiles in the liver of cow throughout pregnancy"	2004	Liver	DNA Microarray	(Herath et al., 2004)
"Temporal gene expression profiling of liver from periparturient dairy cows reveals complex adaptive mechanisms in hepatic function"	2005	Liver	DNA Microarray	(Loor et al., 2005)
"Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling"	2006	Liver	DNA Microarray	(Loor et al., 2006)
"Developmental aberrations of liver gene expression in bovine fetuses derived from somatic cell nuclear transplantation"	2006	Fetal liver	DNA Microarray	(Herath et al., 2006)
"Identification of estrogen-responsive genes in the parenchyma and fat pad of the bovine mammary gland by microarray analysis"	2006	Mammary	DNA Microarray	(Kristensen et al., 2006)
"A gene coexpression network for bovine skeletal muscle inferred from microarray data"	2006	Skeletal muscle and adipose	DNA Microarray	(Reverter et al., 2006)

Table 1.2 (Contd.)

"Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows"	2007	Liver	DNA Microarray	(Loor et al., 2007)
"Target genes of myostatin loss-of-function in muscles of late bovine fetuses"	2007	Muscle	DNA Microarray	(Cassar-Malek et al., 2007)
"Image analysis and data normalization procedures are crucial for microarray analyses"	2008	Muscle and adipose	DNA Microarray	(Kadanga et al., 2008)
"Gene expression patterns during intramuscular fat development in cattle"	2008	Muscle and lean mass (LM) tissue	DNA Microarray	(Wang et al., 2009a)
"Comparative proteomics and transcriptomics analyses of livers from two different Bos taurus breeds: "Chianina and Holstein Friesian""	2009	Liver	DNA Microarray	(Timperio et al., 2009)
"Pleiotropic effects of negative energy balance in the postpartum dairy cow on splenic gene expression: repercussions for innate and adaptive immunity"	2009	Spleen	Affymetrix GeneChip Bovine Genome Array	(Morris et al., 2009)
"Feasibility of a liver transcriptomics approach to assess bovine treatment with the prohormone dehydroepiandrosterone (DHEA)"	2010	Liver	DNA Microarray	(Rijk et al., 2010)
"Negative energy balance and hepatic gene expression patterns in high-yielding dairy cows during the early postpartum period: a global	2010	Liver	Affymetrix GeneChip Bovine Genome Array	(McCarthy et al., 2010)
"Dietary supplementation of selenium in inorganic and organic forms differentially and commonly alters blood and liver selenium concentrations and liver gene expression profiles of growing beef heifers"	2010	Liver	DNA Microarray	(Liao et al., 2011)
"Effect of diet supplementation on the expression of bovine genes associated with fatty acid synthesis and metabolism"	2010	Adipose	Affymetrix GeneChip Bovine Genome Array	(Joseph et al., 2010b)

Table 1.2 (Contd.)

"Omega-6 fat supplementation alters lipogenic gene expression in bovine subcutaneous adipose tissue"	2010	Adipose	DNA Microarray	(Joseph et al., 2010a)
"Altered gene expression in human adipose stem cells cultured with fetal bovine serum compared to human supplements"	2010	Adipose	DNA Microarray	(Bieback et al., 2010)
"Microarray analysis of gene expression profiles in the bovine mammary gland during lactation"	2010	Mammary	Affymetrix GeneChip Bovine Genome Array	(Hou et al., 2010)
"Enhanced mitochondrial complex gene function and reduced liver size may mediate improved feed efficiency of beef cattle during compensatory growth"	2010	Liver	DNA Microarray	(Connor et al., 2010)
"Transcriptomic profiling of bovine IVF embryos revealed candidate genes and pathways involved in early embryonic development"	2010	IVF-derived blastocysts and embryos	DNA microarray	(Huang et al., 2010)
"Comparison of transcriptomic landscapes of bovine embryos using RNA-Seq"	2010	Embryos	RNA-Seq	(Huang and Khatib, 2010)
"SNP discovery in the bovine milk transcriptome using RNA-Seq technology"	2010	Milk somatic cells	RNA-Seq	(Canovas et al., 2010)
"Characterization of the abomasal transcriptome for mechanisms of resistance to gastrointestinal nematodes in cattle"	2011	Fundic abomasum	RNA-Seq	(Li et al., 2011)
"Indistinguishable transcriptional profiles between in vitro- and in vivo-produced bovine fetuses"	2011	Liver and placenta	DNA Microarray	(Jiang et al., 2011)

Table 1.2 (Contd.)

"Global gene expression profiling reveals genes expressed differentially in cattle with high and low residual feed intake"	2011	Liver	DNA Microarray	(Chen et al., 2011)
"Gene expression differences in oocytes derived from adult and prepubertal japanese black cattle during in vitro maturation"	2011	Oocytes	Microarray gene chips	(Dorji et al., 2011)
"Microarray analysis of differentially expressed microRNAs in non-regressed and regressed bovine corpus luteum tissue; microRNA-378 may suppress luteal cell apoptosis by targeting the interferon gamma receptor 1 gene"	2011	Corpus luteum	miRNA microarray	(Ma et al., 2011)
"Transcriptome profiling of bovine milk oligosaccharide metabolism genes using RNA-sequencing"	2011	Milk somatic cells	RNA-Seq	(Wickramasinghe et al., 2011)
"Gene expression in the arcuate nucleus of heifers is affected by controlled intake of high- and low-concentrate diets"	2012	Brain	DNA Microarray	(Allen et al., 2012)
"Endometrial gene expression during early pregnancy differs between fertile and subfertile dairy cow strains"	2012	Endometrial tissue	DNA Microarray	(Walker et al., 2012)
"Gene expression profiling of bovine peripartal placentomes: detection of molecular pathways potentially involved in the release of foetal membranes"	2012	Placentomes	Affymetrix GeneChip Bovine Genome Array	(Streyl et al., 2012)
"Muscle transcriptomic analyses in angus cattle with divergent tenderness"	2012	Muscle	Microarray	(Zhao et al., 2012)
"Transcriptome analysis of subcutaneous adipose tissues in beef cattle using 3' digital gene expression-tag profiling"	2012	Subcutaneous adipose tissue (backfat)	Digital gene expression-tag profiling	(Jin et al., 2012)

Table 1.2 (Contd.)

"Level of nutrient intake affects mammary gland gene expression profiles in preweaned Holstein	2012	Mammary	DNA microarray	(Piantoni et al., 2012)
"Reconstruction of metabolic network in the bovine mammary gland tissue"	2012	Mammary	DNA Microarray	(Wang et al., 2012)
"Cytoskeleton remodeling and alterations in smooth muscle contractility in the bovine jejunum during nematode infection"	2012	Jejunum	RNA-Seq	(Li and Schroeder, 2012)
"Characterization of the longissimus lumborum transcriptome response to adding propionate to the diet of growing Angus beef steers"	2012	Longissimus lumborum muscle	RNA-Seq	(Baldwin et al., 2012)
"Conceptus-endometrium crosstalk during maternal recognition of pregnancy in cattle"	2012	Endometrium tissues	RNA-Seq	(Mamo et al., 2012)
"RNA-Seq analysis uncovers transcriptomic variations between morphologically similar in vivo- and in vitro-derived bovine blastocysts"	2012	Blastocysts	RNA-Seq	(Driver et al., 2012)
"Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis"	2012	Ovarian follicle	RNA-Seq	(Walsh et al., 2012)
"Transcriptional profiling of bovine milk using RNA sequencing"	2012	Milk somatic cells	RNA-Seq	(Wickramasinghe et al., 2012)
"RNA-seq analysis of differential gene expression in liver from lactating dairy cows divergent in negative energy balance"	2012	Liver	RNA-Seq	(McCabe et al., 2012)
"Characterization and comparison of the leukocyte transcriptomes of three cattle breeds"	2012	Leukocytes	mRNA-Seq	(Huang et al., 2012)
"Perturbation dynamics of the rumen microbiota in response to exogenous butyrate"	2012	Rumen epithelium	Pyrosequencing	(Li et al., 2012)

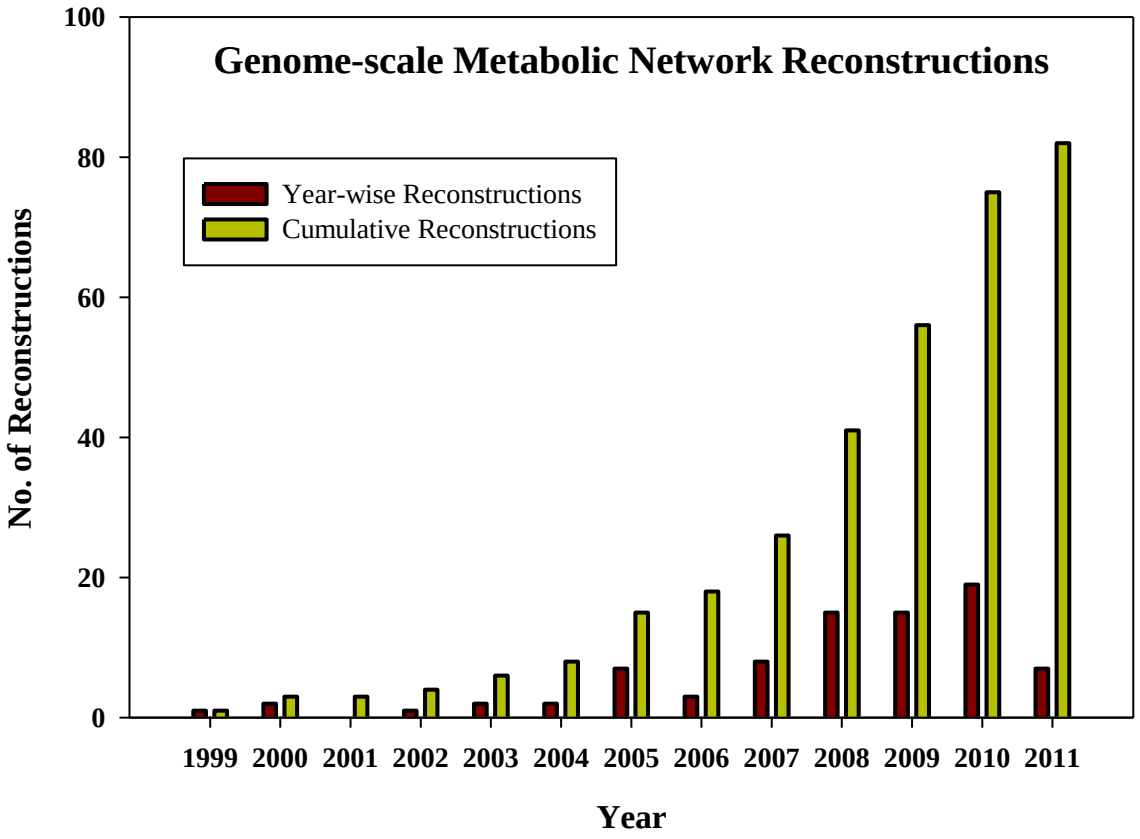

Figure 1.1: Genome-scale metabolic network reconstructions statistics from 1999 to 2011. Year-wise (red) and cumulative (green) studies with respect to total number of reconstructions. The data include a wide range of species from bacteria to eukaryotes.

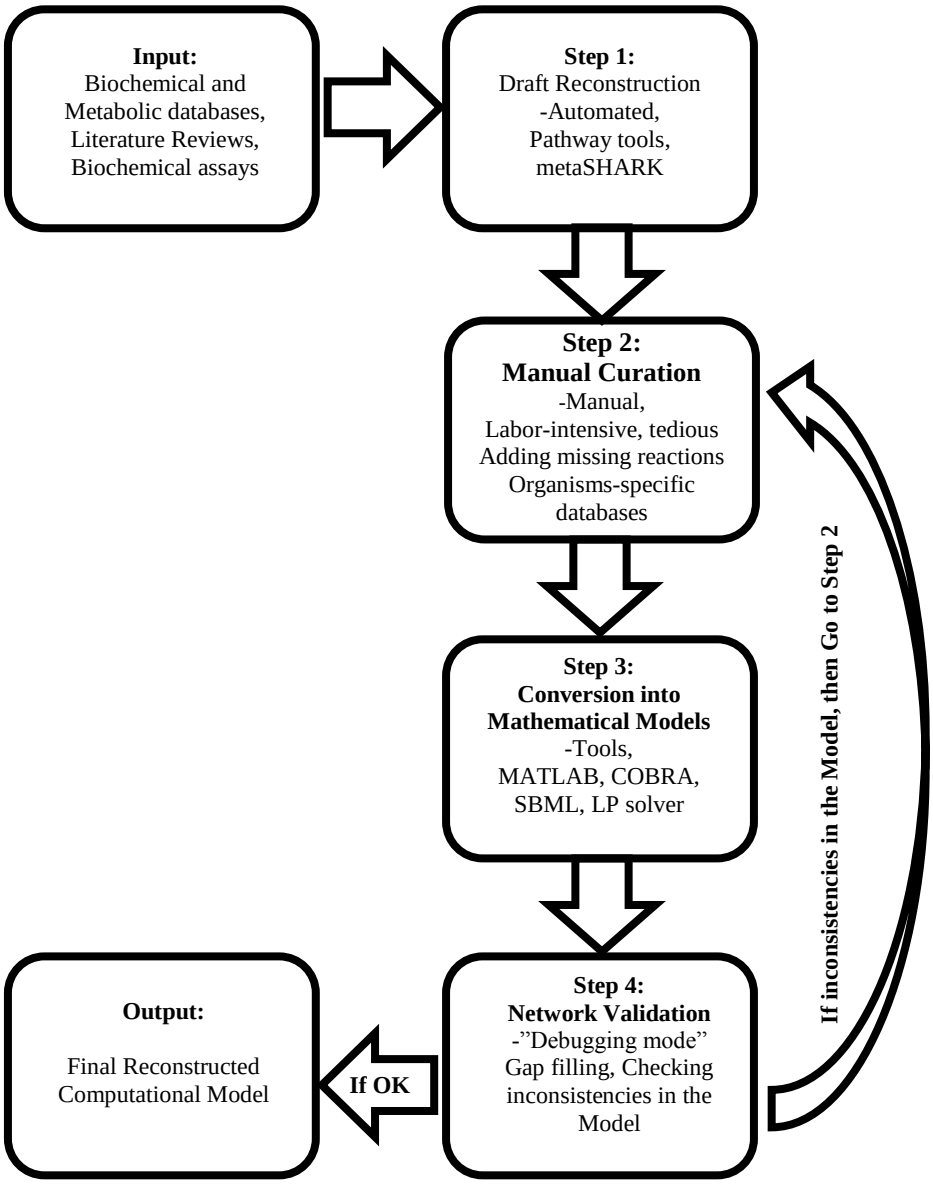

Figure 1.2: A bottom-up systems biology approach. The four conventional steps of modern systems biology are summarized in the figure. Information obtained from biochemical and metabolic databases is given as an input to start building the genome-scale computational models. Step 1 deals with the automated draft reconstruction bioinformatics tools such as pathway tools and metaSHARK. This first stage still leaves some gaps, missing reactions, and dead-end metabolites (i.e., metabolites having unknown reactants or product information). Once the automated draft is created, it needs manual curation, which is completed during step 2. This step involves consulting through organism-specific databases, adding missing reactions, and dealing with dead-end metabolites. Step 3 involves the conversion of the refined draft into mathematical models using stoichiometric calculations. This step involves the application of Matlab-embedded tools (e.g., COBRA, SBML) and linear/quadratic programming solvers to create mathematical models and allows visualization of results on the Matlab interface. Step 4 involves the simulation and evaluation of the reconstructed genome-scale mathematical models under optimal conditions. If there are some inconsistencies in the model, then it is re-evaluated from Step 2. If the model is working correctly in the final stage, then it is considered for further computational applications.

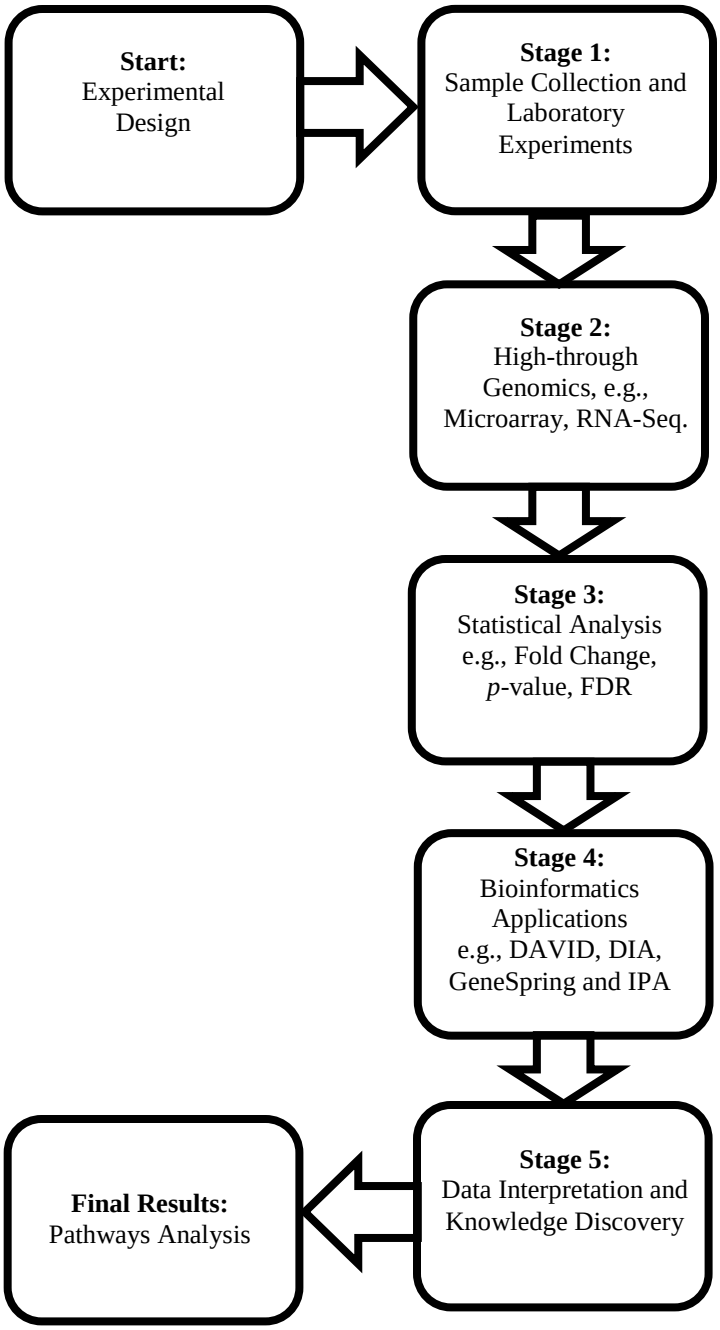

Figure 1.3: A top-down systems biology approach. This approach is categorized into five main stages. After designing an experiment, the first stage involves biological sample collection (e.g. tissue biopsy) of control and treated animals. This is followed by laboratory experiments including RNA extraction, purification, and expression profiling. Stage 2 involves high-throughput genomics using microarray platforms (e.g. Affymetrix) and RNA-Seq. Stage 3 involves data normalization to remove noise and obtain high-quality expression profiling data for statistical analysis utilizing suitable tools (e.g. SAS) and incorporating the key aspects of the experimental design (e.g. time, treatment, and any potential interactions). After the statistical tests, differential expression is determined based on a certain *p*-value criterion. In the stage 4 the significant data are analyzed through bioinformatics techniques. The last stage involves data interpretation and knowledge discovery leading towards the development of new scientific hypothesis.

