

EFFECTS OF WEANING AGE AND MATERNAL NUTRITION ON GENE EXPRESSION  
OF LONGISSIMUS MUSCLE OF BEEF STEERS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy in Animal Sciences  
in the Graduate College of the  
University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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## ABSTRACT

Adipogenic/lipogenic transcriptional networks regulating intramuscular fat (IMF) deposition in response weaning age and dietary starch level were studied. The longissimus muscle (LM) of beef steers on an early weaning (141 days age) plus high-starch diet (EWS) or a normal weaning (NW, 222 days age) plus starch creep-feed diet (CFS) was biopsied at 0 (EW), 25, 50, 96 (NW), 167, and 222 (pre-slaughter) days. Expression patterns of 35 target genes were studied. From NW through slaughter all steers received the same high-starch diet. In EWS steers the expression of PPARG, other adipogenic (CEBPA, ZFP423) and lipogenic (THRSP, SREBF1, INSIG1) activators, and several enzymes (FASN, SCD, ELOVL6, PCK1, DGAT2) that participate in the process of IMF increased gradually to a peak between 96 and 167 days on treatment. Steers in NW did not achieve similar expression levels even by 222 days on treatment, suggesting a blunted response even when fed a high-starch diet after weaning. In conclusion, high-starch feeding at an early age (EWS) triggers precocious and sustained adipogenesis resulting in greater marbling. In a following study, we wanted to test if the exposure to an increased maternal nutrition during late gestation might result in an early increase in the expression of genes and/or other changes due to epigenetic regulation that leads to adipogenesis and lipogenesis in the offspring's LM. A microarray analysis was performed in LM samples of early (EW) and normal weaned (NW) Angus  $\times$  Simmental calves born from cows that were grazing endophyte-infected tall fescue/red clover pastures with no supplement (low plane of nutrition (LPN)), or supplemented with 2.3 kg and 9.1 kg of dried distiller's grains with solubles and soyhulls (70% DDGS/30% soyhulls) (medium and high plane of nutrition (MPN, HPN) respectively) during last 90 days of gestation, at three time points (78, 187 and 354 days of age). Bioinformatics analysis highlighted that offspring transcriptome did not respond markedly to

cow plane of nutrition, resulting in only 13 differentially expressed genes (DEG). However, weaning age and a high-starch diet (EW steers) strongly impacted the transcriptome (DEG = 167), especially causing the activation of the lipogenic program. In addition, between 78 and 187 days of age, EW steers had an activation of the innate immune system due presumably to macrophage infiltration in intramuscular fat. Between 187 and 354 days of age (i.e. the fattening phase), NW steers had an activation of the lipogenic transcriptome machinery, while EW steers had a clear inhibition of the gene transcription machinery. The latter appears to have occurred through the epigenetic control of histone acetylases, which were down-regulated. Higher cow plane of nutrition alone affected 35 DEG in the LM of steers that underscored the presence of a mechanism of macrophage infiltration likely originating from localized oxidative stress as a result of increased levels of hypoplasia and hypertrophy in LM. In conclusion, transcriptome analysis suggests that a macrophage infiltration-related impairment of lipogenesis was produced in LPN steers (especially in EW-LPN steers that had low marbling scores) as a consequence of weaning age and cow plane of nutrition. Finally, gene expression changes without a change in DNA sequence was assessed, mainly focusing in non-coding microRNAs (miRNA) epigenetics control. Results showed that proadipogenic miRNAs were controlling metabolism of LM of NW-MPN steers between 78 and 187 days of age through activation of: a) miR-103 that inhibits CAV1 that destabilizes INSR increasing insulin resistance, b) miR-143 that inhibits DLK1 that inhibits adipocyte differentiation through ERK1/2 activation, c) miR-21 that impaired TGFBR2-induced inhibition of adipocyte differentiation and seems to be related to serum adiponectin concentration. Furthermore, from the selected antiadipogenic miRNAs, cow plane of nutrition impaired miR-34a expression in MPN steers at 78 days of age. MiR-34a has a role on the activation of cell cycle arrest in LM by suppressing SIRT1, which leads to an activation of TP53

between 78 and 187 days of age. In conclusion, in MPN steers, inhibition of miR-34a expression at 78 days of age was the clearest sign of epigenetic regulation of LM of beef offspring due to cow plane of nutrition during late gestation.

DEDICATED WITH EXTREME AFFECTION AND GRATITUDE TO

my parents **Mr. Federico Alejandro Moisés** and **Mrs. Susana Conrad de Moisés**

and

my Argentinean scholarship director **Dr. Rodolfo Cantet**

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## **Chapter 1: Literature Review**

### ***Beef cattle growth and development***

Growth usually is defined as production of new cells. But because growth typically is measured as an increase in mass, growth includes not only cell multiplication (hyperplasia) but also cell enlargement (hypertrophy)<sup>99</sup>. Mature size generally is considered the point at which muscle mass reaches a maximum level of growth and development. During embryonic development, all tissues grow by hyperplasia, but as mammals mature, specialized cells (e.g., nerves, skeletal muscle cells) lose their ability to replicate, and grow only by hypertrophy or incorporation of satellite cells<sup>99</sup>. Net growth is the difference between synthesis and degradation of body tissue which are continuous processes<sup>39</sup>. Most mammals are born with nearly their full complement of skeletal muscle fibers. Muscle hyperplasia occurs primarily prenatally, and muscle fiber numbers increase only slightly postnatally<sup>2</sup>. Skeletal muscle does not have the protein and cell turnover rate than other tissues does, i.e. certain tissues associated with digestion<sup>84</sup>. In beef producer animals, there are differences due to sex and castration in the percentage of total body fat, following this order: heifers > steers > bulls<sup>14</sup>. The partitioning of different carcass fatty tissues at slaughter is similar in steers and heifers of the same genotype, either in early developing breeds like Holstein or Angus<sup>45</sup>, or later developing breeds like Charolais and Limousin<sup>109</sup>. The differences in fat partitioning due to breed and sex are present because the animals with the highest rate of fattening tend to have a higher proportion of subcutaneous and kidney fat than leaner animals compared at the same stage of development<sup>110</sup>. In adipose tissue, there are differences between the fat depots, i.e. visceral adipose tissue is more sensitive to lipolytic stimuli as compared to subcutaneous fat (SC)<sup>50</sup>. Moreover, intramuscular

(IM) depot is a more immature adipose tissue depot than the SC depot. IM depot may contain a greater proportion of preadipocytes and greater rates of hyperplasia than the SC depot<sup>101</sup>. These fact could explain the priority of growth for the different adipose tissues, being the kidney fat the one that is developed first, following by the intermuscular fat, SC and IM<sup>99</sup>. Also, in humans, abdominal obesity confers a higher cardiovascular risk than does a similar fat mass in a SC location due to its very active metabolism<sup>21</sup>. Furthermore, adipose tissue cellularity was different between steers with high (HIM) and low (LIM) marbling score. Steers with LIM had smaller mean diameter adipocytes resulting in greater adipocyte density (cell number/mm<sup>2</sup>) in the IM depot than the HIM steers. Steers that presented high SC fat were likely to undergone more rounds of adipocyte differentiation, to have a greater population of adipocytes capable of accumulating fat<sup>101</sup>.

Early fetal growth largely is genetically regulated, although blood flow later in pregnancy will alter fetal weight and maturity. Growth rate at later fetal stages and after birth but before maturity can be influenced greatly by factors such as plane of nutrition, hormonal status, and environment. Such factors may inhibit cell division to the point that mature body size is below the genetically determined maximum<sup>51</sup>. If earlier maturing cattle (small-framed cattle and heifers) are fed with high-energy diets as calves, they will present enhanced fattening and reduced slaughter weight at a specified fat thickness<sup>31</sup>. In contrast, energy and protein intake restriction during the early hyperplastic phase of growth, leads to a reduction in mature size, probably due to a permanent reduction in either nutrient supply or in satellite cell or muscle fiber number<sup>99</sup>. Moreover, Insulin Growth Factor 1 (IGF-1) response to Growth Hormone (GH) decreased when nutrient status or insulin levels are low<sup>108</sup>. Another class of chemicals that can alter tissue growth and composition are the  $\beta$ -adrenergic agonists. These compounds structurally

are related to epinephrine and norepinephrine. When activated,  $\beta$ -adrenergic receptors in adipose tissue stimulate lipolysis<sup>89</sup> and in muscle tissue they decrease protein degradation<sup>87</sup>. When animals are subjected to a poor quality diet for a long period of time during their growing period, they experienced the so called compensatory growth. The extent of compensatory growth is greater when it follows energy restriction rather than protein restriction. This recover of growth presumably is represented by a rapid hypertrophy of muscle tissue<sup>34</sup>. In heifers, estrogens presumably act through enhancing secretion of growth hormone<sup>11</sup>, which should increase mature size. An increase in mature size automatically reduces fat content and increases growth rate at a specified weight. In contrast, during embryonic life, by speeding up the closure of the growth plate and increasing ossification, estrogens should limit mature size. This could explain the smaller stature of females of many species. In steers, the effects of testosterone or the synthetic anabolic agent, trenbolone acetate, are synergistic with estrogen for enhancing deposition of lean tissue, hence, increasing mature body size<sup>99</sup>.

Cumulative weight plotted against animal age follows a sigmoid curve; this curve is composed of the pre-pubertal (self-accelerating) phase and the post-pubertal (self-inhibiting) phase. It is thought that post-pubertal growth inhibition is due either to a limitation in resources (space, nutrient supply, growth factors) or to accumulation of products or inhibitory factors that restrict cell division.

Specific blood serum proteins can stimulate or inhibit in vitro muscle cell proliferation<sup>29</sup>. Within these blood serum metabolites, glucose, non-esterified fatty acids (NEFA), triacylglycerol (TAG) from diet, leptin, which is secreted by mature adipocytes and regulates appetite, and the hormones of the somatotrophic axis (Insulin, GH and IGF-1) are some of the most important sources of substrates used for growth and development in ruminant animals.

During active growth, GH acts directly on the liver to increase synthesis and release of IGF-1, which, in turn, acts as an indirect feedback regulator of GH. Hormones from the somatotrophic axis promotes translation, transcription, and amino acid uptake. With insulin deficiency, decreases the myogenesis process, whereas with growth hormone deficiency, decreases the number and activity of ribosomes, while the DNA production by muscle tissue ceases. A pause of protein growth occurs simultaneously with decreases in ribosomal and total RNA concentration<sup>48</sup>. With low concentrations of GH, lower levels of IGF-1 circulating in the blood can be expected, though greater concentrations of GH induced by stressors, do not necessarily yield greater IGF-1 concentrations<sup>22</sup>. In an study of beef carcasses, IGF-1 was negatively correlated with the different fat depots development<sup>4</sup> and positive correlated with muscle formation<sup>18</sup>. Muscle cells are thought to produce IGF-1 to promote cell proliferation and differentiation during times of muscle hypertrophy, which would support positive correlations with lean tissue mass. At the same time, GH is known to decrease as animals mature, thus producing fewer stimuli for IGF-1 production during the latter period of time in the feedlot<sup>96</sup>. Moreover, elevated IGF-1 concentrations at weaning have been linked to increased growth rates, final live weight, and G:F in beef cattle<sup>53</sup>.

The genetic potential of a meat producer animal determines their carcass composition at any weight, regardless of the length to reach the end of the finishing phase<sup>78</sup>. Maturity is related to an animal's physiological stage rather than the chronological age<sup>115</sup>

### ***Main nutrient sources for growth and development***

Many studies have estimated the rates of glucose production by ruminants establishing a positive correlation with feed intake. Animals on an *ad libitum* diet produce about 50% more

glucose than animals on a maintenance diet<sup>139</sup>. The most important substrate for glucose synthesis in fed ruminant animals is propionate. Ruminal propionate may account for more than 50% of the substrate used in glucose synthesis<sup>3</sup> and lactate/pyruvate accounts for 15% of the glucose<sup>79</sup>. In fasted animals less propionate is available. Then endogenous sources of substrate should be used for gluconeogenesis, and glycerol from lipolysis becomes a more important glucose precursor; its contribution may reach 40% during fasting<sup>17</sup>. The organs that release glucose into the blood are liver, gut and kidney. The liver is the most important glucose-producing organ in the ruminant (85–90% of whole-body glucose turnover) on a roughage diet<sup>16</sup>. Since the rate of absorption of hexose sugar from the gut is low, the ruminant animal has no glucokinase and little hexokinase, because the need to remove glucose from the portal blood is negligible<sup>7</sup>. The rate of glucose utilization changes between tissues and organs. The muscle extracts 3% of the glucose, which passes through in blood. However, because of the muscle mass, muscle utilization may account for 20–40% of the glucose turnover<sup>98</sup>. Moreover, glucose uptake by muscle is subject to hormonal regulation<sup>64</sup>. It is thought that probably fat may be more efficient at removing glucose than muscle due to a difference in blood flow through the tissues, because lower blood flow through fat may allow a higher extraction ratio. Glucose extraction by the fat pad was also increased by insulin. In both fat and muscle tissue, insulin concentrations are high in blood during feeding and low during fasting<sup>71</sup>.

Lactate is the second major glucose precursor, due to its glucogenic potential in fed ruminants<sup>64</sup>. Endogenous lactate is produced by muscle, which with the exception of a condition of extreme exercise, always has a net output of lactate, and by adipose tissue, which also has a net production of lactate (50% of its glucose uptake)<sup>71</sup>. In intramuscular fat, lactate seems to be the preferred substrate for glycerol synthesis and glucose for fatty acid synthesis<sup>124</sup>. For



subcutaneous adipose tissue, acetate is the main source for fatty acid synthesis<sup>123</sup>. The relationship between lactate and propionate and the differential hormonal response between lactate/pyruvate and propionate in the liver, are undoubtedly related to the differences in their entry into the glucogenic pathway<sup>23</sup>. The conversion of lactate/pyruvate to triose phosphate involves both the pyruvate carboxylase (PC) and the phosphoenolpyruvate carboxykinase (PEPCK) catalyzed reactions (the first reaction is the conversion to oxaloacetate, the second oxaloacetate to triose phosphate), whereas the conversion of propionate to triose phosphate does not involve PC. The activity of PC, but not PEPCK, is responsive to changes in physiological status and hormones<sup>7</sup>.

Quantitatively, acetate is the most important short-chain fatty acid in the ruminant. About 70% of the intra-ruminal turnover or production of acetate can be accounted for by portal absorption of acetate. Acetate is metabolized rapidly by the body<sup>64</sup>. Acetate extraction is lower during fasting and exercise when ketone bodies and long-chain free fatty acids make up the major energy sources<sup>64</sup>. Acetate is a major source of energy for the ruminant. About 2/3 of the acetate is oxidized in the respiratory chain, leaving 1/3 for other uses, such as lipogenesis<sup>100</sup>. Butyrate is the third most important product of carbohydrate fermentation in the rumen. The most important function of butyrate is as a substrate for ketone body production. During absorption butyrate is mostly transformed to ketone bodies in the ruminal epithelium<sup>15</sup>. Ketone body production by the liver is reduced by insulin<sup>24</sup> and is greater when free fatty acids rather than butyrate are available as substrates<sup>67</sup>. More than 80% of the butyrate that is absorbed from the gut is removed in a single step through the liver<sup>106</sup>.

### ***Transcriptional regulation of adipogenesis***

Adipocytes are differentiated from multipotent mesenchymal stem cells. The inducers of adipocyte differentiation are a glucocorticoid agonist, an agent to increase intracellular cAMP and high concentrations of insulin to stimulate insulin-like growth factor receptors (IGF-1R)<sup>88</sup>.

Adipogenesis can be described in two phases: a) determination that involves the commitment of a pluripotent stem cell to the adipocyte lineage and the conversion of the stem cell to a pre-adipocyte, which cannot be distinguished morphologically from its precursor cell but has lost the potential to differentiate into other cell types. b) terminal differentiation, where the pre-adipocyte takes on the characteristics of the mature adipocyte and it acquires the machinery that is necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte-specific proteins<sup>111</sup>. During adipogenesis, auto phosphorylation of insulin/IGF-1 receptor tyrosine kinases in the presence of insulin initiates glucose transport, glucose metabolism, proadipogenic gene transcription, and de novo lipid synthesis<sup>114</sup>. The differentiation of 3T3-L1 preadipocytes is triggered by hormonal agents and carried out by the coordination and precise control of transcriptional cascades. During determination stage of adipocyte differentiation, growth arrest occurs at a confluent state<sup>112</sup>, and proliferating cells will not accumulate lipid droplets in their cytoplasm, even in the presence of hormonal stimulation, until they become confluent. Upon induction of differentiation stage, growth-arrested cells undergo a limited number of additional mitosis, known as mitotic clonal expansion<sup>80</sup> that allows an increase in the final proportion of differentiated fat cells. Mature adipocytes became able to transport large amounts of glucose in response to insulin, to synthesize fatty acids and to store triacylglycerol. In contrast, under the effect of the catecholamine-sensitive pathway, adipocytes hydrolyze triacylglycerol when there is not enough energy available. Adipocytes also has a endocrine

function exerted by the synthesis and secretion of different factors that controls the energy homeostasis, i. e. leptin<sup>43</sup>.

Peroxisome proliferator-activated receptor gamma (*PPAR*  $\gamma$ ) is the master regulator of adipocyte differentiation. *PPAR*  $\gamma$ , which is also known as glitazone receptor or *NR1C3* (nuclear receptor subfamily 1, group C, member 3) is necessary and sufficient to induce adipogenesis<sup>94</sup>. *PPAR* $\gamma$  forms a heterodimer with retinoid  $\times$  receptor  $\alpha$  (*RXR* $\alpha$ ) and binds to peroxisome proliferator response elements (PPREs) on the promoters of its target genes<sup>62</sup>. Therefore, retinoid acids affect adipogenesis via *RXR* $\alpha$  and its interaction with *PPAR* $\gamma$ <sup>147</sup>. Retinoid Acid is biologically represented by two isomers (all-trans RA and 9-cis-RA) that involves the activation of ligand-dependent nuclear receptor proteins and transcription factors, *RAR* and *RXR*<sup>131</sup>. All-trans RA influence gene expression by activating the *RAR* moiety of *RAR/RXR* heterodimers that bind RA response elements. The 9-cis-RA isomer binds *RAR* and *RXR* similar to all-trans RA, but also influences gene expression by activating *RXR* when it interacts with target genes as an *RXR* homodimer or as a heterodimer of *RXR* and other transcription factors (i.e., *PPAR* $\gamma$ )<sup>68</sup>. The effects of vitamin D<sub>3</sub> and vitamin A are mediated by the level of expression of the ligand-dependent transcription factor, vitamin D receptor (*VDR*) and Retinoic Acid Receptor (*RAR*), that block *C/EBP* $\beta$ -dependent activation of expression of *PPAR* $\gamma$  and *C/EBP* $\alpha$  by competing for *RXR* binding, which leads to diminished adipocyte differentiation<sup>68; 20</sup>. This antagonist effect of vitamin D<sub>3</sub> and vitamin A is blocked at the stage in the process of adipocyte differentiation in which adipocytes are irreversibly committed to differentiate<sup>131</sup>.

*PPAR* $\gamma$  is a ligand-activated transcriptional factor. In the inactive state, *PPAR* $\gamma$  is associated with co-repressors to silence its transcription activity. Binding of ligands leads to the replacement of co-repressors with co-activators possessing histone acetyl transferase activity

such as cAMP response element-binding protein (*CBP/p300*)<sup>129</sup>. Acetylation of histones leads to local chromatin decondensation and gene expression. Fatty acids are ligands for *PPAR* $\gamma$ ; oxidized fatty acids activate *PPAR* $\gamma$  with higher potency compared to the native fatty acids<sup>128</sup>. The net effect of *PPAR* $\gamma$  is the enhancing of glucose uptake in muscle and adipose tissue, whilst reducing levels of circulating free fatty-acids<sup>25</sup>. *PPAR* $\gamma$  and CCAAT-enhancer-binding proteins (*C/EBPs*) are some of the most important proadipogenic transcription factors. *PPAR* $\gamma$  is not only crucial for adipogenesis but is also required for maintenance of the differentiated state. Early induction of *C/EBP* $\beta$  and *C/EBP* $\delta$  leads to induction of *C/EBP* $\alpha$  during adipocyte differentiation. *C/EBP* $\alpha$  interacts with cyclin-dependent kinases 2 and 4, preventing in this way the cycling binding, and inducing growth arrest<sup>43</sup>. *C/EBP* $\beta$  could be inactivated by the action of *CHOP* and *C/EBP* $\gamma$  through heterodimerization<sup>28</sup>. The transcription factor *Zfp423* regulates *PPAR* $\gamma$  expression by sensitizing cells to the BMP signals, which are important for commitment to the preadipocyte lineage<sup>56</sup>. *Zfp423* may be involved in the commitment of mesenchymal stem cells (MSCs) to the adipocyte lineage but might not be required for the maintenance of the preadipocyte phenotype<sup>63</sup>. The SMAD-binding domain of *Zfp423* is not necessary for its activity in preadipocyte commitment under basal culture conditions, but it is absolutely required for *Zfp423* modulation of adipogenic activity induced by BMPs<sup>56</sup>. The basic helix-loop-helix protein, adipocyte determination and differentiation-dependent factor-1/sterol regulatory element-binding protein-1 (*ADD-1/SREBP-1*), is another important protein induced during the early stages of adipogenesis<sup>72</sup>. *SREBF-1* is related to lipid metabolism because it is an important activator of fatty-acid synthase (*FASN*) and lipoprotein lipase (*LPL*)<sup>13</sup>. Moreover, reduction of the adipogenic co-repressors like the nuclear receptor (*NCoR1*) and silencing mediator of retinoid and thyroid hormone receptors (*NCoR2* or *SMRT*) promotes adipocyte differentiation<sup>144</sup>.

Binding of Wnt proteins to frizzled receptors initiates signaling through  $\beta$ -catenin-dependent and -independent pathways. Wnt signaling inhibits adipocyte differentiation in vitro by blocking the expression of *PPAR $\gamma$*  and *C/EBP $\alpha$* . Wingless-type 10b (*WNT10b*) is expressed in confluent pre-adipocytes and stromal vascular cells but not in differentiated adipocytes<sup>113</sup>. The well-known Wnt effector,  *$\beta$ -catenin*, binds to the androgen receptor and is translocated to the nucleus in response to testosterone where it interacts with the T cell factor/Lymphoid enhancer factor (*TCF/LEF*) transcription factors to inhibit adipogenesis<sup>119</sup>. Blocking the  $\beta$ -catenin pathway reduces myogenesis<sup>140</sup>. Wnt– $\beta$ -catenin pathway is an important regulator of adipogenesis and mesenchymal-cell fate in vivo, noticeable by the conversion of myometrial tissue into adipose tissue<sup>5</sup>.  *$\beta$ -catenin* also serves as a cofactor of forkhead transcription factor 1 (*FOXO1*). *FOXO1* competes with *TCF* for interaction with  *$\beta$ -catenin*, thereby inhibiting *TCF* transcriptional activity. *FOXO1* overexpression reduces the binding between *TCF* and  $\beta$ -catenin, mostly noticeable under inflammation and oxidative stress conditions<sup>60</sup>, which enhanced the formation of FOXO/ $\beta$ -catenin complex, down-regulating myogenesis and up-regulating adipogenesis<sup>127</sup>. Furthermore, Wnt/ $\beta$ -catenin signaling regulates adipogenesis through chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII* or *NR2F2*). Wnt/ $\beta$ -catenin signaling activates the expression of *NR2F2*, which recruits the silencing mediator for retinoic acid receptor and thyroid hormone receptor (*SMRT* or *NCOR2*) co-repressor complex to inhibit the expression of *PPAR $\gamma$* <sup>95</sup>.

Expression of *DLK1/PREF1* is high in pre-adipocytes and normally declines during differentiation. The expression of *DLK-1* is greater in IM than in SC adipose tissue<sup>59</sup>. *DLK-1* mRNA expression was greater for Wagyu  $\times$  Hereford crosses than Piedmontese  $\times$  Hereford crosses from 3 to 25 months of age, which had a greater population of adipocytes for

differentiation and IM adipose tissue accumulation during the finishing period<sup>134</sup>. *DLK1/PREF1* expression in 3T3-L1 cells blocks adipogenesis by the release of an extracellular moiety during its cleavage, possibly through interaction with Notch signaling pathway<sup>120</sup>. The transcription factor *FOXC2* has a pleiotropic actions producing an inhibition in the adipocyte gene expression transcriptional machinery, increasing the insulin stimulated glucose uptake,  $\beta$ -adrenergic receptor responsiveness and oxygen consumption<sup>26</sup>. Activation of the gene insulin-induced gene 1 (*INSIG1*) exerts a positive effect on *PPAR $\gamma$*  and Steroid response element binding factor 1 (*SREBF1*) which activates a while variety of genes that leads to synthesis of palmitic and oleic acids, and formation of triacylglycerols<sup>54</sup>. Thyroid hormone responsive (*THRSP*) increase rates of lipogenesis in rodent adipose tissue and is partly regulated by thyroid hormone<sup>93</sup>. Both insulin and thyroid hormone can act synergistically in promoting the overall process of adipogenesis in rodents, and thyroid hormone acts synergistically with IGF-1 to promote adipocyte differentiation in pigs<sup>74</sup>. In cattle, *THRSP* was highly correlated with marbling content in breeds widely-known to possess extremely high capacity for marbling like Japanese Black<sup>135</sup> and Wagyu<sup>134</sup>. *THRSP*<sup>145</sup> and Retinoic Acid Receptor-related orphan receptor alpha (*ROR $\alpha$* )<sup>6</sup> have been identified as *PPAR $\gamma$*  coactivators.

*PPAR $\gamma$*  target genes in adipocytes considered as substrate transporter molecules are Cluster of Differentiation 36 (*CD36*), Lipoprotein lipase (*LPL*), Fatty acid binding protein 4 (*FABP4*) and Solute Carrier Family 2 (facilitated glucose transporter), Member 4 (*SLC2A4* or *GLUT4*). *CD36* is a scavenger receptor, which create a positive feedback loop that increases the uptake of oxidized *LDL* and metabolites that function to enhance *PPAR $\gamma$*  transcriptional activity<sup>128</sup>. *LPL* is a water soluble enzyme that hydrolyzes triglycerides in lipoproteins, such as those found in chylomicrons and very low-density lipoproteins (VLDL), into two free fatty acids

and one monoacylglycerol molecule. *LPL* is regulated in a tissue-specific manner by nutrients and hormones. As preadipocytes begin differentiating into mature adipocytes, *LPL* is one of the first genes expressed due to its role in de novo lipogenesis<sup>55</sup>. Stress decrease *LPL* activity in white adipose tissue but increase the *LPL* activity through the effect of catecholamines in skeletal muscle<sup>107</sup>. Adipose tissue *LPL* activity is high in fed animals and low when animals are fasted, but the opposite occurs in skeletal muscle. Moreover, high-carbohydrate exposure increases the *LPL* response to feeding in both adipose tissue and skeletal muscle<sup>143</sup>. Insulin has a major effect on *LPL* activity in adipose tissue during adipocyte differentiation by increasing *LPL* gene transcription<sup>116</sup>. In mature adipocytes, insulin not only increases the level of *LPL* mRNA but also regulates *LPL* activity through both posttranscriptional and posttranslational mechanisms<sup>105</sup>. Glucose also increases adipose tissue *LPL* activity. The glucose stimulatory effect appears to be mostly through the glycosylation of *LPL*, which is essential for *LPL* catalytic activity and secretion<sup>97</sup>. GH and sex steroid hormones inhibit adipose tissue *LPL* activity and promote lipid mobilization but increase skeletal muscle *LPL* activities<sup>19</sup>. Fatty acid binding protein 4 (*FABP4*) also called adipocyte Protein 2 (*aP2*) is primarily expressed in adipocytes and macrophages. *FABP4* is a small cytosolic protein that specifically binds and transport long chain fatty acids and retinoic acid intracellularly<sup>81</sup>. In studies in beef cattle, *FABP4* protein contents were greater in Wagyu and Angus genotypes, which deposit much more intramuscular fat, than for Piedmontese x Hereford and Limousin breed, which produces a leaner meat<sup>66; 77; 134</sup>. Recently *FABP4* was converted in a strong candidate gene for marbling among adipose-specific genes<sup>137</sup>. Finally, *SCL2A4* is the insulin-regulated glucose transporter found in adipose tissues and skeletal muscle that is responsible for insulin-regulated glucose translocation into the cell. In a study using

Holstein steers, *SLC2A4* was decreasing in level of expression throughout life<sup>1</sup>. *SLC2A4* had a greater temporal expression in Angus × Simmental steers that were fed a low starch diets<sup>54</sup>.