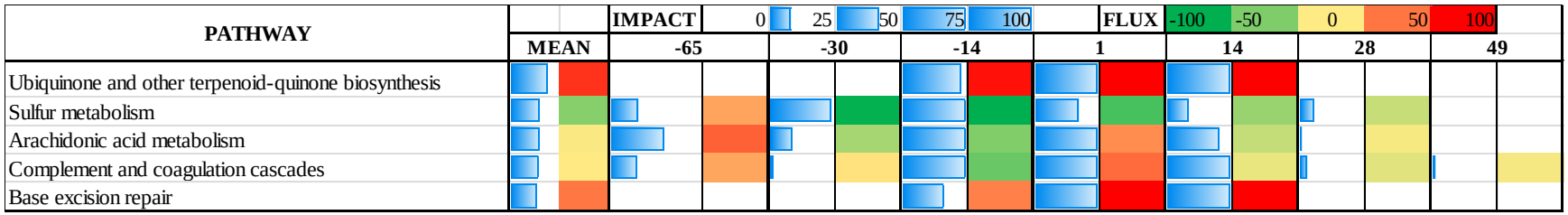

Figure 1.4: Top 5 impacted pathways sorted by overall impact in response to overfeeding (OF) versus restricting dietary energy (RE) during the prepartum period in dairy cattle. The data correspond to days -65, -30, -14, +1, +14, +28 and +49 relative to parturition. The impact values are shown in light-blue colored horizontal bars (from 0 to 100 based on the biological perturbation in a pathway), while flux values are depicted in red (activated/induced) to green (inhibited/reduced) shades of color (-100 to 100). The impact corresponds to the overall perturbation in a pathway while flux corresponds to the direction of the impact. The "mean" column represents the overall change of impact and flux from day -65 to day +49.

CHAPTER 2: BOVINE LIVER

In-depth study of bovine liver transcriptome dynamics as affected by prepartal plane of nutrition

2.1. INTRODUCTION

Liver is an important organ in the ruminant that performs essential metabolic functions under several physiological conditions. These include gluconeogenesis and glycogen synthesis, synthesis of several plasma proteins (e.g., albumin) encompassing clotting factors and acute phase proteins (e.g., haptoglobin), metabolism of amino acids and lipid including fatty acids oxidation and cholesterol synthesis, and ammonia detoxification (Loor et al., 2005). The importance of liver becomes critical during the peripartum period (a.k.a., the transition period) ranging from three weeks prepartum to three weeks postpartum (Drackley, 1999). The diet can have a great impact during this period, particularly on the liver. Several studies on the bovine liver indicate that overfeeding energy during prepartum results in lower feed intake early postpartum that generates a higher negative energy balance making the animals more susceptible to several metabolic disorder such as fatty liver and ketosis, (Drackley et al., 2005, Loor et al., 2006, McCarthy et al., 2010, Naesens et al., 2007). One of the causative agents of fatty liver and ketosis is the higher lipolysis of the adipose tissue. This process results in increased level of serum non esterified fatty acids (NEFA). The increased plasma NEFA leads to higher triacylglycerol synthesis and accumulation in the liver (Bobe et al., 2004) and greater synthesis of ketone bodies. Restricted feed energy intake prepartum on the other hand is considered a way to prevent dairy cows from those metabolic disorders (Douglas et al., 2006).

Previous studies from our group have identified the hepatic gene expression signatures during the transition from pregnancy into lactation in dairy cows (Loor et al., 2005) and in response to overfed or restricted energy prepartum (Loor et al., 2006). Both studies were performed using bovine-specific cDNA microarray platform to evaluate the transcriptomics adaptation of the liver during the transition period. Due to reduced availability of functional

analytical tools for large microarray data, the dynamic adaptation of the transcriptome was analyzed using the *k*-mean cluster approach; however, the main discussion was based on specific genes and a model was proposed that encompassed the transcriptomics data plus the physiological measurements. The data uncovered that overfed cows had significant higher insulin prepartum and larger hepatic lipid accumulation post-partum. Overall the analysis indicated that moderate overfeeding of energy pre-partum results in transcriptional changes predisposing cows to fatty liver and potentially compromising liver health early post-partum. Those transcriptomics changes were likely driven by the evident hyperinsulinemia.

Recent advances in bioinformatics tools for analysis of large scale transcriptome studies opened up unprecedented opportunity. Several bioinformatics tools, many of those freely available (Huang et al., 2009a), exist today in order to perform the functional analysis of large list of genes in more biologically meaningful way. The approach more commonly used in most of those tools is the enrichment analysis or overrepresented approach (Huang et al., 2009a). This approach is robust and computationally efficient for single list of genes; however, it does not allow the comparison of results from analysis of multiple gene lists; *de facto* limiting the capability for functional analysis of transcriptomics data from time course or multiple treatments experiments (Bionaz et al., 2012, Huang et al., 2009a).

In the present study we have taken advantage of the advances in bioinformatics tools for analysis of microarray data. In order to unravel the biological effect of prepartum energy diet in liver of periparturient cows, we have re-analyzed statistically the transcriptomics dataset previously published (Loor et al., 2006). The differentially expressed genes (DEG) between the two treatment in each time point considered during the transition period were analyzed using the novel bioinformatics approach called Dynamic Impact Approach (DIA) developed by Bionaz et

al. (2012) and the classical enrichment analysis by mean of Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009b).

2.2. MATERIALS AND METHODS

Experimental Design

The information about the ethics statement, sampling, RNA extraction, and microarray data were previously described (Loor et al., 2006). Briefly, 8 Holstein dairy cows were randomly assigned to two groups with different dietary energy level during the last two months prepartum. Same energy level in the diet was provided from the day of parturition until end of the trial. The cows were either assigned to a high energy ad-libitum or overfed (>150% of net energy requirements; n=4; **OF**) and restricted energy (80 % of net energy requirements; n=4; **RE**) prepartum consisting of corn silage and alfalfa. The biopsies were taken at day -65, -30, -14, +1, +14, +28, +49 relative to parturition. The microarray data were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) with accession number GSE3331.

Statistical Analysis

Microarray spots with median intensity ≥3 standard deviation above the median of the background and GenePix 6 flag >100 were applied as filters to ensure high quality data. A total of 106 microarrays were adjusted for dye and array effect (Loess normalization and array centering), duplicated spot intensities were not averaged and were subsequently used for statistical analysis. A mixed model with repeated measures was then fitted to the normalized log2-transformed adjusted ratios (sample/reference standard) using Proc MIXED (SAS, SAS Inst. Inc., Cary, NC). The model included the fixed effects of time (-65, -30, -14, 1, 14, 28, 49 DIM), diet (OF and RE), and interactions of time × diet. Cow was considered as random effect. 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 relative gene expression were considered significant at an FDR-adjusted P ≤0.05 for time × diet. Post-hoc P ≤0.05 was considered significant between diets at each time point.

Dynamic Impact Approach (DIA) and Enrichment Analysis

The detailed methodology for data analysis using DIA was previously described (Bionaz et al., 2012). For the purpose, the whole dataset with Entrez gene ID, fold-change and the significance (p-values) of gene expression between the two diets at each time point, plus the overall FDR adjusted p-values were uploaded into DIA.

The KEGG pathways and all three GO categories (i.e., Biological process [BP], Cellular components [CC], and Molecular functions [MF]) were analyzed with the DIA. The KEGG analysis was performed using the automatic system built in Excel; while for the GO category a manual approach was taken using the annotation database gently provided by the team of DAVID upon request (Bionaz et al., 2012). For all the analyses a minimum of 20% genes in the annotated microarray vs. whole genome was used. For the enrichment analysis the lists of DEG (overall time × diet FDR<0.05 and P-value plus the whole annotated microarray as background were uploaded into DAVID bioinformatics resource database (http://david.abcc.ncifcrf.gov/).

2.3. RESULTS AND DISCUSSION

The goal of this study was to identify the functions differentially affected by prepartal dietary energy level in the liver of dairy cows by means of transcriptomics data in association with bioinformatics approaches. The transition period in dairy cows is very critical for the future performance of the dairy cow. The liver plays a pivotal role in such adaptations (Bionaz and Loor, 2012). Several studies using transcriptome profiling have been performed to investigate the biochemical and physiological adaptation of the liver during the transition period (Loor et al., 2005, Loor et al., 2006, McCarthy et al., 2010). The level of dietary energy prepartum plays a pivotal role in the physiological adaptations to the onset of parturition both in dairy (Dann et al., 2006) and beef cows (Sullivan et al., 2009), with high-energy in the far-off period (first ~20-30 d of the dry period) resulting in more detrimental effect in the subsequent lactation. The present work was performed in order to identify the functional differences determined by the two extreme dietary approaches using longitudinal transcriptomics data.

Gene Expression pattern in OF and RE groups

A mixed model ANOVA with FDR correction resulted in 4,790 oligo array IDs with a time x diet interaction (FDR ≤ 0.05). Out of these Oligo IDs, 4,111 were annotated with bovine Entrez gene IDs (3,460 unique Entrez gene IDs). These 4,111 differentially expressed genes (DEG) between OF and RE at each time point with a *p*-value ≤ 0.05 were used for the analysis. The number of DEG (Figure 2.1) indicated that there was a marked difference in expression of genes due to dietary energy level prepartum. The large number of DEG was observed through 2 weeks after cows were back on the same lactation diet (i.e., carryover effect); however, the carryover effect was of brief duration because at 28 d after parturition few DEG between the two

groups of cows were observed. Interestingly, there was not a difference in number of genes more expressed in one group vs. the other except at d 1 and, more pronounced, at 14 DIM where there was a greater number of genes more expressed in OF vs. RE (Figure 2.1A). By applying a ≥2-fold change threshold cut-off (Figure 2.1B), it can be observed that most of the DEG had a large difference in expression only between -14 and 14 DIM with a greater number of genes with ≥2-fold larger expression in RE vs. OF at -14 DIM and OF vs. RE at 1 DIM.

KEGG pathway analysis

The impact values in DIA represent the estimated perturbations in a biological pathway, while the direction of the impact or flux values represent the overall direction of the perturbation (the pathway can be overall activated or inhibited) (Bionaz et al., 2012). The DIA provides two different outputs when the KEGG pathways are analyzed: the summary of the KEGG pathways in main categories and sub-categories, which allows for a quick overview of the most impacted categories of pathways (Figure 2.2).

As mentioned above, the DAVID bioinformatics resource performs enrichment analysis of large gene lists. In our case (Figure 2.3), it uncovered oxidative phosphorylation as the most significantly enriched (FDR < 0.05) pathway followed by ribosome and proteasome in RE. Without multiple correction (i.e., raw p-value < 0.05) other enriched pathways in RE vs. OF included fatty acid metabolism, lysosome, glycan biosynthesis, complement and coagulation and basal transcription factors, were enriched in genes more induced; whereas, base-excision repair, ubiquitination, and ECM receptor were enriched in genes more induced by OF vs. RE.

Summary of KEGG pathways

The summary of KEGG pathways results describe the impact and direction of the impact (or flux) of each category and sub-category of KEGG pathways (Figure 2.2). In accord with the number of DEG (Figure 2.1) all the categories of KEGG pathways were more impacted from -14 to 14 DIM paralleled with the other time comparisons, with the 'Metabolism' followed by the 'Genetic Information Processing' being the most impacted. The 'Metabolism' category, and relative sub-categories with exception of 'Metabolism of terpenoids and polyketides', and 'Organismal System' category were more induced in RE vs. OF at -14 DIM with some subcategories such as 'Energy metabolism' and 'Amino acid metabolism' remaining more induced in RE vs. OF up until 14 DIM. Several pathways evidently more induced in OF vs. RE from 1 to 14 DIM, such as 'Glycan biosynthesis and degradation', and 'Metabolism of cofactors and vitamins' (Figure 2.2). The 'Genetic Information Processing', 'Environmental Information Processing' and 'Cellular Processes', with related sub-categories with the exception of 'Folding, sorting, and degradation' and 'Membrane transport', were evidently more induced in OF compared to RE at 1 and 14 DIM. The 'Human Disease' category of pathways results was not considered in the results and discussion.

Biological Interpretation of most impacted KEGG pathways during transition

The most impacted KEGG pathways (Figure S1) along with their categories and sub-categories are discussed below. The results show that two different dietary effects started at -65 DIM has greater impact on the liver from -14 DIM to parturition as prepartal dietary plane, and 14 DIM as carryover effect. These results indicate a larger induction of liver transcriptome (and

potentially alteration in function) likely as a way for the dairy cow to prepare for milk synthesis. In order to provide a biological interpretation of the biological pathways we have divided the discussion in main KEGG pathway categories.

1. Metabolism

Metabolism of carbohydrate (Baird, 1981), energy, lipid (Dale et al., 1979, Grum et al., 1996, Petit et al., 2007), and amino acids (Overton et al., 1998) is very critical in the liver of dairy cows during transition period. Liver has a great flexibility to adapt to metabolic changes occurring during the transition period (Donkin, 2012). The most impacted metabolic pathways are discussed below:

Carbohydrate Metabolism: Among the Carbohydrate metabolism, 'Pyruvate metabolism', 'Fructose and mannose metabolism', 'Glycolysis/gluconeogenesis', and 'Citrate cycle (TCA)' were the most impacted pathways. Pyruvate Metabolism was more induced in OF vs. RE group until -14 DIM, while it was more induced in RE vs. OF after parturition until 14 DIM as carryover effect. The pyruvate metabolism along with 'Glycolysis and gluconeogenesis' pathways (Figure S1) indicates a larger use of glucose and/or propionate for metabolism in OF compared to RE cows before parturition, particularly due to a larger expression of pyruvate kinase, but a larger use of propionate and lactate in RE vs. OF after parturition with a concomitant larger induction of 'TCA cycle'.

The increased rate of acetoacetyl-CoA synthesis indicate greater rate of fatty acid oxidation into their respective acyl-CoA (Nafikov et al., 2006). The data indicated a larger induction of 'Pentose phosphate pathway' in the liver of OF cows. This may suggests the

utilization of glucose 6-P by the pentose phosphate pathway for NADPH generation (Wilson, 2003).

The ‘Fructose and mannose metabolism’ was more induced in RE vs. OF cows before parturition; however, it was evidently more induced in OF vs. RE after parturition. The main source of fructose and mannose is glucose. Both are major components of glycoproteins. The mannose (M-6-P) is generated through glucose (G-6-P) and fructose (F-6-P) molecules by energy consumption and enzymatic reactions (Herman, 1971). Several studies in rat liver have indicated that excess amount of carbohydrates such as glucose, sucrose and fructose lead to several disorders such as somatic mutations, metabolic syndromes, fatty liver disease and type II diabetes (Hansen et al., 2008, Roncal-Jimenez et al., 2011).

Energy metabolism: The ‘Sulfur metabolism’ was the most impacted of all analyzed pathways and was more induced in RE vs. OF from -30 to 28 DIM. The role of sulfur metabolism in the liver of periparturient dairy cows has not been investigated thoroughly yet. However, it is used to balance the cation-anion concentrations in the liver because of the anionic property of the sulfur compounds (Tucker et al., 1991). It may also be involved in the synthesis of sulfur containing amino acids (Spears et al., 2011). Sulfur metabolism in sulfur-containing compounds (e.g., enzymes, hormones and xenobiotic compounds) can play an important role in the regulation of different cellular processes and drug metabolism in the liver (Hebbring et al., 2007).

The ‘Oxidative phosphorylation’ was overall more induced in RE vs. OF from two weeks prepartum to two weeks postpartum as shown by gene enrichment analysis (Figure 2.3). The lower rate of this pathway in OF vs. RE indicates a lower energy utilization in the formed

compared to the latter. The increased rate of oxidative phosphorylation in RE cows might have been associated with increased energy synthesis (Burgess et al., 2007).

Lipid metabolism: The lipid metabolism involved several pathways including fatty acid biosynthesis, cholesterol biosynthesis, signaling molecules, production of hormones, nutrient transportation, lipoproteins and cellular membrane biosynthesis (Bach and Wachtel, 2003, Fahy et al., 2009, Higgins et al., 2004, Nguyen et al., 2008, Subramaniam et al., 2011). Among the most impacted pathways were 'Arachidonic acid metabolism', 'Biosynthesis of unsaturated fatty acids', 'Synthesis and degradation of ketone bodies' and 'Fatty acid metabolism'. All pathways were more induced in RE vs. OF from -14 to 14 DIM except for 'Arachidonic acid metabolism' and 'Biosynthesis of unsaturated fatty acids' which were more induced at 1 DIM in OF vs. RE. The 'Glycerol metabolism' was however, more induced in OF vs. RE after parturition.

Arachidonic acid is a long chain polyunsaturated fatty acid (Daley et al., 2010). The larger induction of 'Arachidonic acid metabolism' may have potential role in insulin signaling regulation in hepatocytes (Metz et al., 1983). However, 'Synthesis and degradation of ketone bodies' and 'Fatty acid metabolism' more induced in RE vs. OF cows (Figure S1). The synthesis and degradation of ketone bodies and higher rate of fatty acid metabolism after calving indicate negative energy balance condition.

Amino acids and other amino acids metabolism: Amino acids (**AA**) can play an important role during early lactation to meet the glucose requirements through hepatic gluconeogenesis (Bell et al., 2000). In addition, certain amino acids such as alanine, aspartate and glutamate may play an important role in hepatic gluconeogenesis during starvation (Muller and Seitz, 1981). The data

indicated a larger utilization of AA for gluconeogenesis in RE vs. OF, as is the case for ‘Alanine, aspartate and glutamate metabolism’ that was more induced at -14 DIM in RE vs. OF cows, likely due to higher glucose requirement in this group as consequence of restricted feed intake (Lobley, 1992).

Glycan biosynthesis and metabolism: Glycans are carbohydrate molecules that are linked with lipids and protein moieties to form glycolipids and glycoproteins and also act as signaling molecules (Etzler and Esko., 2009). The most impacted glycan pathways included ‘Other glycan biosynthesis’, and ‘N Glycan Biosynthesis’, ‘Glycosphingolipid biosynthesis – lacto and neolacto series’, and ‘O-Glycan biosynthesis’. Except the last two, all the “Glycan Biosynthesis and Metabolism’ related pathways were more induced in RE vs. OF at 2 weeks prepartum and more induced in OF vs. RE postpartum (Figure S1).

The potential role of N-Glycan biosynthesis in liver may lie in its ability to handle the misfolded proteins in ER during stressed condition (Fagioli and Sitia, 2001). The function of ER is affected by both intracellular and extracellular stimuli during protein synthesis leading to ER stress. This results in accumulation of misfolded proteins in ER lumen (Lu et al., 2006).

The glycosphingolipid biosynthesis - lacto and neolacto series pathway was observed as more induced from -14 d to 14 DIM in OF vs. RE. Glycosphingolipids are glycolipids with an amino alcohol sphingosine molecule attached to them (Chalfant and Poeta, 2010). The activation of glycosphingolipids biosynthesis during -14 to 14 DIM in OF vs. RE might have played a role in the larger hepatic lipid accumulation in OF vs. RE, as suggested by previous findings in mice by Jennemann et al. (2010). In another study it has also been observed that lack of lacto or

neolacto glycolipids enables B cell activation (Togayachi et al., 2010), potentially triggering an immune response.

Metabolism of cofactors and vitamins: The ubiquinone and other terpenoid-quinone biosynthesis was more induced in OF vs. RE during transition but was due to the different expression of only 1 genes (at the last at 1 and 14 DIM). Despite this pathway has a potential role in oxidative phosphorylation. as part of the cellular respiratory chain that transfers electrons from complex I or II to complex III (Kawamukai, 2002); however, the oxidative phosphorylation was less induced overall in OF vs. RE. This result suggests an important role of oxidative phosphorylation during transition in dairy cows.

The 'Folate biosynthesis', was more overall more induced in OF vs. RE, particularly postpartum indicated a larger transformation of folate in its active form (i.e., tetrahydrofolate) in OF vs. RE. This pathway acts in DNA synthesis and repair during pregnancy, lactation and plays an important role in methionine metabolism (Ragaller et al., 2009). Deficiency of folate can hinder proper DNA synthesis and affect the metabolism of several AA, including methionine (Ragaller et al., 2009). The large production of active folate might be related to the larger apparent cell proliferation in OF vs. RE.

Metabolism of Terpenoids and Polyketides: 'Terpenoid backbone biosynthesis' was more inhibited at -65 DIM, and after that it was gradually induced until -14 DIM in OF vs. RE. Then it was more induced after parturition in RE vs. OF until 14 DIM (Figure S1). In bacteria, the role of this pathway is to synthesize peptidoglycan in the cell wall, and in mammals to synthesize cholesterol via the mevalonate pathway (Xu et al., 2011). However, in the liver of dairy cows its

role is likely as associated with cholesterol biosynthesis through the mevalonate pathway (Schlegel et al., 2012).

2. Genetic Information Processing

Overall this category of pathway was more induced in OF vs. RE after parturition (Figure 2.2). The details of the pathways clearly support a larger induction of transcription, RNA metabolism, and replication and repair of DNA postpartum in OF vs. RE; however, even though the 'Ribosome biogenesis in eukaryotes' was more induced in OF vs. RE, the 'Ribosome' KEGG pathway (i.e., protein synthesis machinery components) was more induced in RE vs. OF. In addition, the 'Protein export', 'Protein processing in endoplasmic reticulum (ER)' and 'Proteasome' were overall more induced in RE vs. OF except 'Protein processing in ER' and 'Ubiquitin mediated proteolysis' which were greater in OF vs. RE. Those data appear to indicate that, in one hand, the overall amount of RNA (and/or number of different transcripts), including the rRNA, was larger in OF vs. RE, as also supported by the number of DEG (Figure 2.1), but in the other end the overall protein synthesis and export was higher in RE vs. OF.

Transcription: The liver cells proliferate because of several internal and external stimuli including nutritional induced responses (Costa et al., 2003). The liver cell proliferative stimuli activate several growth factors, signaling pathways and cytokines leading to immune system regulation, cell division, proliferation and apoptotic events (Friedman, 2008). Our results indicate greater transcription and translation rate due to OF during transition as shown in Figure 2.2. Specifically, the basal transcription factors were more impacted and induced from -14 to 14

DIM. These transcription factors work with RNA-polymerase II enzyme to transcribe the DNA into RNA (Reese, 2003).

Translation: RNA transport was more induced in OF vs. RE, while ribosomal translation was evidently induced in RE vs. OF during transition as shown in Figure 2.3 from enrichment analysis results. The RNA transport is an essential mechanism for different types of gene expressions (Kohler and Hurt, 2007). The RNA after transcription is transported from nucleus to cytoplasm through nuclear pore complexes (Kohler and Hurt, 2007). However, in the similar fashion, mRNA surveillance pathway was also induced in OF cows. The main role of this pathway is to degrade abnormal mRNAs due to several reasons including inefficient or inaccurate metabolic processes (Isken and Maquat, 2007). On the other hand, the ribosomal translational process was actively involved in protein synthesis in RE cows. The activation of ribosomal subunit has also been verified in several physiological states including starvation (Yamada et al., 1997).

Folding, sorting and degradation: Protein export, protein processing in endoplasmic reticulum (ER) and proteasome were overall induced in RE vs. OF during prepartum to postpartum except protein processing in ER at 14 DIM and proteasome at -14 DIM. While ubiquitin mediated proteolysis was overall induced in OF vs. RE cows during transition from -14 to 14 DIM.

The main role of the pathways under this sub-category is to remove misfolded proteins by the ER- associated degradation pathway (Vashist and Ng, 2004). The activation of protein export and processing in ER during transition can be associated with the higher rate of ribosomal translation machinery. However, the proteasome-mediated degradation of protein became more

induced in RE cows after parturition. The proteasome performs a protein degradative function in the nucleus and cytoplasm (Lingbeck et al., 2003). But the ubiquitin mediated proteolysis was more induced in OF cows. The activation of this pathway may indicate higher energetic state of the cell. This pathway also regulates several cellular processes, including but not limited to, gene expression, cell cycle, stress response, DNA repair and translation (Ciechanover and Schwartz, 1994).

Replication and Repair: The genome surveillance system helps to control the inconsistencies in the DNA replication using different enzymatic processes to maintain the genome integrity (Chalker and Yao, 2011). The results indicate that DNA replication and repair was mostly active in OF compared to RE cows. Among these, base excision repair (BER), non-homologous end-joining (NHEJ) and mismatch repair (MMR) were the most impacted pathways by DIA.

The gradual activation of BER pathway from -14 through 14 DIM may indicate a potentially greater degree of DNA damage because of its central role in repairing the damaged DNA (Liu et al., 2007) and controlling the cell proliferation (Reese, 2003). BER mainly checks the non-helix distorting base lesions during replication process and excises them out of DNA (Charlet-Berguerand et al., 2006). During this process different DNA glycosylases, endonucleases and polymerases are involved. The main enzyme involved is DNA-glycosylase which removes incorrect bases (Wyatt et al., 1999). Like BER, NHEJ was also gradually induced two weeks prepartum to two weeks post-partum in the liver cells. It mainly plays an important role in repairing the DNA double strand breaks, failure of which leads to cell proliferation through cancer or invokes cell death (Sharma et al., 2011).

3. Environmental Information Processing

This category includes ‘membrane transport’, ‘signal transduction’ and ‘signaling molecules and interaction’ as sub-categories that were exposed as the most impacted ones.

Membrane Transport: In membrane transport molecules, ATP binding cassette (ABC) transporters were significantly induced in RE group. They are used to transport various molecules across the cell membranes during hepatic metabolism (Adachi et al., 2009, Nicolaou et al., 2012). They also play an important role in tissue defense and remove xenobiotic compounds through bile salts (Huls et al., 2009).

Signal Transduction: Notch signaling pathway was significantly induced during the two weeks prepartum to two weeks postpartum in RE vs. OF. This pathway is involved in various gene regulation functions that are involved in cell proliferation, differentiation, and apoptosis (Gazave et al., 2009). In the liver of mammalian cells it appears to play a role in bile duct development (Zong et al., 2009). In one of study conducted in the rat liver, it was also concluded that the inhibition of notch signaling pathway can affect the functioning of oval cells (stem cells) to repair liver (Darwiche et al., 2011).

Signaling Molecules and Interaction: Extra cellular matrix (ECM)-receptor interaction pathway was more induced in RE vs. OF two weeks prepartum; whereas its significant induction was observed in OF vs. RE after parturition until two weeks postpartum. This pathway is involved in cell growth, development, maintenance and morphogenesis of different tissues and organs (Rosso et al., 2004). This might also regulate cell proliferation by interacting with cyclic

adenosine monophosphate (cAMP) (Saxena et al., 1999). This cAMP ultimately interacts with mitogen-activated protein (MAP)-kinase which in response activates a variety of cellular functions such as cell division, gene expression, cell survival, and apoptosis (Pearson et al., 2001).

4. Cellular Processes

The cellular processes were overall more induced in OF vs. RE postpartum. Among these the pathways from 'transport and catabolism' are briefly discussed below.

Transport and Catabolism: Among the cellular processes, peroxisome was more induced at -14 DIM in RE vs. OF cows while it was then induced postpartum in OF vs. RE cows. The peroxisome is a ubiquitous cell organelle performing a key role in lipid metabolism and in detoxifying reactive oxygen species (Tower et al., 2011). It breaks down long-chain fatty acids through beta-oxidation and it is associated with synthesis of plasmogen, cholesterol and bile salts, and also metabolic processes such as glyoxylate and reactive oxygen species (Mueller et al., 2002, van den Bosch et al., 1992).

It can be inferred that activation of peroxisome may be involved in beta oxidation of free fatty acids to meet the energy requirements in RE cows. The second most impacted pathway under this sub-category is lysosome. It was more induced in RE group prepartum as indicated by Figure 2.3. However, it was more induced after parturition in OF cows. Lysosome contains acid hydrolases that are used to remove unwanted molecules or cells using different fusion mechanisms (Luzio et al., 2007). Like ubiquitin-proteasome system, it also plays an important role in energy dependent proteolysis system (Ciechanover, 2005). In RE cows, due to less

availability of cellular energy, this process could have been less induced; whereas its activation in OF cows may indicate higher availability of energy in the form of ATP.

5. Organismal Systems

Among the organismal system, immune system, endocrine system, circulatory system and excretory systems were the most impacted during the transition period.

Immune system: The immune system is usually invoked as a response to eliminate internal or external pathogens (Ulevitch, 1999). In this case the complement and coagulation cascade and nod-like receptor signaling pathways were among the most impacted pathways in the periparturient period. The complement system invokes the innate immune response in the cells against cell injuries or shocks while coagulation system acts as homeostatic role in immune response development (Amara et al., 2008). This pathway usually becomes more sensitive in mounting an inflammatory response (Carroll, 2004).

A greater induction of the complement and coagulation cascade before parturition in RE cows indicated the potential existence of signals within the tissue that triggered preparation of an immune response ahead of parturition. In contrast, its significant induction after parturition in OF cows indicates a potential role in repairing cell injury. The Nod-like receptor signaling pathway was also actively involved in the immune system of the liver tissue. Its induction was observed in RE cows from -14 to 14 DIM. This pathway is mainly responsible for detecting pathogens and elicits the innate immune response to kill the pathogens via signaling cascades that trigger apoptosis (Sirard et al., 2007). Its activation in RE cows indicates a stronger immune response against pathogens in conjunction with ubiquitin-mediated proteolysis.

Endocrine system: Among the 'Endocrine systems', the 'Renin-angiotensin system' and the 'PPAR signaling' were the most impacted. The 'Renin-angiotensin system' mainly regulates the blood pressure, the homeostasis of sodium and water, tissue trauma and helps to combat inflammation and fibrosis in liver (Bataller et al., 2005, Lubel et al., 2008). In cows fed RE, this system was induced at -14 DIM, while in OF cows it was more induced after parturition (1 to 14 DIM). This induction suggests an active role in regulation of blood circulation through the tissue and liver diseases such as inflammation and fibrosis.

The second most-impacted pathway under endocrine system is the peroxisome proliferator-activated receptor (PPAR) signaling. It was more induced in RE cows two weeks prepartum and two weeks postpartum except immediately after parturition as seen at 1 DIM. However, its induction was observed in OF cows at 1 DIM. Depending on the specific isoform (α, δ, γ), PPAR signaling regulates a variety of cellular functions such as lipid catabolism, adipogenesis, cell proliferation, differentiation, tissue repair, and inflammation (Wahli and Michalik, 2012). Its activation in RE cows at -14 DIM and 14 DIM may indicate an anti-inflammatory role. The PPAR signaling may have been activated as a response to long-chain fatty acid influx, its derivatives, and/or as a response to inflammation caused by metabolic disorders as proposed previously (Loor et al., 2006).

Most Impacted and Enriched Gene Ontology (GO) Terms

In Figure 2.4 are reported the 10 most impacted GO terms in each GO category resulted after DIA analysis. GO terms provides more holistic view of the gene expression profilings data in terms biological processes (BP), celular components (CC) and molecular functions (MF). GO

terms describe the relationship gene products (BP) to their fucntions (MF) in their particular compartments (CC). The detailed informaion at different time points is provided. The following section describes the 10 most enriched pathways in each GO term.

A) Biological Processes (BP)

The most enriched BP observed was the 'negative regulation of megakaryocyte differentiation'. This process indicates that the differentiation of megakaryocyte was extensively downregulated in OF vs. RE cows. The megakaryocytes produce platelet cells which act as clotting factors (Geddis, 2010). The second most enriched term is the 'activation of MAPKKK activity' which is activated by insulin and as a response activates other growth factor signaling pathways (Ueki et al., 1994).

Other terms such as 'cell separation during cytokinesis', 'cytokinesis, completion of separation', 'cytokinetic process', 'cell migration in hindbrain', 'hindbrain tangential cell migration', 'granule cell precursor tangential migration' and 'centrosome localization' strongly support a greater degree of cell division and proliferation in liver cells in OF vs. RE cows (Alcantara et al., 2000, de Anda et al., 2005, Kulesa and Fraser, 2000, Roncero and Sanchez, 2010).

The 'macrophage activation during immune response' supports the process of immune response activation through macrophages. This is a primary response activated by the immune system in response to pathogens, blood antigens, protein synthesis and metabolism for hepatic immune surveillance (Knolle and Gerken, 2000). All these processes were induced during two weeks prepartum and then sustained their activation during transition to two weeks postpartum in OF vs. RE cows.

B) Cellular Components (CC)

In CC, 'Chiasma', 'MutLalpha complex', 'Sin3 complex', 'Sin3-type complex', 'PCAF complex', 'nucleotide-excision repair complex' and 'nucleolus organizer region' correspond to several cellular processes such as cell division and proliferation, histone modification, DNA repair, replication, and transcription factors (de Laat et al., 1999, Jones, 1984, Kanao et al., 2009, Kuzmichev et al., 2002, Martin et al., 2005, Nagy and Tora, 2007). The 'MHC class I peptide loading complex' belongs to the immune system and functions as antigen processing in liver cells (Chen et al., 2005). These were more induced postpartum at 14 DIM in OF cows. The GO terms 'integral to peroxisomal membrane' and 'intrinsic to peroxisomal membrane' refers to the peroxisomal membrane synthesis (Fujiki et al., 1984). Some of the GO terms have been discussed briefly in different sections. These GO terms cover the induction from -14 to 14 DIM in OF vs. RE cows.

C) Molecular Functions (MF)

In MF, all the 10 most enriched categories were induced in OF cows except 'thiol oxidase activity' and 'aryl sulfotransferase activity', which were induced in RE cows during the transition period. Among these terms, 'pyruvate kinase activity' was induced in OF cows as this activity is involved in the conversion of phosphoenolpyruvate to pyruvate to regulate gluconeogenesis (Moreno et al., 1976). 'Vitamin D binding' protein is a serum glycoprotein found on the surface of B and T cells in the liver (Cooke, 1986) which indicates an immune response. The 'exo-alpha-sialidase activity' and 'alpha-sialidase activity' are involved in glycoproteins (Achyuthan and Achyuthan, 2001).

The ‘kinetochore binding’, in chromosomal segregation indicates cell division (Chan et al., 1998). The ‘thiol oxidase activity’ is involved in oxidation and hydroxylation reactions (Karunakaran et al., 2005). Its deactivation may cause oxidative stress in liver cells. Aryl sulfotransferase activity catalyzes the sulfuric acid esterification reactions which causes cancer in the liver; however it was more induced in RE vs. OF cows.

The involvement of ‘lysozyme activity’ in injured hepatic cells increases to combat with destroyed leucocytes (Manifold et al., 1982). The ‘ATP citrate synthase activity’ plays a role in oxaloacetate synthesis in mitochondria (Shepherd and Garland, 1969) and ‘siRNA binding’ in RNA interference during protein synthesis (Doench et al., 2003) was higher in OF vs. RE dairy cows.

2.4. SUMMARY and CONCLUSION

In figure 2.5 is reported an overview of the most important findings from our analysis. The DIA and enrichment analyses of KEGG pathways and GO terms in OF vs. RE prepartum suggests that overfeeding compared to restricted diet elicited a stronger transcriptional response potentially leading to greater cell signaling and interaction, liver cell proliferation, tissue remodeling, and lipid synthesis. In contrast, those data suggest a lower degree of energy utilization by reducing ATP production. Such effects persisted after calving, i.e. had a strong carryover effect until two weeks postpartum. A larger accumulation of liver lipid and lower degree of energy utilization in OF vs. RE were also supported by in vitro metabolic data from the same samples (Loor et al., 2006).

In addition, data indicated an inhibition of protein synthesis together with lower amino acid metabolism, protein export, and degradation in OF vs. RE during the transition period. The larger effect in RE vs. OF as it relates to oxidative phosphorylation, ribosome, and proteasome was suggestive of higher energy production, protein synthesis and degradation due to restricting energy intake prepartum. The data indicated a higher catabolic capacity in RE vs. OF coupled with a greater reliance on protein turnover; the significance of the latter is not apparent, but could indicate a greater degree of enzyme turnover. In contrast, the analysis of these pathways suggested that OF resulted in greater production of NADPH through the combination of glycolysis/gluconeogenesis and pentose phosphate pathway. NADPH is also a source of energy for glycerolipid synthesis.

Overall, these results indicate that OF vs. RE prepartum elicited a stronger transcriptional response potentially leading to alterations in immune system, metabolism, and DNA damage and

repair, increased NEFA during first week post-partum. It can be concluded that moderate overfeeding of energy prepartum results in transcriptional changes predisposing cows to fatty liver and potentially compromising liver health during early postpartum.

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2.6. FIGURES

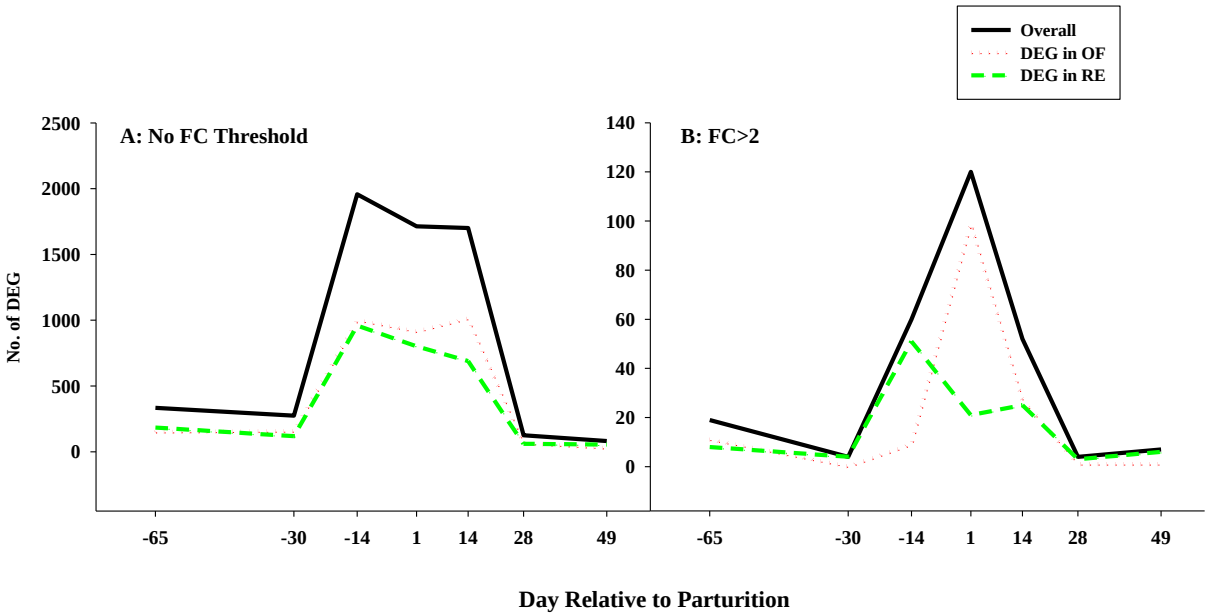

Figure 2.1: Number of differentially expressed genes (DEG) in bovine liver due to overfed (OF) vs. restricted energy (RE) prepartum. A). DEG with no fold change (FC) threshold cutoff. A larger number of DEG between the two diets was observed from the two weeks prepartum to two weeks postpartum with a peak at -14 DIM. There was overall a similar number of genes more expressed in OF vs. RE compared to RE vs. OF, with exception of 14 DIM, where there was a larger number of genes more expressed in OF vs. RE compared to RE vs. OF. B). DEG with 2-fold change (FC) cutoff. The total number of DEG between the two diets with ≥2-fold difference in expression was <7% of total DEG. Most of the large differences in gene expression between OF *vs*. RE occurred during -14 to 14 DIM. At day -14 there was the largest number of DEG more expressed in RE *vs*. OF and at day 1 the largest number of DEG more expressed in OF *vs*. RE.