### ***Lipogenesis***

Lipogenesis is the process by which acetyl-CoA is converted to fat. By means of this process, the energy can be efficiently stored in the form of fats. Lipogenesis includes the processes of fatty acid synthesis and esterification with glycerol to form fat<sup>69</sup>. In terms of fat depot location, it was established that intramuscular fat, which has less sensitivity for dietary manipulation, have smaller adipocytes and lower lipogenic rates as compared to subcutaneous adipose tissue<sup>124</sup>. In ruminants, adipocytes are the principal site for lipogenesis<sup>12</sup>. Fatty acids are synthesized from the two-carbon precursors, acetate or lactate, or from glucose through glycolysis in non-ruminants or pre-ruminants, after it has been converted to pyruvate by glycolysis<sup>32</sup>. The nature of the mechanism that minimizes lipogenic use of glucose by ruminants remains unclear. It is believed that the main reason for this is the lack of ATP-citrate lyase (ACLY) and NADP-malate dehydrogenase in bovine adipose tissue<sup>57</sup>. However, later it was discovered that lactate, after being converted to pyruvate, was used by bovine adipose tissue, at rates similar to those of acetate. Consequently, lactate also requires the enzymes ACLY and NADP-malate dehydrogenase in order to be converted to fatty acids. The activity of ACLY in bovine adipose tissue, even though lower than that in rat tissues, is still sufficient to allow the observed rates of lactate conversion to fatty acids<sup>44</sup>.

Microbial catabolism of glucose in the rumen results in the production of pyruvate and acetate, which is the main precursor for lipogenesis in these animals. The synthesis of long-chain fatty acids occurs in the cytoplasm by the repeated addition of two carbons to a 'primer' molecule, which is usually acetyl-coenzyme A (acetyl-CoA)<sup>61</sup>. Acetyl CoA Carboxylase (ACC)



is a biotin-dependent enzyme that catalyzes the initial, rate-limiting step of lipogenesis. This results in the conversion of acetyl-CoA to malonyl-CoA. Malonyl-CoA then acts as the donor of two-carbon acetyl groups to the growing fatty acid chain<sup>58</sup>. Increased circulating insulin concentrations, in response to dietary energy, induce ACC gene transcription and translation resulting in an increase in ACC and increased rates of lipogenesis and fatty acid formation. After the formation of malonyl-CoA, fatty acid synthase (*FASN*) catalyzes the synthesis of Palmitate from Acetyl CoA and Malonyl-CoA into long chain saturated fatty acids, in the presence of NADPH<sup>122</sup>. Palmitic acid is the primary product of lipogenesis in adipose tissue. In the endoplasmic reticulum, palmitic acid (16:0) is converted to stearic acid (18:0) by the enzyme fatty acid elongase (*ELOVL6*) which catalyzes the elongation of saturated and monounsaturated fatty acids with 12, 14, and 16 carbons<sup>83</sup>. Stearoyl-CoA desaturase (*SCD* or  $\Delta 9$  desaturase) converts a portion of stearic acid (18:0) into oleic acid (18:1)<sup>136</sup>. The presence of oleic acid, which has a lower melting point than stearic acid, ensures the fluidity of lipids. The long-chain fatty acids that are synthesized during lipogenesis are then esterified to a glycerol backbone. Concentrations of non-esterified fatty acids are very low in the body and the formation of triacylglycerol provides a non-toxic storage form for lipids. In ruminants, non-saturated fats derived from the diet are quickly converted to saturated fats in the rumen by a process known as rumen biohydrogenation<sup>102</sup> before intestinal absorption and, as a result, ruminant fatty acids are, in general, more saturated than those of non-ruminants. Other enzymes that regulate the lipogenesis process are diacylglycerol O-acyltransferase 2 (*DGAT2*) that catalyzes the final step of triacylglycerol synthesis; MID1 interacting protein 1 (*MID1IP1*) required for an efficient lipid biosynthesis, including triacylglycerol, diacylglycerol and phospholipid; and

phosphoenolpyruvate carboxylase (*PCK1*) is a cytosolic enzyme regulated by insulin, glucagon and diet, that is responsible for the synthesis of glycerol.

### ***Lipolysis***

Lipolysis is the metabolic process by which is produced the breakdown of fat cells and involves the hydrolysis of triglycerides into free fatty acids followed by further degradation into acetyl units by  $\beta$ -oxidation<sup>37</sup>. Epinephrine, norepinephrine, glucagon, growth hormone, testosterone, and cortisol are the hormones that induce the process of lipolysis. Hormonal stimulation triggers a G-protein-coupled specific receptor which activates adenylate cyclase. The result is an increased production of cAMP, which activates protein kinase A, which subsequently activates lipases found in adipose tissue. Triglycerides are transported through the blood to i.e. adipose or muscle tissue by lipoproteins such as very low-density-lipoproteins (VLDL). Triglycerides present on the VLDL undergo lipolysis by the cellular lipases of target tissues, which yields glycerol and free fatty acids. Free fatty acids released into the blood binds to albumin which is the main transporter of free fatty acids in the blood<sup>103</sup>. Long chain fatty acids are bound to albumin in the circulation, and to fatty acid binding proteins (FABPs) within cells due to its cytotoxic effect.

There are several lipolytic states; under prolonged fasting there is an increased dependence on lipolysis for provision of fatty acids as an energy source, and by low circulating levels of insulin and catecholamines. Under acute stress, the lipolysis is triggered by catecholamines. Under obesity conditions, body energy depots “overflow”, and the increased rate of lipolysis occurs in the context of high circulating levels of triglycerides, glucose and insulin<sup>133</sup>. Three main organs produce and export fatty acids (FA): white adipose tissue (WAT),

the intestine and the liver. Intestine and liver esterify FAs prior to their release as triacylglycerols (TAG). Adipocytes release non-esterified fatty acids (NEFAs)<sup>133</sup>. Lipolysis is the first of three main control points in lipid energy metabolism. The others are located at the carnitine palmitoyl transferase shuttle, which controls FA entry into mitochondria for  $\beta$ -oxidation, and hepatic mitochondrial 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase, which controls entry to the ketogenic pathway via HMG, which is essentially liver-specific<sup>47</sup>.

There are two important lipases that regulate the lipolysis process. The hormone sensitive lipase (*HSL*) and the patatin-like phospholipase domain containing 2 or Adipocyte triglyceride lipase (*PNPLA2* or *ATGL*). *HSL* can cleave fatty acyl esters of various compounds, including cholesterol, steroids and retinoic acid as well as glycerol. *HSL* has greater affinity for diacylglycerols (DAG) than TAG or monoacylglycerols (MAG)<sup>46</sup>. *HSL* activation is associated with its displacement to the surface of the lipid droplet<sup>38</sup>, and its action is enhanced by the presence of *FABP4*<sup>121</sup>. *PNPLA2*, also called desnutrin, has been implicated in both basal and  $\beta$ -adrenergic stimulated lipolysis<sup>146</sup>. In contrast to *HSL* which acts on a variety of lipid substrates, *PNPLA2* selectively mediates lipolysis of triglycerides and it is induced by fasting and glucocorticoids<sup>132</sup>.

### ***Early weaning***

At birth and for around 25 days afterwards, calves depend exclusively on the milk of the cow, and so these young ruminants are in effect non-ruminants<sup>92</sup>. Milk is a particularly nutritious feed, compared with forages, and during the first 3-4 weeks of life, there is a rapid development of both total muscle mass and adipose tissue<sup>130</sup>. During the growing phase, which is the period that follows after weaning, nutrients are partitioned in favor of skeletal and muscle

growth while the rate of fat deposition is relatively low. In contrast, during the finishing phase, fat deposition was the highest response due to finishing diets. Body growth and fat deposition is influenced by how the animal is weaned<sup>91</sup>.

Early weaning systems encourage the calves to eat dry feed sooner. Calves can be weaned successfully at 4 to 5 weeks of age if they receive good management. An adaptation period is required, starting by offering to the calf a small amount of good quality starter right after feeding milk. The starter should have high protein content (18 to 20% of dry matter) and sweeteners like molasses added to obtain a better palatability, improve the intake and reduce the dustiness. The period of adaptation for the starter should end when the calf is eating at least 453g of starter per day. It also is recommended to cut the milk feeding to about the half during the last week before early weaning.

Early weaning is a technique used to improve cow body condition. During lactation, there is loss of body condition due to the increased nutrient requirements associated with lactation. By early weaning the cow's nutrient requirements for lactation disappear, and cows are able to increase or maintain their body condition scores. Also early weaning (between 45 to 105 days) can improve conception rates because cows have a greater opportunity to rebreed in an optimum time frame. Moreover, early weaning improved forage availability for the cow because there is a reduction of the cow dry matter intake and also, it eliminates the demand on the forage from the calf. Consequently, the cows remaining on the pasture have access to more forage and demands on the pasture are reduced, which can enhance sustainability and forage production in the future.

By weaning early and providing a highly nutritious diet, calves can reach their genetic potential to marbling and have increased quality grade at slaughter<sup>86</sup>. One of the disadvantages of

early weaning is that instead of their mother's milk and pasture, early weaned calves will eat high quality grains, protein supplements and/or commercially prepared feedstuffs, and consequently the calves requires greater attention to proper nutrition and health. Moreover, results of a study comparing animal performance for both weaning techniques determined that during the finishing period normal weaned steers ate more, gained more and were more efficient than early weaned steers. Furthermore, weaning calves earlier will result in increased expenses for the beef cattle producer<sup>118</sup>.

### ***Creep feeding***

Creep feeding is the managerial practice of providing concentrated-based supplemental feed to the nursing calf. Creep feeding maximizes the calf's full growth potential, when the calf is not getting enough nutrients from the cow's milk or because a lack of pasture due to drought. Feedstuffs are provided in a creep feeder or some type of physical barrier, which allows the calf, but not the cow, to have access to the supplemental feed <sup>41</sup>. Creep feeding could be based on starch or fiber. Calves allowed to consume grain while nursing their dams, has a much larger rumen by 6 weeks of age than calves kept on milk only, and the development of the rumen papillae was more prominent if they received creep feeding<sup>10; 33</sup>. Feeding only milk does not stimulate development of the reticulum and rumen epithelium. This is because most of the milk consumed is shunted directly to the omasum and then abomasum by closure of the reticular groove and the presence of the sulcus omasii. In calves of the same age, that were fed with starter grain, the closure of the reticular groove is not complete and the grain eaten by the calf enters the rumen and is fermented by the developing rumen microbial population, so the calf that received creep feeding, can use the VFA produced in the rumen as an energy source<sup>33</sup>. Creep feed could be also based on fiber, i. e. soyhulls. There is too little carbohydrate in soyhulls that is easily

fermentable by the immature rumen microbial population, where cellulolytic bacteria are not yet established and functional in these calves; hence fermentation of cellulose occurs at a very low rate. Therefore, calves that received fiber creep is unable to ferment much of the cellulose, and subsequently, less energy could be taken from the supplementation.

Providing a creep ration to nursing calves grazing forage has been a management strategy to increase weaning weight<sup>82</sup>. Although, it was established that the efficiency of usage of forage for gain may be decreased if creep feed exceeds 25% of the DM entering the rumen<sup>27</sup>. Multiple studies showed that the use of creep feeding produce higher weights at weaning<sup>42; 126; 75</sup>. However, statistical differences were not observed ( $P>0.10$ ) in dry matter intake, average daily gain (ADG) or gain:feed (G:F) between calves that received creep feed or not<sup>42</sup>. During the finishing period, creep-fed calves converted feed to gain less efficiently than those not creep-fed<sup>42</sup>. However, the creep-fed calves had a statistical advantage in quality grade by means of the marbling score ( $P<0.05$ ), with no difference in yield grade, ribeye area, or backfat<sup>126; 117</sup>. Calves that received starch creep had a higher quality grade than calves that were fiber creep-fed<sup>42</sup>. Moreover, there were no differences in ADG or daily DMI between the normal weaned steers that received starch and fiber creep, but the fiber creep steers were 5% more efficient than the starch creep steers. An explanation for this could be that the fiber creep steers compensating for their poorer performance during the growing period<sup>118</sup>.

### ***Epigenetic regulation***

The transcriptional or pre-translational controls (related to gene expression), translational controls (related to protein biosynthesis) and post translational processes (related to protein processing and folding) are the events needed for regulation of gene expression and enzymatic

activity. The control of the gene expression at the RNA level is called post-transcriptional regulation. It occurs between the transcription and the translation of the gene. Epigenetics is a term that refers to gene expression changes without a change in DNA sequence<sup>138</sup>. The conformational change of the structure of histones is the main epigenetic mechanism that plays an important role in the regulation of gene expression by processes that mediates transcriptional activation, chromatin remodeling, nucleosome loosening, chromatin condensation, and chromatin stabilization<sup>90</sup>. Non-coding microRNAs (miRNA) are also included within these mechanisms<sup>85</sup>.

A non-coding RNA (ncRNA) is a functional RNA molecule that is not translated into a protein. An example of non-coding RNA is the microRNAs (miRNAs), which is a short ribonucleic acid molecule of ~21–23 nucleotides. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression, target degradation and gene silencing<sup>8</sup>. The primary transcripts of the miRNA genes are the pri-miRNAs which are processed in the nucleus by Drosha, a member of the RNase III superfamily, to yield precursors of ~70 nucleotides (pre-miRNAs) that have the capacity to form hairpin-like structures and are exported from the nucleus by Exportin-5<sup>73</sup>. MicroRNA synthesis is produced by an endoribonuclease in the RNase III family called Dicer that cleaves double-stranded RNA (dsRNA) and pre-microRNA (miRNA) into short double-stranded RNA fragments called small interfering RNA (siRNA)<sup>70</sup>. MiRNA is biochemically indistinguishable from another small RNA species that is known as small interfering RNA (siRNA). The inhibitory mechanism of miRNAs on gene expression is based on their partial complementarity, at a site in the 3' UTR, to a part of one or more messenger RNAs (mRNAs) hence, the annealing of the miRNA to the target mRNA inhibits protein translation<sup>142</sup>.

The mechanism of action of miRNAs of preventing translation by degrading the mRNA was discarded because the target mRNA remains intact after miRNA binding<sup>49</sup>. Only the degree of complementarity between a miRNA and its RNA target determines its function. Though an imperfectly matched miRNA-mRNA pair with central bulges leads to translational inhibition followed by deadenylation and degradation of the mRNA, a perfectly complementary pair tends to promote mRNA slicing by the Argonaute protein<sup>30</sup>.

The microRNA profile from beef cattle showed different pattern according to the fat depot location, with 80, 66 and 63 miRNAs detected respectively in abdominal subcutaneous, back subcutaneous and rump subcutaneous tissues by qRT-PCR<sup>65</sup>. Also, within an specific fat depot location, they were found by qRT-PCR analysis, variations in microRNA profiles with 42 differentially expressed microRNAs, according to fat traits (high backfat thickness vs. low backfat thickness), being miR-378 the most differentially expressed<sup>9</sup>.

Other examples of epigenetic control of gene expression are at the chromatin level. The basic unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapping around eight histone proteins that presented two copies each of H2A, H2B, H3, and H4. These tail of the histones, undergo a variety of posttranslational modification processes like methylation of lysines and arginines, phosphorylation of serines and threonines, acetylation, ubiquitinylation, SUMOylation (small ubiquitin-related modifier) and ADP-ribosylation<sup>104</sup>. DNA-binding domains with functions involving DNA structure have biological roles in conformational changes of DNA, such as methylation.



### ***Fetal programming***

Fetal programming is the response to a specific challenge to the mammalian organism during a critical developmental time window that alters the trajectory of development qualitatively, quantitatively, or both, with resulting persistent effects<sup>35</sup>. The prenatal periods during which the organism is susceptible to environmental stimuli leading to programming are the early period (embryonic phase of the development), the mid-gestation period (organogenesis), and late gestation (rapid growth)<sup>40</sup>.

In a review on fetal programming and skeletal muscle development in the ruminant<sup>35</sup> it was concluded that adipogenesis is initiated during mid-gestation. At this time, a pool of undifferentiated mesenchymal stem cells is present. From this pool, either myocytes or adipocytes are able to differentiate from committed mesenchymal cells to become skeletal muscle or adipose tissue<sup>141</sup>. The increase in number of stem cells throughout middle-to-late gestation led to the hypothesis that nutritional management has the potential to be more effective during the prenatal period rather than the postnatal portion of an animal's life<sup>35</sup>. However, it remains to be determined if plane of nutrition during late-pregnancy can elicit carryover effects acquired through programming.

The effects of maternal nutrition on fetal growth and its carry-over effects on offspring growth and development were reviewed recently<sup>35</sup>. Maternal nutrition seems to elicit different outcomes in the offspring depending on the gestational stage during which treatments begin. For example, protein supplementation of grazing cows during late gestation in the winter enhanced feedlot performance and carcass quality of the offspring<sup>76</sup>. There are several maternal-nutrition

studies utilizing real-time RT-PCR to evaluate specific target genes in adipose tissue or longissimus muscle (LM) of beef<sup>125</sup>, lamb<sup>36</sup> and sheep<sup>52</sup> offspring.

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## Chapter 2: Central role of the PPAR $\gamma$ gene network in coordinating beef cattle intramuscular adipogenesis in response to weaning age and nutrition

### ABSTRACT

Adipogenic/lipogenic transcriptional networks regulating intramuscular fat deposition (IMF) in response weaning age and dietary starch level were studied. The *longissimus* muscle (LM) of beef steers on an early weaning (141 days age) plus high-starch diet (EWS) or a normal weaning (NW, 222 days age) plus starch creep-feed diet (CFS) was biopsied at 0 (EW), 25, 50, 96 (NW), 167, and 222 (pre-slaughter) days. Expression patterns of 35 target genes were studied. From NW through slaughter all steers received the same high-starch diet. In EWS steers the expression of *PPARG*, other adipogenic (*CEBPA*, *ZFP423*) and lipogenic (*THRSP*, *SREBF1*, *INSIG1*) activators, and several enzymes (*FASN*, *SCD*, *ELOVL6*, *PCK1*, *DGAT2*) that participate in the process of IMF increased gradually to a peak between 96 and 167 days on treatment. Steers in NW did not achieve similar expression levels even by 222 days on treatment, suggesting a blunted response even when fed a high-starch diet after weaning. High-starch feeding at an early age (EWS) triggers precocious and sustained adipogenesis resulting in greater marbling.

**Keywords:** adipogenesis, nutrition, transcriptomics, marbling, weaning

## Introduction

Complex biological processes such as adipogenesis and lipogenesis are governed by a vast number of enzymes that act together along with key hormones and metabolites to regulate fat cell metabolism. Differentiation of adipocyte precursors is driven by a cascade of events controlled by transcription regulators, coactivators, and cell-cycle controls. This entire process is closely regulated at the transcriptional level. Preadipocytes can be induced to differentiate in vitro by means of an external cue such as insulin, glucocorticoids and/or molecules that can increase intracellular cAMP<sup>1</sup>. These cues trigger the beginning of a transcriptional cascade composed of a network of proteins that mediate the functions of adipocytes.

The master regulator of adipocyte differentiation in monogastrics is peroxisome proliferator activated receptor gamma (*PPARG*), a member of the nuclear receptor superfamily which is both necessary and sufficient for adipogenesis<sup>2</sup>. Several CCAAT/enhancer-binding proteins (*CEBP*) are important adipogenic and lipogenic transcription regulators. They work as part of a cascade of events with early induction of *CEBPB* and *CEBPD* leading to induction of *CEBPA*. *CEBPA* is a transactivator of *PPARG*, and both transcription regulators act together to promote adipogenesis<sup>3</sup>. The overall outcome of in vitro *PPARG* activation in bovine adipose tissue is to upregulate gene targets that allow for differentiation of pre-adipocytes into mature adipocytes, i.e. cells that can store triacylglycerol (TAG)<sup>4,5</sup>.

Contrary to the vast data in monogastrics, little is known about the environmental factors (including nutrition) that regulate adipogenesis in growing ruminants that are raised for meat production. Previous work indicated that during the “growing phase” in beef cattle (i.e. prior to weaning at ~205 days of age), nutrients are partitioned in favor of skeletal and muscle growth while the rate of fat deposition is relatively low. In contrast, during the “finishing phase” fat

deposition has the greatest response primarily because of the high content of non-structural carbohydrate (starch) of the diet<sup>6</sup>.

Body growth and fat deposition in cattle also are influenced by the age at which animals are weaned. “Early weaning” (EW) systems encourage the offspring to eat a high-non-structural carbohydrate diet that has been hypothesized would allow them to reach their genetic potential to accrete intramuscular fat (“marble”)<sup>7</sup>, thus, achieve a greater meat quality grade at slaughter<sup>8</sup>. Clearly, knowledge about the fundamental processes controlling adipogenesis in beef cattle has important biological and consumer implications.

The hypothesis for this study was that weaning at an early age compared with “normal weaning” (NW) in combination with a high starch diet would trigger upregulation of the *PPARG* signaling network and cause long-term activation of adipogenesis, which would be reflected in the intramuscular fat content of the animal during growth and also at slaughter. The specific aim was to assess the temporal expression profiles of *PPARG* target genes as well as other important components of the adipogenic/lipogenic program (Figure 1) in *longissimus* muscle (LM) of two breeds of beef cattle. Body composition and selected blood biomarker data were used to determine the phenotypic effect on intramuscular fat deposition of altered *PPARG* gene network expression due to EW.

## **Materials and Methods**

### ***Animal management***

A subset of fourteen purebred Angus (A) and Angus × Simmental (SA) steers from the University of Illinois cattle herd were selected from a larger group of animals utilized in a companion study<sup>9</sup>. The steers were randomly divided in two groups (EWS and CFS). Seven

animals were early-weaned at an age of  $141 \pm 31$  days and housed in buildings at the Beef Field Research Laboratory at the University of Illinois. The buildings are open to the south with bird screens and the inside temperature fluctuates with the outside temperature. This point marked the beginning of the present study (day = 0). The animals had a period of adaptation to the new diet of two weeks.

Early-weaned steers were housed in buildings on concrete slatted floors (covered with rubber) and fed from fence-line concrete bunks. Another seven animals continued nursing their dams while they were on pasture (fescue grass, orchard grass, bluegrass, white clover, red clover, and alfalfa) and received a high-starch “creep supplement” until they were weaned at about 222 days of age. Beginning on 222 days of age, both groups joined the feedlot and received a high-starch finishing-phase diet until slaughter. Individual feed intake data were collected during this phase using the GrowSafe® system (GrowSafe Systems Ltd, Alberta, Canada). Average daily gain (ADG) was calculated using each animal’s body weight, which was recorded close to biopsy time. Individual daily dry matter intake (DMI) and ADG were used to estimate feed conversion efficiency (gain/feed; kg/kg; Table 2) during the finishing phase.

Ultrasound measurements (500 V Aloka ultrasound with 3.5-MHz transducer) of back fat thickness, muscle depth and intramuscular fat were obtained at the beginning of the finishing phase and at 44 day intervals to determine the end-point of the fattening period (target harvest back fat thickness of 1.2 cm). At ~140 days of age all the steers received a dose of 4 pellets containing 100 mg progesterone and 10 mg estradiol benzoate (Synovex C, Pfizer Animal Health). In the finishing phase (day 97 after early weaning), steers were implanted with a silicone rubber implant containing a total of 25.7 mg of estradiol + 0.5 mg of Oxitetracycline (Compudose 200, Elanco).

### ***Biopsy procedure and blood sampling***

The biopsy procedure was previously approved by the University of Illinois Institutional Animal Care and Use Committee under protocol No. 05095 and was described in detail previously<sup>10</sup>. Briefly, biopsies were harvested under local anesthesia at time of EW (0 day) then at 25, 50, and 96 days (NW time) of the growing phase and at half-way through the finishing phase (167 days after EW) and one week prior to slaughter (222 days after EW). It is important to mention that NW steers required 20 more days to reach target back fat thickness of 1.2 cm. Animal identification was noted so that the same animals were biopsied at each time point to allow for repeated measures sampling.

A total of fourteen A and SA steers within the EW (n = 4 A, 3 SA) and NW (n = 3 A, 4 SA) groups were selected for biopsy. The first biopsy was collected from a section between the 12<sup>th</sup> and 13<sup>th</sup> rib on the right side of the animal. Subsequent biopsies were collected ca. 5 cm from the previous one moving towards the head. Over 0.5 g of tissue was obtained from each steer at each time point and was stored in liquid-N<sub>2</sub> until RNA extraction.

Blood was collected from the jugular vein prior to biopsies (ca. 0900 h) to isolate serum for insulin (Bovine Insulin ELISA kit, Cat No. 10-1201-01, Mercodia AB, Uppsala, Sweden), glucose and non-esterified fatty acids (NEFA) (Diagnostics Laboratory, College of Veterinary Medicine, University of Illinois), and growth hormone (GH), leptin (LEP), and insulin-like growth factor-1 (IGF-1) via radioimmunoassay<sup>11,12</sup>. Animals had free access to feed and consumed ca. 6 meals per day, thus, minimizing the potential for postprandial effects on blood metabolite concentrations.

### ***RNA extraction and qPCR analysis***

These procedures were exactly as described previously<sup>13</sup>. Briefly, muscle tissue sample was weighted (~0.3-0.5 g) and immediately homogenized with Trizol reagent (Invitrogen Corp.) and linear acrylamide (Ambion® Cat. No. 9520) as coprecipitant to proceed with RNA extraction. Genomic DNA was removed from RNA with DNase using RNeasy Mini Kit columns (Qiagen, Germany). The RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA (A260/A280) for all samples was above 1.81. Moreover, RNA integrity number (RIN) was measured using the Bioanalyzer platform (Agilent Technologies, Inc., Santa Clara, CA, USA). The final data were normalized using the geometric mean of *UXT*, *MTG1* and *RPS15A*, which were validated as suitable internal control genes in bovine LM. During the analysis of the qPCR results it was determined that *WNT10b* and *MLXIPL* had a very low level of expression, thus, we concentrated the cDNA (dilution 1:3) and reanalyzed those genes along with the internal controls.

### ***Statistical analysis***

Quantitative PCR data were analyzed using the MIXED procedure of SAS (SAS 9.1 Institute, Cary, NC, USA). Before statistical analysis, normalized qPCR data (using the geometric mean of *UXT*, *MTG1* and *RPS15A*) were transformed to fold-change relative to day 0 (i.e. day of EW). To estimate standard errors at day 0 and prevent biases in statistical analysis, normalized qPCR data were transformed to obtain a perfect mean of 1.0 at day 0, leaving the proportional difference between the biological replicate. The same proportional change was calculated at all other time points to obtain a fold-change relative to day 0. Fixed effects in the statistical model for each variable analyzed (i.e. genes, production, carcass, blood metabolite) included treatment, breed, time on experiment, treatment × breed, treatment × time on

experiment and breed  $\times$  treatment  $\times$  time on experiment interactions when appropriate (e.g. mRNA expression over time). Gene expression data analysis included a repeated-measures statement with an autoregressive covariate structure. Animal performance, carcass quality parameters, blood metabolites and ultrasound data were also analyzed using the MIXED procedure of SAS, and treatment was the fixed effect in the statistical model. The random effect in all models was steer within treatment.

The statistical model used was:  $Y_{ijklm} = \mu + C_i + T_j + B_k + S_l + (C \times T)_{ij} + (T \times B)_{jk} + (C \times B)_{ik} + (T \times C \times B)_{ijk} + \epsilon_{ijklm}$ ; where,  $Y_{ijklm}$  is the background-adjusted normalized fold change or blood data value;  $\mu$  is the overall mean;  $C_i$  is the fixed effect of time (6 levels);  $T_j$  is the fixed effect of treatment (2 levels);  $B_k$  is the fixed effect of breed (2 levels);  $S_l$  is the random effect of steer nested within treatment;  $C \times T$ ,  $T \times B$ ,  $C \times B$  are the interactions of time by treatment, treatment by breed and time by breed, respectively;  $T \times C \times B$  is the interaction or third order for the main effects; and  $\epsilon_{ijklm}$  is the random error ( $0, \sigma_e^2$ ) associated with  $Y_{ijklm}$ . A likelihood ratio test was used in order to examine if the main effects were non-significant, and if they could have an impact on the logarithm of convergence of the original model. Breed effect had no significant impact on the logarithm of convergence of the model, thus, breed remained in the model. Moreover, carcass categorical data (YG and QG) were analyzed using the GENMOD procedure of SAS. Statistical differences for animal performance and carcass data were declared at  $P \leq 0.10$  due to the scarce degrees of freedom that came out from the subset of steers from which skeletal muscle were used for gene expression analysis<sup>9</sup>. For gene expression, ultrasound data and blood metabolites analysis, statistical significant differences were declared at  $P \leq 0.05$ . Lastly, partial Pearson correlation analysis among genes and between genes and ultrasound data,



adjusted for the fixed effects, was conducted using the PROC CORR procedure of SAS (Table 7, 7 and 8).