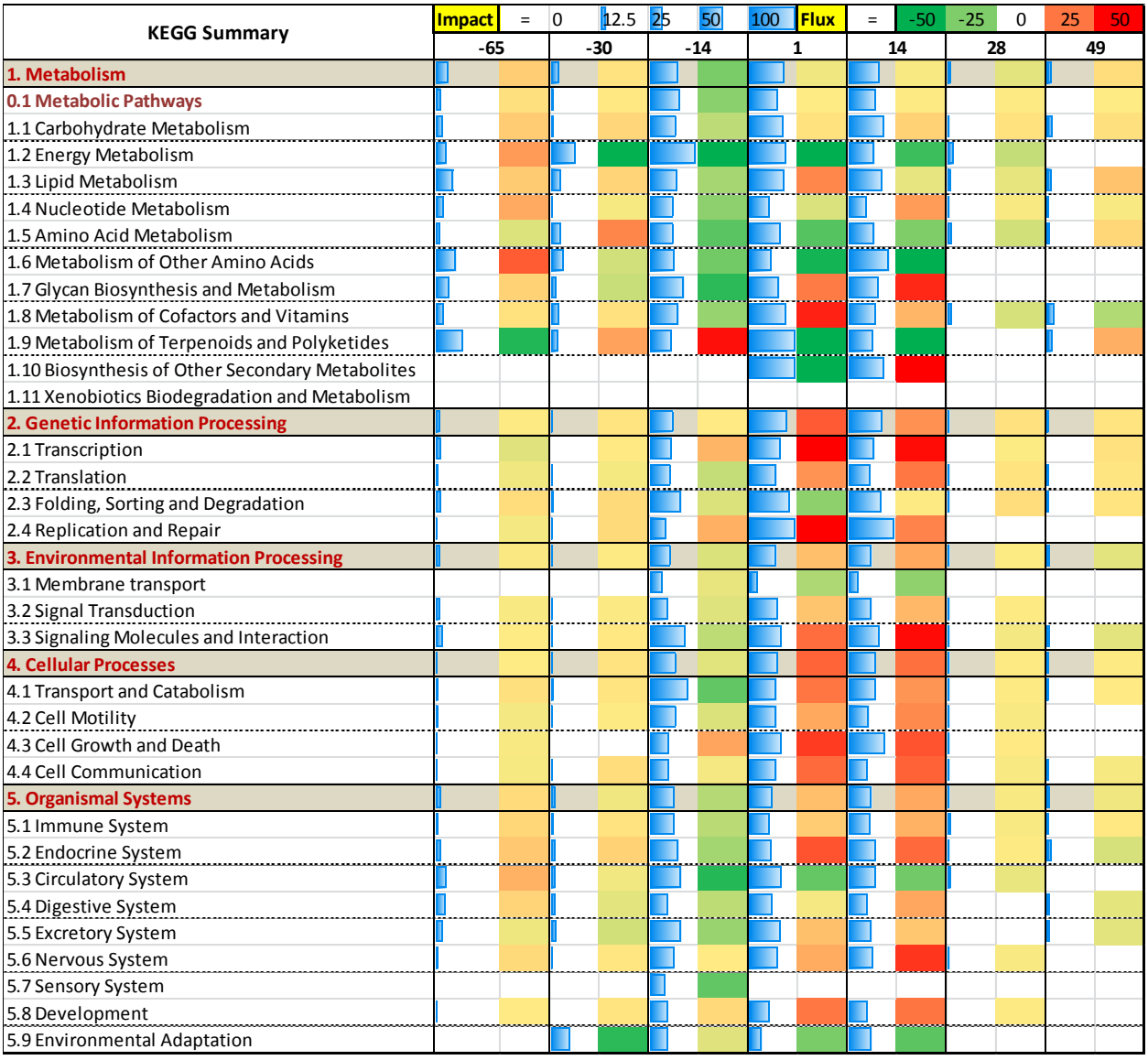

Figure 2.2: KEGG summary of bovine liver consisting of categories and sub-categories of KEGG pathways analyzed through the dynamic impact approach (DIA). The days -65, -30, -14, 1, 14, 28 and 49 are related to the parturition. The 'Impact' values are represented in the light blue bars ranging from 0 (no change) to 75 (maximum change). The 'Direction of the impact' values are shown by green (more induced in RE vs. OF) or red (more induced in OF vs. RE).

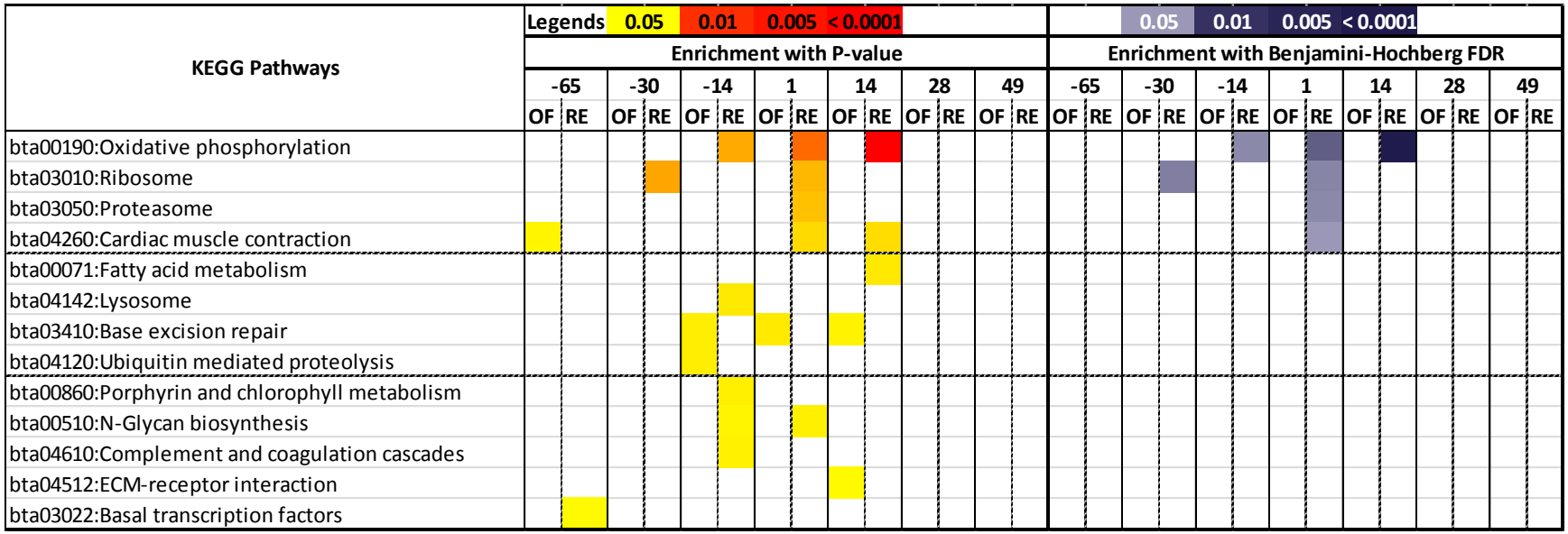

Figure 2.3: Enriched DAVID KEGG pathways due to high energy or overfed (OF) and restricted energy (RE) prepartum dietary plans. The enrichment raw p-values and Benjamini-Hochberg FDR are colored according to the respective legends. The analysis was performed using the DEG more expressed in one group vs. the other (i.e., OF = DEG more expressed in OF vs. RE; RE = DEG more expressed in RE vs. OF.

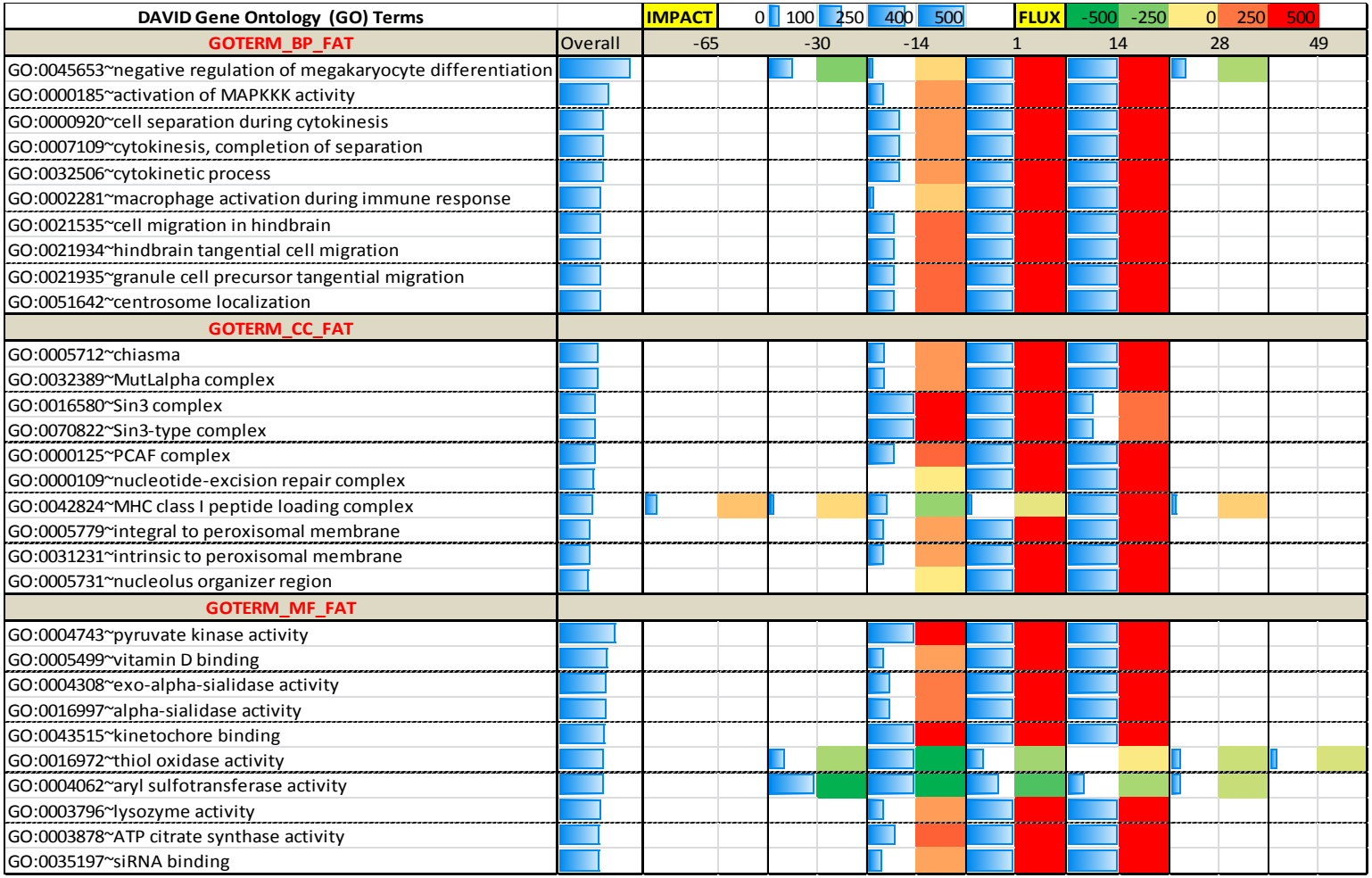

Figure 2.4: The 10 most impacted Gene Ontology (GO) terms [Biological Processes (BP), Cellular Components (CC) and Molecular Functions (MF)] as uncovered by DIA analysis. The days -65, -30, -14, 1, 14, 28 and 49 are relative to the parturition. The 'Impact' values are represented in the light blue bars ranging from 0 (no change) to 500 (maximum change). The 'Direction of the impact' is denoted by the green (more induced in RE vs. OF) and red (more induced in OF vs. RE). The "Overall" column represents the overall Impact and was used to rank the GO terms for each category.

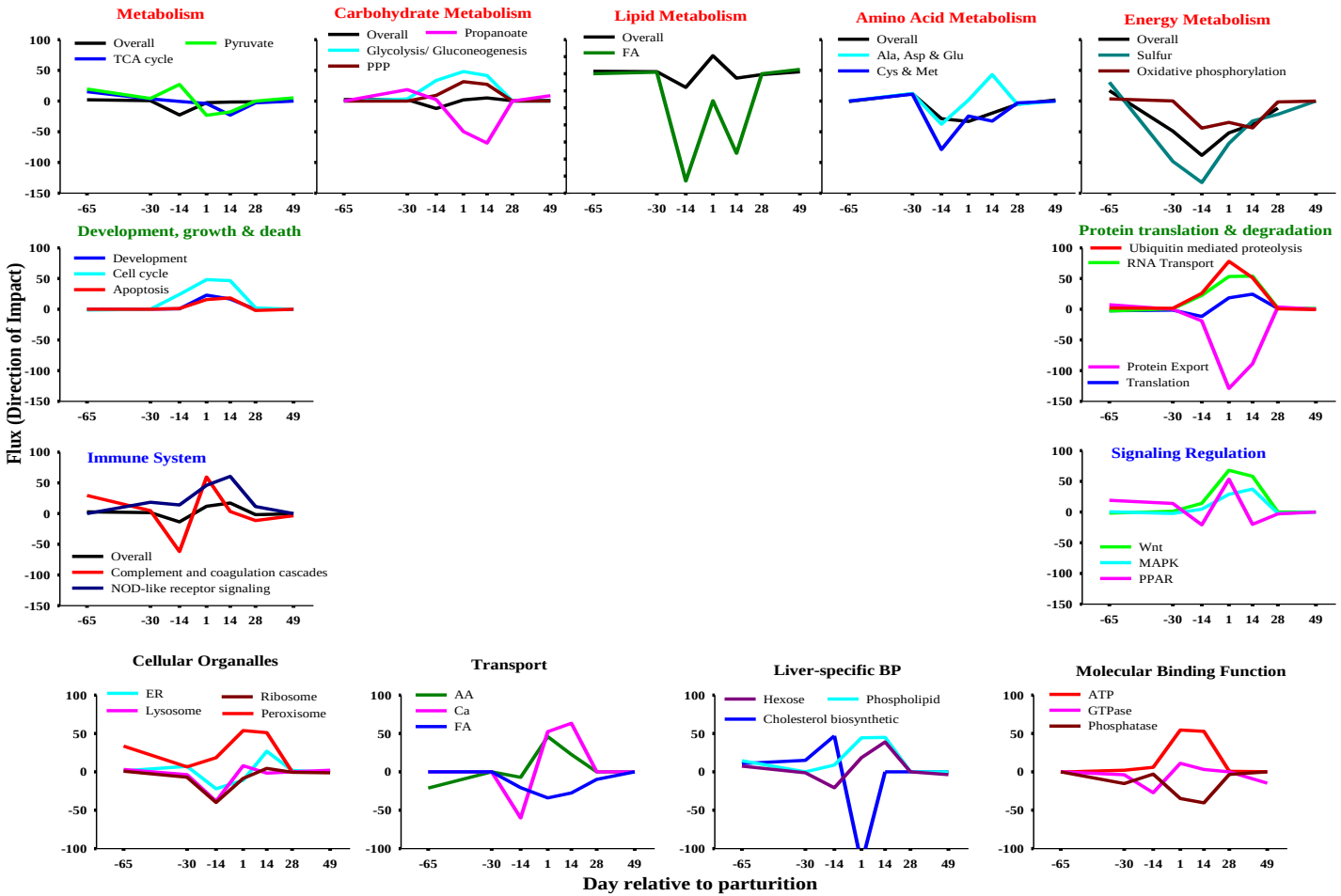

Figure 2.5: The figure summarizes the most relevant biological terms affected in liver by energy level prepartum in dairy cows as revealed by the Dynamic Impact Approach (DIA) analysis of KEGG pathways and Gene Ontology (GO) terms. The data on the X-axis correspond to the days relative to parturition, while the data on the Y-axis correspond to the 'Direction of impact'. The red titles indicate the sub-category of pathways related to metabolism.

Legends: AA = amino acid; Ala = Alanine; Asp = Aspartate; ATP = Adensine Triphosphate; BP= Biological Processes; Ca = Calcium; Cys = Cysteine; ER = endoplasmic reticulum; FA = fatty acids; Glu = Glutamate; MAPK = mitogen-activated protein kinase; Met = methionine; NOD = Nucleotide Oligomerization Domain; Notch = Notch signaling pathway; PPP= Pentose phosphate pathway; PPAR = peroxisome proliferator-activated receptor signaling pathway; RNA = Ribonucleic Acid; TCA cycle = tricarboxylic acid cycle; Wnt = Wingless signaling.

CHAPTER 3: BOVINE ADIPOSE TISSUE

In-depth study of bovine adipose tissue transcriptome dynamics during the transition period

3.1. INTRODUCTION

The metabolic and physiological adaptations during transition period in dairy cows involve several complex biological interactions between key tissues such as liver, mammary glands, muscles and adipose tissue etc. (Loor et al., 2011). The metabolism of adipose tissue in establishing an effective lactation cycle during early postpartum has been well studied in both human and animals (Stuebe and Rich-Edwards, 2009). During transition period, the rate of lipolysis increases, while the rate of lipogenesis decreases in adipose tissues resulting in free fatty acids (Vernon, 1980). The released fatty acids, also known as non-esterified fatty acids (NEFA) are directed to liver and mammary tissues to generate energy through TCA cycle and oxidative phosphorylation and glucose synthesis through gluconeogenesis for body requirements (McNamara, 1991). At this stage, animal becomes susceptible to different metabolic disorders such as ketosis, acidosis, and negative energy balance etc. In the liver, NEFA are oxidized to CO2, converted to ketone bodies or (esterified to) triacylglycerol (TAG) (Drackley, 1999). This is the time, when energy demands for lactation and body maintenance supersedes the energy supplies, leading to a common disease called negative energy balance (NEB) (McCarthy et al., 2010). When an animal is in NEB, then it faces several health disorders including ketoacidosis, immunosuppression, reduction in milk production and reproductive performance (Gumen et al., 2011). Adipose tissue also plays and important role in initiating the supply of adipokines, cytokines, immune related proteins, and inflammatory responses during transition to combat with metabolically stressed conditions (Loor et al., 2006).

An efficient study of metabolic changes during transition period is indispensable to improve milk production and reproductive performance of animals (McNamara, 2011). To get

deeper understanding of metabolic and physiological changes occurring in animals during transition period, a study of key tissues (e.g., liver, adipose tissue, mammary gland, and muscles) using systems biology approaches is indispensable. In this article we have investigated the role of adipose tissue using the concept of dynamism. A systematic study of adipose tissue can provide a holistic view of metabolic changes occurring from prepartum to postpartum. These metabolic changes play an important role for providing vital foundations in lactation onset (Sumner-Thomson et al., 2011). To check the dynamic role of adipose tissue during transition period relatively fewer transcriptome profiling studies have been conducted (Mukesh et al., 2010; Sumner-Thomson et al., 2011; Shahzad and Loor, 2012) . These initial findings indicate that systems level information has not been fully explored yet.

In this article we have used the microarray dataset from -21 d prepartum to 28 d postpartum (viz., -21, -7, 7, 28), and compared the dynamism of metabolic changes from pregnancy to lactation (viz., -7vs-21, 7vs-21, 28vs -21, 7vs-7 and 28vs7) to explore the systems level information. We have employed the Dynamic Impact Approach (DIA) developed by Bionaz et al. (2012b) to measure the systematic differences between different time point comparisons. The DIA utilized the information from KEGG pathways (Kanehisa and Goto, 2000) obtained from KEGG database (http://www.genome.jp/kegg/pathway.html) and DAVID bioinformatics resource (Huang da et al., 2009b, a) to study the physiological changes. KEGG database provide the information of metabolic pathways, while DAVID database provide the information of several biological pathways including KEGG and Gene Ontology (GO) terms with respect to user defined gene lists. DAVID bioinformatics resource relies on a well-known overrepresented approach (ORA) (Huang da et al., 2009b). Even though, this approach has some limitations as well, e.g., it precludes the full investigation of dynamism about functional

adaptations in time-course experiments and it counts enrichment analysis based on likelihood *p*-values. The DIA approach has potentially overcome these limitations and provided a dynamic visualization of functions/pathways of time series data. It also accounts for the ratio of differentially expressed genes (DEG) compared to the genes present in array and fold change values in addition to likelihood *p*-values (Bionaz et al., 2012a).

We have applied the DIA approach on KEGG pathways and GO terms retrieved from the KEGG database and "Functional annotation chart" of DAVID bioinformatics resource database (http://david.abcc.ncifcrf.gov/) respectively. Our studies highlight the major changes occurring during relative dynamism from pregnancy into lactation with respect to adipose tissue irrespective of dietary treatments in dairy cows.

3.2. MATERIALS AND METHODS

Holstein dairy cattle from Washington State University Knott Dairy Center (Pullman) were used for the systematic analysis of adipose tissue.

Ethics Statement

All procedures for animal handling prior to and after adipose tissue biopsy were followed in accordance with USDA guidelines and protocols approved by the Washington State University Institutional Animal Care and Use Committee under protocol # 3364.

Animal Sampling and RNA Extraction

A total of 24 cows were selected for adipose tissue extraction. All cows were fed with the same ration from -21 d prepartum to 28 d postpartum as mentioned in (Sumner and McNamara, 2007). The adipose tissue samples were collected under local anesthesia from the tailhead region. After animal sampling the RNA was extracted using a standard protocol as described in (Sumner-Thomson et al., 2011). Briefly, the adipose tissue biopsies were taken at -21, -7, 7, 28 d relative to parturition. These biopsies were collected under local anesthesia from the tailed region. Then laboratory protocols were followed for RNA cleaning and extraction.

Microarray

The RNA was hybridized to Affymetrix Bovine Gene Array™ (Affymetrix, Santa Clara, CA) containing 14,200 spots. The standard procedures were followed as described by manufactures at the Washington State University Microarray Core Facility. After RNA hybridization to Affymetrix Bovine Gene Array, washing and staining, the arrays were scanned

using Affymetrix GeneChip Scanner 2700. The scanned images were quantified using Affymetrix GeneChip Operating Software (GCOS, Affymetrix). A total of 24 microarray experiments were conducted (-21d= 4, -7d = 8, 7d = 4, 28d = 8).

Statistical Analysis

The array data were normalized using GeneSpring GX (v. 7.3) software. For the background information and annotation, a complete bovine genome Affy annotation (3' IVT Expression Analysis Arrays) array was used. This annotation file was downloaded from the Affymetrix website (http://www.affymetrix.com/support/technical/annotationfilesmain.affx). After data normalization, fold change (FC), raw p-values and false discovery rate (FDR) for multiple hypothesis correction were calculated. For the FC values, six time points were selected viz., '-7vs-21', '7vs-21', '28vs-21', '7vs -7', 28vs-7 and '28vs7' for comparisons. For statistical analysis, a parametric test with unequal variance assumption (Welch t-test) under 1-way ANOVA was used.

Dynamic Impact Approach (DIA) and Enrichment Analysis

We applied the DIA on both KEGG pathways and GO terms. For the DIA analysis, we employed the methodology as described in (Bionaz et al., 2012b). Briefly, the whole dataset consisting of Affy IDs and Entrez gene IDs along with FC, raw p-values, and FDR-corrected *p*-values was uploaded to the excel-based DIA software. Then the impacted KEGG pathways and KEGG summary results along with flux (direction of impact) were downloaded after DIA calculations has been completed. Inside the DIA, we used *p*-values ≤ 0.05 and FDR ≤ 0.05 parameters. At least 30% genome representation with respect to the genes present in the Affymetrix array was selected for further analysis. The enrichment analysis was performed by

the DAVID database using the KEGG pathways. A probability criterion with p-value ≤ 0.05 was considered for the enriched pathways. The default results from the DAVID pathways were downloaded with our Affymetrix background. For this purpose, 'Functional Annotation Chart' tool was used. To interpret the role of GO terms using DIA, three GO terms, viz., Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF) were used from the default results.

3.3. RESULTS AND DISCUSSION

In this article we have analyzed the role of biological pathways along with the concept of dynamism using DIA. Bioinformatics tools that are based on overrepresented approach (ORA), are unsuitable for capturing dynamism of tissues along different time points. To study the role of dynamic changes along different time points, DIA has uncovered the role of most significant biological changes occurring in adipose tissue during transition into lactation. The transcriptomic profiling technique covering the whole genome is helpful in increasing our understanding of adipose tissue biology during transition period. This technology has led us to link even small level changes occurring in mRNA to the metabolic and physiological adaptation with respect to different time point associations.

Statistical Results and overall gene expression pattern

The ANOVA with FDR correction resulted in 2160 differentially expressed genes (DEG, FDR$\leq$ 0.1) by time across different groups. Among these 2160 DEG, 1802 (1644 unique) were annotated with Entrez gene IDs. DEGs with different time point comparisons are shown in Figure 3.1. Without any FC threshold, the DEGs with respect to six time comparisons are represented in Figure 3.1A. The overall gene expression was gradually increased from one week prepartum to 28 DIM as compared to -21 DIM (-7vs-21, 7vs-21, 28vs-21 and 28vs-7). However, this expression was decreased at 7 DIM compared to -7 DIM and 28 DIM compared to 7 DIM. The individual gene expression of up- and down-regulated genes was almost similar at all the time comparisons. They were increased gradually from –7 to 7 and 28 DIM compared to -21

DIM. In Figure 3.2B FC ≥ 2 threshold criteria is adapted to show the DEGs. The apparent results show that the overall gene expression was higher at 7vs-21, 28vs-21 and 28vs-7 d postpartum. The overall expression was gradually decreased to 7vs-7 and 28vs7 d postpartum. The results with FC ≥ 2 indicate a larger number of genes down regulation at -7vs-21, 7vs-21, 28vs-21 and 28vs-7 d compared to the up-regulated genes. These genes were than decreased gradually at 7vs-7 and 28vs7 d postpartum.

The Overall Summary of KEGG Pathways

The DIA analysis over KEGG pathways resulted in two types of fallouts, the overall summary results encompassing categories and sub-categories and the most-impacted pathway (Figure S2). These results consist of impact and flux values against each pathway under their respective time comparison. The impact values correspond to the overall change in a pathway while flux values correspond to the direction of the impact. The KEGG database hierarchy divides the biological pathways into categories, and sub-categories. Figure 3.2 summarizes the KEGG pathways at main categories and sub-categories levels. The main categories include 'Metabolism', 'Genetic Information Processing', 'Environmental Information Processing', 'Cellular Processes' and 'Organismal Systems'. The 'Human Disease' has been removed from our analysis because it has very little to do with analysis of bovine adipose tissue. The results indicate that Metabolism category is overall inhibited from prepartum to postpartum. Even though, its activation was seen gradually as the cows were shifting from dry period into lactation.

The Metabolism category consists of 11 sub-categories, and most of them were inhibited except the metabolism of nucleotide, glycan biosynthesis and biosynthesis of other secondary metabolites. The metabolism of nucleotide and glycan biosynthesis was overall slightly induced

at -7, 7 and 28 DIM compared to -21 DIM. The biosynthesis of other secondary metabolites was seen as induced significantly during the first week of lactation compared to -21 d prepartum, but after that its inhibition was observed as the lactation was proceeding especially at 28vs7 d postpartum. However, the metabolism of other amino acids was significantly induced at 28vs-7 and 28vs7 d postpartum.

Other categories including 'Genetic Information Processing', 'Environmental Information Processing' and 'Cellular Processes' were slightly induced from one week prepartum to four weeks postpartum as compared to -21 d prepartum. Among these translation, replication and repair, membrane transport, signaling molecules and interaction, cell motility, and cell growth and apoptosis were significantly induced suggesting a mechanism of protein synthesis and cell replenishment (Klaus et al., 1990; McNamara, 1991; Lefterova and Lazar, 2009; White and Stephens, 2010).

The 'Organismal System' category was gradually inhibited from one week prepartum to postpartum. Among this category, the immune system was induced after parturition onward. The development process was also induced postpartum. However, the rest of the sub-categories such as endocrine system, circulatory system, digestive system, excretory system, nervous system and sensory system were either slightly or greatly inhibited postpartum as compared to -21 d prepartum.

Biological Interpretation of the Most-Impacted KEGG pathways:

DIA analysis revealed 211 KEGG pathways from the KEGG database that were impacted as an overall in adipose tissue. To discuss this much pathways is beyond of this article discussion. So we have discussed the most impacted pathways that were significantly affected

from prepartum to postpartum in adipose tissue as shown in Figure S2. The DAVID enrichment analysis results are shown in the Figure 3.3. According to these results, ‘valine, leucine and isoleucine degradation’, ‘oxidative phosphorylation’, ‘TCA cycle’, ‘pyruvate metabolism’, ‘propanoate metabolism’, ‘butanoate metabolism’ tryptophan metabolism and several other pathways as shown in Figure 3.3 were overall down-regulate postpartum compared to -7 and -21 d prepartum. However, complement and coagulation cascades and inositol phosphate metabolism were among the induced pathways likely postpartum.

The adipose tissue primarily serves as fatty acids depots so that it can be used during starvation or early lactation period to provide the required energy needs (Sumner and McNamara, 2007). During transition the required energy is compensated by either lipolysis in the adipose tissue or proteolysis in the muscle tissue (Zurek et al., 1995). The most impacted metabolic and physiological changes from transition into lactation including lipids metabolism as a major are discussed in the following sections.

1. Metabolism

Most of the metabolic pathways in adipose tissue were inhibited early postpartum, and clearly by 7 and 28 DIM. Among the inhibited pathways were, e.g., pyruvate, citrate cycle, propanoate, butanoate, fatty acid biosynthesis and metabolism, and amino acid degradation and metabolism. The induced pathway at 7vs-21 and 28vs-21 d postpartum include riboflavin metabolism. Together, this suggested a lower utilization of glucose, amino acids and fatty acids by adipose tissue.

Carbohydrate Metabolism: The metabolism of dietary carbohydrate provide major source of energy for body maintenance, growth, development and production of milk in dairy cows (Nafikov and Beitz, 2007). Among the carbohydrates, glucose is the major precursor for fatty acid biosynthesis in adipose tissues and milk lactose synthesis in mammary glands (Whitehurst et al., 1978; Nafikov and Beitz, 2007; Mellenberger et al., 2009). It also provides the energy in the form of NADPH through pentose phosphate pathway for triglyceride synthesis in liver (Fabregat et al., 1985). In adipose tissue major carbohydrate pathways such as pyruvate metabolism, citrate cycle (TCA cycle), metabolism of propanoate, butanoate, glyoxylate and dicarboxylate were significantly inhibited during transition into lactation (Figure S2).

Pyruvate is the major source for fatty acid synthesis in adipose tissue (Coore et al., 1971). It provides oxaloacetate through pyruvate carboxylase, which is then converted to citrate. Citrate is then converted into acetyl units for further fatty acid synthesis (Martin and Denton, 1971). The inhibition of this pathway indicates the shutdown of fatty acid biosynthesis during transition period. With the inhibition of pyruvate metabolism, the intermediate supply for TCA cycle was also less induced (Cannon and Nedergaard, 1979) as shown by enrichment analysis in Figure 3.3. The inhibition of TCA cycle in the same fashion indicates very lower rate of ATP generation, because of very low or unavailability of substrates postpartum compared to prepartum. The propanoate and butanoate including acetate are short chain fatty acids that constitute major source of energy in ruminant metabolism (Leng and Annison, 1963). Propanoate plays an important role by promoting the conversion of pyruvate into Glyceride-glycerol for long chain fatty acid synthesis during fating (Reshef et al., 1967). It has been observed in rat adipose tissues that both propanoate and butanoate stimulate the conversion of lactate to CO_2 or fatty acid

synthesis (Patel et al., 1971). The short chain fatty acids also regulate the leptin hormone (Lin et al., 2012). Leptin hormone is secreted from adipose tissue, which further regulate the lipid metabolism by promoting lipid oxidation and protein synthesis by cutting down the lipid synthesis (Buettner et al., 2008). Our results suggest that these pathways were more induced prepartum. In contrast, the inhibition mechanism may potentially suggest the increased level of NEFA in the blood and accumulation of triglycerides in the liver leading to several potential metabolic disorders such as negative energy balance, ketosis and fatty liver (Rukkwamsuk et al., 2000; Loor et al., 2007; Fenwick et al., 2008; Wathes et al., 2009) .

The next most impacted metabolic pathway among carbohydrate metabolism in adipose tissue is 'glyoxylate and dicarboxylate metabolism'. This pathway remained inhibited from prepartum to lactation like other metabolic pathways compared to -21 d prepartum. In bacterial studies, it has been shown that glyoxylate pathway is also used for energy synthesis by either being converted into malate by malate synthase enzyme or converted into acetyl CoA (in TCA cycle) or phosphoenolpyruvate (in dicarboxylic acid cycle) through 3-Phosphoglycerate by glyoxylate carboligase enzyme (Ornston and Ornston, 1969). Our results indicate that this pathway may have been induced prepartum compared to postpartum for energy synthesis.

Energy Metabolism: The oxidative phosphorylation was also significantly inhibited postpartum compared to prepartum as shown in the Figure 3.3. The main role of oxidative phosphorylation is to produce ATP through electron transport chain (Kelly et al., 2011). The reduced efficiency of oxidative phosphorylation after postpartum indicate lower dependency of adipose tissue for

energy synthesis. However, from the results it can be inferred that this process was more induced prepartum for meeting the energy requirements.

Lipid Metabolism: Lipid is stored in the adipose tissue in the form of fats, while its storage increases during mid pregnancy due to extra energy production (Drackley, 2004). During late pregnancy and early lactation, the energy utilization in cows increases leading to the increased fat mobilization through lipolysis (Bell et al., 1987). This process continues until peak lactation and after body fat recovery process starts (McNamara, 1989). Our results of fatty acid biosynthesis with most impacted and inhibited during late pregnancy to early lactation compared to -21 d prepartum are also in consistent with these findings. The earlier findings in carbohydrate metabolism indicate that with the shutting down of major carbon source pathways, the biosynthesis of fatty acids was also reduced.

Fatty acid biosynthesis is negatively correlated with the lipolysis during transition into early lactation due to copious milk synthesis (Block et al., 2001). At this point the demand for dry matter intakes (DMI) decreases, while the rate of lipid mobilization from adipose tissue to liver increases in the form NEFA (Drackley et al., 2006). The metabolism of 'Glycerolipid' and 'Glycerophospholipid' was gradually inhibited from -7 d prepartum to 28 d in milk as compared to the -21 d prepartum. These pathways are involved in lipid synthesis such as triacylglycerol or other major lipid sources (Bouyekhf et al., 1992).

The inhibition of glycerolipid and glycerophospholipid metabolic pathways in conjunction with propanoate inhibition support the evidence that lipogenesis was shut down during early lactation. During late pregnancy the demand for energy utilization increases leading

to increased rate of lipid release from body fat reserves leading to triacylglycerol accretion in the liver tissue (Rukkwamsuk et al., 2000; Loor et al., 2006). The biosynthesis of unsaturated fatty acids such as n-6 fatty acids (e.g., linoleic acid, arachidonic acid) and n-3 fatty acids, was also inhibited postpartum (Figure 3.3). Ether lipids function as ubiquitous and are synthesized by peroxisomal enzymes. These ether lipids are further used in membrane biosynthesis (Hajra et al., 1988).

The induction in the fatty acid elongation in mitochondria was observed at -7vs-21 d prepartum. After subsequent metabolic changes around parturition its inhibition was observed particularly at 28vs-21 d. The *de no* fatty acids synthesis during one week prepartum can be related with the access availability of acetate precursors in adipocytes (Vernon, 1980). However, its inhibition postpartum may suggest an access availability of free fatty acids, because of feedback mechanism (Bergen and Mersmann, 2005). The ether lipid metabolism was inhibited at -7, 7 and 28 d as compared to -21 d, but it was induced at 7vs-7 and 28vs7. Its active role after parturition may indicate cell signaling and membrane transport and lipid metabolism (Bartz et al., 2007).

Amino Acid Metabolism: The metabolism of amino acids is very essential for several metabolic reactions and most important of them is gluconeogenesis for maintaining glucose and energy levels in the body (Aschenbach et al., 2010). The most impacted metabolisms of amino acids include phenylalanine, tyrosine, histidine, and tryptophan (Figure S2 and Figure 3.3). The first three are gluconeogenic amino acids while the fourth one is ketogenic amino acid (Hamana et al., 2010). These amino acids contribute in energy metabolism through different routes of TCA

cycle at different entry points (Brosnan, 2000). The deficiency in any of these amino acids can make animal susceptible to many diseases (e.g., oxidative damage, muscular atrophy) (Valerio et al., 2011). The degradation of several amino acids takes place in adipose tissue. The proteolysis mechanism of different amino acids was studied in details in obese women by (Patterson et al., 2002). Valine, leucine and isoleucine degradation was significantly inhibited at 7 and 28 postpartum compared to -21 d prepartum (Figure 3.3). In bovine, it has been indicated that plasma concentration of histidine, valine, leucine and isoleucine directly affect by decreasing the concentrations of phenylalanine, tyrosine, methionine, lysine and arginine (Korhonen et al., 2002).

Metabolism of Cofactors and Vitamins: Cofactors and vitamins play an important role in regulation of several physiological processes including metabolic and energy reactions, biosynthetic processes, gene expression, cellular growth and development (Che et al., 2003; van Herwaarden et al., 2007). Among these cofactors and vitamins, biotin and riboflavin were significantly impacted in the adipose tissue. Metabolism of biotin (vitamin H) was significantly inhibited at -7, 7, and 28 d vs. -21 d prepartum.

Biotin also serves as a cofactor in lipid biosynthetic pathways. In chicken, it has been observed that deficiency of biotin in the diet may lead to fatty liver disease (Whitehead et al., 1976). The inactivation of this vitamin may indicate liver lipid infiltration and very low or no symptoms of lipogenesis in adipose tissue as indicated from the above findings. Riboflavin (vitamin B12) metabolism is one of pathway that sustained its induction from prepartum to postpartum. The induction of riboflavin activity during transition indicates greater rate of beta oxidation in adipose tissue (Patterson and Bates, 1989). Purine nucleotide, especially GTP is an

essential precursor of riboflavin biosynthesis (Jimenez et al., 2005). The riboflavin further serves as precursor for two enzymes such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These enzymes are further involved in several electron transfer reactions (Ross and Hansen, 1992).

Xenobiotics Biodegradation and Metabolism: The metabolism of xenobiotics was inhibited overall. Among these, the most impacted pathways include 'drug metabolism - cytochrome P450' and 'metabolism of xenobiotics by cytochrome P450'. Cytochromes are major enzymes responsible for drug metabolism (Furge and Guengerich, 2006; Guengerich, 2008). These are used to remove xenobiotic compounds that cause cell toxicity. The expression level of cytochrome P450 is increased as response to metabolize the drugs. This pathway is affected by several lipophilic environmental pollutants such as dioxins etc. These expression studies were conducted in white adipose tissue of rat by Yoshinari et al. (2004). In our case, the inhibition of this pathway may suggest an inactivation of drug metabolizing hormones in the adipose tissue specifically during postpartum.