## **Results and Discussion**

### ***Animal performance***

Because it was not feasible to measure DMI of animals on pasture, data for all groups are only available from 96 days after early weaning until slaughter. There was no significant treatment  $\times$  breed interaction for DMI in any of the treatments (Table 2). However, compared with CFS steers, the EWS steers consumed more feed in the first two weeks after normal weaning (Figure 10). Feed efficiency (FE) in the finishing phase was unaffected by the pre-weaning high-energy concentrate feeding during the nursing period, which is similar to results in a previous study<sup>14</sup>. Moreover, there were significant differences in ADG in response to treatments between birth and EW day (Table 2).

Least means squares for ultrasound data are reported in Figure 2. Ultrasound marbling score (Figure 2) and carcass marbling score (Table 1) are represented using the USDA scale that goes through traces (200 - 299), slight (300 - 399), small (400 - 499), modest (500 - 599), moderate (600 - 699), slightly abundant (700 - 799) to moderately abundant (800 - 899). Ultrasound marbling score (i.e. a measure of intramuscular fat deposition) had a significant breed  $\times$  treatment  $\times$  time interaction ( $P = 0.01$ ) and a time effect ( $P < 0.01$ ) due to the response in Angus steers (Figure 2). Muscle depth had a significant breed  $\times$  treatment  $\times$  time interaction ( $P < 0.01$ ), with higher values for CFS-SA at 187 days after early weaning (Figure 2).

There were no significant breed  $\times$  treatment  $\times$  time interactions for carcass marbling score; however, marbling scores were greater ( $P = 0.10$ ) in EWS (Table 1). Moreover, there were no differences between treatments in carcass weight ( $P = 0.32$ ), back fat thickness ( $P = 0.80$ ) and

rib-eye area ( $P = 0.95$ ). Percentage of kidney, pelvic and heart fat (KPH) also did not differ ( $P = 0.51$ ) (Table 1). It is noteworthy that for EWS as compared to CFS carcasses there was a treatment effect ( $P = 0.08$ ) for a greater percentage of carcasses with quality grades greater than or equal to "High Choice" and a treatment  $\times$  breed interaction for a greater percentage of carcasses with quality grades greater than or equal to "Average Choice" (Table 1). Quality grade is a carcass quality parameter that is mainly based on marbling score and skeletal maturity and both can be used as indicators of the degree of beef tenderness<sup>15</sup>.

In this study, feed intake could have played a role in promoting adipogenesis because CFS steers still consumed less feed during the entire finishing phase despite the fact that they compensated during the second half of this stage. Therefore, the dietary nutrient level itself could have driven the observed precocity for adipogenesis discussed in the sections below. Because of this, "epigenetic" regulation (e.g. DNA methylation) and its interaction with plane of nutrition is an issue that has to be addressed in future experiments. In this study, the plane of nutrition effect is clearly underscored by the fact that EW steers had greater rates of intake during the first five weeks of the finishing phase (Figure 10), which correlated with a further upregulation of the adipogenic program through 167 days. Over the same time frame, the greater intake of nutrients in CFS steers was unable to compensate for this lack at an early age, thus, only a few of the adipogenic genes responded in spite of these animals eating at the same rate as EWS steers. To support these associations, it is important to underscore that the average time required to harvest the steers was quite similar across treatments, i.e.  $371 \pm 8$  and  $363 \pm 8$  days for EW and NW steers.

### ***Blood biomarker concentration***

Growth hormone (GH) and insulin-like growth factor 1 (IGF-1) had a significant breed  $\times$  treatment  $\times$  time interaction. Concentration of GH during the finishing phase was greater for EWS compared with CFS steers. In contrast, IGF-1 concentration was greater for CFS steers compared with EWS steers. Serum IGF-1 concentration is indicative of nutritional and metabolic status, and consequently it increases in response to an increment in nutrient intake<sup>12</sup>. Moreover, in beef cattle an elevated IGF-1 concentration at weaning has been linked with increased growth rates, final live weight, and gain to feed ratio<sup>16</sup>.

The CFS steers seemed to have an advantage in terms of growth rate, likely due to the greater IGF-1 concentration. In contrast, throughout the study EWS-A steers had greater concentrations of GH but stable concentration of IGF-1. The GH concentration decreases as animals mature, thus, reducing the stimuli for production of IGF-1 during the latter period of time in the feedlot<sup>17</sup>. It is well-known that the IGF-1 response to GH decreases when nutrient status or insulin levels are low<sup>18</sup>, but results of this study do not agree with those conclusions. Serum leptin concentration had a noticeable increase during the growing and the finishing phase but without a significant breed  $\times$  treatment  $\times$  time interaction (Figure 3). That response agrees with the gradual increase in fat deposition as the animal ages, i.e. more adipocytes able to produce and secrete leptin or adipocytes growing in size.

### ***Expression of adipogenic activators***

Results of adipogenic activators indicated that *ZFP423* was the only gene that did not have a significant breed  $\times$  treatment  $\times$  time interaction (Figure 4). Within this group, *PPARG*, *CEBPA*, *BMP2*, and *HEY1* had the highest level of upregulation with a marked increase from 0 until 167 days after EW. Furthermore, the response was greater for EWS steers compared to CFS

steers. *CEBPB* and *CEBPD* expression had an opposite pattern, being greater in CFS steers. Moreover, there were more significant correlations at 167 d for CFS steers between muscle depth and adipogenic activators (*PPARG*, *CEBPA*, *CEBPD* and *BMP2*). Additionally, there were more significant correlations at 167 d for EWS steers between back fat thickness and adipogenic activators (*ZFP423*, *BMP2* and *SMADI*). The correlation between marbling and *SMADI* at 222 d was positive for EWS steers and between marbling and *BMP2* at 167 d it also was positive for CFS steers.

The early feeding of starch in the diet along with early weaning induced a clear change between EWS and CFS steers in the mRNA expression of adipogenic activators. A vast number of studies in monogastrics have demonstrated that *PPARG*<sup>19</sup> and *CEBPA*<sup>20</sup> are the principal inducers of the adipocyte differentiation process in preadipocytes. These two genes, together with bone morphogenetic protein 2 (*BMP2*), had the highest difference in expression between EWS-A and EWS-SA steers at the beginning of the growing phase and at the end of the finishing phase. *BMP2* upregulation was higher in EWS compare with CFS steers, with a noticeable increase during the finishing phase, suggesting that this protein does not participate in the early programming of adipogenesis and it is not essential to sustain this process during the finishing phase. The decrease in *BMP2* expression close to slaughter was expected due to the fact that low *BMP2* expression promotes adipogenesis, while high *BMP2* expression promotes osteogenesis<sup>21</sup>. In contrast, this protein might serve to revamp this process in CFS steers during the finishing phase, i.e. it might represent a sort of compensatory mechanism in response to the higher-energy feeding prior to slaughter<sup>22</sup>.

Initial studies suggested that *BMP2* induced adipocyte differentiation in a Smad-1 independent fashion via the induction of *PPARG*<sup>23</sup>. However, it is now clear that BMPs activate

*PPARG* during adipogenesis through the interaction of *ZFP423* and *SMAD1*<sup>24</sup>. *SMAD* family member 1 (*SMAD1*) is a signal transducer that strengthens the association between the transcription regulator with DNA, furnishing transcriptional activation capacity<sup>25</sup>. Zinc finger protein 423 (*ZFP423*) is the DNA binding protein that associates with *SMAD1*<sup>26</sup>. This protein is a downstream target of BMP signaling and a zinc finger transcription regulator, currently recognized as an essential functional determinant of preadipocyte commitment<sup>27</sup>. The *SMAD*-binding domain of *ZFP423* is absolutely required for *ZFP423* to elicit a role during the committed stage of adipocyte differentiation induced by BMPs<sup>27</sup>. Our data for *ZFP423* did not reach statistical significance, but it is important to note that this transcription factor followed the same pattern of expression as *PPARG* and *CEBPA*. Moreover, among all treatments the changes in *SMAD1* expression were statistically significant but relatively small, suggesting that it might have been dampened by a co-repressor<sup>28</sup> (i.e. *SMAD6*), hence, diminishing the effect of *SMAD1* as an adipogenic signal.

During adipocyte differentiation the early induction of *CEBPB* and *CEBPD* leads to an upregulation of *CEBPA*<sup>29</sup>. The response to nutrition and weaning age for *CEBPB* and *CEBPD* was exactly the opposite of other adipogenic transcription activators, i.e. there was marked temporal upregulation in CFS steers compared with EWS. By virtue of relying on forage as dietary source, those animals likely had a greater proportion of acetate available in blood as a nutrient source, thus, suggesting that *CEBPB* and *CEBPD* in growing ruminants might be activated through acetylation. Previous work provided some evidence that acetylation may contribute to adipogenesis<sup>30,31</sup>. From these results, it would appear that the type and quantity of nutrients supplied in early postnatal life coupled with the right hormonal profile are necessary to permanently blunt the negative effect of *CEBPB*, *CEBPD*, and *BMP2*. These combined results

lead us to propose that 1) there might be a particular type of preadipocyte phenotype formed in response to the early exposure to a high starch diet; 2) there might be an adipocyte phenotype less sensitive to a high-starch diet during the finishing phase; or 3) there could be an epigenetic effect (e.g. methylation, acetylation) influencing our results.

The expression of *HEY1* followed a similar pattern across all treatments with a peak in expression (~1.5 fold) mid-way through the finishing phase. Hairy/enhancer-of-split related with YRPW motif 1 (*HEY1*) is a basic helix-loop-helix protein and a member of the HES-related repressor protein family that belongs to the Notch signaling pathway. In model organisms, *HEY1* has a pro-adipogenic role via the inhibition of *FOXC2* that it is still not well understood<sup>32</sup>. The modest change in expression in *HEY1* relative to other transcription regulators seems to suggest that this protein may play a secondary role, if any, on adipogenesis.

The dynamics of the *PPARG* and *CEBPA* response along with that of *SREBF1* and the lipogenic enzymes studied provide evidence of a coordinated pro-adipogenic response in EWS animals likely as a result of enhanced nutrient supply from the high dietary starch. In other words, CFS steers had a delay in the ability of the adipose cells to gain the adipogenic phenotype.

Taken together, these responses clearly underscore that the environmental effect of nutrition on marbling deposition, either due to more intake or more intake of a specific nutrient, overrides the genetic ability of the animal to accumulate intramuscular fat. This is supported by the fact that Angus cattle are known to deposit more intramuscular fat than Angus × Simmental steers on the same plane of nutrition<sup>33</sup>.

### ***Expression of adipogenic repressors***

In the case of the adipogenic repressors (Figure 5), there was a significant breed  $\times$  treatment  $\times$  time interaction for *NCOR1*, *NCOR2*, *WNT10B*, *FOXC2*, and *NR2F2*. Expression of *NCOR1* did not change during the growing phase but increased markedly between 96 and 222 days, which constituted the most robust response among all the adipogenic repressors studied. This was particularly the case in EWS-SA steers.

The nuclear receptor corepressors *NCOR1* and *NCOR2* encode proteins that mediate ligand-independent transcriptional repression of thyroid-hormone and retinoic-acid receptors via histone deacetylation that leads to chromatin condensation<sup>34</sup>. In the absence of ligand binding, *PPARG* forms a protein complex with its corepressors leading to reduced transcriptional activation of target genes<sup>35</sup>. The marked increase of *NCOR1* expression during the finishing phase could partly explain the decrease in expression of all pro-adipogenic transcriptional activators between 167 and 222 days, which was most evident in EWS steers fed high-starch. Although the role *NCOR2* in adipocyte function is unclear, a recent study suggested that it can diminish insulin sensitivity in adipocytes and also decrease *LEP* expression, in that way limiting the ability of fat mass to expand with increase caloric intake<sup>36</sup>.

A link between *NCOR2* and *LEP* in our study is unclear except for EWS-A steers which had a relatively constant expression of *NCOR2* but clearly had a decrease in *LEP* expression by 222 days. This result could mean that the pro-adipogenic program is rather sensitive during this early age but highly-dependent on a proper nutrient and hormonal milieu. Therefore, the different feed intake response between treatments during the first two weeks of the growing phase also is noteworthy in terms of the corepressors because their mRNA expression did not

seem to be sensitive to plane of nutrition. The similar pattern of expression of *NCOR1* and *NCOR2* seems to argue against the existence of different adipocyte phenotypes<sup>37</sup>.

These corepressors could be nutrient-independent-like sensors which are programmed genetically to control and/or terminate adipogenesis. In fact, it would appear based on *CEBPB* and *CEBPD* expression that a lack of nutrients (i.e. non-structural carbohydrate) at an early age could “deregulate” the process and cause a robust upregulation noticeable from 96 days until 167 days after EW in spite of CFS steers achieving greater DMI during the first half of the finishing phase<sup>38</sup>. Accordingly, the adipogenic response in those steers induced during the growing phase appeared to have been dampened in a discrete fashion after NW, first through upregulation of *NCOR2* during the initial portion of the finishing phase and then through upregulation of *NCOR1* during the second half of the finishing phase. Whether the adipokines *LEP* or *ADIPOQ* (Figure 6) contributed to that response is unclear and merits further study.

In model organisms, chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) also called nuclear receptor subfamily 2, group F, member 2 (*NR2F2*) is expressed at the early stages of white adipocyte development<sup>39</sup>. *NR2F2* positively regulates adipogenesis at an early expansion phase, promoting formation of small adipocytes; whereas, the persistent expression of *NR2F2* during differentiation may be inhibitory to the increase in adipose tissue mass<sup>40</sup>. Similar to a previous study<sup>10</sup>, the temporal response in expression of *NR2F2* was modest. However, the 1-fold increase of *NR2F2* expression in NW-SA steers between 50 and 96 days after EW and EWS-A steers at 167 days after EW (Figure 5) could be a signal of greater amounts of small preadipocytes.

Wnt signaling through  $\beta$ -catenin and TCF maintains preadipocytes in a non-differentiated stage<sup>41</sup>. Wnt/ $\beta$ -catenin signaling activates the expression of *NR2F2* which recruits the *NCOR2*



corepressor complex to *PPARG*. The formation of an *NR2F2/NCOR2* complex maintains the chromatin in a hypoacetylated state, which inhibits *PPARG* expression and blocks adipogenesis<sup>42</sup>. The greater expression of *WNT10B* and adipogenic activators in EWS-SA steers suggested that *WNT10B* may not have dampened the ability of adipocytes to differentiate.

Forkhead Transcription Factor 2 (*FOXC2*) blocks adipogenesis by increasing the sensitivity of the  $\beta$ -adrenergic-cAMP-protein kinase A (PKA) signaling pathway<sup>43</sup> and by inhibiting the capacity of *PPARG* to promote the expression of genes recognized as markers of mature adipocytes (*CEBPA*, *ADIPOQ*, and *FABP4*)<sup>44</sup>. In our study, *FOXC2*, like *NCOR2* and *NR2F2*, did not experience marked changes in expression. However, there appear to have been interesting patterns in the response of this gene, for example, the gradual increase in expression until 96 days after EW in all treatments, followed by the decrease by 222 days after EW in expression without differences between treatments. It could be possible that the expression level of *FOXC2* in CFS-SA steers was sufficient to generate an inhibitory effect on the *PPARG* targets studied.

### ***Expression of adipokines***

Among all the adipokines analyzed (*LEP*, *LEPR*, *ADIPOQ* and *ADIPOR2*), *LEPR* was the only one without a breed  $\times$  treatment  $\times$  time interaction (Figure 6). Of these genes, the response in *LEP* expression in EW steers was the most marked, i.e. expression increased until 167 days after EW followed by a decrease by 222 days.

Administration of a diet rich in energy promotes angiogenesis because the increment in fat mass generated by this type of diet leads to an increase in capillary networks<sup>45</sup>. These capillary networks supply nutrients to fat cells allowing the adipocytes to actively secrete adipokines (e.g. leptin) at a rate strongly related with adiposity<sup>46</sup>. While adipocytes increase in

size and mass, concentrations of leptin (*LEP*) in blood also increase<sup>47</sup>. Moreover, a positive correlation exists between adipocyte diameter and *LEP* mRNA levels in crossbred steers<sup>48</sup>.

A previous study<sup>49</sup> reported a significant effect of breed and fat depot anatomical site, with greater *LEP* mRNA expression in the intramuscular fat of Angus steers than in subcutaneous fat. The opposite was observed in Limousin steers<sup>49</sup>. In our study, peak expression level for *LEP* was 250-fold in EWS-A steers and 170-fold in EWS-SA steers at 167 days after EW. Subsequently, *LEP* decreased dramatically. Despite the robust response observed for *LEP*, changes in expression of *LEPR* in EWS steers were quite modest, and occurred primarily during the growing phase. This was the opposite in CFS steers, which had greater expression during the early stages of the growing phase with a smaller upregulation of *LEP*. It could be possible that these differences are functionally related to leptin sensitivity in adipocytes.

Adiponectin (*ADIPOQ*) is an anti-inflammatory and insulin-sensitizing protein secreted from adipose tissue. *ADIPOQ* promotes adipocyte differentiation and enhances lipid accumulation in mature adipocytes<sup>50</sup>. Upon action of *ADIPOQ*, adipogenesis is accelerated by ensuring a marked and prolonged expression of *PPARG*<sup>51</sup> and *CEBPA*<sup>50</sup>. In fully differentiated adipocytes<sup>50,52</sup>, this response increases the rate of cytoplasmic lipid droplet accumulation by enhancing the efficiency of triacylglycerol synthesis and, also insulin-responsive glucose transport through increases in glucose transporter 4 (*SLC2A4*) expression.

In contrast to *LEP*, smaller adipocytes secrete more *ADIPOQ*, resulting in enhanced adipocyte differentiation and lipid accumulation<sup>50</sup>. Coinciding with previous results<sup>10</sup>, EW and high dietary starch resulted in the greatest response in *ADIPOQ* expression. This is important because *ADIPOQ* must be activated ahead of *LEP* to induce the adipogenic program<sup>53</sup>. The

*ADIPOR2* response, however, might have been linked with the marked *ADIPOQ* expression because *ADIPOQ* overexpression in adipocytes was reported to decrease lipid accumulation<sup>50</sup>.

All treatments led to an increase in level of expression of *ADIPOR2* between 96 and 167 days after EW. That was followed by a sharp decrease in expression during the second half of the finishing phase, especially for CFS-A steers (Figure 6). The EWS steers seemed to have enhanced the ability of adipose tissue to respond to *ADIPOQ* particularly during the first half of the finishing phase when the peak in expression of *ADIPOR2* was observed. It could be possible that the combined response of *ADIPOQ* and its receptor in EWS animals blocked part of the negative effect of co-repressor upregulation (e.g. *NCOR2*).

### ***Expression of lipogenic regulators***

With the exception of *SREBF1*, all the genes classified as lipogenic transcription regulators (Figure 7) had a breed  $\times$  treatment  $\times$  time interaction. *SREBF1* was affected by weaning age. In EWS steers *THRSP* at 167 days, *MLXIPL* at 96 days and *INSIG1* at 167 days had the highest fold changes in expression relative to 0 days. *FOXO1* was the only lipogenic regulator that had greater expression in CFS steers and at 167 days after EW.

Thyroid hormone responsive (*THRSP*) had the greatest change in expression compared to other lipogenic regulators. Our results for *THRSP* are quite similar to those obtained in a previous study<sup>54</sup>. Moreover, a recent study<sup>55</sup> confirmed an association of bovine fatty acid composition with a nucleotide polymorphism in *THRSP*. *THRSP* was highly-correlated with marbling content in breeds widely-known to possess extremely high capacity for marbling such as Japanese Black<sup>56</sup> or Wagyu<sup>57</sup>. All of these responses support the existence of an important role of *THRSP* in terms of bovine lipogenic regulation.

In rodents there is well-established evidence that *SREBF1* is central for the control of hepatic lipogenesis<sup>58</sup>. Carbohydrate response element binding protein (ChREBP) or Max-like X interacting protein-like (*MLXIPL*) is a glucose-responsive transcription factor that is affected by acetylation (i.e. if glucose is present, there is no acetylation of the histones). Acetylation of histones leads to local chromatin decondensation and gene expression. *MLXIPL* plays a critical role in hepatic lipogenesis in response to high carbohydrate diet, converting excess glucose to storage lipid by mediating the activation of several regulatory enzymes of glycolysis and lipogenesis including pyruvate kinase, acetyl-CoA carboxylase, and fatty acid synthase<sup>59</sup>. Our results agree with a previous study<sup>10</sup>, where *MLXIPL* expression was greater in EWS Angus steers consuming a high starch diet. Furthermore, in our study, at 96 days *MLXIPL* had a 100-fold greater level of expression in EWS-SA steers as compared to EWS-A steers. This could be one of the reasons why most of the genes analyzed in this study were activated earlier in EWS-SA steers as compared to EWS-A steers.

A recent study provided evidence that *INSIG1* can block proteolytic activation of SREBPs, hence, reducing the activation of a wide variety of genes that leads to synthesis of palmitic and oleic acids, and formation of triacylglycerol<sup>60</sup>. Our study, however, supports previous results demonstrating that in EWS-A steers with high marbling score *INSIG1* expression was greater as compared to crossbreed steers<sup>54</sup>.

Forkhead box protein O1 (*FOXO1*) can have both a positive and a negative effect on adipogenesis. Insulin-induced phosphorylation inactivates *FOXO1*<sup>61</sup> and holds it in the cytoplasm, thus, preventing binding to DNA and reducing the *FOXO1-PPARG* interactions<sup>62</sup>. During insulin resistance, there is reduced phosphorylation and increased nuclear accumulation of *FOXO1*, which is coupled to lower expression of *PPARG* target genes. In adipocyte

progenitor cells, the suppression of *FOXO1* activity by insulin prevents the activation until growth has subsided of the cell cycle arrest-associated factor p21<sup>63</sup>. At this stage of adipogenesis, *FOXO1* is activated by a glucose-derived O-linked  $\beta$ -N-acetylglucosamine (O-GlcNAc)<sup>64</sup>, and cooperates with *PPARG* to trigger a post-mitotic growth arrest, which is required for terminal differentiation. Our results revealed a significant activation of *FOXO1* in CFS-A steers at 167 days after EW. These results could be taken as evidence of both a glucose effect on *FOXO1* in CFS-A steers, or a lack of insulin leading to an inhibition of *FOXO1* gene expression. Correlation analysis at 222 days after EW revealed a strong negative correlation between *FOXO1* and muscle depth for EWS steers.

The MID1 interacting protein 1 (*MIDIIP1*) binds and forms a heterodimer with *THRSP*<sup>65</sup>. In the absence of *THRSP*, *MIDIIP1* forms a homodimer, a process required for efficient fatty acid<sup>66,67</sup> and lipid biosynthesis, including triacylglycerol, diacylglycerol and phospholipid<sup>68</sup>. The expression of this gene had the lowest level of expression but a similar pattern as compared with other lipogenic regulators analyzed in this study. Analysis revealed a positive correlation at 167 days after EW between *MIDIIP1* and back fat thickness for EWS steers, a negative correlation at 222 days after EW with marbling for EWS steers, and a positive correlation at 167 days after EW with muscle depth for CFS steers.

### ***Expression of lipogenic enzymes***

#### ***Substrate uptake***

The insulin-sensitive transporter *SLC2A4* was the only gene among those related with lipolysis and lipogenesis that did not have a breed  $\times$  treatment  $\times$  time interaction (Figure 11). Moreover, all these genes had a similar pattern of upregulation (always greater for EWS steers compared to CFS steers) between 0 to 167 days after EW followed by a decrease by 222 days.

Among all the lipogenic genes *SCD* and *FASN* had the greatest fold change by 167 days. *LPL* and *CD36* had the lowest degree of change in expression within this group of genes (Figure 8), but always greater in EWS steers.

In beef cattle intramuscular fat, lactate is the preferred substrate for glycerol synthesis, and glucose for fatty acid synthesis<sup>69</sup>. For subcutaneous adipose tissue, acetate is the main source for fatty acid synthesis<sup>70</sup>. Lactate requires the enzymes ATP-citrate lyase (*ACLY*) and NADP-malate dehydrogenase in order to be converted to fatty acids<sup>71</sup>. The activity of *ACLY* in bovine adipose tissue, even though lower than that in rat tissues, is still sufficient to allow for conversion of lactate to fatty acids. Our results revealed that *ACLY* had greater upregulation in EWS-SA steers but also a higher drop in the level of expression from 167 to 222 days after EW. In contrast, correlation analysis at 167 days after EW revealed a positive correlation for *ACLY* with marbling and muscle depth in CFS steers.

#### *De novo synthesis*

Fatty acid synthase (*FASN*) catalyzes the synthesis of palmitate from acetyl-CoA and malonyl CoA<sup>72</sup>. In our study, *FASN* had a clear difference of expression between CFS and EWS steers, with greater levels of expression in EWS steers (Figure 7). Fatty acid binding protein 4 (*FABP4*) delivers long-chain fatty acids (LCFA) and retinoic acid to their cognate receptors in the adipocyte nucleus<sup>73</sup>. *FABP4* followed a similar pattern of expression as *FASN*, with an earlier and stronger response in EW-SA as compared with EWS-A (Figure 8). Thus, the *FABP4* response, especially for EWS-SA steers between 50 and 96 days after EW, underscored the importance of LCFA, eicosanoids and retinoic acid uptake (or LCFA recycling during basal lipolysis) to sustain the a robust lipogenic process<sup>74</sup>. Moreover, increased *FABP4* expression is associated with increased marbling in cattle<sup>73</sup> and increased TAG content<sup>75</sup>.

### *Desaturation*

Stearoyl-CoA desaturase (*SCD* or  $\Delta 9$  desaturase) converts a portion of stearic acid (18:0) into oleic acid (18:1)<sup>76</sup>. Several studies have indicated that time on a corn-based finishing diet increases muscle and adipose tissue *SCD* gene expression<sup>77</sup>. This was reflected also in our study, with impressive differences in *SCD* expression in EWS as compared with CFS steers. Moreover, in a previous study where *SCD* expression was measured in Angus and Wagyu steers, corn-fed Angus steers had greater *SCD* expression compared with Wagyu steers<sup>78</sup>. Our results are similar in that there was a lower degree of change in *SCD* expression in SA steers which is a breed with a tendency to have lower marbling as compared with a breed such as Angus.

### *Elongation*

In the endoplasmic reticulum of the adipocyte, elongase of long chain fatty acids family 6 (*ELOVL6*) catalyzes the elongation of saturated and monounsaturated fatty acids with 12-, 14- and 16-carbons. *ELOVL6* is expressed in lipogenic tissues and in monogastrics is regulated by sterol regulatory element binding factor 1 (*SREBF1*)<sup>79</sup>. At all-time comparisons in our study, *ELOVL6* had a greater upregulation in EWS steers, but at 96 days after EW the expression in EWS-SA steers was 20-fold greater compared with EWS-A steers. This result is similar to a study where lean and fat pig breeds were compared<sup>80</sup>, thus, underscoring the role of this enzyme in the elongation of 16:0 likely to provide 18:0 for the *SCD* reaction that precedes the latter stages of TAG synthesis.

### *Glyceroneogenesis*

Phosphoenolpyruvate carboxykinase (*PCK1*) is a cytosolic enzyme that along with GTP, catalyzes the formation of phosphoenolpyruvate from oxaloacetate<sup>81</sup>. In monogastrics, *PCK1* expression is regulated by insulin, glucagon and diet, and it is responsible for the synthesis of

glycerol-3-phosphate<sup>82</sup> and re-esterification of free fatty acids (FFA) to generate TAG<sup>83</sup>.