2. Organismal Systems (OS)

Among the OS category, immune system, endocrine system, digestive system and excretory systems were among the most impacted during transition.

Immune System: The complement and coagulation cascades pathway was activated at -7, 7, and 28 d compared to -21 and at 7vs-7 d; whereas this pathway was inhibited at 28vs7 d during lactation (Figure S2). The complement system usually invokes during proinflammatory responses in adipocytes (Zhang et al., 2007). This pathway is central to the host defense during

innate immune response, which is activated by the coagulation system and as response, complement cascade is initiated to combat with cell injuries or external pathogens (Amara et al., 2008). The activation of this pathway during transition into lactation may suggest a greater induction of immune response either through invasion of external pathogens or internal cell injuries.

Endocrine System: Two pathways were impacted under endocrine system, which are renin-angiotensin system and PPAR signaling. The renin-angiotensin system was greatly inhibited during the whole transition. The potential role of renin-angiotensin system is to regulate body fat mass, insulin resistance, systematic blood pressure and metabolic functions (Stiefel et al., 2011). In adipose tissue, it has the ability to modulate the adiposity and initiate obesity by preadipose cell differentiation and lipogenesis (Darimont et al., 1994; Kim et al., 2006).

The inhibition of this pathway also supports lipolysis in adipocytes. The second pathway is PPAR signaling, which was overall inhibited gradually from prepartum to postpartum until 28 DIM (Figure 3.3). This pathway is involved in several biological pathways such as lipid metabolism, pre-adipocyte differentiation, and gluconeogenesis and also in transcription initiation during differential gene expression (Taniguchi et al., 2008). The inhibition of this pathway may suggest an increased rate of lipolysis and accumulation triglycerides in liver leading to fatty liver disease as studied in mice (Barak et al., 1999).

Gene Ontology (GO) Terms

The DIA approach was applied over GO terms and the 10 most enriched GO terms from each BP, CC and MF were selected for discussion as shown in the Figure 3.4. The DIA over GO terms resulted in 2557 BP, 420 CC, and 896 MF.

Biological Processes (BP)

The 10 most enriched BP (Figure 3.4) include ‘thyroid hormone transport’, ‘activation of transmembrane receptor protein tyrosine kinase activity’ , ‘adult feeding behavior’, ‘regulation of response to feed’, ‘negative regulation of appetite’, ‘regulation of dopamine secretion’, ‘regulation of catelcholamine secretion’, and ‘multicellular organismal protein metabolic process’. The some other BP include ‘fatty acid elongation’, ‘triglyceride biosynthetic process’, ‘alditol catabolic process’, ‘glycerol catabolic process’, ‘glycerol-3-phosphate catabolic process’, ‘polyol catabolic process’, ‘regulation of antigen processing and presentation’. All of these were inhibited except the last GO term that is related to the immune system.

These enriched BP belong to biological functions such as protein metabolism, quality control and phosphorylation (multicellular organismal protein metabolic process, activation of transmembrane receptor protein tyrosine kinase activity), lipid biosynthesis (fatty acid elongation, triglyceride biosynthetic process), and glycerol breakdown (glycerol catabolic process, glycerol-3-phosphate catabolic process) (Hubbard and Miller, 2007; Joseph et al., 2010; Zhao et al., 2010; Bar-Lavan et al., 2012). Thyroid hormone transport is mainly involved in energy regulation, balancing glucose level, and maintaining body temperature (Skarulis et al., 2010). Adipose tissue generate heat through oxidation of fatty acid and glucose when they need extra heat (Cannon and Nedergaard, 2004). Inactivation of this hormone transport may indicate extra lipolysis to maintain thermogenesis in adipocytes. These results have also been validated by a similar study of maintaining energy balance and rate of lipolysis in mice adipocytes as a consequence of obesity (Martinez-Botas et al., 2000).

Aditol and polyol catabolic processes were also inhibited. These pathways are involved in catabolism of glycerides and monosaccharide derived polyhydric compounds, and polyhydric alcoholic compounds (MetaCyc, accessed on June 23, 2012b, a). The inhibition of this pathway indicates an alternative route for energy synthesis instead of glyceride breakdown. However, regulation of antigen processing and presentation was highly active indicating an activation of immune system response specifically during early lactation. These results also in accord to the above mentioned results of KEGG pathways.

Cellular Processes (CP)

Among the CC (Figure 3.4), 'NADPH oxidase complex', 'glycerol-3-phosphate dehydrogenase complex', 'platelet dense tubular network', 'fibrinogen complex', 'Golgi lumen', 'chylomicron', 'germinal vesicle', '1-phosphatidylinositol-4-phosphate 3-kinase, class IA complex', 'intermediate-density lipoprotein particle' and 'axolemma'.

These CC showed an overall combined response of activation and inhibition during lactation. These results showed an inactivation of glycerol-3-phosphate dehydrogenase complex that plays it role in glyceride catabolism (Zhao et al., 2010).

In contrast, it showed an activation of NADPH oxidase complex activity postpartum (7vs-7 and 28vs7) indicating an increased level consumption of NADPH during lactation (Jitrapakdee et al., 2008). The activity of chylomicrons was induced after postpartum specifically during first week of lactation 7vs-21 and 7vs-7. Chylomicrons are major lipid carries for lipid transportation (Erkelens, 1989). It has been indicated that these are hydrolyzed quickly as they are released from the adipose tissue. The enzyme responsible for hydrolysis of triglyceride in

chylomicron is lipoprotein lipase present in adipose tissue (Scow, 1977). Intermediate-density lipoprotein particle is also a lipid carrier that was affected mainly postpartum rather than prepartum for lipid molecules transportation (Erkelens, 1989). Immune related components include 'platelet dense tubular network', and 'fibrinogen complex' that were significantly induced postpartum. These indicated a greater level platelets aggregation and homeostasis (Litvinov et al., 2011) in adipocytes. The cellular component '1-phosphatidylinositol-4-phosphate 3-kinase, class IA complex' was inhibited -7, 7 and -28 vs. -21 d, however, its induction was observed at 28vs7 d postpartum.

The key role of this pathway is to activate a complex series of phosphorylation cascades (e.g., tyrosine kinase signaling) which is further used in several diverse biological functions e.g., cell growth, differentiation and proliferation, cell motility and survival etc. (Foster et al., 2003). Inhibition of Golgi lumen at -7 and 7 vs. -21 indicates less utilization of this organelle in processing proteins and fats while its activation at 28vs7 indicates greater synthesis of protein and lipids molecules. The main function of Golgi complex is to process lipids and protein molecules that have been generated through endoplasmic reticulum for transportation to other destinations (Munro, 2011; Carolg and Palade, 1964). The paraspeckles compartment of cell was inhibited at -7 and 7 compared to -21 d. However, it was induced at 28vs7 d postpartum during lactation. The main function of this dynamic compartment is transcriptional regulation and ordered localization of protein components in a cell (Schuldt, 2002). Its inhibition one week prepartum to one week postpartum indicates less amount protein synthesis, which increased gradually as seen at 28vs7 d postpartum. The peculiar induction of germinal vesicles indicates cellular differentiation process after parturition at 28vs-21, 7vs-7 and 28vs7 (Palma et al., 2012).

Molecular Functions (MF)

Like BP, all MF were inhibited except 'hemoglobin binding' fibroblast growth factor 2 binding and fibrinogen binding GO term, which was activated postpartum compared to -7 and -21 DIM prepartum (Figure 3.4). Among the MF, 'thyroid hormone binding', 'prolactin receptor activity', 'vitamin D binding', 'acetyl-CoA carboxylase activity', 'biotin carboxylase activity', 'butyrate-CoA ligase activity' '[acyl-carrier-protein] S-acetyltransferase activity', '[acyl-carrier-protein] S-malonyltransferase activity', '3-oxoacyl-[acyl-carrier-protein] reductase activity', '3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase activity', were strongly inhibited postpartum as compared to prepartum, while among these, one molecular function 'hemoglobin binding' was greatly induced during first week postpartum, afterwards it was inhibited at 28 d compared to 7 d postpartum. Hemoglobin is an oxygen carrying molecule (Hawkey et al., 1991) that may support the evidence of fatty acid oxidation during first week of lactation (Nakamura and Nishida, 1971).

The most impacted and enriched GO term under MF is the 'prolactin receptor activity', which was overall inhibited from prepartum to postpartum in adipocytes. The prolactin receptor activity is mostly expressed in mammary glands of lactating animals during lactation (Djiane et al., 1981). Its increased inhibition in adipose tissue may suggest its potential activation in mammary glands for milk synthesis from early lactation to onwards. Thyroid hormone binding was among the inhibited functions as discussed earlier. The main role of vitamin D is to modulate calcium homeostasis and bone metabolism and to combat with macrophages, while this activity is mainly observed in bone cells and blood serum (Chun et al., 2012). Vitamin D binding inhibition in adipocyte during postpartum (7vs-21, 28vs-21, 7vs-7 and 28vs-7 d) may indicate its activation in bone cells or blood serum. The inhibitory role of biotin ('biotin carboxylase

activity') has also been discussed earlier. The activity of acetyl-CoA carboxylase is dependent on biotin. Its functional role is to convert acetyl-Co A to malonoyl CoA for fatty acid biosynthesis (Tong, 2005).

However, because of an access availability of fatty acids during first week of lactation, it was inhibited potentially due to feedback mechanism. In the same series of events the next four terms viz., '[acyl-carrier-protein] S-acetyltransferase activity', '[acyl-carrier-protein] S-malonyltransferase activity', '3-oxoacyl-[acyl-carrier-protein] reductase activity', and '3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase activity', were also inhibited during lactation to stop the biosynthesis of fatty acids and cholesterol as compared to three weeks (-21 d) prepartum. It is obvious from the activity names that these carry acyl group for transferring it from one molecule to another (Ferrer et al., 2008).

3.4. SUMMARY AND CONCLUSION

We have applied the well-established overrepresented approach (ORA) via DAVID and newly-developed approach (DIA) for functional analysis of transcriptional adaptations in adipose tissue occurring during three weeks prepartum through early lactation. Bioinformatics tools strictly based on ORA (e.g., Ingenuity Pathway Analysis) are unsuitable for capturing the dynamism of tissues as they adapt to changes in several different physiological states. Hence, there is a need for alternative and more biology-based informatics approaches. DIA, one of the bioinformatics approach, elegantly solves this limitation. During the analysis, DAVID enrichment results uncovered pathways such as 'oxidative phosphorylation', 'amino acid degradation', 'pyruvate metabolism', 'TCA cycle', 'fatty acid metabolism' and 'glycerolipid metabolism', which were markedly inhibited postpartum compared to prepartum; whereas, 'complement and coagulation cascades', 'inositol phosphate metabolism' and 'spliceosome' were among the pathways with sustained induction postpartum compared to prepartum. The DIA analysis revealed that the onset of lactation resulted in a gradual decrease in adipose tissue utilization (metabolism) of glucose, lactate, and acetate to produce energy (e.g. most impacted pathways included 'pyruvate metabolism', 'propanoate metabolism', 'TCA cycle'). This would also support the preference for the use of these molecules by mammary gland for milk production. Due to greater availability of fatty acids in serum, these become the major source of energy requirements during early lactation. Most of the pathways in adipose tissue were inhibited early postpartum, and clearly by 28 DIM. Together, this suggested lower utilization of glucose, amino acids and fatty acids by the tissue. The summarized results of KEGG pathways and GO terms are presented in the Figure 3.5.

Overall, the combined results from this bioinformatics approach indicated that the adipogenic capacity of adipose tissue is quite robust during late pregnancy while the innate immune response and ability to begin the cellular replenishment process of the tissue are novel features of the system during early lactation. The immune responsiveness of adipose may be driven at least in part by stressors including cytokines/hepatokines, NEFA, and/or pathogens, which characterize the early postpartal period. The role of immune pathways in the mechanisms associated with tissue remodeling cannot be discounted.

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3.6. FIGURES

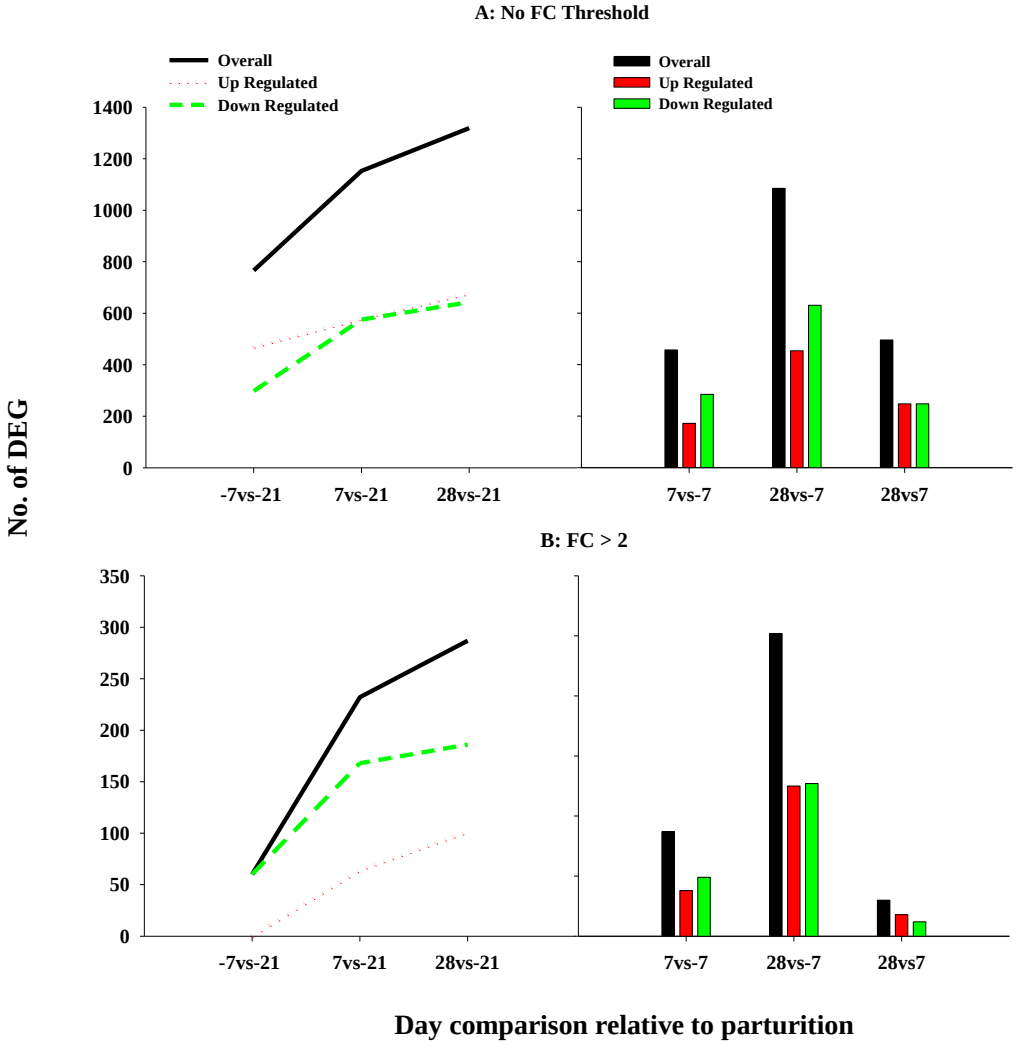

Figure 3.1: Differentially expressed genes (DEG) in adipose tissue during lactation. A). DEG with no fold change (FC) threshold. B). DEG with fold change (FC) threshold ≥ 2. The line plot represents days comparison relevant to -21 d prepartum (-7vs-21, 7vs-21, 28vs-21), while vertical bar chart represents the comparison of days after lactation compared to one week prepartum (7vs-7 and 28vs-7) and four weeks compared to first week postpartum (28vs7). The numbers of DEG with FC and without FC thresholds were calculated separately. Most of the differences in expression occurred from pregnancy to lactation. The graph shows that most of the gene expression changes (overall) were taken place at 7 vs. -21 and 28 vs. -21 d during transition.

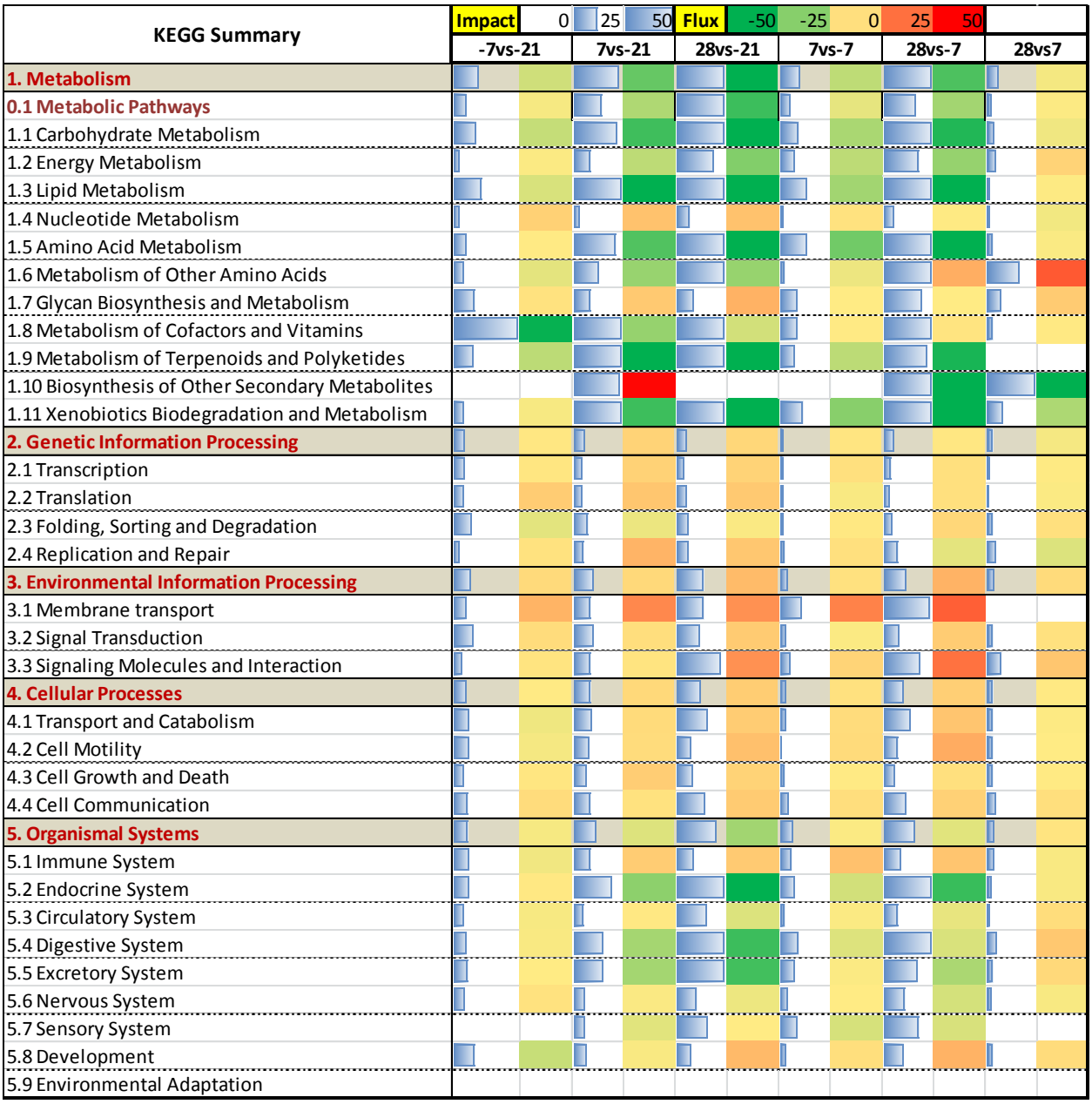

Figure 3.2: The overall view of KEGG summary of bovine adipose tissue encompassing impacted categories and sub-categories during transition into lactation. Under each comparison, the horizontal bars on the left in light-blue color denote the overall impact while the bars on the right denote the direction of the impact (red = induced; green = inhibited). The analysis of these pathways revealed that overall carbohydrate, lipid and amino acid metabolic pathways, metabolism of terpenoids and polyketides, xenobiotics degradation, and endocrine, digestive and excretory systems were mostly inhibited during postpartum vs. prepartum in adipose tissue.

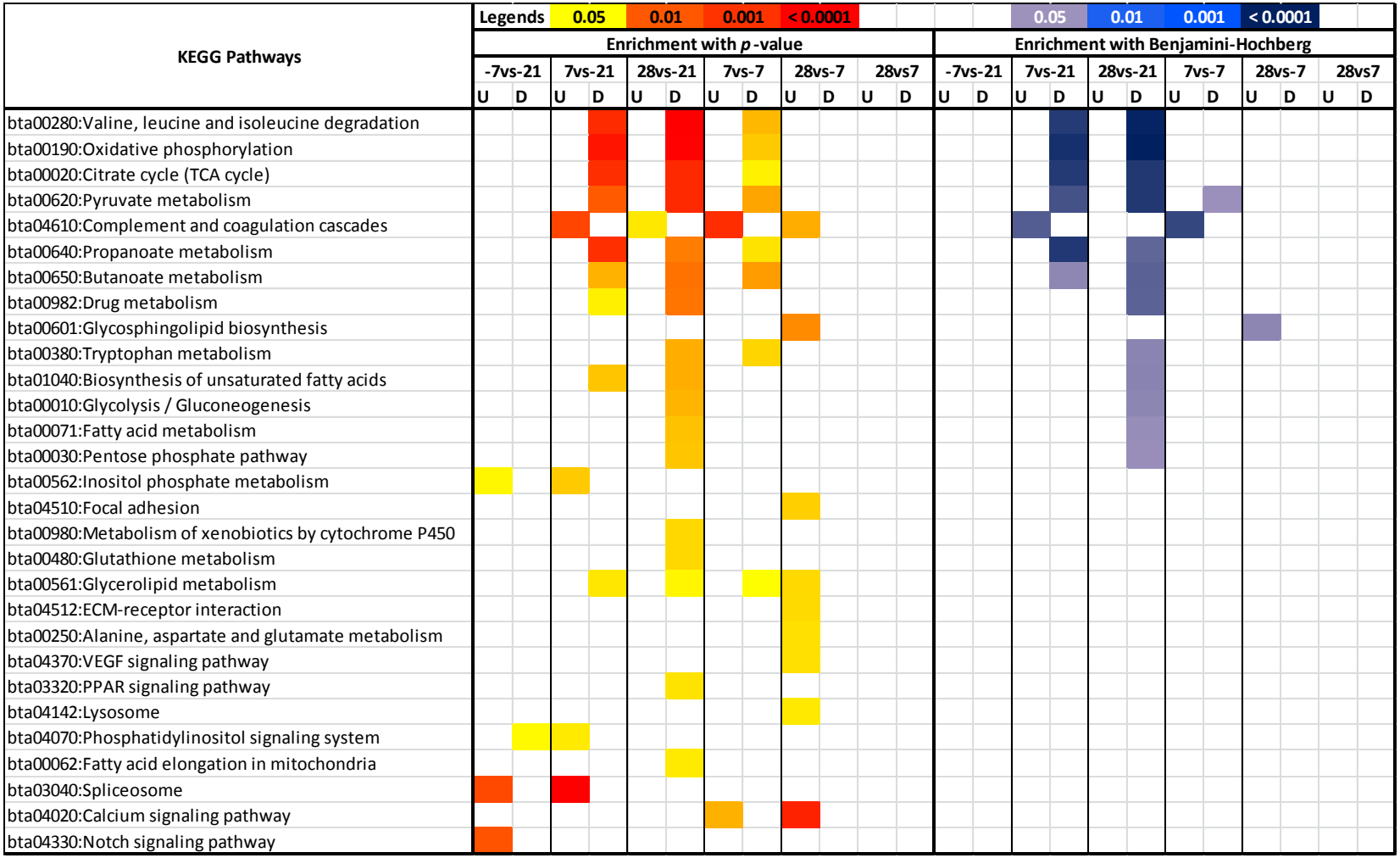

Figure 3.3: DAVID Gene set enrichment analysis. The enrichment p-values of each KEGG pathway are tinted from yellow (0.05) to red (<0.001). The Benjamini and Hochberg enrichment p-values of each KEGG pathway are tinted from light blue (0.05) to dark blue (<0.001).

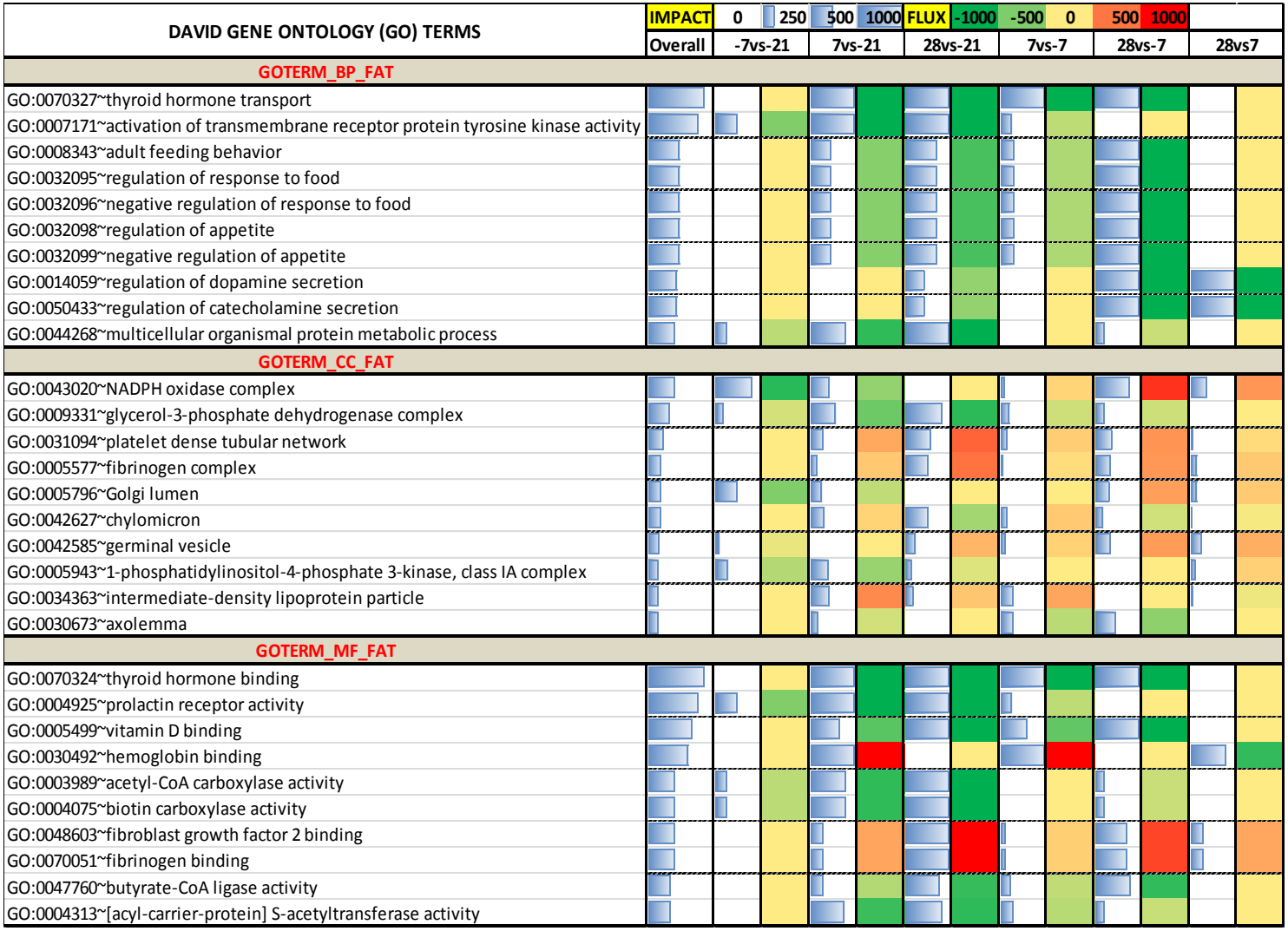

Figure 3.4: The 10 most impacted Gene Ontology (GO) terms revealed after DIA implication for each Biological Processes, Cellular Components and Molecular Functions. Under each time comparison, the horizontal columns on the left (light-blue) denote the overall impact and the squares on the right denote the overall direction of the impact (red = induced; green = inhibited).

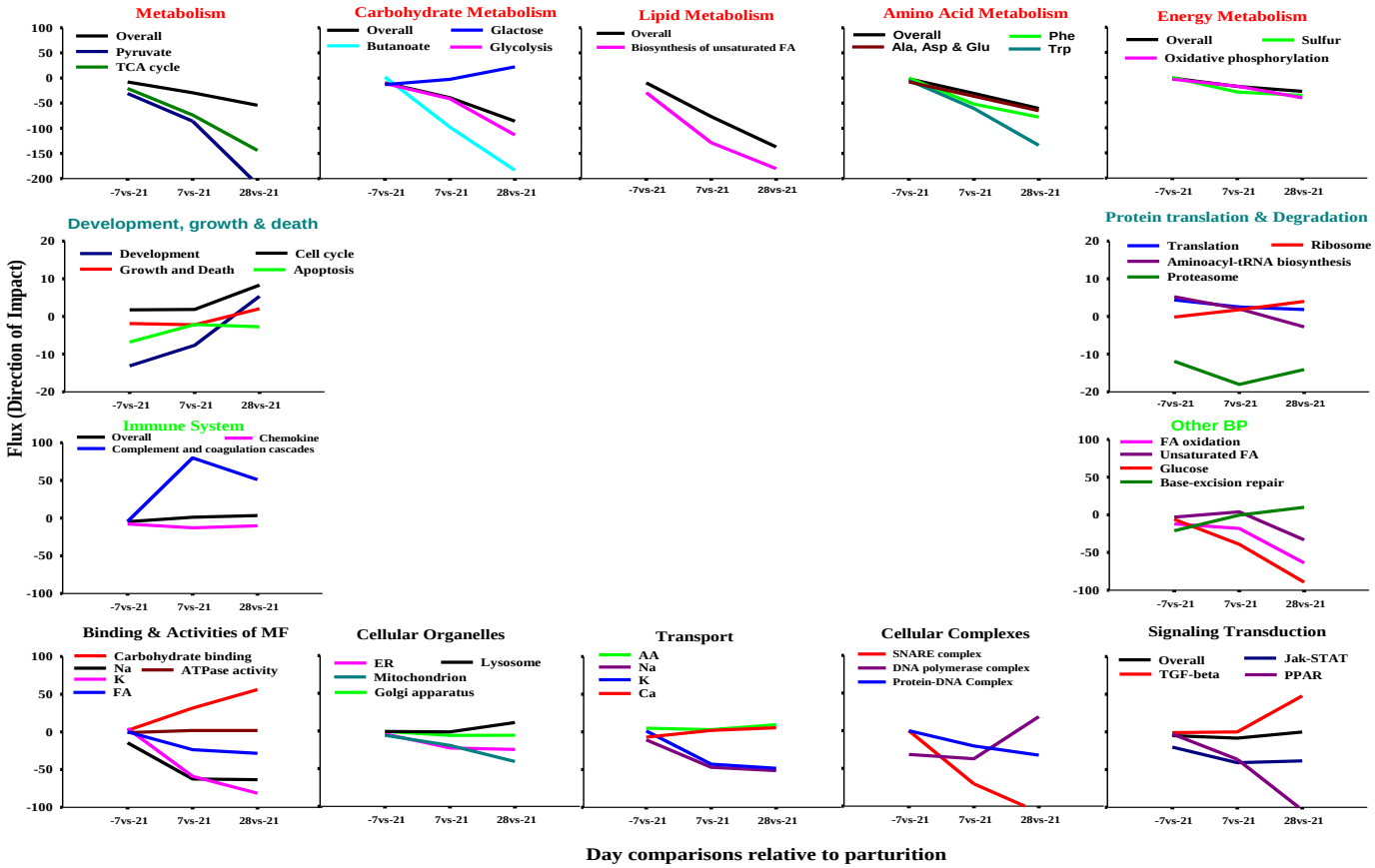

Figure 3.5: The summarized model of the most relevant biological functions in the bovine Adipose tissue during pregnancy into lactation as revealed by the dynamic impact approach (DIA) analysis of KEGG pathways and Gene Ontology (GO) terms. The time comparisons covered are -7, 7, and 28 DIM compared to -21 DIM. The data on the x-axis correspond to the days comparisons relative to parturition, while the data on the y-axis correspond to the flux (direction of impact) values. The red titles are most relevant to the hepatic tissues metabolic process as revealed by the DIA application on the KEGG pathways. Other titles correspond to the main hepatic functions during the transition period.

Legends: AA = Amino Acid; Ala = Alanine; Asp = Aspartate; BP = Biological Processes; Ca = Calcium; ER = Endoplasmic Reticulum; FA = Fatty Acids; Glu = Glutamate; Jak-STAT = Janus kinase-Signal Transducer and Activator of Transcription; K = Potassium; MF = Molecular Functions; Na = Sodium; PPAR = Peroxisome Proliferator-Activated Receptor signaling pathway; Phe = Phenylalanine; SNARE = SNAP (Soluble NSF Attachment Protein) REceptor; TCA cycle = Tri-Carboxylic Acid cycle; TGF-beta = Tumor growth factor beta; Trp = Tryptophan.

CHAPTER 4: RUMINANT PHYSIOME MICROARRAY DATABASE

4.1. INTRODUCTION

Bioinformatics databases are the main resources for presenting enormous amount of biological data generated through scientific experiments and high throughput genomics techniques such as PCR, microarrays, RNA-Seq. etc. These databases ease the access and retrieval process of the required data through online resources. These databases are linked with user interactive interface called web browsers. At the backhand of these browsers, different algorithms are embedded through several different software (Stein, 2003). These user-interactive web browsers are designed according to the availability of data inside the databases. Since 1990's onward, the trends in the biological database development have been increased tremendously. There are several biological databases, these are, but not limited to, genes or genome specific databases, e.g., NCBI (Cates, 2006), OMIM (Hamosh et al., 2000), GeneCard (Rebhan et al., 1998), EMBL (Kulikova et al., 2007), protein or proteome specific databases, e.g., Swiss-Prot , PIR (Wu et al., 2003), PDB (Bernstein et al., 1977), mitoProteome (Cotter et al., 2004), metabolic and enzymatic databases, e.g., KEGG (Kanehisa and Goto, 2000), BRENDA (Schomburg et al., 2004), Reactome (Matthews et al., 2009), and organism specific databases e.g., FlyBase (McQuilton et al., 2012), Wormbase (Harris et al., 2010), SGD (Dwight et al., 2002), BiGG (Schellenberger et al., 2010) and BioCyc (http://biocyc.org/).

Every database is focused on providing particular information through its web resources. Among these several database provide bioinformatics tools for data retrieval such as FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990Altschul et al., 1990) etc. These tools search the relevant biological information with a database or through cross reference databases.

In this article we focused on developing a web resource that is dedicated to ruminant's 'omics' and physiology (http://ruminantphysiome.ansci.illinois.edu/). Initially, we have populated the database with microarray data information of bovine liver and adipose tissue and swine mammary gland. Basically, we used the MySQL database system as our database design. For accessing the data, we used the PHP code at the backhand side for connecting the user interface with the database system. We have categorized our database web-hosting system into two main parts, based on the general information available on the webpage and ruminant's physiome information provided through the link 'Ruminant Physiome menu'. This link is provided on the 'Home' and every subsequent page. Further the structure of this database and hosting system is described in details in the following sections.

4.2. MATERIALS AND METHODS

To create ruminant physiome database, we have utilized the ACES college services. We had been given an access to a Drupal based management system for web and database development. The Drupal package provides us with the following facilities as mentioned on the ACES site (http://www.itcs.uiuc.edu/charges/web_hosting.html).

- "Web Server: Apache HTTP Server"
- "Host OS: Linux"
- "Includes one MySQL database"
- "Server Side Scripting: Perl, PHP, Python, ColdFusion"
- "Disk Space: 1 GB"
- "Maintenance Access: SFTP/SCP"
- "Data Backup: daily with four week retention"
- "Site Statistics: provided within disk space allotment"
- "DNS Registration: single .illinois.edu domain included, www. subdomains and legacy .illinois.edu domain included at no additional charge upon request".

The Ruminant Physiome database development system is divided into two phases:

A. User-interactive interface Development phase

B. Database Development phase

A. User-interactive interface Development phase:

This phase is divided into two modes: (1) Development site and (2) Production site.

1. Development site

The main changes are performed on the development site. This is a developer's interactive mode. All the new developments and changes are implemented here before updating into main site. We can also call this as developer's testing site. To access this site the URL is given below:

http://ruminantphysiome-dev.ansci.illinois.edu/

After finalizing all the updates to this mode, it is published to a production site through a command line interface by typing the following command:

publish-to ruminantphysiome.ansci.illinois.edu

2. Production site

The production site is the mirror image of a development site. This is a user's interactive mode. This site is aimed at providing the public access to the stored data. To access this site, the URL is given below:

http://ruminantphysiome.ansci.illinois.edu/

The both sites can also be accessed using command line interface commonly called command prompt shell. For this purpose, puTTY software must be installed on the machines. In

puTTY software, we give the host name or IP address (e.g., site-maint.itcs.illinois.edu) and port as 22. The connection type must be SSH (default) as shown in the Figure 4.1. Then we hit the 'open' button to open the command prompt. In the start we provide our user name and password to access the remotely located website data for both development and production sites.

B. Database Development:

For database development system, we are using MySQL (v.5) database access system. This database, like user-interactive development site, has also two modes for uploading the data i.e., production site and development site. This database system can be accessed through two ways which are given below:

1. Command line tool
2. phpMyAdmin instance available at the following URL (Figure 4.2):
   https://mysql-admin-beta.itcs.illinois.edu/phpMyAdmin/

Installing an extra module inside the Drupal Environment:

Under Drupal environment different basic modules are installed by default. The user can also install its own modules based on the website requirements. For instance, we installed two modules inside the Drupal environment. These are CKEDITOR (7.x-1.9) and IMCE (7.x-1.5). 'ckeditor' is a text editor, while an 'imce' is an image or file uploader. For ckeditor module, ckeditor software (3.6.3) is required. Image editor module works within ckeditor module environment. Inside the ckeditor module, we copied the both ckeditor software and imce module according to the given instructions. Then we transferred this module into Drupal modules environment by using command line interface to make it accessible through development site.

4.3. RESULTS AND DISCUSSION

Our web-hosting database system is divided into the following two sections:

A. General Information

B. Ruminant Physiome Database

These two sections are discussed below in details:

A. General Information

The general information menu provides the general information about the database, projects, publications and people working on it (Figure 4.3). We have also included an extra functionality called 'Forums' inside our web-system. Further details are described below:

Home: The home section provides the updated information about aims and projects and research activities that are being carried on by our lab.

Ruminant Physiome: The ruminant physiome will be discussed with details in the next section.