Interestingly, our results revealed a singular expression for EWS steers. In EWS-A steers, *PCK1* was upregulated for a longer time than EWS-SA steers, which had a peak of expression at 96 days after EW, followed by a marked decrease until the end of the study. These results suggest that in EWS-A steers compared with EWS-SA the flux of glucose via glyceroneogenesis might have been greater. Moreover, *PCK1* did not seem to respond to EW until the last portion of the growing phase; thus, this suggest that the pro-adipogenic program begins earlier and the maturation program afterwards when there is a need to generate plenty of glycerol-3-phosphate for esterification (and re-esterification) of LCFA and subsequent lipid droplet formation.

### *Esterification*

The expression of lipoprotein lipase (*LPL*), encoding a water-soluble enzyme that hydrolyzes triglycerides into two free fatty acids and one monoacylglycerol molecule, reached peak expression in EWS-SA steers at 96 days and at 167 days in EWS-A steers (Figure 8). As preadipocytes begin differentiating into mature adipocytes, *LPL* is one of the first genes expressed due to its role in providing LCFA for intracellular esterification<sup>1</sup>. High-carbohydrate diets increase the *LPL* expression in both adipose tissue and skeletal muscle<sup>29</sup>. By increasing *LPL* gene transcription<sup>84</sup> insulin has a major effect on *LPL* activity during adipocyte differentiation.

Another enzyme that is essential for lipid droplet formation is diacylglycerol acyltransferase 2 (*DGAT2*), which catalyzes the final step of triacylglycerol synthesis<sup>85</sup>. In a previous study using Hereford × Angus steers, *DGAT2* activity was higher in subcutaneous adipose tissue compared with IMF or muscle tissue<sup>86</sup>. The difference in *DGAT2* expression



between treatments may be associated with differences in the positional distribution of fatty acids in the triacylglycerol molecule<sup>87</sup>.

Even though we did not measure the fatty acid composition and adipocyte size in the LM, the combined results for the lipogenic enzymes lead us to conclude that intramuscular fat composition might vary within breeds, treatments and age in accordance with previous studies<sup>73,84</sup>. In this study, there was a time-window between 50 and 96 days after EW where gene expression data reveals higher uptake of lipogenic sources to promote intramuscular fat deposition especially for EWS-SA steers.

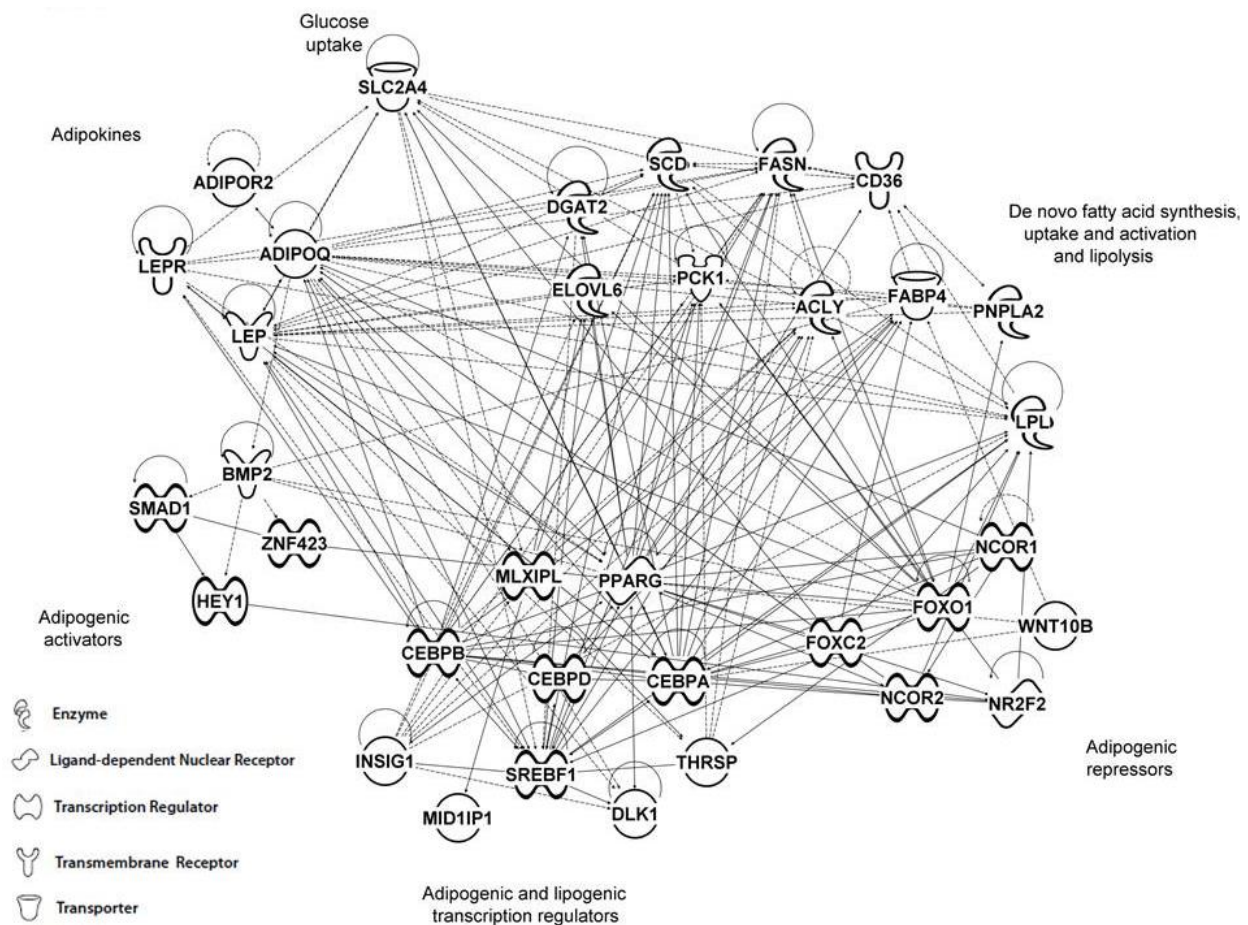
## Conclusions

Transcription network analysis of LM during EW and NW revealed that precocious and sustained activation of the *PPARG* and its target genes is one factor leading to greater intramuscular fat deposition and consequently more carcasses grading greater than or equal to “High Choice”. The combination of EW and high dietary starch leads to a strong programming effect in skeletal muscle tissue with both *PPARG* and *CEBPA* as the central coordinators of the response. From a practical standpoint, results provide additional evidence that EW is a valuable management strategy to the beef cattle producer. Furthermore, the response observed in Angus × Simmental steers underscores the potential for fine-tuning adipogenesis in this breed of animals. Angus × Simmental compared with Angus EW steers had a precocious and greater expression level of most of the genes analyzed, which was contrary to our expectations because Angus animals typically have greater capacity to marble. Despite this, the application of the EW husbandry approach even in Angus calves appeared to induce precocious pre-adipocyte differentiation and lipid filling through the *PPARG* and *CEBPA* network.

## Figures and Tables

**Figure 1.** Currently-known relationships among genes analyzed based on manually-curated examination of the published literature within the Ingenuity Pathway Analysis ([www.ingenuity.com](http://www.ingenuity.com)) knowledge base.

**Notes:** Genes are grouped by the predominant process they play in lipid metabolism. Different shapes denote the type of protein encoded by the specific genes, including enzymes, ligand-dependent nuclear receptors, transmembrane receptors, transcription regulators, and transporters. From Ingenuity Pathway Analysis.



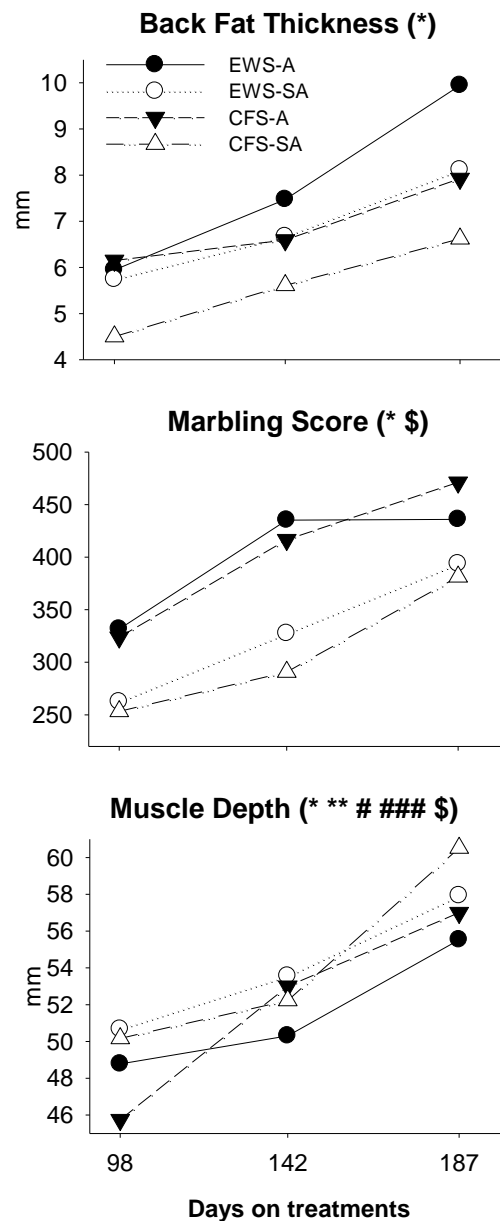
**Table 1.** Carcass performance traits for early weaned (EWS) and normal weaned (CFS) Angus (A) and Angus × Simmental (SA) steers (n = 7/treatment). All steers were on a common diet from 96 to 222 days.

Item	Group				SEM	<i>P</i> value <sup>a</sup>		
	EWS-A	EWS-SA	CFS-A	CFS-SA		T	B	T×B
Carcass weight (kg)	293	332	339	345	16.0	0.09	0.18	0.32
Marbling <sup>b</sup>	553	567	520	408	55.7	0.10	0.38	0.27
Back fat (cm) <sup>c</sup>	1.30	0.98	1.43	1.14	0.1	0.09	<0.01	0.80
REA (cm <sup>2</sup> )	70.37	74.71	72.89	77.69	3.5	0.43	0.21	0.95
KPH (%)	2.44	2.12	2.25	2.05	0.1	0.20	0.02	0.51
USDA Yield Grade (YG)								
YG 2 (%)	33	67	33	75	-	0.87	0.16	0.87
YG 3 (%)	67	33	67	25	-	0.87	0.16	0.87
Quality Grade (QG)								
≥ Low Prime (%)	33	0	0	0	-	0.20	0.20	0.20
≥ High Choice (%)	33	33	0	0	-	0.08	1.00	1.00
≥ Ave Choice (%)	33	67	67	0	-	0.45	0.45	0.04
≥ Low Choice (%)	100	100	100	50	-	0.12	0.12	0.12

**Notes:** <sup>a</sup>Statistical values for the effect of treatment (EWS or CFS; T), breed (A or SA; B), or interaction of treatment and breed (T×B); <sup>b</sup>Marbling evaluated based on scores: 300-399 – Slight (High Select); 400-499 – Small (Low Choice); 500-599 – Modest (Average Choice); 600-699 – Moderate (High Choice) and 700-799 – Slightly Abundant (Low Prime). <sup>c</sup>Target back fat thickness was 1.2 cm. USDA YG = 2.5 + (2.5 × 12th rib fat thickness in inches) + (0.0038 × hot carcass weight in pounds) + (0.2 × %KPH) - (0.32 × rib eye area in square inches); REA=Rib eye area in centimeters square; KPH = Percent kidney, pelvic & heart fat.

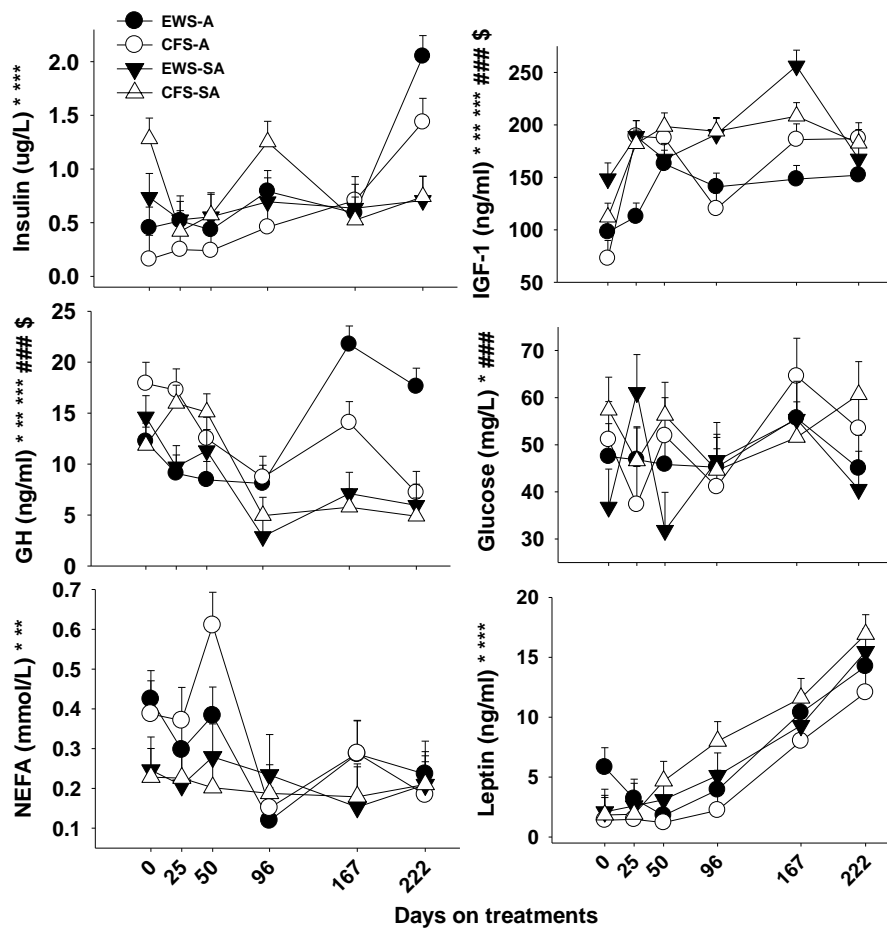
**Figure 2.** Assessment of muscle depth, subcutaneous and intramuscular fat deposition in early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus  $\times$  Simmental (EWS-SA, CFS-SA) steers (n = 7/treatment). All steers were fed a common diet from 96 to 222 days.

**Notes:** Marbling evaluated based on scores: traces (200 – 299), slight (300 - 399), small (400 – 499), modest (500 – 599). Statistical differences due to \*time, \*\*treatment, \*\*\*time  $\times$  treatment, #breed, ###treatment  $\times$  breed and \$ breed  $\times$  treatment  $\times$  time interaction.



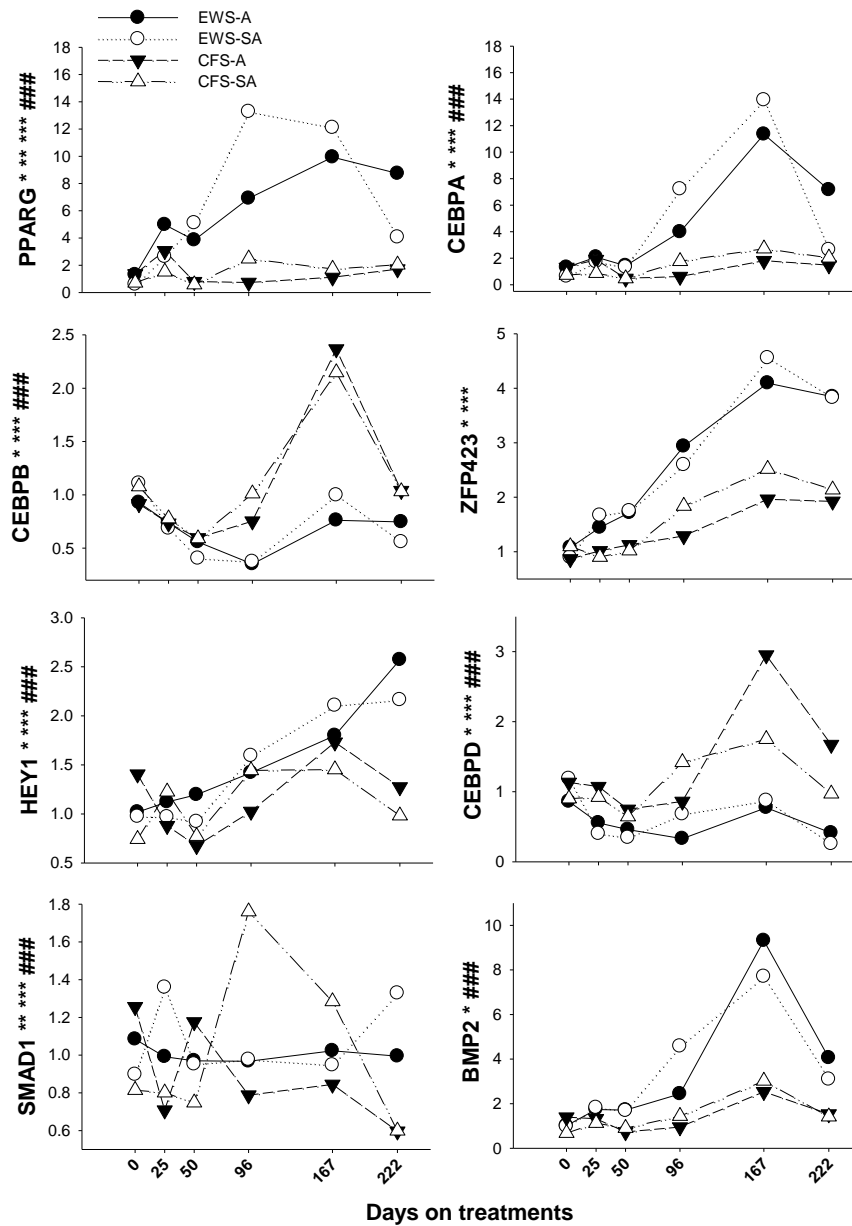
**Figure 3.** Blood serum concentration of insulin, insulin-like growth factor-1(IGF-1) and growth hormone (GH), non-esterified fatty acids (NEFA), glucose and leptin in early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus  $\times$  Simmental (EWS-SA, CFS-SA) steers.

**Notes:** All steers were fed a common diet from 96 to 222 days. Statistical difference due to \*time, \*\*treatment, \*\*\*time  $\times$  treatment, #breed, ##treatment  $\times$  breed, ### time  $\times$  breed and \$ breed  $\times$  treatment  $\times$  time interaction.



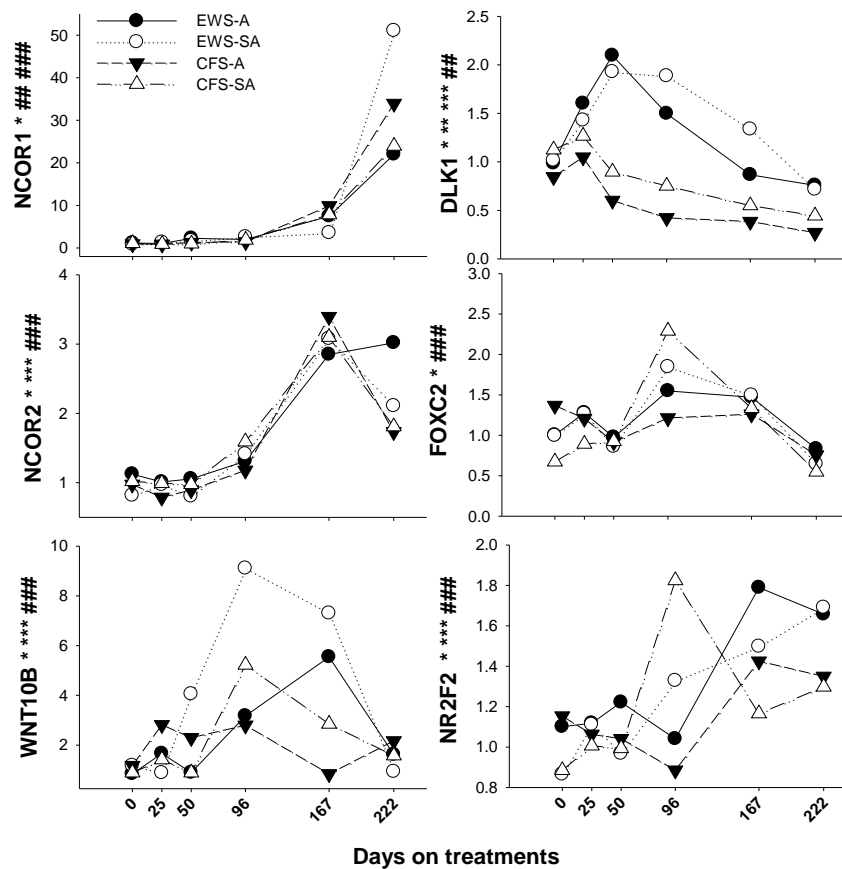
**Figure 4.** Patterns of mRNA expression (fold-change) of adipogenic activators in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus  $\times$  Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

**Notes:** Statistical difference due to \*time, \*\*treatment, \*\*\*time  $\times$  treatment, #breed, and ### breed  $\times$  treatment  $\times$  time interaction. The largest standard error of the mean for *PPARG*, *CEBPA*, *CEBPB*, *ZFP423*, *HEY1*, *CEBPD*, *SMAD1*, and *BMP2* was 1.9, 1.4, 0.3, 0.5, 0.3, 0.3, 0.3, and 0.8, respectively.



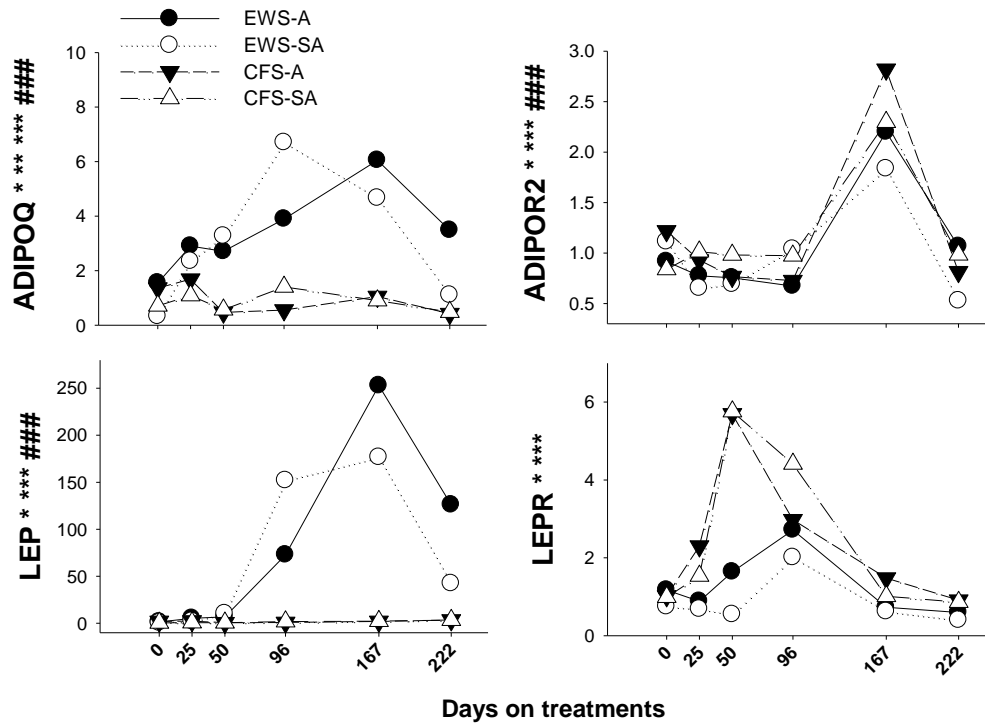
**Figure 5.** Patterns of mRNA expression (fold-change) of adipogenic repressors in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus  $\times$  Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

**Notes:** Statistical difference due to \*time, \*\*treatment, \*\*\*time  $\times$  treatment, ##treatment  $\times$  breed, and ### breed  $\times$  treatment  $\times$  time interaction. The largest standard error of the mean for *NCOR1*, *DLK1*, *NCOR2*, *FOXC2*, *WNT10B*, and *NR2F2* was 4.7, 0.3, 0.3, 0.3, 1.5, and 0.2, respectively.



**Figure 6.** Patterns of mRNA expression (fold-change) of adipokines in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus  $\times$  Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

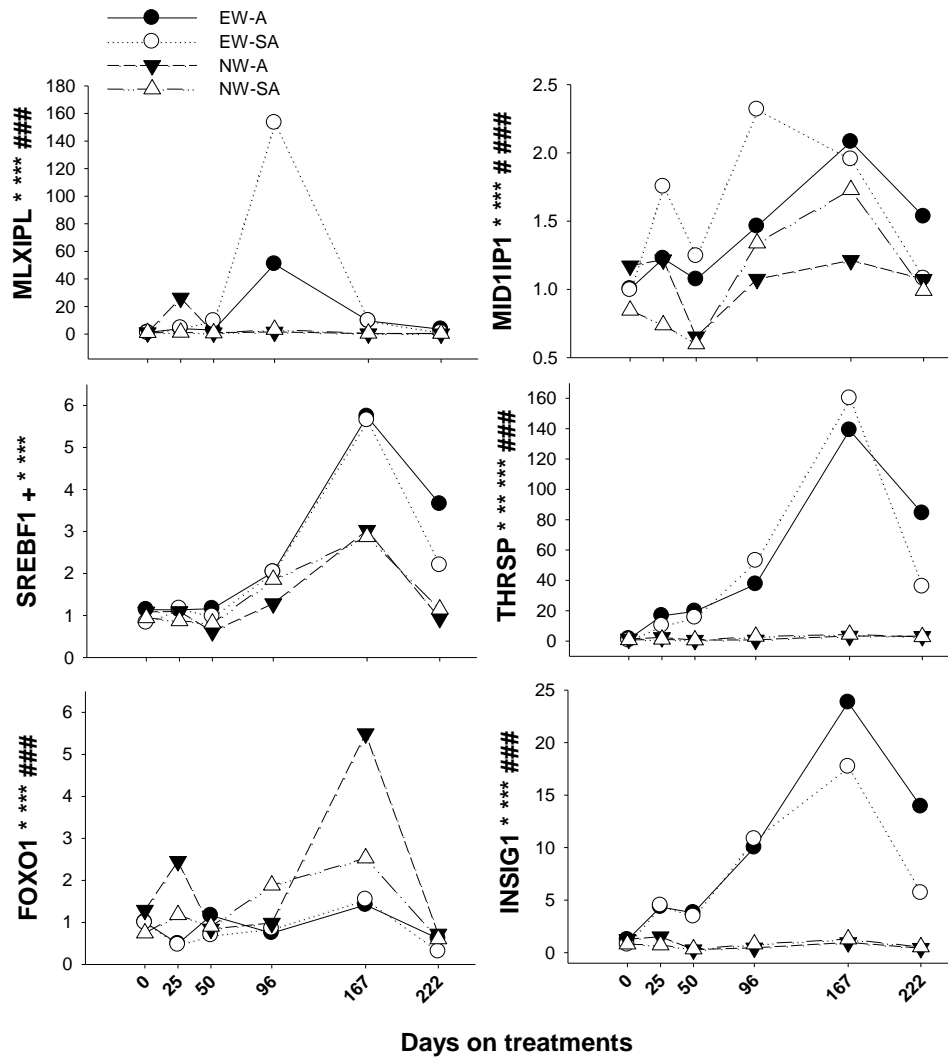
**Notes:** Statistical difference due to \*time, \*\*treatment, \*\*\*time  $\times$  treatment, and ### breed  $\times$  treatment  $\times$  time interaction. The largest standard error of the mean for *ADIPOQ*, *ADIPOR2*, *LEP*, and *LEPR* was 0.7, 0.3, 42, and 1, respectively.





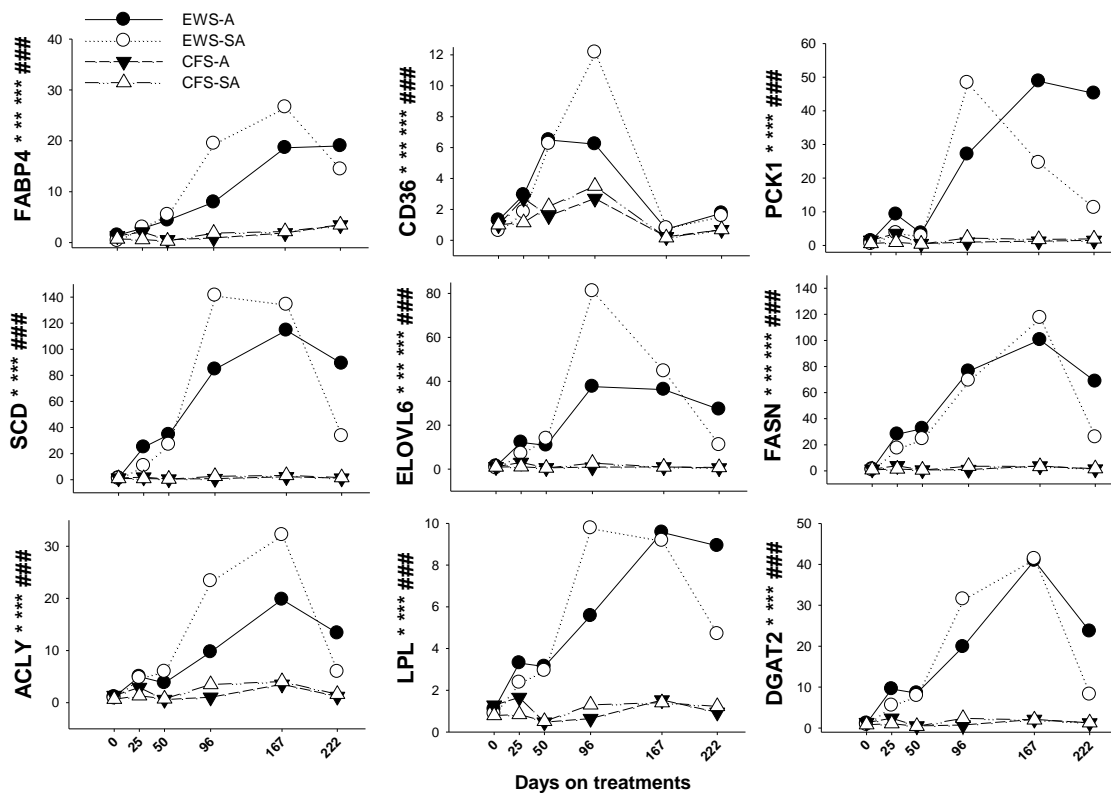
**Figure 7.** Patterns of mRNA expression (fold-change) of lipogenic regulators in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus × Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

**Notes:** Statistical difference due to \*time, \*\*treatment, \*\*\*time × treatment, #breed, and ### breed × treatment × time interaction. The largest standard error of the mean for *MLXIPL*, *MID1IP1*, *SREBF1*, *THRSP*, *FOXO1*, and *INSIG1* was 21, 0.3, 0.6, 19, 0.5, and 3, respectively.



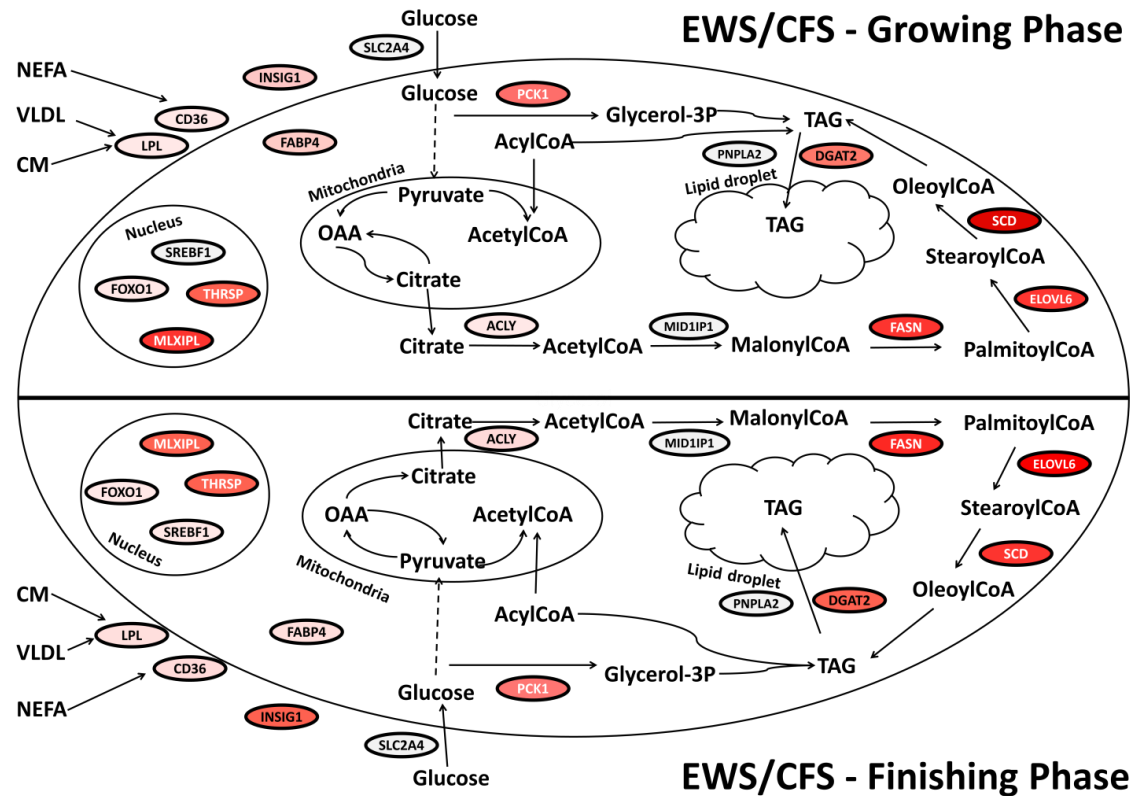
**Figure 8.** Patterns of mRNA expression (fold-change) of lipogenic enzymes in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus  $\times$  Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

**Notes:** Statistical difference due to \*time, \*\*treatment, \*\*\*time  $\times$  treatment, and ### breed  $\times$  treatment  $\times$  time interaction. The largest standard error of the mean for *FABP4*, *CD36*, *PCK1*, *SCD*, *ELOVL6*, *FASN*, *ACLY*, *LPL*, and *DGAT2* was 3.9, 1.3, 8.8, 26, 12, 17, 4, 1.6, and 6.3, respectively.



**Figure 9.** Putative model of transcriptional adaptations during the growing and finishing phase in response to plane of nutrition.

**Notes:** Gene names with differences in color intensity denote expression level: darker red color (high expression) and white color (slightly expressed). Non-esterified fatty acids (NEFA), very low density lipoprotein (VLDL) and chylomicron (CM).



**Table 2.** Performance of Angus and Angus  $\times$  Simmental steers ( $n = 7/\text{treatment}$ ) in response to early weaning plus a high-starch diet (EW), normal weaning plus corn-grain supplement (NW) during the growing phase (0 to 100 d) followed by a common high-starch diet during finishing phase (100 to 300 d). Different letters means significant difference between treatments.  $P$  value  $< 0.05$ .

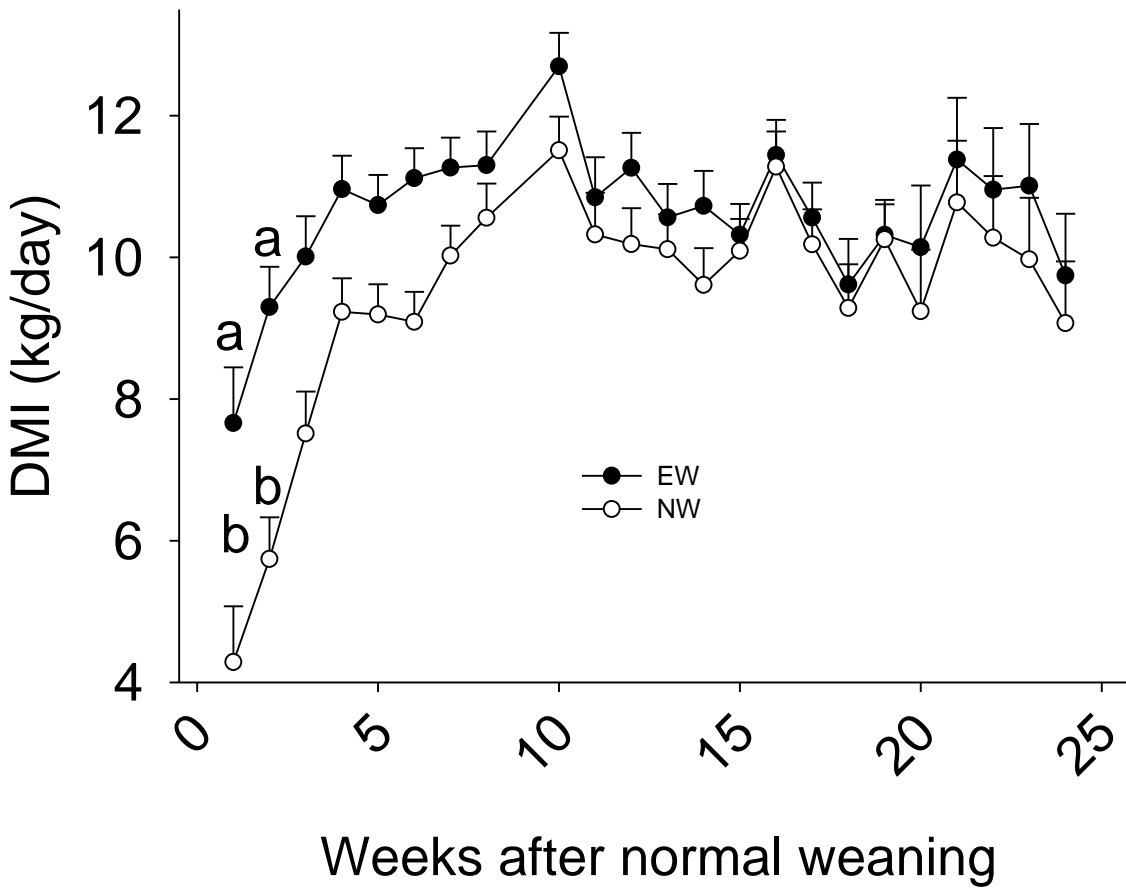
Item	Time	Group				SEM	P value			
		EW-A	EW-SA	NW-A	NW-SA		Trt	Breed	Age	T $\times$ B
Body weight	Birth	33	38	38	40	4.6	0.56	0.18	0.86	0.60
	0 days	183	199	149	127	7.5	<0.01	0.48	<0.01	<0.01
	25 days	228	252	192	160	17.1	0.02	0.61	<0.01	<0.01
	50 days	269	298	230	190	19.1	0.01	0.52	<0.01	<0.01
	100 days	366	402	277	250	21.6	<0.01	0.66	<0.01	0.01
	200 days	464	522	402	391	24.9	0.01	0.09	0.01	0.01
	300 days	484	573	535	545	50.5	0.87	0.07	0.85	0.15
Average daily gain	Birth-0 d	1.053	1.485	2.938	2.798	0.21	<0.01	0.20	<0.01	0.02
	0 – 25 d	1.789	2.071	1.761	1.290	0.61	0.64	0.76	0.27	0.24
	25 – 50 d	1.613	1.823	1.649	1.185	0.38	0.57	0.52	0.17	0.10
	50 – 100 d	2.087	2.260	1.022	1.329	0.40	0.10	0.25	0.89	0.74
	100 – 200 d	1.622	1.894	1.771	1.657	0.31	0.90	0.61	0.30	0.23
	200 – 300 d	0.881	1.492	1.561	1.650	0.27	0.31	0.04	0.30	0.13
	0 – 100 d	1.902	2.115	1.339	1.282	0.21	0.04	0.48	0.12	0.23
	100 – 300 d	1.417	1.677	1.509	1.490	0.23	0.88	0.31	0.34	0.24
	0 – 300 d	1.654	1.873	1.429	1.382	0.18	0.18	0.35	0.11	0.16
Feed Efficiency	100 – 200 d	0.15	0.16	0.23	0.22	0.02	0.06	0.64	0.79	0.57
	200 – 300 d	0.11	0.10	0.14	0.14	0.03	0.33	0.79	0.43	0.84
	100 – 300 d	0.14	0.12	0.16	0.17	0.02	0.13	0.85	0.64	0.27

**Table 3.** Least mean square values for carcass performance traits for early weaned (EW) and normal weaned (NW) Angus (A) and Angus × Simmental (SA) steers.

	EW-A	EW-SA	NW-A	NW-SA	SEM	T	Breed	Age	T×B
REA (cm <sup>2</sup> )	77.45	73.83	71.15	74.39	4.5	0.67	0.95	0.43	0.27
KPH (%)	2.21	2.25	2.19	2.27	0.2	0.99	0.61	0.64	0.83
Camera YG	3.16	3.22	3.31	3.47	0.3	0.68	0.60	0.70	0.82
USDA YG	2.64	2.46	2.54	2.44	0.4	0.91	0.61	0.89	0.89

USDA YG =  $2.5 + (2.5 \times 12\text{th rib fat thickness in inches}) + (0.0038 \times \text{hot carcass weight in pounds}) + (0.2 \times \% \text{KPH}) - (0.32 \times \text{rib eye area in square inches})$ ; REA=Rib eye area in centimeters square; MARBLING Score: 400-499 – Small; 500-599 – Modest; 600-699 – Moderate; Target back fat thickness = 1.1 cm; KPH = Percent kidney, pelvic & heart fat. T= Treatment, T×B= Treatment × Breed interaction. Different letters means significant difference between treatments ( $P < 0.05$ ).

**Figure 10.** Weekly dry matter intake (DMI) in kilograms per day during finishing phases for early weaning with program fed high-concentrate diet (EW), normal weaned with corn-based creep supplement (NW) treatments. Different superscript letters denote significant difference ( $P < 0.05$ ) due to treatment  $\times$  time interactions.



**Table 4.** qPCR performance among the 34 genes measured in skeletal muscle

<b>Gene</b>	<b>Median Ct<sup>1</sup></b>	<b>Median <math>\Delta</math>Ct<sup>2</sup></b>	<b>Slope<sup>3</sup></b>	<b>(R<sup>2</sup>)<sup>4</sup></b>	<b>Efficiency<sup>5</sup></b>	<b>relative mRNA abundance<sup>6</sup></b>
<i>ACLY</i>	25.616	0.966	-3.680	0.990	1.870	0.546
<i>ADIPOQ</i>	22.010	-2.815	-3.346	0.997	1.990	6.939
<i>ADIPOR2</i>	23.982	-0.618	-3.240	0.996	2.035	1.552
<i>ALAS1</i>	22.785	-2.014	-3.100	0.990	2.102	4.465
<i>BMP2</i>	28.426	3.850	-2.990	0.989	2.160	0.052
<i>CD36</i>	26.718	1.926	-2.985	0.985	2.163	0.226
<i>CEBPA</i>	27.906	2.953	-3.505	0.992	1.929	0.144
<i>CEBPB</i>	24.442	-0.347	-2.965	0.982	2.174	1.309
<i>CEBPD</i>	26.520	1.836	-3.845	0.995	1.820	0.333
<i>CLOCK</i>	27.089	2.500	-3.140	0.993	2.082	0.160
<i>DGAT2</i>	22.610	-1.953	-3.590	0.993	1.899	3.500
<i>DLK1</i>	27.609	2.862	-3.390	0.991	1.972	0.143
<i>ELOVL6</i>	27.603	3.090	-4.010	0.990	1.776	0.170
<i>FABP4</i>	25.016	0.239	-3.295	0.993	2.011	0.846
<i>FASN</i>	21.294	-3.215	-3.380	0.994	1.976	8.939
<i>FOXC2</i>	29.517	5.061	-2.780	0.993	2.289	0.015
<i>FOXO1</i>	26.808	2.133	-3.420	0.991	1.961	0.238
<i>HEY1</i>	29.193	4.540	-4.375	0.991	1.693	0.092
<i>INSIG1</i>	25.745	0.828	-3.420	0.993	1.961	0.573
<i>LEP</i>	29.493	4.737	-2.900	0.994	2.212	0.023
<i>LPL</i>	23.245	-1.514	-3.435	0.993	1.955	2.758
<i>MID1IP1</i>	25.876	1.471	-2.950	0.981	2.183	0.317
<i>NCOR1</i>	26.985	2.334	-3.400	0.992	1.968	0.206
<i>NCOR2</i>	26.279	1.709	-3.910	0.991	1.802	0.366
<i>NR2F2</i>	27.121	2.537	-3.535	0.995	1.918	0.192
<i>PCK1</i>	28.024	3.251	-3.355	0.992	1.986	0.107
<i>PPARG</i>	27.552	2.790	-3.465	0.991	1.944	0.157
<i>SCD</i>	20.685	-3.948	-3.350	0.994	1.988	15.081
<i>SLC2A4</i>	23.069	-1.524	-3.480	0.989	1.938	2.740
<i>SMAD1</i>	26.300	1.696	-2.630	0.991	2.400	0.227
<i>SREBF1</i>	25.685	1.385	-3.295	0.992	2.011	0.380
<i>THRSP</i>	24.982	0.439	-3.840	0.991	1.821	0.769
<i>WNT10B</i>	33.641	8.821	-3.445	0.972	1.951	0.003
<i>ZFP423</i>	28.010	3.207	-3.390	0.989	1.972	0.113

**Table 4.** qPCR performance among the 34 genes measured in skeletal muscle

<sup>1</sup> The median is calculated considering all time points and all steers.

<sup>2</sup> The median of  $\Delta Ct$  is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each steer.

<sup>3</sup> Slope of the standard curve.

<sup>4</sup>  $R^2$  stands for the coefficient of determination of the standard curve.

<sup>5</sup> Efficiency is calculated as  $[10^{(-1 / \text{Slope})}]$ .

<sup>6</sup> relative mRNA abundance =  $1 / \text{Efficiency}^{\text{Median } \Delta Ct}$



**Table 5.** Gene ID, GenBank accession number, hybridization position, sequence and amplicon size of primers for *Bos taurus* used to analyze gene expression by qPCR.

Gene ID	Accession #	Gene	Primers <sup>1</sup>	Primers (5'-3') <sup>2</sup>	bp <sup>3</sup>
511135	NM_001037457.1	<i>ACLY</i>	F.2287	GTTCTCCTCCGAGGTCCAGTT	104
		<i>ACLY</i>	R.2390	CAAACACTCCAGCCTCCTTCA	
282865	NM_174742.2	<i>ADIPOQ</i>	F.261	GATCCAGGTCTTGTGGTCCTAA	65
		<i>ADIPOQ</i>	R.325	GAGCGGTATACATAGGCACCTTCTC	
407234	NM_001040499.1	<i>ADIPOR2</i>	F.25	CATCCACCCTCCCAAGAAGAA	120
		<i>ADIPOR2</i>	R.144	AGCTGGCTCTGGAGTCTTGCT	
615037	NM_001099141.1	<i>BMP2</i>	F.724	GGAAACATATGCCCCGAAGCTT	110
		<i>BMP2</i>	R.833	TCTCGTCACAGGGAAGTGGGA	
281052	X91503	<i>CD36</i>	F.743	GTACAGATGCAGCCTCATTTCC	81
		<i>CD36</i>	R.823	TGGACCTGCAAATATCAGAGGA	
281677	NM_176784.2	<i>CEBPA</i>	F.	GCAAAGCCAAGAAGTCCG	46
		<i>CEBPA</i>	R.	GGCTCAGTTGTTCCACCCGCTT	
338319	NM_176788.1	<i>CEBPB</i>	F.	CGACAGTTGCTCCACCTTCT	65
		<i>CEBPB</i>	R.	CTCGCAGGTCAAGAGCAAG	
281678	NM_174267.2	<i>CEBPD</i>	F.1440	AGGAGATGGAAAGGACAGTCACA	100
		<i>CEBPD</i>	R.1539	AACGACTTTATTTATTCGTCCAGGTT	
404129	AJ519787	<i>DGAT2</i>	F.399	ATTCTGCACCGATTGCTGG	85
		<i>DGAT2</i>	R.483	CCTGCCA <u>C</u> TTTCTTGGGT	
281117	BC120429.1	<i>DLK1</i>	F.1163	GGTGGAATTGCTCTCCTTTGT	103
		<i>DLK1</i>	R.1265	TTCATCTGGCAATTACGTTGGTT	
533333	BC148954.1	<i>ELOVL6</i>	F.439	AGCACCCGAAGT <u>A</u> GAGATACAAT	102
		<i>ELOVL6</i>	R.540	TACCAGGAGTACAGAAGCACAGTGA	
281759	DV778074	<i>FABP4</i>	F.402	TGGTGCT <u>G</u> GAATGTGTCATGA	102
		<i>FABP4</i>	R.559	TGGAGTTCGATGCAAACGTC	
281152	CR552737	<i>FASN</i>	F.6383	ACCTCGTGAAGGCTGTGACTCA	92
		<i>FASN</i>	R.6474	TGAGTCGAGGCCAAGGTCTGAA	
507300	XM_867203.3	<i>FOXC2</i>	F.417	CAACGAGTGCTTCGTCAAGGT	103
		<i>FOXC2</i>	R.519	GCTGCCGTTCTCGAACATGT	
506618	XM_583090.4	<i>FOXO1</i>	F.716	GCTCCTGGTGGATGCTCAAT	105
		<i>FOXO1</i>	R.820	GGCCTCGGCTCTTAGCAAAAT	
408005	NM_001001172.2	<i>HEY1</i>	F.179	CATCGAGGTGGAAAAGGAGAGT	100
		<i>HEY1</i>	R.278	TTTCCTGGCCAAGATCTGTGA	

**Table 5.** Continuation

Gene ID	Accession #	Gene	Primers <sup>1</sup>	Primers (5'-3') <sup>2</sup>	bp <sup>3</sup>
511899	CX736793	<i>INSIG1</i>	F.82	CATCGACAGTCACCTTGGAGA	108
		<i>INSIG1</i>	R.189	TCCAGTTTAGCACTAGCGTGGT	
280836	NM_173928.2	<i>LEP</i>	F.2243	CAGGGCACGTCAGCATCTATT	100
		<i>LEP</i>	R.2342	GTCTGCTGTTATGGTCTTAGGTATTTTC	
497205	NM_001012285.2	<i>LEPR</i>	F.1350	ACCCAATGCAATCCAATCACTT	120
		<i>LEPR</i>	R.1469	AAGTGGCAATCTTTGGGTTC	
280843	NM_001075120.1	<i>LPL</i>	F.715	CAGGATAACCGGCTTAGATCCA	115
		<i>LPL</i>	R.829	CCCCCTGGTGAATGTGTGTAA	
615572	NM_001076383.1	<i>MID1IP1</i>	F.1300	GAGGCCACAGTGTTGCTAAGG	102
		<i>MID1IP1</i>	R.1401	AGAGCAAGGAGGAGATTCGAAA	
788534	XM_001255565.2	<i>MLXIPL</i>	F.2529	CCTGACGGATCCAGACTGTATACC	91
		<i>MLXIPL</i>	R.2619	AGGGTCTGTCCAGCACTATAAAGATT	
509768	AK074976	<i>MTG1</i>	F.696	CTTGGAATCCGAGGAGCCA	101
		<i>MTG1</i>	R.796	CCTGGGATCACCAGAGCTGT	
507623	XM_866711.3	<i>NCOR1</i>	F.6292	AGCTCAGACTCTTCCAGTAGCTTATCTT	100
		<i>NCOR1</i>	R.6423	CACGACCTCCGGCTGGTA	
100337057	XM_581058.3	<i>NCOR2</i>	F.30	CGGAGCAGGCCTCATGAC	100
		<i>NCOR2</i>	R.129	TTTACCCATGAGCGCCTTTCT	
281945	EU822301.1	<i>NR2F2</i>	F.1162	CGGATCTTCCAAGAGCAAGTG	103
		<i>NR2F2</i>	R.1264	CACAGGCATCTGAGGTGAACA	
282855	NM_174737	<i>PCK1</i>	F.601	AAGATTGGCATCGAGCTGACA	120
		<i>PCK1</i>	R.720	GTGGAGGCACTTGACGAACTC	
508493	NM_001046005.1	<i>PNPLA2</i>	F.765	CACCAGCATCCAGTTCAACCT	102
		<i>PNPLA2</i>	R.866	CTGTAGCCCTGTTTGCACATCT	
281993	Y12420	<i>PPARG</i>	F.1356	GAGCCCAAGTTCGAGTTTGC	100
		<i>PPARG</i>	R.1455	GGCGGTCTCCACTGAGAATAAT	
619131	BC108231	<i>RPS15A</i>	F.31	GAATGGTGCGCATGAATGTC	101
		<i>RPS15A</i>	R.131	GACTTTGGAGCACGGCCTAA	
280924	AY241933	<i>SCD</i>	F.665	TCCTGTTGTTGTGCTTCATCC	101
		<i>SCD</i>	R.765	GGCATAACGGAATAAGGTGGC	
282359	BC120057	<i>SLC2A4</i>	F.1582	AGGCCTACCTCAGCGGTGA	101
		<i>SLC2A4</i>	R.1682	CACGTTCTCGCCTTTCCAG	
540488	NM_001076223.2	<i>SMAD1</i>	F.1053	GAGCGTGTTGGTGGATGGTT	100
		<i>SMAD1</i>	R.1152	GTTTTCGATGGTGGAATTTGC	
511279	XM_864907.2	<i>SMAD6</i>	F.1806	TCTCCTCGCGACGAGTACAA	100
		<i>SMAD6</i>	R.1905	ATTCACCCGGAGCTGTGATG	

**Table 5.** Continuation

Gene ID	Accession #	Gene	Primers <sup>1</sup>	Primers (5'-3') <sup>2</sup>	bp <sup>3</sup>
539371	CO885324	<i>SREBF1</i>	F.168	AGCCTGGCAATGTGTGAGAAG	84
		<i>SREBF1</i>	R.251	GGCCTTGTCAATGGAGCTGT	
515940	AY656814	<i>THRSP</i>	F.266	CCGAGGGAGCTGAGACTGAA	101
		<i>THRSP</i>	R.366	AGCGAAGTGCAGGTGGAAC	
525680	BQ676558	<i>UXT</i>	F.323	TGTGGCCCTTGGATATGGTT	101
		<i>UXT</i>	R.423	GGTTGTCGCTGAGCTCTGTG	
539337	XM_586498.4	<i>WNT10B</i>	F.25	TCAAGCCAGGCAAGTTCGAT	100
		<i>WNT10B</i>	R.124	CATGTCTCTTTGCCTCAGTTTCC	
534353	NM_001098081.1	<i>YYI</i>	F.1118	ACGACACCAACTGGTCCATACTG	100
		<i>YYI</i>	R.1217	CACATGTGTGCGCAAATTGA	
508025	NM_001101893.1	<i>ZFP423</i>	F.4490	CGTGATGTGATTGCTTGGCTATT	110
		<i>ZFP423</i>	R.4599	CATAGTTTAAATCACTGTCCGGTGAA	

<sup>1</sup> Primer direction (F – forward; R – reverse) and hybridization position on the sequence.

<sup>2</sup> Exon-exon junctions are underlined.

<sup>3</sup> Amplicon size in base pair (bp).

**Table 6.** Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (<http://www.ncbi.nlm.nih.gov>) are shown. Similar information for *ADIPOQ* and *FABP4* was reported previously

(Bionaz and Loor, 2007a; Piantoni et al., 2008).