Download: The download page currently provides the links for processed microarray data. The information about the FDR is provided on this page along the download link. We have also provided the most updated version of DIA developed and described by Bionaz et al. (Bionaz et al., 2012a; Bionaz et al., 2012b). In the start, we have provided the processed microarray data along with annotation files of bovine liver (prepartal energy plane), adipose tissue (pregnancy into lactation) and swine mammary glands (late pregnancy). The annotation files are up to-date and are specifically designed to provide the required information about the concerning microarray data. These customized annotation files provide the information about the Oligo or Affymetrix IDs used for experiments, their annotation with Entrez gene IDs and respective

symbols. The complete annotation files are also provided in the given links to annotate any required microarray data.

Abstracts: The section of the website provides the information about the published abstracts in the national and international conferences under ruminant physiome group.

Publications: Publication section is aimed to provide the updates about the research productivities at national and international level journals. The aim of our research group is to conduct high level research to address current and future challenges.

People: The people section provides the information of active and former members, and potential collaborators who are and were actively involved with the ruminant's physiome research group and their research activities. This page also is aimed to introduce about our lab members to potential candidates in the same field.

Contact: The contact page helps the audience to contact with concerning their questions. The users can also give us their feedback about the active research and can ask web-development type queries.

Forums: The 'Forums' is a new and interactive discussion platform, which allows the audience to debate about our active research, to answer their queries and to highlight the research holes that needs to be focused carefully.

B. Ruminant Physiome Database

This menu is the main menu to show our research productivities, data mining results and detailed level description about our projects. We have presented our results in user-interactive form. Initially we have given the three aforementioned results (bovine liver and adipose tissue, swine mammary glands) and their descriptions. In each section we have provide the KEGG and GO terms results that are processed by DIA. The analysis results can be downloaded using or viewed if more detailed insight into the given descriptive results is required.

4.4. REFERENCES

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4.5. FIGURES

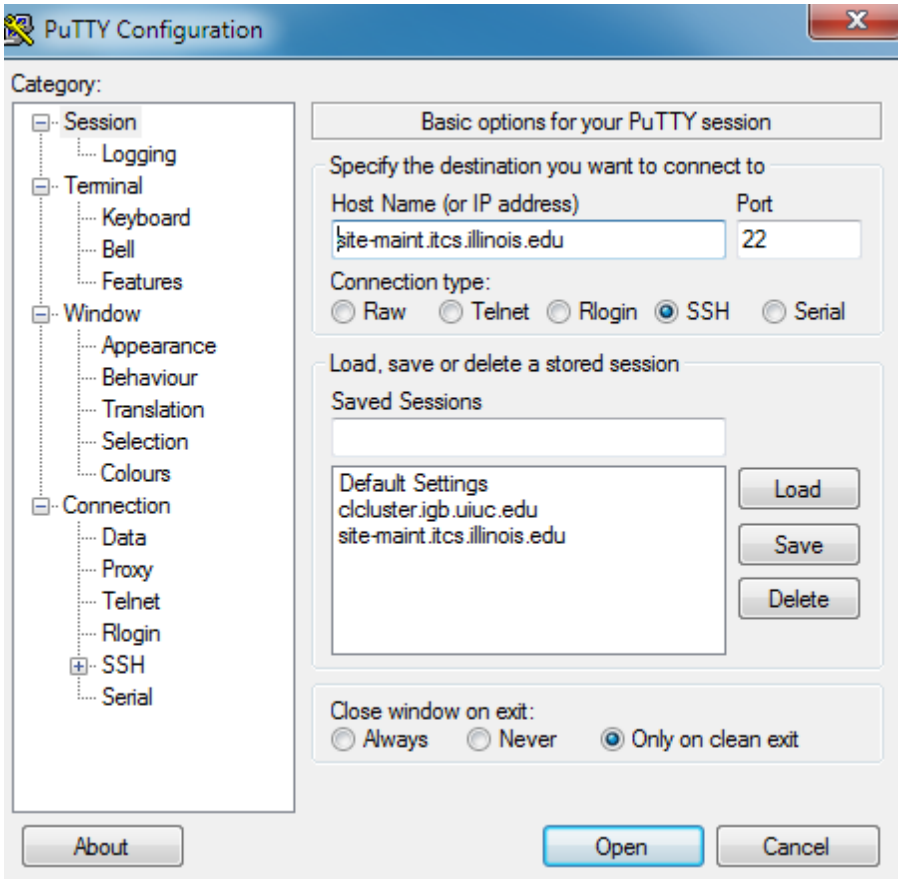

Figure 4.1: The snapshot of 'PuTTY configuration' to access the website through command line interface.

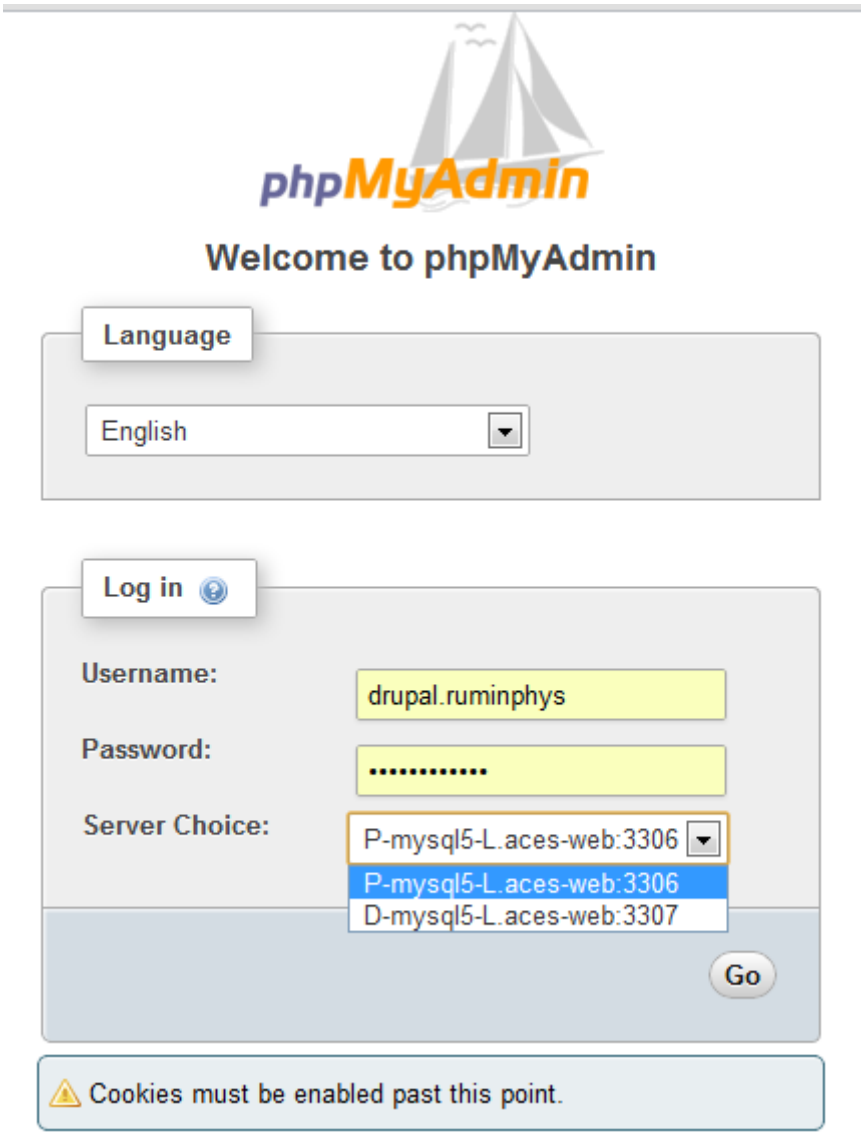

Figure 4.2: The snapshot of phpMyAdmin database login system.

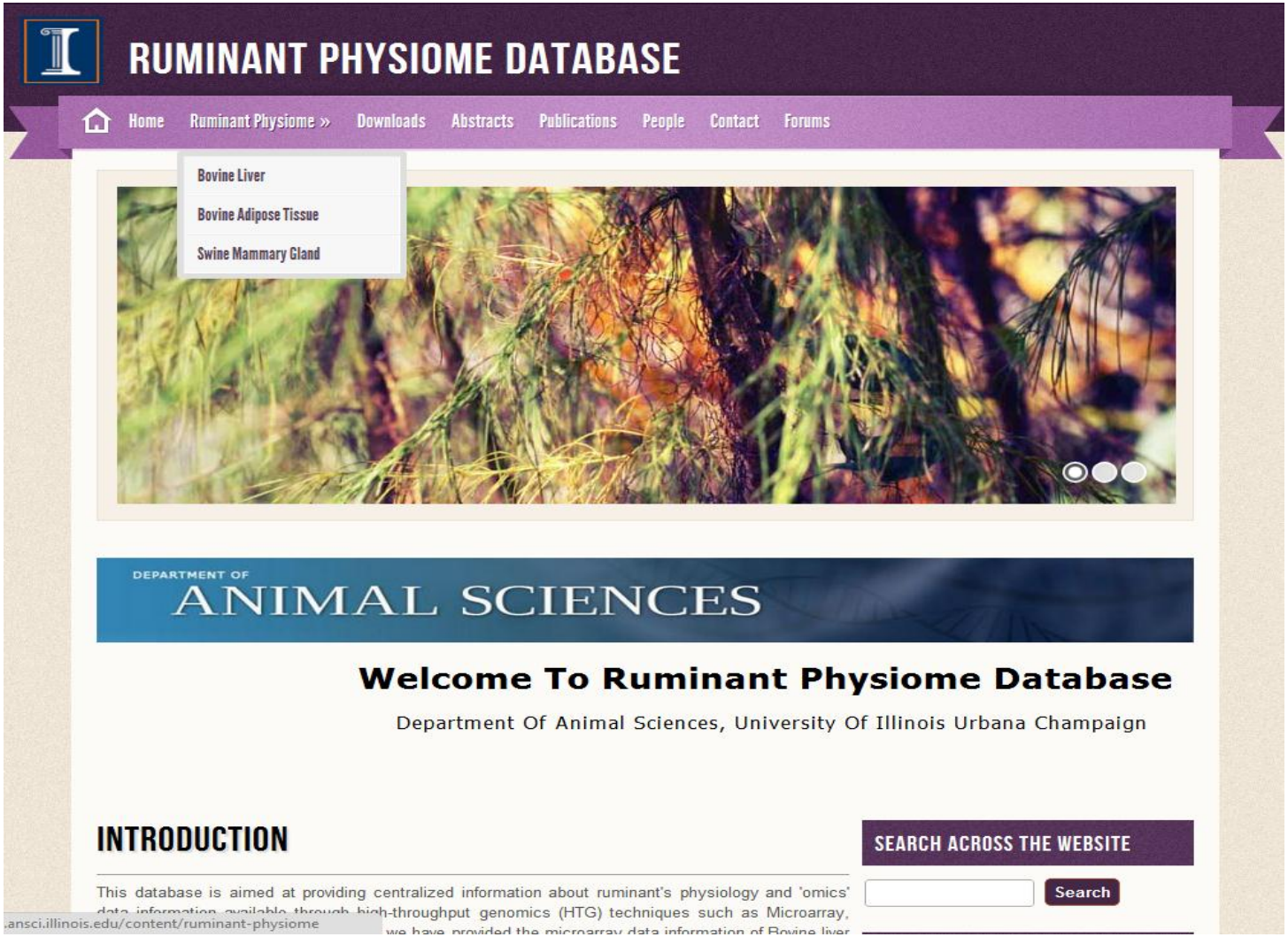

Figure 4.3: The snapshot of Ruminant Physiome website's main page.

APPENDIX: SUPPLEMENTARY MATERIALS

Liver

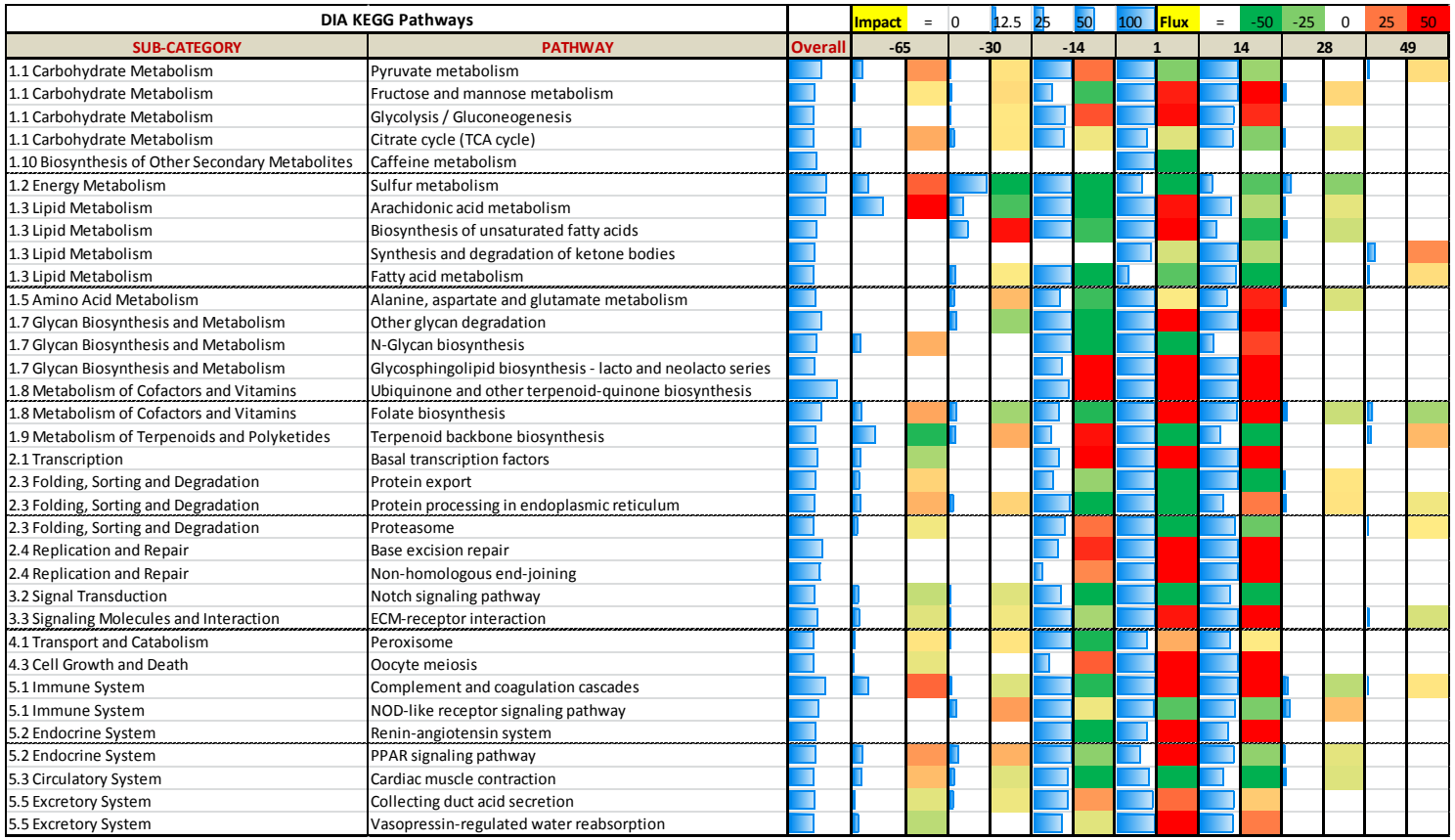

Figure S1: Most Impacted KEGG Pathways of Liver analyzed through Dynamic Impact Approach (DIA). The days -65, -30, -14, 1, 14, 28 and 49 are relative to parturition. The pathways are arranged according to the sub-category and then overall impact values. The blue bars indicate the impact values while the red to green ranged bars indicate the pathway expression from Overfed to restricted energy diet. The bar color and ranges are defined at the top of the figure in the legends.

Adipose Tissue

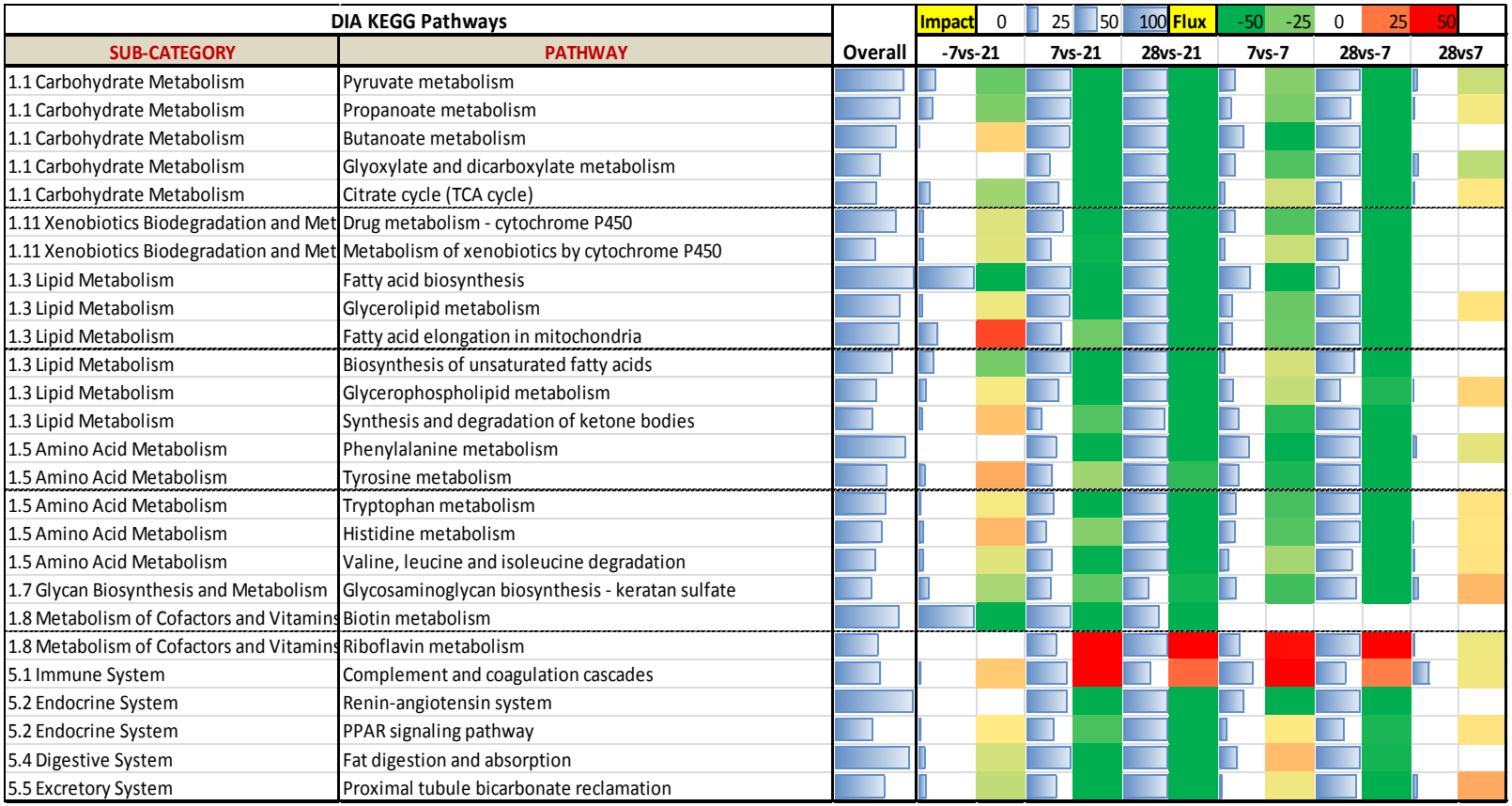

Figure S2: Most Impacted KEGG Pathways of Adipose Tissue analyzed through Dynamic Impact Approach (DIA). The days -21, -7, 7, and 28 are relative to parturition. The pathways are arranged according to the sub-category and then overall impact values. The blue bars indicate the impact values while the red to green ranged bars indicate the pathway expression from Overfed to restricted energy diet. The bar color and ranges are defined at the top of the figure in the legends.