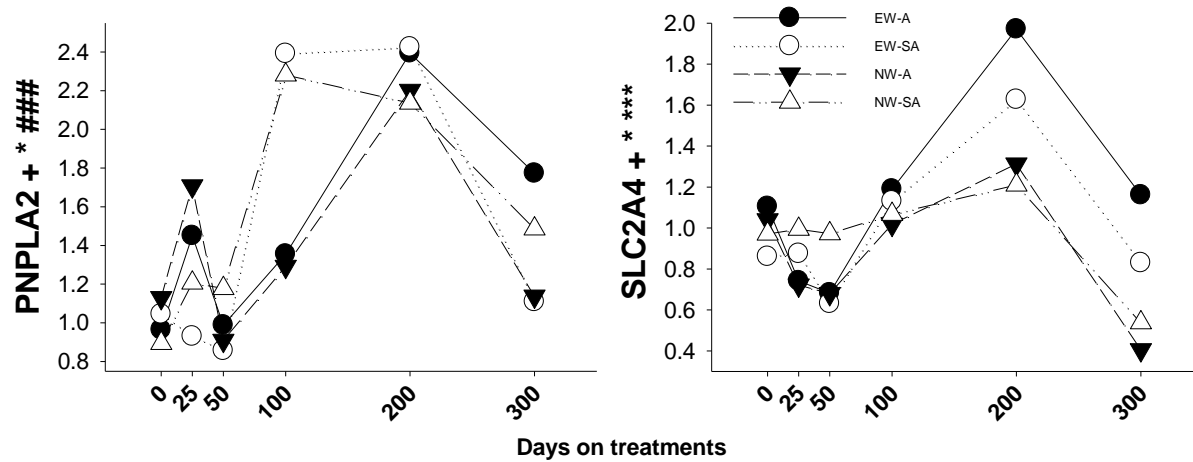
Gene	Sequence
<i>ACLY</i>	CTGGACTGTGCCACCAGGCTTCTGAAACCTGCAGTTGCCAAGAACCAGGCCTTGAAGGAGGCTGGAGTGCTTTGGA
<i>ADIPOR2</i>	GGGAAAACCTTTGTTCGAAAGCCCTTGGGGTTCCCATGAACGAGACAGAGGAAAACCGGCTGGAGCACAGCAAGACTCCAGAGCCAGCTAA
<i>BMP2</i>	AATTATATTNATGAAATTTATAAAACCTGGCCACAGCTAACTCCAAGGTTTCCCTGTGACGAGAAAAA
<i>CEBPA</i>	ATATCGGGTTCGGCGGAGCGCAACAACATCGCGGTGCGCAAGGAGCCGGGACAAGGCCAAGCA GCGCAACGTTGGAGACGCAGCAGAAGGTGCTGG
	AGCTGACCAGTGACAATGACCGCTGCGCAAGCGGGTGAACAACCTGAGCCA
<i>CEBPB</i>	GCTGCGTCTCCAGGTTGCGCATCTTGGCCTTGTGCGGCTCTTGGCACC GCGATGTTGTTGCGCTCCCGCCGGATCTTGTACTCGTCGCTGTG
	CTTGTCCACCGTCTTCTTGGCCTTGCTCTTGACCTGCGAGA
<i>CEBPD</i>	ACTTACGTGCTCCTGTGCCGTA CTGTGTATATTTCAAAGATTAACCTGGACGAATAAATAAAGTCGTTTAA
<i>DGAT2</i>	GTCGTGCGTCCTTCTTCCCGTGC GTGTTGACTGGAACACACCCCCAAGAAAGGTGGGCAGGAGGTCACAGTAATA
<i>DLK1</i>	AGCCGTTGGACAGCCATCGCCTCCCTCCTGCGCTTTGCGCCTGCCGGTGTGTGTGCAACCCAACGTAATTGCCAGAATGAAAA
<i>ELOVL6</i>	CAGAAGCTGATCTTCTGCACTGGTACCACCACATCACTGTGCTTCTGTACTCCTGGTAAA
<i>FOXC2</i>	CCGGCAAGACGGGCAGGGCAGCTACTGGACCTGGACCCGGACTCCTAGGAACATTGGTTTCGAGAACGGCAGCAA
<i>FOXO1</i>	GAGGAGAGTGATCCCCCGGAGAGAGCCGCATCCATGGACAACAACAGTAAATTTGCTAAGAGCCGAGGCCAAA
<i>HEY1</i>	GGGTAAGGGACTGTGGATTGCTTTAGGGTTCCAGTGTCCCCAACTACAATCTTCACAGATCTTGGGCCAGGGAAAAA
<i>LEP</i>	GGCCCCCTTGTAGCGGGATAGCTAGAATTTAATAAGAAGGAAAAATACCTTAAGACCCAT
<i>LEPR</i>	GGGAGCATGGAGCAGTAGGATCATAGGAGCAGCCGTGTA CTGTTCTGGATGGTTCCGTCTATTATCATCTGGTATCTGAACCCAAAGATTGGCCACTTTAA
<i>LPL</i>	GCAACTCGGGAGCAGAAGCTCCAAGTCGCCTTTCTCCTGATGATGCGGATTTTGTAGACGTTTACACACATTACACAGGGGGA
<i>MLXIPL</i>	CGAGCCGGGCGGTACAGAGGCACCCCTTGGCAATCTTTATAGTGTGGACAGACCCCTTGA
<i>NCOR1</i>	CCCTGGCTTAACCCCGTGGGCGATGCTTATTTT GAGGGTGATAAGCCCCGCCAGCTTCTTCCCGCACC GCCCCAGGAGAAGGCTTGAACGGCCTTACCAGCCGGAAGGTCGTGGA
<i>NCOR2</i>	GCACGGGGTCAGGACAGTCCAGGCCACCAACCAGTGGGGCTGGAGGGCTATTATTAGAAAGGGGCGCTCATGGGTAAAA
<i>PCK1</i>	GCCATGTGTACAGCAGTCGCATCATGACGAGGATGGGCACCAGCGTCCTGGAAGCGTGGGGGACGGCGAGTTCGTCAAGTGCCTCCACAAA
<i>PNPLA2</i>	GCCTCGCCTTCAGGCCTGTTCGCCCCGAGCCCTGGTNC TTCGAGAGATGTGCAAACAGGGCTACAGAACCC
<i>SMAD1</i>	CCCACAGACCGCTTCGCTCGGGGCTGCTCTCCACGTTAACCCGAAATTTCCACCATCGGAAAACTCACCCTTTG
<i>SMAD6</i>	GTGTATGTGATTCAATTGTCTTTACACTGAAACAGGAAGGGCCGGCCAAC TCCCTTCATCACAGCCTCCGGGGTGGAATTAGAACTTT
<i>SREBF1</i>	GCGTCAGTCGTCGTCCCCACGTGTCGTGCGTAGTAATGGGGCTGCTGGGTCTCTTCTCCTTGGCACTTCTCTTTGTAAA
<i>WNT10B</i>	CTCTTTTGGTCAAGCACCTTCCAGTCACTCAGCTATCGGTGGGGGAAACTGAGGCAAAGAGACATGATTTTTG
<i>YY1</i>	GGACCTTCGGTCCGCTTCAAGGCTGCGGGGAAACGCTTTTCACTGGACTTCAATTTGCGCACACATGTGA
<i>ZFP423</i>	CGTACACCTTTTATTTTATTTAAGACCTAATGCCTTTGATTGGGATTTGCCAGTTCACCCGGACAGTGATTA AAACTATTGGA

**Table 7.** Sequencing results of genes using BLASTN from NCBI

against nucleotide collection (nr / nt) with total score.

<b>Gene</b>	<b>Best hit in NCBI</b>
<i>ACLY</i>	<i>Bos taurus</i> ATP citrate lyase ( <i>ACLY</i> ), mRNA >gb/BC108138.1/ <i>Bos taurus</i> A
<i>ADIPOQ</i>	<i>Bos taurus</i> adiponectin, <i>CIQ</i> and collagen domain containing ( <i>ADIPOQ</i> ), mRNA >gb/AF269230.1/
<i>BMP2</i>	<i>Bos taurus</i> bone morphogenetic protein 2 ( <i>BMP2</i> ), mRNA >gb/BC142129.1/
<i>CEBPA</i>	<i>Bos taurus</i> CCAAT/enhancer binding protein ( <i>C/EBP</i> ), alpha
<i>CEBPB</i>	<i>Bos taurus</i> CCAAT/enhancer binding protein ( <i>C/EBP</i> ), beta ( <i>CEBPB</i> ), mRNA
<i>CEBPD</i>	<i>Bos taurus</i> CCAAT/enhancer binding protein ( <i>C/EBP</i> ), delta ( <i>CEBPD</i> ), mRNA >gb/BC133581.1/
<i>DGAT2</i>	<i>Bos taurus</i> diacylglycerol O-acyltransferase 2 ( <i>DGAT2</i> ), mRNA >gb/BT030532.1/
<i>DLK1</i>	<i>Bos taurus</i> delta-like 1 homolog ( <i>Drosophila</i> ), mRNA (cDNA clone MGC:143417 IMAGE:8287009)
<i>ELOVL6</i>	<i>Bos taurus</i> ELOVL fatty acid elongase 6 ( <i>ELOVL6</i> ), mRNA >gb/BC148954.1/
<i>FOXC2</i>	<i>Bos taurus</i> forkhead box C2 ( <i>MFH-1</i> , mesenchyme forkhead 1) ( <i>FOXC2</i> ), mRNA
<i>FOXO1</i>	PREDICTED: <i>Bos taurus</i> forkhead box O1 ( <i>FOXO1</i> ), mRNA
<i>HEY1</i>	<i>Bos taurus</i> hairy/enhancer-of-split related with YRPW motif 1 ( <i>HEY1</i> ), mRNA >gb/BC112574.1/
<i>LEP</i>	<i>Bos taurus</i> leptin ( <i>LEP</i> ), mRNA >gb/BT020625.1/ <i>Bos taurus</i> leptin (obesity homolog, mouse) ( <i>LEP</i> )
<i>LEPR</i>	<i>Bos taurus</i> leptin receptor ( <i>LEPR</i> ), mRNA >dbj/AB199589.1/
<i>LPL</i>	<i>Bos taurus</i> lipoprotein lipase ( <i>LPL</i> ), mRNA >gb/BC118091.1/
<i>MID1IP1</i>	<i>Bos taurus</i> MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish)) ( <i>MID1IP1</i> )
<i>MLXIPL</i>	<i>Bos taurus</i> MLX interacting protein-like ( <i>MLXIPL</i> ), mRNA
<i>NCOR1</i>	<i>Bos taurus</i> nuclear receptor corepressor 1 ( <i>NCOR1</i> ), mRNA
<i>NCOR2</i>	PREDICTED: <i>Bos taurus</i> nuclear receptor corepressor 2 ( <i>NCOR2</i> ), mRNA
<i>PCK1</i>	<i>Bos taurus</i> phosphoenolpyruvate carboxykinase 1 (soluble) ( <i>PCK1</i> ), mRNA >gb/AY145503.1/
<i>PNPLA2</i>	<i>Bos taurus</i> adipose triglyceride lipase ( <i>ATGL</i> ) mRNA, complete cds
<i>SMAD1</i>	<i>Bos taurus</i> SMAD family member 1, mRNA (cDNA clone MGC:138917 IMAGE:8091343)
<i>SMAD6</i>	PREDICTED: <i>Bos taurus</i> Smad6-like ( <i>SMAD6</i> ), mRNA
<i>WNT10B</i>	PREDICTED: <i>Bos taurus</i> wingless-type MMTV integration site family, member 10B-like ( <i>WNT10B</i> )
<i>YY1</i>	<i>Bos taurus</i> YY1 transcription factor ( <i>YY1</i> ), mRNA >gb/BC140676.1/
<i>ZFP423</i>	<i>Bos taurus</i> zinc finger protein 423 ( <i>ZNF423</i> ), mRNA >gb/BC148946.1/

**Figure 11.** Patterns of mRNA expression (Fold change) of lipogenic enzymes in Longissimus tissue from early weaned (EW) and normal weaned (NW) Angus (EW-A, NW-A) and Angus  $\times$  Simmental (NW-SA, NW, SA) steers. All steers were fed a common diet from 100 to 300 days. Statistical difference due to \*time, \*\*treatment, \*\*\*time  $\times$  treatment, and ### time  $\times$  treatment  $\times$  breed.



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### **Chapter 3: Maternal Plane of Nutrition during Late Gestation and Weaning Age Alter Offspring Transcriptome Profiles of Longissimus Muscle In Angus × Simmental Steers**

#### **ABSTRACT**

We evaluated if a higher plane of maternal nutrition during late gestation and weaning age alter the offspring's *Longissimus* muscle (LM) transcriptome. A microarray analysis was performed in LM samples of early (EW) and normal weaned (NW) Angus × Simmental calves born to grazing cows receiving no supplement (low plane of nutrition (LPN)), or supplemented with 2.3 kg of dried distiller's grains with solubles and soyhulls (70% DDGS/30% soyhulls) (medium plane of nutrition (MPN)) during the last 90 days of gestation. Biopsies were harvested at 78, 187 and 354 days of age. Microarray analysis was performed using the Agilent Bovine Oligo Microarray v2 platform. Bioinformatics analysis highlighted that offspring transcriptome did not respond markedly to cow plane of nutrition, resulting in only 13 differentially expressed genes (DEG). However, weaning age and a high-starch diet (EW steers) strongly impacted the transcriptome (DEG = 167), especially causing the activation of the lipogenic program. In addition, between 78 and 187 days of age, EW steers had an activation of the innate immune system due presumably to macrophage infiltration in intramuscular fat. Between 187 and 354 days of age (i.e. the fattening phase), NW steers had an activation of the lipogenic transcriptome machinery, while EW steers had a clear inhibition of the gene transcription machinery. The latter appears to have occurred through the epigenetic control of histone acetylases, which were down-regulated. Higher cow plane of nutrition alone affected 35 DEG in the LM of steers that underscored the presence of a mechanism of macrophage infiltration likely originating from localized oxidative stress as a result of increased levels of hypoplasia and hypertrophy in LM. In conclusion, transcriptome analysis suggests that a macrophage infiltration-related impairment of

lipogenesis was produced in LPN steers (especially in EW-LPN steers that had low marbling scores) as a consequence of weaning age and cow plane of nutrition.

**Keywords:** beef, fetal programming, weaning, transcriptome, microarray

## Introduction

The prenatal periods during which the organism is susceptible to environmental stimuli leading to programming are the embryonic phase, the mid-gestation period (organogenesis), and late gestation (rapid growth). In a review on fetal programming and skeletal muscle development in the ruminant<sup>6</sup> it was concluded that adipogenesis is initiated during mid-gestation. At this time, a pool of undifferentiated mesenchymal stem cells is present. From this pool, either myocytes or adipocytes are able to differentiate from committed mesenchymal cells to become skeletal muscle or adipose tissue<sup>66</sup>. The increase in number of stem cells throughout middle-to-late gestation led to the hypothesis that nutritional management has the potential to be more effective during the prenatal period rather than the postnatal portion of an animal's life<sup>6</sup>. However, it remains to be determined if plane of nutrition during late-pregnancy (last 90 days) can elicit carryover effects acquired through programming.

The effects of maternal nutrition on fetal growth and its carry-over effects on offspring growth and development were reviewed recently<sup>6</sup>. Maternal nutrition seems to elicit different outcomes in the offspring depending on the gestational stage during which treatments begin. For example, protein supplementation of grazing cows during late gestation in the winter enhanced feedlot performance and carcass quality of the offspring<sup>26</sup>. There are several maternal-nutrition studies utilizing real-time RT-PCR to evaluate specific target genes in adipose tissue or longissimus muscle (LM) of beef<sup>58</sup>, lamb<sup>7</sup> and sheep<sup>15</sup> offspring. Despite these efforts, to the best of our knowledge, there

are no published studies of whole-transcriptome profiles in LM of offspring from mothers fed high or low planes of nutrition during late-pregnancy.

Our general hypothesis was that a high-plane of nutrition of the cow during late gestation would result in early activation of genes associated with myogenesis, adipogenesis, lipogenesis and the synthesis of adipokines in the offspring's skeletal muscle. Furthermore, the overnutrition of the pregnant mother also would elicit changes associated with epigenetic regulation of gene expression. The objectives of this study were to assess the effect of maternal plane of nutrition and early weaning to a high-starch diet on the skeletal muscle transcriptome of the offspring.

## **Materials and Methods**

A subset of 20 Angus × Simmental beef cows from the University of Illinois Dixon Springs Agriculture Center (DSAC) in Simpson, IL, were selected from a group of animals utilized in a parallel study<sup>54</sup>. Main effects evaluated were maternal plane of nutrition during late gestation and postnatal management of the offspring. Three months prior to the projected parturition date cows were assigned to treatments (low or medium plane of nutrition) in a split-plot design. Low plane of nutrition (LPN) was achieved by grazing endophyte-infected tall fescue/red clover pastures during July, August, and September with no supplement. Medium plane of nutrition cow diet (MPN) was achieved by grazing endophyte-infected tall fescue/red clover pastures supplemented with 2.3 kg of dried distiller's grains with solubles and soyhulls (70% DDGS/30% soyhulls).

Composition of the diet (DM basis) fed to early weaned (EW) steers upon arrival

to the feedlot and prior to normal weaning (NW), and the feedlot diet fed to EW and NW steers after normal weaning consisted on modified wet distiller's grains with solubles (45%), dry whole corn (25%), corn husklage (20%), ground corn (7.3%), limestone (2.5%), trace mineral salt (0.1%), Rumensin 90 (198 g monensin/kg Rumensin 90; Elanco Animal Health, Greenfield, IN) (0.018%), Tylosin 40 (88 g tylosin/kg Tylosin 40; Elanco Animal Health, Greenfield, IN) (0.012%), soybean oil (0.076%). Trace Mineral Salt contained 8.5% Ca (as  $\text{CaCO}_3$ ), 5% Mg (as  $\text{MgO}$  and  $\text{MgSO}_4$ ), 7.6% K (as  $\text{KCl}_2$ ), 6.7% Cl (as  $\text{KCl}_2$ ) 10% S (as  $\text{S}_8$ , prilled), 0.5% Cu (as  $\text{CuSO}_4$  and Availa-4 (Zinpro Performance Minerals; Zinpro Corp, Eden Prairie, MN)), 2% Fe (as  $\text{FeSO}_4$ ), 3% Mn (as  $\text{MnSO}_4$  and Availa-4), 3% Zn (as  $\text{ZnSO}_4$  and Availa-4), 278 ppm Co (as Availa-4), 250 ppm I (as  $\text{Ca}(\text{IO}_3)_2$ ), 150 Se ( $\text{Na}_2\text{SeO}_3$ ), 2,205 KIU/kg vitamin A (as retinyl acetate), 662.5 KIU/kg vitamin D (as cholecalciferol), 22,047.5 IU/kg vitamin E (as DL- $\alpha$ -tocopheryl acetate), and less than 1% CP, fat, crude fiber, and salt. Analyzed nutrient content of the EW diet was CP %, 17.3, NDF %, 23.9, ADF %, 14.1, and crude fat %, 5.3. Cow supplementation was initiated at  $103 \pm 11$  days prepartum while on pasture and it was halted at the midpoint of parturition ( $2 \pm 11$  days postpartum). More information about cow supplementation is reported elsewhere<sup>54</sup>.

Angus  $\times$  Simmental steer calves were randomly assigned to early or normal weaning (EW or NW) treatments within each gestational treatment. This allowed for 10 animals for each postnatal treatment and 5 animals of each of the interactions of gestational  $\times$  postnatal treatments. At  $78 \pm 2$  days postpartum, EW offspring were weaned, transported to Urbana Beef Unit, and adapted to a high-starch diet until they had *ad libitum* consumption. At  $187 \pm 2$  days postpartum, NW offspring were weaned

and transported to Urbana Beef Unit. All offspring were co-mingled among treatments. LM biopsies were sampled from a subset of 5 animals per gestational  $\times$  postnatal treatment at ~78 days of age, ~187 days, and during the last week prior to harvest (~354 days). Blood was collected from the jugular vein at 78, 187 and 296 days of age to isolate serum for insulin (Bovine Insulin ELISA kit, Cat No. 10–1201–01, Mercodia AB, Uppsala, Sweden), glucose (Diagnostics Laboratory, College of Veterinary Medicine, University of Illinois) and adiponectin (Millipore, LA, USA). After normal weaning, all offspring were placed on a common, corn grain-based high-starch finishing diet that is typical of industry management (CP %, 18.1, NDF %, 25.3, ADF %, 14.3, crude fat %, 5.1). All the offspring in the study were harvested at a commercial packing plant when they reached the selected end point target back fat thickness of 1.1 cm. Reported final body weight (BW) was calculated from hot carcass weight using a 62% dressing percentage.

Selection of steer progeny for biopsy was performed based on 3 criteria: first, offspring for biopsy were selected based on their dam's performance. Only offspring from cows whose BW and BW change during late gestation was within  $\frac{1}{2}$  of a standard deviation on either side of the mean (LPN or MPN) were considered for biopsy. Selecting based on cow BW and BW change ensured that only calves from cows that were representative of their treatment were utilized for transcriptomics. The final selection of steers for biopsy was based on steer BW. Only steers whose BW was within  $\frac{1}{2}$  of a standard deviation on either side of the mean were utilized. This selection strategy minimized the effects of variation in dam's milk production, which was not significantly different between LPN and MPN treatments<sup>54</sup>.

Transcriptomics was performed with a transcriptome-wide bovine microarray (Agilent-015354 Bovine Oligo Microarray-4x44K) that contains 21,475 unique genes and transcripts of *Bos Taurus*, with two probes per gene. The methods used for hybridization and scanning were according to manufacturer's protocols and Loores *et al*<sup>31</sup>. 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 GSE65560.

### ***Data Mining***

The entire microarray data set with associated statistical *P*-values were imported into Ingenuity Pathways Analysis ® (IPA, [www.ingenuity.com](http://www.ingenuity.com)) in order to examine the number of activated and inhibited differentially expressed genes (DEG). Entrez Gene IDs were used to identify individual sequences.

### ***Statistical analysis***

Data from the microarray analysis were normalized for dye and microarray effects (i.e., Lowess normalization and array centering) and used for statistical analysis. The MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. Fixed effects were treatment (EW, NW), diet (LPN, MPN), time (78, 187, and 354 days of age), first, second and third order interactions between diet, time and treatment, and dye (Cy3, Cy5) and random effects included steer and microarray. Raw *P* values were adjusted using Benjamini and Hochberg's false discovery rate (FDR).

The statistical model used was:  $Y_{ijklm} = \mu + T_i + D_j + W_k + S_l + (T \times D)_{ij} + (D \times W)_{jk} + (T \times W)_{ik} + (D \times T \times W)_{ijk} + \epsilon_{ijklm}$ ; where,  $Y_{ijklm}$  is the background-adjusted



normalized fold change value;  $\mu$  is the overall mean;  $T_i$  is the fixed effect of time (3 levels);  $D_j$  is the fixed effect of cow plane of nutrition (2 levels);  $W_k$  is the fixed effect of wean (2 levels);  $S_l$  is the random effect of steer nested within treatment;  $T \times D$ ,  $D \times W$ ,  $T \times W$  are the interactions of time by diet, diet by wean and time by wean, respectively;  $D \times T \times W$  is the interaction of third order for the main effects; and  $\varepsilon_{ijklm}$  is the random error ( $0, \sigma_e^2$ ) associated with  $Y_{ijklm}$ . All means were compared using the PDIF statement of SAS (SAS Institute, Inc., Cary, NC, USA). Statistical significance was declared at  $P \leq 0.05$  and  $FDR \leq 0.10$ . Animal feedlot performance, carcass quality parameters, ultrasound and blood data were also analyzed using the MIXED procedure of SAS, and treatment was the fixed effect in the statistical model. The random effect in all models was steer within treatment.

#### ***Dynamic impact approach (DIA)***

Bioinformatics analysis of microarray data was performed using DIA<sup>4</sup> and information from the freely-available online databases Kyoto Encyclopedia of Genes and Genomes (KEGG) and Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 databases. A list of gene identifiers (Entrez Gene IDs) was uploaded all at once to extract and summarize functional annotations associated with groups of genes or with each individual gene. The significance value associated with biological processes and pathways is a measure of the likelihood that the distribution of DEG in these pathways and biological processes is due to chance. The significance is expressed as a  $P$ -value, which is calculated using the right-tailed Fisher's Exact Test and adjusted using FDR. Details of the DIA approach and its validation have been reported

previously<sup>4</sup>. The interpretation of the bioinformatics analysis was performed following the same approach as our previous study<sup>34</sup>.

## **Results**

### ***Animal Performance***

Performance data for the entire group of animals on study are reported elsewhere<sup>54</sup>. Feedlot performance of EW and NW steers used for transcriptomics is presented in Table 8. Only initial BW at the time steers entered the feedlot was significant ( $P < 0.01$ ) with EW steers being heavier as compared with NW steers. Dry matter intake (DMI) was higher for MPN as compared with LPN steers ( $P = 0.03$ ). Ultrasound at the time of EW (78 days of age) revealed no significant differences ( $P > 0.05$ ) between treatments (i.e. all possible combinations between weaning age and cow plane of nutrition) for marbling and back fat thickness (Table 8).

Hot carcass weight did not differ ( $P > 0.10$ ) (Table 8). Calculated yield grade had a weaning effect ( $P = 0.03$ ) with lower values for NW as compared with EW steers. Marbling score was greater ( $P = 0.07$ ) for EW-MPN steers. Lastly, there was a weaning effect ( $P = 0.01$ ) associated with EW steers reaching the back-fat thickness target end-point earlier than NW steers (Table 8).

Serum glucose concentration had a significant time effect ( $P < 0.05$ ) with lower values at 296 days of age. Adiponectin concentration had a significant cow plane of nutrition (diet), time, and diet  $\times$  time interaction ( $P < 0.05$ ) with increasing concentrations between 78 and 187 days of age and a switch to decreasing concentrations after 187 days of age. Insulin concentration was affected by weaning age,

time, and weaning  $\times$  time ( $P < 0.05$ ) with a noticeable increase in concentration between 187 and 296 days of age (Figure 12).

### ***Microarray analysis***

At an FDR  $< 0.10$  (raw  $P$  value  $< 0.05$ ), there were 13 differentially expressed genes (DEG) for the time  $\times$  wean  $\times$  diet interaction, 43 DEG for the time  $\times$  diet, 31 DEG for the wean  $\times$  diet interaction, and 167 DEG for the wean  $\times$  time interaction. In addition, a total of 35 DEG were affected by the cow plane of nutrition, 145 DEG were affected by weaning time and 7,639 DEG were affected by time.

### ***Cow plane of nutrition, weaning age and time effect***

There were only 13 genes in the offspring LM muscle affected (FDR  $P < 0.10$ , raw  $P < 0.05$ ) by the interaction of cow plane of nutrition during the last 90 days of gestation, weaning age and time. A brief description of those 13 DEG can be found in Table 9.

### ***Cow plane of nutrition effect***

There were 35 genes in the offspring LM muscle affected (FDR  $P < 0.10$ , raw  $P < 0.05$ ) by the cow plane of nutrition during the last 90 days of gestation. A brief description of those 35 DEG can be found in Table 10.

### ***Weaning age by time effect***

When we focus only on the 167 DEG due to time  $\times$  treatment interaction, the highest number of DEG, 91 down-regulated and 44 up regulated ( $P$  value  $< 0.05$ ; FDR  $< 0.10$ ), were detected in EW steers between 78 and 187 days of age (Figure 13). During the same time-frame, NW steers had the lowest DEG with 53 downregulated and 4

upregulated genes ( $P$  value  $< 0.05$ ; FDR  $< 0.10$ ). It is noteworthy that the number of DEG in NW steers increased markedly between 187 and 354 days of age. Thus, when comparing the responses between 78 and 354 day of age, EW and NW steers had a similar number of DEG (Figure 13).

The DIA analysis was performed with DEG ( $n = 167$ ) at a  $P$  value = 0.05 and an FDR = 0.10. Table 4 contains the summary of KEGG pathways results from DIA. The top-three most impacted canonical pathways are reported in Table 5. For biological processes, only those with an impact value higher than 50% of the maximum total impact value for each time comparison for EW and NW steers are discussed (Table 13).

The DIA analysis revealed that fatty acids biosynthetic process, biosynthesis of unsaturated fatty acids, and insulin signaling were highly-activated in EW steers between 78 and 187 days of age (Table 12). Analyses further revealed that activation of these pathways was namely due to upregulation of *FASN* (lipogenic enzyme) and *PCK2* (involved in glyceroneogenesis) (Table 12). In contrast to EW steers, between 78 and 187 days of age NW steers had a higher impact with no apparent direction of the cytochrome P450-related pathways and Glutathione Metabolism.

Between 187 and 354 days of age in EW steers there was a significant negative impact on Pyrimidine metabolism (Nucleotide Metabolism Subcategory within the Metabolism KEGG Category), RNA polymerase (Transcription Subcategory within the Genetic Information Processing KEGG Category) and Cytosolic DNA sensing pathway (Immune System Subcategory within the Organismal System KEGG Category) (Table 12). Polymerase (RNA) III (DNA directed) polypeptide A, 155kDa (*POLR3A*) was the

only DEG that could explain the inhibition of these pathways. In the case of NW steers between 187 and 354 days of age, Biosynthesis of unsaturated fatty acids, Fatty acid biosynthesis and Glycerophospholipid metabolism (all within the KEGG Lipid Metabolism Subcategory) were the most-impacted and activated pathways (Table 12). Between 78 and 354 days of age, Fatty acid biosynthesis and Biosynthesis of unsaturated fatty acids were the most-impacted and activated pathways in both EW and NW, but the activation of these pathways took place at different time points (Table 12).

When we focus on the biological processes (BP), between 78 and 187 days of age in EW steers there was a clear activation of the BP related to adipogenesis and lipogenesis. Biosynthetic process and fatty acid biosynthetic process had the highest impact, with a lower impact detected for fatty acid elongation of saturated fatty acids, long-chain fatty acid biosynthetic process and regulation of triglyceride biosynthetic process (Table 13). Between 78 and 187 days of age in EW steers, most of the biological processes affected by weaning age that had an impact value higher than 50% of the maximum total impact were processes related to inflammation, innate immune response and lipogenesis. In contrast, in NW steers only keratinocyte differentiation and negative regulation of cell migration had an impact level greater than 50% of the maximum (Table 13).

Between 187 and 354 days of age, EW steers had an inhibition of histone H3, H4-K5, H4-K8 and H4-K12 acetylation, retrograde vesicle-mediated transport from Golgi to endoplasmic reticulum, Golgi vesicle prefusion complex stabilization and organization, intra-Golgi vesicle-mediated transport, cell growth, iron-sulfur cluster assembly, and other pathways that had a lower impact value (Table 13).

## Discussion

### *Animal performance and blood metabolites*

The greater initial body weight in EW steers was a result of feeding of the high-starch (finishing) diet at an earlier age<sup>17</sup>. NW-MPN steers gained more kg of BW at the feedlot compared with other treatments because they were less efficient in terms of fat accumulation, hence, remained longer at the feedlot until they reached back-fat target finishing end-points. On average, EW-LPN steers ate less but reached back-fat endpoints earlier. The strong impact of cow plane of nutrition (LPN or MPN) on the performance of EW steers is striking.

The decrease in glucose concentration between 187 and 296 days of age might reflect a greater rate of glucose uptake from the bloodstream. Higher plasma insulin concentrations between 187 and 296 days of age are common in animals fed diets that result in greater production of propionate (i.e. high-starch diets). Adiponectin is exclusively secreted from adipose tissue and levels of this adipokine are usually inversely correlated with body fat percentage and positively correlated with body size in humans. In non-ruminants, adiponectin is involved in regulating systemic glucose levels as well as fatty acid oxidation<sup>30</sup>. In a previous study, a higher adiponectin concentration was associated with a decrease in insulin sensitivity in humans<sup>60</sup>. The minor changes in serum insulin and glucose concentrations between 78 and 187 days of age in all steers appear to indicate an effect of adiponectin on glucose homeostasis due to its increasing levels during that period of time.

### *Cow plane of nutrition, weaning age and time*

The DEG affected by the interaction of cow plane of nutrition (diet), weaning age and time encompass various biological processes (Table 9). For instance, *NME1* is a kinase that has a role in the activation of G proteins by GDP phosphorylation to GTP<sup>24</sup> that in turn is used for dynamin-dependent fission of coated vesicles during endocytosis<sup>44</sup> at the caveolae or lipid rafts present in adipocytes. The down-regulation of *NME1* suggests a potential inhibition of the above-mentioned biological processes. *MAPK4* is a kinase that promotes adipogenesis in 3T3-L1 adipocytes by activating the JNK1 and inhibiting the p38MAPK pathway<sup>11</sup>. The down-regulation of *MAPK4* due to time, diet and weaning suggests a negative effect on adipose proliferation and differentiation. There are some DEG for which a biological role in skeletal muscle has not, to our knowledge, been reported. Thus, further research will have to be conducted to uncover the biological role of *OR51F1*, *IMPG1*, *KIAA0232*, *KRTDAP* and *SLC25A5* as it relates to cow plane of nutrition, time, and weaning age.

The change in expression of several transcription factors could reflect an overall decrease in gene transcription in LM, the overall effect of which could not be discerned in the present study. Every known SWI/SNF chromatin-remodeling complex incorporates an ARID-DNA binding domain-containing subunit. *ARID1A* associates with *E2F4* and *E2F5* and contribute to down-regulation of target promoters<sup>36</sup>. Up-regulation of *ARID1A* in our study could reflect a blockage of transcription potentially leading to cell cycle arrest<sup>12</sup> in the LM of rapidly growing steers. *PLAGL1* encodes a growth suppressor protein and it shares with p53 the ability to induce both apoptosis and cell cycle arrest<sup>50</sup>. The up-regulation of *PLAGL1* could be taken as an indication of LM

growth inhibition (Table 9). Along the same line, the up-regulation of *OTX1* plays a critical role during lactating mammary gland remodeling<sup>43</sup>. Hence, the observed down-regulation of *OTX1* suggests a possible inhibition of the transcriptional machinery that leads to LM development in rapidly growing beef steers.

In a previous study it was demonstrated that the induction of *EPAS1* allowed cells to accumulate intracellular lipid droplets<sup>52</sup>. Thus, the down-regulation of *EPAS1* in the present study could have limited a pro-adipogenic response leading to increased intramuscular fat accumulation (i.e. marbling). Although it is well-known that myocyte differentiation no longer occurs postnatally, the down-regulation of *BTG1* in LM also could have been related with intramuscular fat deposition because a previous study reported that *BTG1* has a role in regulating adipose-derived stem cell differentiation to osteocytes and myocytes<sup>47</sup>. Overall, the responses observed for all transcription regulators indicated a state of inhibition of cell proliferation and differentiation. It remains to be determined if those responses have a biological effect in terms of LM growth.

### ***Cow plane of nutrition effect***

#### ***Adipose tissue inflammation***

The LM in LPN steers had signs of an inflammatory response due to the up-regulation of signal peptide, CUB domain, EGF-like 1 (*SCUBE1*) and caspase recruitment domain family, member 14 (*CARD14*). *CARD14* interacts with T- and B-cell receptor complexes present in lipid rafts<sup>14</sup> and it functions to assemble these complexes at the plasma membrane to transduce distinct upstream stimuli to the



activation of *BCL10* and nuclear factor  $\kappa$ B (*NF- $\kappa$ B*)<sup>62</sup>. The strong up-regulation of *SCUBE1* and *CARD14* in LPN steers as compared to MPN steers suggests a localized inflammatory response due to the low plane of nutrition in utero (Table 10). The trigger for such localized inflammatory response could be associated with expression of Dynein (*DYNLL1*). Dynamitin is a subunit of dynactin, which is important for linking the microtubule dependent motor protein dynein to vesicle membranes in macrophages<sup>68</sup>. Formation of microtubule cytoskeleton is important for macrophage spreading; therefore, *DYNLL1* up-regulation in LPN steers might be one factor allowing macrophage infiltration in intramuscular fat of LM.

The feeding of a diet that facilitates a rapid expansion of adipose tissue mass could lead to hypoxia, which ultimately promotes adipocyte apoptosis and the infiltration of macrophages to phagocytize cell death-related leftovers<sup>57</sup>. Ubiquitination is a process that leads to proteasome degradation. *MARCH7* functions as an ubiquitin ligase E3 that generates a polyubiquitin chain to promote the proteasomal degradation of caspases upon binding<sup>38</sup>. The down-regulation of *MARCH7* in LPN compared with MPN steers suggests that proteasomal degradation might have been impaired.

### *Macrophage infiltration*

Cell migration is dependent on the continuous organization of the actin cytoskeleton, which is regulated by members of the small Rho GTPase family<sup>1</sup>. *ARAP1* and *IQGAP1*<sup>61</sup> encodes domains for Arf guanosine triphosphatase-activating protein (GAP) and Rho GAP<sup>67</sup>. The Arf family of GTP-binding proteins are regulators of membrane trafficking and actin remodeling. If ADP-ribosylation does not occur, actin

cytoskeleton assembly and recycling is inhibited<sup>40</sup>. The actin cytoskeleton is a key mediator of cell polarization and help direct migration of macrophages and neutrophils into tissues<sup>21</sup>. Together, the down-regulation of *ARAP1*, *IQGAP1* and *ART3* (which modifies the function of proteins by the addition or removal of ADP-ribose to an arginine residue) suggest an inhibition of actin cytoskeleton remodeling due to impaired ADP-ribosylation. Such response could have been associated with an inhibition of LM remodeling by macrophage infiltration in LM of LPN steers (Table 10).

Despite signs of impaired innate immune response in LPN steers, the up-regulation of *EHD4* suggests a potential activation of endocytosis, perhaps of apoptotic debris from adipocytes infiltrated macrophages<sup>16</sup>. *EHD4* controls recycling by regulating the transport of receptors and it co-localizes with vesicular and tubular structures, implying roles in the internalization of receptors, cytoskeletal dynamics and their transport to early endosomes<sup>37</sup>. The biological role of *EHD4* and the endocytosis process in LM from growing steers is still unknown.

The activation of the innate immune response in EW steers between 78 and 187 days of age highlighted in our transcriptome analysis is mainly due to macrophage infiltration after LM was challenged by a greater rate of lipogenesis<sup>16</sup>. One of the first symptoms of inflammation in adipose tissue is hypoxia (i.e. due to a high degree of cell proliferation, oxygen availability does not supply cell requirements), leading to deregulated production and secretion of adipocytokines<sup>41</sup>. Thus, at a certain point during the fattening period, hyperplastic fat depots and inflammation could potentially reduce the capacity of adipose tissue for lipid storage and secretion of adipokines<sup>32</sup>.

The inhibition of the receptor for Fc fragment of IgA (*FCAR* or *CD89*) which is expressed on immune cells (i. e. macrophages) suggests an inhibition of the immune response in LPN steers compared with MPN. IgA binds to *FCAR* and forms a complex that initiates a downstream cascade of events that trigger a variety of inflammatory responses including phagocytosis, release of inflammatory cytokines, oxidative burst, and antibody-dependent cell-mediated cytotoxicity<sup>42</sup>. The down-regulation of *KRT74* could have decreased the formation of keratinocyte-derived chemokines which are highly expressed in preadipocytes<sup>39</sup>. Although they do not have an effect on adipogenesis, they induce adipocyte expression of inflammatory factors such as *IL1*, *IL6*, and *TNF*<sup>39</sup>.

Insulin insensitivity could be a consequence of oxidative stress. In healthy animals, long-chain fatty acid infusions cause an increase in oxidative stress and insulin resistance that is reversed by the administration of antioxidants such as glutathione<sup>46</sup>. Glutathione S-transferase, alpha 4 (*GSTA4*) is involved in detoxification and protection of cells against chemical and oxidative stress<sup>48</sup>. *GSTA4* catalyzes the detoxification of hydroxynonenal (HNE) and related lipid peroxides by conjugation to glutathione (GSH)<sup>3</sup>. The down-regulation of *GSTA4* in LPN compared with MPN steers suggests that they were not undergoing an overt oxidative stress condition that would be expected in MPN steers due to the greater hypertrophy and hyperplasia of adipocytes in LM.

The conversion of methionine residues in actin to methionine-R-sulfoxide and back-conversion to methionine by methionine sulfoxide reductase B1 (*MSRB1*)<sup>27</sup> might be one of the mechanisms utilized by macrophages to support actin assembly during macrophage recruitment into adipose tissue in animals at an advanced stage of fattening.

Therefore, *MSRBI* down-regulation in LPN steers is suggestive that the process of adipose tissue remodeling via hypertrophy and hyperplasia did not reach a maximum. As such, there were fewer oxidative stress signals that could enhance macrophage infiltration in these animals.

### *Glucocorticoid Receptors*

Mitochondrial glucocorticoid receptor (GR) regulates some enzymes with specific roles in the oxidative phosphorylation pathway<sup>45</sup>. In the absence of glucocorticoids, the glucocorticoid receptor (GR) resides in the cytosol (inactive) complexed mainly with heat shock protein 90 (hsp90) and the heat shock protein 70 (hsp70)<sup>35</sup>. The translocase of outer mitochondrial membrane 34 (*TOMM34*) has a tetratricopeptide repeat domain (TPR1) that specifically binds Hsp70. *TOMM34* represents a novel scaffolding co-chaperone of Hsp70 and Hsp90, which may facilitate Hsp70/Hsp90 cooperation during protein folding or keep the complex in an unfolded mitochondrial protein import compatible state<sup>10</sup>. The up-regulation of *TOMM34* in LPN compared with MPN steers might have enhanced the role of GR in maintaining normal mitochondrial functions (i.e. oxidative phosphorylation) even under oxidative stress conditions (Table 10).

### *Regulation of transcription*

The overall process of gene transcription could have been affected by the cow plane of nutrition by means of changes in expression of 3 DEG: hepatic leukemia factor (*HLF*), nuclear receptor subfamily 2, group C, member 2 (*NR2C2*) and enhancer of polycomb homolog 1 (*EPC1*). The knockdown of *HLF* significantly reduced lipid

content in *Drosophila*<sup>8</sup>, thus, the up-regulation of *HLF* in LPN steers as compared to MPN steers could have been associated with differences in intramuscular fat deposition (Table 10). Because *NR2C2* can act as a negative regulator of the retinoid signaling pathway<sup>28</sup>, the down-regulation of *NR2C2* in LPN steers as compared to MPN steers could have played a role in the overall control of intramuscular fat deposition (Table 10). Finally, *EPC1* is part of a core repressor complex, with *E2F6* and *DPI1*. In proliferating cells, *EZH2* binds to *EPC1*<sup>2</sup>. *EZH2* has histone methyltransferase activity and it was proven to elicit a pro-adipogenic effect<sup>63</sup>. Overall, these data suggest that at the transcriptional level a low maternal plane of nutrition favored intramuscular fat development in the offspring (Table 10).

#### *Lipid metabolism*

Lipid metabolism was affected by cow plane of nutrition by means of the activation in LPN steers of ATP citrate lyase (*ACLY*) and the slight down-regulation of 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6*). The former is a key link between the metabolism of carbohydrates and the production of fatty acids<sup>55</sup>. The role of *AGPAT6* is to esterify the acyl-group from acyl-ACP to the sn-1 position of glycerol-3-phosphate, thus, participating in triacylglycerol synthesis<sup>5</sup>. In ruminant adipose tissue, *ACLY* activation (together with NADP-malate dehydrogenase) provides the necessary NADPH for fatty acid production from lactate<sup>55</sup>. Taken together, fatty acid metabolism in LM of LPN steers seems to have been more active in terms of lipogenesis.

### *Weaning age and time effect*

In the present study, the effect of feeding a high-starch diet at a young age was confirmed, i.e. EW steers had an overall activation of the fatty acid biosynthetic process soon after early weaning, between 78 and 187 days of age. In contrast, only after the NW steers joined the EW steers at the feedlot (around 187 days of age) their LM had an activation of these metabolic pathways (Table 13). Thus, high-dietary starch at an early age is a consistent trigger of adipogenesis and lipogenesis.

### *Innate immune response*

The transcriptome response between 78 and 187 days of age revealed a clear activation of the innate immune system within LM in EW compared with NW steers. It is noteworthy that none of the EW steers biopsied had to be treated for bovine respiratory disease as compared to the herd from where these biopsied steers were selected<sup>54</sup>. Thus, the overall activation of the innate immune response in LM of EW steers might have been a normal response to the greater dietary energy, i.e. more nutrient availability to cells (Table 14).

Among the innate immune pathways affected, the activation of the MyD88-dependent toll-like receptor signaling pathway in EW steers between 78 and 187 days of age could denote an inflammatory response elicited by circulating cytokines acting within LM in a MyD88-dependent manner<sup>33</sup>. There is previous evidence demonstrating that chronic feeding of high-starch diets could elicit an increase in circulating endotoxin/LPS which can then trigger a pro-inflammatory response<sup>23</sup>. The activation of the LPS pathways leads to the LPS binding to the CD14/TLR4/MD2 receptor complex

in many cell types such as monocytes, macrophages and B cells, which promotes the secretion of pro-inflammatory cytokines<sup>13</sup>. Another sign of the activation of the innate immune response in EW steers was the activation of the JNK cascade which is an intermediary molecule in the cascade of events that leads to the synthesis of cytokines. JNK is one of the four well-characterized subfamilies of MAP kinases (MAPKs)<sup>18</sup>. MAPKs phosphorylate transcription factors and other targets to regulate gene transcription and immune responses.

The normal activation of the innate immune response is exerted when fat depots reach a plateau in which excessive hyperplasia and hypertrophy and diminished oxygen availability inhibit adipogenesis and increase lipolysis and fatty acid release<sup>57</sup>. These processes of adipose tissue remodeling at advanced stages of fat accumulation, as can be surmised for EW and NW steers, lead to a situation of obesity-related chronic subclinical inflammation in adipose tissue<sup>57</sup>. This pseudo-inflammation is characterized by the massive infiltration to the inflammatory site of exogenous immunoreactive cells such as macrophages, lymphocytes, eosinophils, and mast cells, and the subsequent phagocytic activity and release of inflammatory mediators<sup>65</sup>.

Interleukins promote the development and differentiation of T and B lymphocytes, and hematopoietic cells. B cells are a type of lymphocyte in the humoral immunity of the adaptive immune system<sup>59</sup>. *IL-6* production activates B cell differentiation into plasma cells that are responsible for antibody secretion. *IL-17* maintains cytokine production and *IL-23* has a role in the maintenance of *IL-17* producing cells<sup>19</sup> (Table 13). Taken together with the positive regulation of interleukin production (*IL6*, *IL17* and *IL23* specifically) in EW steers between 78 and 187 days of

age the present data suggest that activation of lymphocyte proliferation and positive regulation of cytokines and interleukins are normal mechanisms that contribute to adipose tissue expansion. The present study confirms the existence of an innate immune response in LM of EW steers after weaning likely due to increased intramuscular fat accumulation.

Microsomal glutathione S-transferase 1 (*MGST1*) transcription, similar to some glutathione peroxidases, also is susceptible to oxidative stress<sup>22</sup>. In the present study, *MGST1* appears particularly important in the LM response to weaning because the bioinformatics analysis indicates that it influences several pathways in NW steers between 78 and 187 days of age. Among these pathways, those related to glutathione metabolism and cytochrome P450 had a slight inhibition in NW steers. In contrast, these pathways were markedly activated and with a higher impact in EW steers. It is plausible that activation of these pathways was related to the initial stages of an inflammatory process in EW steers due to the earlier deposition of fat during growing phase<sup>16</sup>.

#### *Myogenesis and adipogenesis*

Chloride intracellular channel 4 (*CLIC4*) downregulation likely was responsible for the inhibition in NW steers of keratinocyte differentiation and negative regulation of cell migration between 78 and 187 days of age (Table 13). *CLIC4* is considered a growth inhibitory protein, which in vivo is located in the nucleus. By altering the Cl<sup>-</sup> and pH of the nucleus *CLIC4* contributes to cell cycle arrest and the specific gene expression program associated with conversion of fibroblasts into myofibroblasts<sup>49</sup>. It can be speculated that *CLIC4* exerts a similar function in the LM of NW steers. The fact that



keratinocyte differentiation was the biological process with the highest impact in NW steers between 78 and 187 days of age supports a role for *CLIC4* in LM. However, because the LM biopsy likely contained a combination of myocytes and adipocytes also could mean that *CLIC4* is important in the overall process that inhibits cell differentiation that could control muscle and fat growth in young steers.

#### *Epigenetic regulation of transcription*

Contrary to the precocious response in EW steers between 78 and 187 days of age, the LM of NW steers had a marked activation of the lipogenic biological processes between 187 and 354 days of age. In fact, during the same time frame EW steers appeared to undergo an inhibition in the regulation of transcription through inhibition of histone acetylation. Histone acetylation is an epigenetic-regulated process that produces changes through chemical reactions in gene expression caused by certain base pairs in DNA, or RNA, being "shutdown" or "turned on" again<sup>9</sup>. In our study, EW steers had an inhibition of histone H3 and H4 acetylation. Acetylation of histone H3 occurs at several different lysine positions in the histone tail and is performed by a family of enzymes known as histone acetyltransferases (HATs)<sup>53</sup>. The inhibition of histone H4-K5 acetylation is catalyzed by several enzymes, including *KAT5* and CBP/p300 proteins in mammals<sup>25</sup>. CBP/p300 are important transcriptional activators that serve to open chromatin transcriptional start site by acetylating histones.

Regulation of acetylation by transcription coactivator CBP/p300 has been related to an autoimmune response<sup>51</sup> suggesting that in our study transcriptional inhibition might have been triggered by the activation of the innate immune response between 78

and 187 days of age in EW steers. Histone H4-K8 acetylation and histone H4-K12 acetylation were also inhibited in EW steers between 187 and 354 days of age.

### *Lipid droplet formation*

Nascent lipid droplets are secreted and transported from the endoplasmic reticulum (ER) to the Golgi apparatus<sup>64</sup>. The transport of lipid droplets requires the localization and activity of proteins that create ER exit sites, coat proteins to collect cargo and to change membrane's shape into a transport container, and address tags (SNARE proteins) to target the vesicles specifically to the Golgi apparatus. ER export factors, SNAREs, and misfolded proteins used during the release of lipid droplets must be retrieved from the Golgi to the ER again<sup>56</sup>. If this process is impaired, Golgi vesicle prefusion complex stabilization occurs (Table 13). Lipid droplet dynamics, trafficking and dispersion was demonstrated to be produced by homotypic fusion between lipid droplets using SNAREs<sup>29</sup>. Although translocation of resident proteins from the Golgi to the ER, and endosomal trafficking to the biosynthetic/secretory compartments (so called retrograde trafficking)<sup>20</sup>, was inhibited between 187 and 354 days of age in EW steers potentially leading to a lack of Golgi organization and a diminished intra-Golgi vesicle-mediated transport (Table 14). If such processes occurred it might have led to less lipid droplet formation, hence, less marbling in LM. The inhibition of the mechanism of retrograde trafficking could imply a negative impact on the lipid droplet formation for EW steers between 187 and 354 days of age.

## Conclusions

Cow plane of nutrition during the last 90 days of gestation resulted in small changes in the offspring longissimus transcriptome. Among these genes the bioinformatics analysis revealed an inflammatory process in offspring born from cow with medium plane of nutrition due to a suggested higher metabolic rate in terms of lipogenesis and fat accumulation. Early weaning induced a robust activation of the lipogenic program accompanied by activation of the innate immune system. The latter appeared related to the greater lipogenesis during the growing phase. Subsequently during the finishing phase, alterations of genes in the Golgi complex organization suggest an inhibition of lipid droplet formation potentially diminishing intramuscular fat accumulation in these steers. The inflammatory response in adipose tissue detected between 78 and 187 days of age in EW steers when only genes affected by weaning age were considered, was also detected when only DEG affected by cow plane of nutrition was considered; in this case, with signs of adipose tissue inflammation for both, MPN and LPN steers. We suggest that these mechanisms had a higher impact on EW-LPN steers due to their lower marbling scores. Overall, results from this work underscore the need to conduct further studies to understand better the functional outcome of the transcriptome changes. Additional knowledge on molecular and functional outcomes induced by the combination of prenatal and postnatal nutritional management would help produce more efficient beef cattle.

## Tables and Figures

**Table 8:** Feedlot performance, ultrasound measurements and carcass quality parameters for Angus x Simmental steers (n=23) from cows that received a low (LPN) or a medium (MPN) plane of nutrition (D) during the late gestation period. Weaning times (W) are early weaning (EW) and normal weaning (NW). Different letters denoted significant differences ( $P < 0.05$ ).

Item	Treatments				SEM	P-value <sup>1</sup>		
	EW		NW			D	W	D*W
	LPN	MPN	LPN	MPN				
Feedlot Performance								
Initial BW	258	278	212	204	18.25	0.73	<0.01	0.42
Final BW*	548	581	524	533	19.78	0.26	0.07	0.52
ADG (kg/d)	1.74	1.66	1.81	1.69	0.15	0.40	0.56	0.85
DMI (kg/d)	7.84	9.33	8.18	8.48	0.41	0.03	0.52	0.14
Ultrasound measurements								
BF at EW (cm)	0.31	0.35	0.33	0.34	0.02	0.13	0.71	0.52
BF at NW (cm)	0.33	0.33	0.31	0.33	0.02	0.54	0.57	0.74
Marbling at EW	427	388	434	419	27.80	0.30	0.48	0.65
Marbling at NW	330	409	376	418	40.41	0.13	0.47	0.63
Carcass quality parameters								
HCW (kg)	340	360	325	331	12.27	0.27	0.07	0.51
Calculated YG	3.0	3.0	2.5	2.3	0.27	1.00	0.03	1.00
LM area (cm <sup>2</sup> )	77.3	82.5	78.6	79.4	3.27	0.34	0.76	0.48
Marbling	418	573	480	482	43.13	0.07	0.72	0.07
Back fat thickness (cm)	1.24	1.37	1.30	1.12	0.13	0.83	0.41	0.22
KPH (%)	2.1	2.1	2.3	2.1	0.12	0.29	0.28	0.70
Days to harvest	367	377	392	412	11.10	0.16	0.01	0.59

\*Based on 62% dressing percentage

<sup>1</sup>D = cow plane of nutrition effect (diet), W = weaning age effect, D × W = diet × weaning interaction

**Table 9.** Symbol, entrez gene ID, log ratio expression value, type of molecule and localization in the cell for the 13 differentially expressed genes affected by the weaning  $\times$  diet  $\times$  time interaction

Symbol	Entrez Gene Name	Log Ratio	p-value	Location	Type(s)
ARID1A	AT rich interactive domain 1A (SWI-like)	2.885	2.04E-04	Nucleus	TR
BTG1	B-cell translocation gene 1, anti-proliferative	-1.262	2.07E-03	Nucleus	TR
EPAS1	endothelial PAS domain protein 1	-1.797	2.08E-01	Nucleus	TR
HSBP1	heat shock factor binding protein 1	1.064	2.38E-02	Nucleus	TR
IMPG1	interphotoreceptor matrix proteoglycan 1	1.577	2.22E-05	Extrac. Space	other
KIAA0232	KIAA0232	1.889	3.33E-04	Extrac. Space	other
KRTDAP	keratinocyte differentiation-associated protein	-12.672	8.49E-01	Extrac. Space	other
MARK4	MAP/microtubule affinity-regulating kinase 4	-1.247	2.99E-02	Cytoplasm	kinase
NME1	NME/NM23 nucleoside diphosphate kinase 1	-5.373	1.17E-01	Cytoplasm	kinase
OR51F1	olfactory receptor, family 51, subfamily F, member 1	-1.393	4.66E-04	Plasm. Memb.	G receptor
OTX1	orthodenticle homeobox 1	-3.683	2.31E-01	Nucleus	TR
PLAGL1	pleiomorphic adenoma gene-like 1	1.313	5.06E-03	Nucleus	TR
SLC25A5	solute carrier family 25, member 5	-4.401	2.36E-01	Cytoplasm	transporter

Extrac. Space = extracellular space, Plasm. Membrane = plasma membrane, TR = transcription regulator; G receptor = G protein coupled receptor

**Table 10.** Symbol, entrez gene ID, log ratio expression value (LPN vs. MPN), type of molecule and localization in the cell for the 35 differentially expressed genes affected by cow plane of nutrition





































<b>Symbol</b>	<b>Entrez Gene Name</b>	<b>Log Ratio</b>	<b>p-value</b>	<b>Location</b>	<b>Type(s)</b>
ABHD11	abhydrolase domain containing 11	0.30	8.74E-06	Cytoplasm	enzyme
ACLY	ATP citrate lyase	0.35	7.74E-05	Cytoplasm	enzyme
AGPAT6	1-acylglycerol-3-phosphate O-acyltransferase 6	-0.22	5.90E-05	Cytoplasm	enzyme
ARAP1	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1	-0.44	1.53E-04	Cytoplasm	other
ART3	ADP-ribosyltransferase 3	-0.78	4.64E-05	Plasm. Memb.	enzyme
ATP5S	ATP synthase, H <sup>+</sup> transporting, mitochondrial Fo complex, subunit s	0.33	1.09E-04	Cytoplasm	transporter
C15orf40	chromosome 15 open reading frame 40	-0.21	1.72E-04	Other	other
C8orf48	chromosome 8 open reading frame 48	-0.72	2.18E-05	Other	other
CARD14	caspase recruitment domain family, member 14	0.96	4.31E-05	Cytoplasm	other
CHST12	carbohydrate (chondroitin 4) sulfotransferase 12	0.78	5.32E-05	Cytoplasm	enzyme
DEXI	Dexi homolog (mouse)	0.29	1.50E-04	Other	other
DHDH	dihydrodiol dehydrogenase (dimeric)	0.40	8.49E-08	Other	enzyme
DYNLL1	dynein, light chain, LC8-type 1	0.47	2.29E-05	Cytoplasm	other
EDC3	enhancer of mRNA decapping 3	0.55	2.31E-05	Cytoplasm	other
EHD4	EH-domain containing 4	0.60	1.39E-04	Plasm. Memb.	enzyme
ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2	0.53	7.24E-05	Cytoplasm	enzyme
EPC1	enhancer of polycomb homolog 1 (Drosophila)	-0.28	9.13E-05	Nucleus	TR
FCAR	Fc fragment of IgA, receptor for	-0.52	6.40E-05	Plasm. Memb.	other
GSTA4	glutathione S-transferase, alpha 4	-1.74	1.37E-05	Other	enzyme
HLF	hepatic leukemia factor	0.72	1.69E-04	Nucleus	TR
IQGAP1	IQ motif containing GTPase activating protein 1	-0.55	5.28E-05	Cytoplasm	other
KRT74	keratin 74	-1.01	7.04E-05	Cytoplasm	other
LOC789391	tRNA methyltransferase catalytic subunit TRMT61A like	-0.76	1.07E-04	Nucleus	enzyme

**Table 10.** Continued

MARCH7	membrane-associated ring finger 7, E3 ubiquitin protein ligase	-0.62	7.57E-09	Extrac. Space	other
MSRB1	methionine sulfoxide reductase B1	-0.49	3.86E-05	Other	other
NR2C2	nuclear receptor subfamily 2, group C, member 2	-0.27	7.21E-05	Nucleus	LDNR
NSMCE4A	non-SMC element 4 homolog A ( <i>S. cerevisiae</i> )	0.34	1.67E-04	Nucleus	other
PNMAL1	paraneoplastic Ma antigen family-like 1	0.89	1.31E-04	Other	other
PSPH	phosphoserine phosphatase	0.74	1.62E-04	Cytoplasm	phosphatase
PYCR1	pyrroline-5-carboxylate reductase 1	0.47	8.97E-05	Cytoplasm	enzyme
SCUBE1	signal peptide, CUB domain, EGF-like 1	1.05	1.03E-04	Plasm. Memb.	transm. receptor
SERF1A	small EDRK-rich factor 1A (telomeric)	0.41	1.48E-04	Other	other
SRSF5	serine/arginine-rich splicing factor 5	-0.53	9.98E-05	Nucleus	other
TAGLN3	transgelin 3	0.34	1.17E-04	Extrac. Space	other
TOMM34	translocase of outer mitochondrial membrane 34	0.36	1.14E-04	Cytoplasm	other

Extrac. Space = extracellular space, Plasm. Membrane = plasma membrane, Transm. Receptor = transmembrane receptor; TR = transcription regulator; G receptor = G protein coupled receptor, LDNR – ligand-dependent nuclear receptor

**Table 11.** Results of the 3 most impacted pathways during growing phase (187 vs. 78 d), finishing phase (354 vs. 187 d) and the whole experiment (354 vs. 78 d) uncovered by the Dynamic Impact Approach (DIA) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways database analysis of the bovine muscle transcriptome. Flux represent the direction of the pathway (green color = inhibition, yellow color = stable, red color = activation with different color intensities according with the level of up-regulation or down-regulation). Blue lines show the impact of each pathway. DEG present in each pathway is listed follow by it ratio value (P value < 0.05; FDR < 0.10).







































































































WEANING	TIME	CATEGORY	SUBCATEGORY	PATHWAY	IMPACT	FLUX	DEG/Ratio
EW	187vs78	Metabolism	Lipid Metabolism	Fatty acid biosynthesis			FASN (3.32)
		Metabolism	Lipid Metabolism	Biosynthesis of unsaturated fatty acids			SCD (3.20), ELOVL6 (3.07), HSD17B12 (-1.10)
		Organismal Systems	Endocrine System	Insulin signaling pathway			FASN (3.32), PCK2 (1.20)
	354vs187	Metabolism	Nucleotide Metabolism	Pyrimidine metabolism			POLR3A (-2.78)
		Genetic Information Processing	Transcription	RNA polymerase			POLR3A (-2.78)
		Organismal Systems	Immune System	Cytosolic DNA-sensing pathway			POLR3A (-2.78)
NW	187vs78	Metabolism	Lipid Metabolism	Fatty acid biosynthesis			FASN (2.99)
		Metabolism	Lipid Metabolism	Biosynthesis of unsaturated fatty acids			SCD (3.59), ELOVL6 (3.64), HSD17B12 (-1.11)
		Organismal Systems	Endocrine System	Insulin signaling pathway			FASN (2.99), PCK2 (-1.09)
	354vs187	Metabolism	Xenobiotics Biodegradation and Metabolism	Metabolism of xenobiotics by cytochrome P450			MGST1 (-1.64)
		Metabolism	Xenobiotics Biodegradation and Metabolism	Drug metabolism - cytochrome P450			MGST1 (-1.64)
		Metabolism	Metabolism of Other Amino Acids	Glutathione metabolism			MGST1 (-1.64)
	354vs78	Metabolism	Lipid Metabolism	Biosynthesis of unsaturated fatty acids			SCD (-3.23), ELOVL6 (-1.11), HSD17B12 (-1.09)
		Metabolism	Lipid Metabolism	Fatty acid biosynthesis			FASN (-1.64)
		Metabolism	Lipid Metabolism	Glycerophospholipid metabolism			AGPAT2 (-1.11)
	354vs78	Metabolism	Lipid Metabolism	Fatty acid biosynthesis			FASN (3.18)
		Metabolism	Lipid Metabolism	Biosynthesis of unsaturated fatty acids			SCD (3.86), ELOVL6 (4.25), HSD17B12 (-1.09)
		Metabolism	Xenobiotics Biodegradation and Metabolism	Metabolism of xenobiotics by cytochrome P450			MGST1 (2.19)



**Table 12.** Significant biological processes (BP): in this table are described the biological processes BP for each DEG present in the pathways of Table 4 for each time comparison. Flux represent the direction of the pathway (green color = inhibition, yellow color = stable, red color = activation with different color intensities according with the level of up-regulation or down-regulation). Blue lines show the impact of each pathway (P value < 0.05; FDR < 0.10).

DEG	Biological Process	NORMAL WEANING						EARLY WEANING					
		187 vs78		354vs187		354vs78		187 vs78		354vs187		354vs78	
FASN	fatty acid biosynthetic process (GO:0006633)												
SCD	fatty acid biosynthetic process (GO:0006633)												
ELOVL6	fatty acid elongation, saturated fatty acid (GO:0019367) long-chain fatty acid biosynthetic process (GO:0042759)												
HSD17B12	fatty acid biosynthetic process (GO:0006633) positive regulation of cell-substrate adhesion (GO:0010811)												
PCK2	gluconeogenesis (GO:0006094)												
POLR3A	innate immune response (GO:0045087)												
MGST1	glutathione metabolic process (GO:0006749) response to lipopolysaccharide (GO:0032496) protein homotrimerization (GO:0070207)												
AGPAT2	positive regulation of cytokine production (GO:0001819) phosphatidic acid biosynthetic process (GO:0006654)												

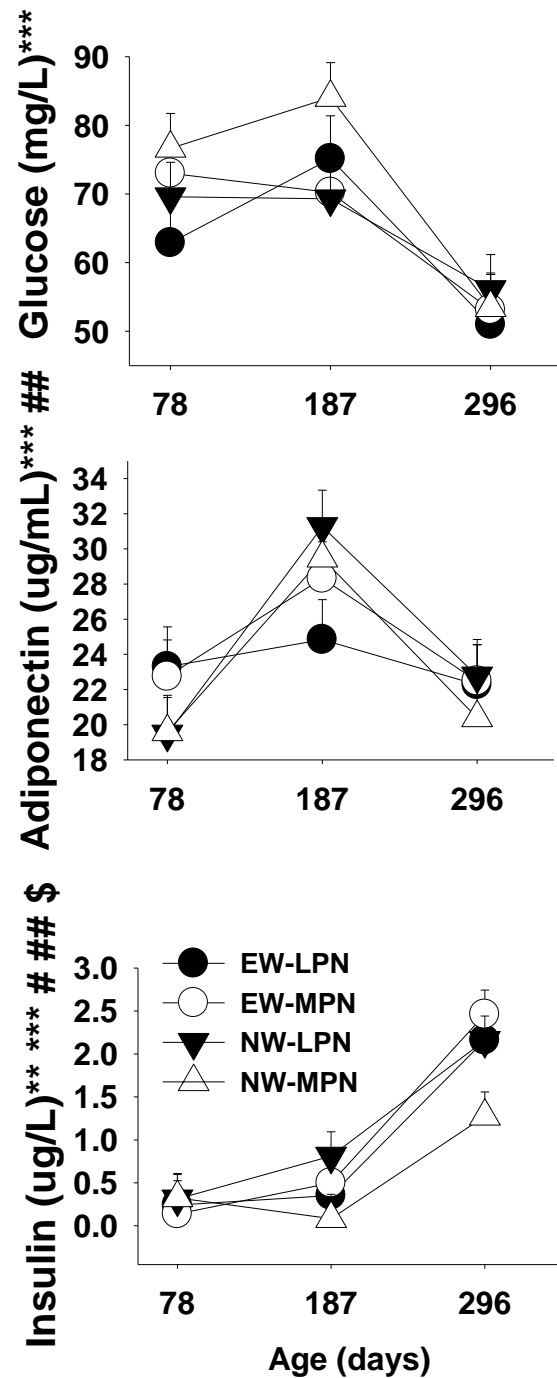
**Table 13.** Significant biological processes (BP): in this table are described BP for EW and NW steers between 78 and 187 days of age and 187 and 354 days of age that presented more than 50% of the maximum total impact. Furthermore, the time comparison for each BP is sorted from high impacted to less impacted. Flux represent the direction of the BP (green color = inhibition, yellow color = stable, red color = activation with different color intensities according with the level of up-regulation or down-regulation). Blue lines show the impact of each pathway (P value < 0.05; FDR < 0.10).

WEANING	TIME COMPARISON	BIOLOGICAL PROCESSES	IMPACT	FLUX
EARLY WEANED	187 vs 78 days of age	GO:0009058~biosynthetic process		
		GO:0006633~fatty acid biosynthetic process		
		GO:0002755~MyD88-dependent toll-like receptor signaling pathway		
		GO:0016064~immunoglobulin mediated immune response		
		GO:0031663~lipopolysaccharide-mediated signaling pathway		
		GO:0032494~response to peptidoglycan		
		GO:0032496~response to lipopolysaccharide		
		GO:0032740~positive regulation of interleukin-17 production		
		GO:0032747~positive regulation of interleukin-23 production		
		GO:0032755~positive regulation of interleukin-6 production		
		GO:0032760~positive regulation of tumor necrosis factor production		
		GO:0042127~regulation of cell proliferation		
		GO:0045080~positive regulation of chemokine biosynthetic process		
		GO:0045351~type I interferon biosynthetic process		
		GO:0046330~positive regulation of JNK cascade		
		GO:0050671~positive regulation of lymphocyte proliferation		
		GO:0050727~regulation of inflammatory response		
		GO:0050830~defense response to Gram-positive bacterium		
EARLY WEANED	354 vs 187 days of age	GO:0019367~fatty acid elongation, saturated fatty acid		
		GO:0042759~long-chain fatty acid biosynthetic process		
		GO:0010866~regulation of triglyceride biosynthetic process		
		GO:0030216~keratinocyte differentiation		
		GO:0030336~negative regulation of cell migration		
		GO:0043966~histone H3 acetylation		
		GO:0043981~histone H4-K5 acetylation		
		GO:0043982~histone H4-K8 acetylation		
		GO:0043983~histone H4-K12 acetylation		
		GO:0006890~retrograde vesicle-mediated transport, Golgi to ER		
		GO:0048213~Golgi vesicle prefusion complex stabilization		
		GO:0007030~Golgi organization		
		GO:0006891~intra-Golgi vesicle-mediated transport		
		GO:0016049~cell growth		
		GO:0016226~iron-sulfur cluster assembly		
		GO:0043488~regulation of mRNA stability		
		GO:0000002~mitochondrial genome maintenance		
NORMAL WEANED	354 vs 187 days of age	GO:0000165~MAPK cascade		
		GO:0006366~transcription from RNA polymerase II promoter		
		GO:0048311~mitochondrion distribution		
		GO:0070375~ERK5 cascade		
		GO:0006529~asparagine biosynthetic process		
		GO:0006541~glutamine metabolic process		
		GO:0042149~cellular response to glucose starvation		
		GO:0045931~positive regulation of mitotic cell cycle		
		GO:0006633~fatty acid biosynthetic process		
		GO:0019367~fatty acid elongation, saturated fatty acid		
		GO:0042759~long-chain fatty acid biosynthetic process		
		GO:0008343~adult feeding behavior		
		GO:0032099~negative regulation of appetite		
		GO:0030644~cellular chloride ion homeostasis		
		GO:0009058~biosynthetic process		
		GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway		

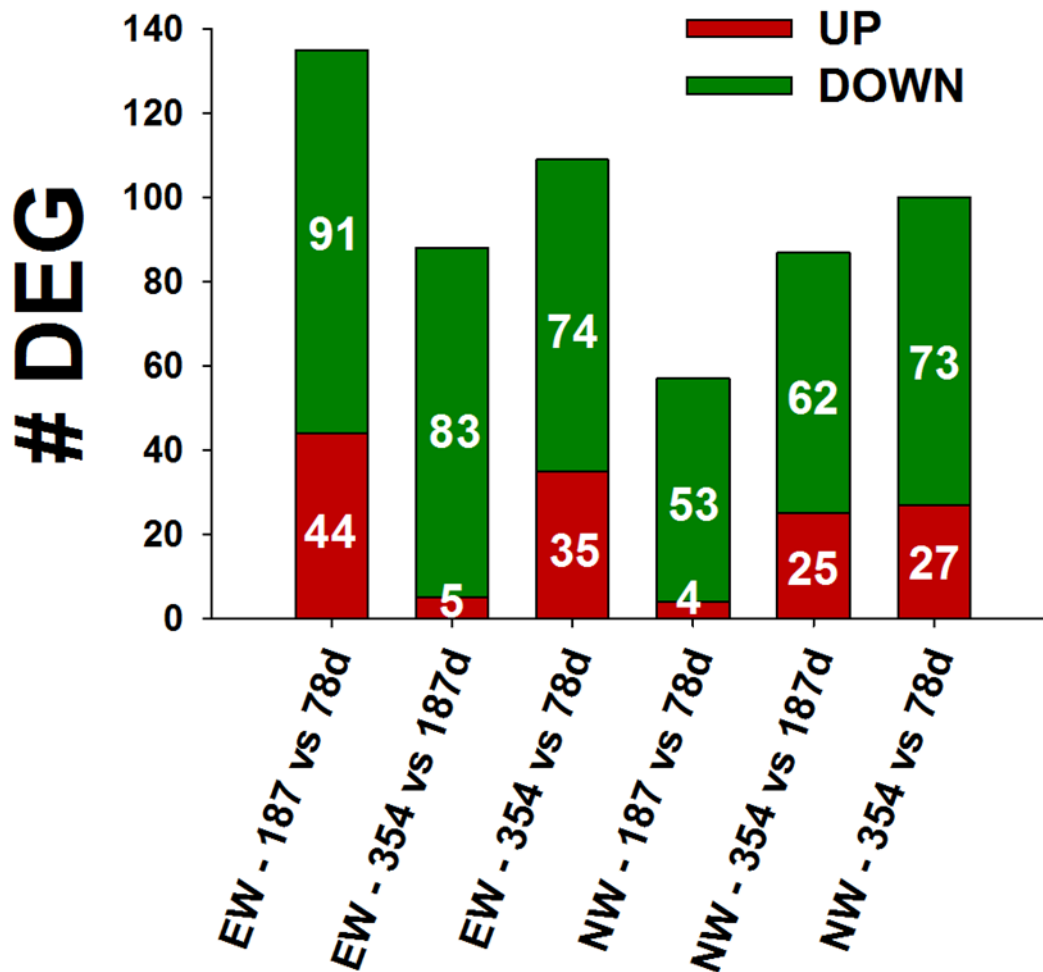
**Table 14.** Results of flux and impact uncovered by the Dynamic Impact Approach (DIA) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways database analysis of the bovine muscle transcriptome of early and normal weaned steers for each life period. Flux represent the direction of each category and the corresponding subcategory (green color=inhibition, yellow color=stable, red color=activation with different color intensities according with the level of up-regulation or down-regulation). Blue lines show the impact of each category and the corresponding subcategories (P value < 0.05; FDR < 0.10).

Category	EARLY WEANING				NORMAL WEANING			
	187 vs 78 days of age	354 vs 187 days of age	187 vs 78 days of age	354 vs 187 days of age	187 vs 78 days of age	354 vs 187 days of age	187 vs 78 days of age	354 vs 187 days of age
<b>1. Metabolism</b>								
0.1 Metabolic Pathways								
1.1 Carbohydrate Metabolism								
1.2 Energy Metabolism								
1.3 Lipid Metabolism								
1.4 Nucleotide Metabolism								
1.5 Amino Acid Metabolism								
1.6 Metabolism of Other Amino Acids								
1.7 Glycan Biosynthesis and Metabolism								
1.8 Metabolism of Cofactors and Vitamins								
1.9 Metabolism of Terpenoids and Polyketides								
1.10 Biosynthesis of Other Secondary Metabolites								
1.11 Xenobiotics Biodegradation and Metabolism								
<b>2. Genetic Information Processing</b>								
2.1 Transcription								
2.2 Translation								
2.3 Folding, Sorting and Degradation								
2.4 Replication and Repair								
<b>3. Environmental Information Processing</b>								
3.1 Membrane transport								
3.2 Signal Transduction								
3.3 Signaling Molecules and Interaction								
<b>4. Cellular Processes</b>								
4.1 Transport and Catabolism								
4.2 Cell Motility								
4.3 Cell Growth and Death								
4.4 Cell Communication								
<b>5. Organismal Systems</b>								
5.1 Immune System								
5.2 Endocrine System								
5.3 Circulatory System								
5.4 Digestive System								
5.5 Excretory System								
5.6 Nervous System								
5.7 Sensory System								
5.8 Development								
5.9 Environmental Adaptation								

**Figure 12.** Glucose, Adiponectin and Insulin serum levels for Angus x Simmental steers from cows that received a low (LPN) or medium (MPN) plane of nutrition during the late gestation period. Weaning times are early weaning (EW) and normal weaning (NW). \* Weaning, \*\* Diet, \*\*\* Time, # weaning × diet, ## weaning × time, ### diet × time and \$ time × weaning × diet interaction. Significant differences are declared at  $P < 0.05$ .



**Figure 13.** Percentage of differentially expressed genes (DEG) for early (EW) and normal weaned (NW) steers during the growing (78 to 187 days of age), finishing (187 to 354 days of age) and growing and finishing phases (78 to 354 days of age). Total number of molecules = 15,000 (P value < 0.05; FDR < 0.10)



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## **Chapter 4: Postnatal consequences of the plane of nutrition during late gestation on microRNA and metabolic target gene expression in longissimus muscle of Angus × Simmental offspring**

### **ABSTRACT**

In the following study, we wanted to test if the exposure to different planes of maternal nutrition during late gestation result in an early increase in the expression of genes and/or other changes due to epigenetic regulation that leads to adipogenesis, lipogenesis or muscle growth in the offspring's *Longissimus* muscle (LM) at 78, 187 and 354 days of age. Gene expression changes that not affect DNA sequence were assessed, mainly focusing in non-coding microRNAs (miRNA) epigenetics control. Selected pro and anti adipogenic miRNAs were analyzed by quantitative RT-qPCR in LM samples of early (EW) and normal weaned (NW) Angus × Simmental calves born from cows that were grazing endophyte-infected tall fescue/red clover pastures with no supplement (low plane of nutrition (LPN)), or supplemented with 2.3 kg and 9.1 kg of dried distiller's grains with solubles and soyhulls (70% DDGS/30% soyhulls) (medium and high plane of nutrition (MPN, HPN) respectively) during last 90 days of gestation. Results showed that proadipogenic miRNAs were controlling metabolism of LM of NW-MPN steers between 78 and 187 days of age through activation of: a) miR-103 that inhibits *CAVI* that destabilizes *INSR* increasing insulin resistance, b) miR-143 that inhibits *DLK1* that inhibits adipocyte differentiation through *ERK1/2* activation, c) miR-21 that impaired *TGFBR2*-induced inhibition of adipocyte differentiation and seems to be related to serum adiponectin concentration. Furthermore, from the selected antiadipogenic miRNAs, cow plane of nutrition impaired miR-34a expression in MPN steers at 78 days of age. MiR-34a has a role on the

activation of cell cycle arrest in LM by suppressing *SIRT1*, which leads to an activation of *TP53* between 78 and 187 days of age. In conclusion, in MPN steers, inhibition of miR-34a expression at 78 days of age was the clearest sign of epigenetic regulation of LM of beef offspring due to cow plane of nutrition during late gestation.

**Keywords:** beef, fetal programming, plane of nutrition, epigenetics, microRNAs



## Introduction

Nutritional programming of the offspring can occur via epigenetics, i.e. changes in gene expression unrelated to the DNA sequence of the animal<sup>67</sup>. The conformational change of the structure of histones is a key epigenetic mechanism that regulates gene expression by changes in transcriptional activation, chromatin remodeling, nucleosome loosening, chromatin condensation, and chromatin stabilization<sup>47</sup>. In contrast, non-coding microRNA (miRNA), a functional RNA molecule that is not translated into a protein, also is considered an important mechanism of epigenetic regulation of gene expression<sup>43</sup>. A miRNA is a short ribonucleic acid molecule of ~21–23 nucleotides. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression, target degradation and gene silencing<sup>2</sup>.

Studies of adipose tissue miRNA profiling in beef cattle revealed different patterns according to fat depot location, with 80, 66 and 63 miRNAs, respectively, detected in abdominal subcutaneous, back subcutaneous and rump subcutaneous tissues<sup>23</sup>. Fat depot features also influence miRNA profiles, i.e.: high backfat thickness vs. low backfat thickness; 42 miRNAs were differentially expressed between these two groups, being miR-378 the most differentially expressed<sup>3</sup>.

In beef cattle, it is well-established that late gestation (rapid fetal growth) is a physiological point at which maternal nutrition could elicit programming of adipose tissue in the offspring<sup>8</sup>. Thus, the hypothesis we sought to test was that maternal plane of nutrition during late gestation leads to greater expression of skeletal muscle metabolic target genes related to adipogenesis, lipogenesis, and adipokine synthesis in the offspring at least in part through

alterations in the expression of proadipogenic and antiadipogenic miRNAs. The objective of this study was to assess the effect of a high, medium, and low plane of nutrition of the cow during the last 90 days of pregnancy on selected proadipogenic and antiadipogenic miRNAs and its selected target genes in LM samples of offspring that were early weaned or normal weaned followed by a common high-starch diet during the fattening period.

## **Materials and methods**

### ***Treatments and animals selected for skeletal muscle biopsy***

A subset of 20 Angus × Simmental beef cows from the University of Illinois Dixon Springs Agriculture Center (DSAC) in Simpson, IL, were selected from a group of animals utilized in a parallel study<sup>61</sup>. Main effects evaluated were maternal plane of nutrition during late gestation and postnatal management of the offspring. Three months prior to the projected parturition date cows were assigned to treatments (low, medium or high plane of nutrition) in a split-plot design. Low plane of nutrition (LPN) was achieved by grazing endophyte-infected tall fescue/red clover pastures during July, August, and September with no supplement. Medium and high plane of nutrition cow diets (MPN, HPN) were achieved by grazing endophyte-infected tall fescue/red clover pastures supplemented with 2.3 kg and 9.1 of dried distiller's grains with solubles and soyhulls (70% DDGS/30% soyhulls) respectively.

Composition of the diet (DM basis) fed to early weaned (EW) steers upon arrival to the feedlot and prior to normal weaning (NW), and the feedlot diet fed to EW and NW steers after normal weaning consisted on modified wet distiller's grains with solubles (45%), dry whole corn

(25%), corn husklage (20%), ground corn (7.3%), limestone (2.5%), trace mineral salt (0.1%), Rumensin 90 (198 g monensin/kg Rumensin 90; Elanco Animal Health, Greenfield, IN) (0.018%), Tylosin 40 (88 g tylan/kg Tylosin 40; Elanco Animal Health, Greenfield, IN) (0.012%), soybean oil (0.076%). Trace Mineral Salt contained 8.5% Ca (as  $\text{CaCO}_3$ ), 5% Mg (as MgO and  $\text{MgSO}_4$ ), 7.6% K (as  $\text{KCl}_2$ ), 6.7% Cl (as  $\text{KCl}_2$ ) 10% S (as  $\text{S}_8$ , prilled), 0.5% Cu (as  $\text{CuSO}_4$  and Availa-4 (Zinpro Performance Minerals; Zinpro Corp, Eden Prairie, MN)), 2% Fe (as  $\text{FeSO}_4$ ), 3% Mn (as  $\text{MnSO}_4$  and Availa-4), 3% Zn (as  $\text{ZnSO}_4$  and Availa-4), 278 ppm Co (as Availa-4), 250 ppm I (as  $\text{Ca}(\text{IO}_3)_2$ ), 150 Se ( $\text{Na}_2\text{SeO}_3$ ), 2,205 KIU/kg vitamin A (as retinyl acetate), 662.5 KIU/kg vitamin D (as cholecalciferol), 22,047.5 IU/kg vitamin E (as DL- $\alpha$ -tocopheryl acetate), and less than 1% CP, fat, crude fiber, and salt. Analyzed nutrient content of the EW diet was CP %, 17.3, NDF %, 23.9, ADF %, 14.1, and crude fat %, 5.3. Cow supplementation was initiated at  $103 \pm 11$  days prepartum while on pasture and it was halted at the midpoint of parturition ( $2 \pm 11$  days postpartum). More information about cow supplementation is reported elsewhere<sup>61</sup>.

Angus  $\times$  Simmental steer calves were randomly assigned to early or normal weaning (EW or NW) treatments within each gestational treatment. This allowed for 10 animals for each postnatal treatment and 5 animals of each of the interactions of gestational  $\times$  postnatal treatments. At  $78 \pm 2$  days postpartum, EW offspring were weaned, transported to Urbana Beef Unit, and adapted to a high-starch diet until they had *ad libitum* consumption. At  $187 \pm 2$  days postpartum, NW offspring were weaned and transported to Urbana Beef Unit. All offspring were co-mingled among treatments. LM biopsies were sampled from a subset of 5 animals per gestational  $\times$  postnatal treatment at  $\sim 78$  days of age,  $\sim 187$  days, and during the last week prior to harvest ( $\sim 354$  days). Blood was collected from the jugular vein at 78, 187 and 296 days of age to

isolate serum for insulin (Bovine Insulin ELISA kit, Cat No. 10–1201–01, Mercodia AB, Uppsala, Sweden), glucose (Diagnostics Laboratory, College of Veterinary Medicine, University of Illinois) and adiponectin (Millipore, LA, USA). After normal weaning, all offspring were placed on a common, corn grain-based high-starch finishing diet that is typical of industry management (CP %, 18.1, NDF %, 25.3, ADF %, 14.3, crude fat %, 5.1). All the offspring in the study were harvested at a commercial packing plant when they reached the selected end point target back fat thickness of 1.1 cm. Reported final body weight (BW) was calculated from hot carcass weight using a 62% dressing percentage.

Selection of steer progeny for biopsy was performed based on 3 criteria: first, offspring for biopsy were selected based on their dam's performance. Only offspring from cows whose BW and BW change during late gestation was within  $\frac{1}{2}$  of a standard deviation on either side of the mean were considered for biopsy. Selecting based on cow BW and BW change ensured that only calves from cows that were representative of their treatment were utilized for transcriptomics. The final selection of steers for biopsy was based on steer BW. Only steers whose BW was within  $\frac{1}{2}$  of a standard deviation on either side of the mean were utilized. This selection strategy minimized the effects of variation in dam's milk production, which was not significantly different between treatments<sup>61</sup>.

### ***Jugular blood sampling and analyses***

Blood was collected from the jugular vein at 78, 187 and 296 days of age to isolate serum for insulin (Bovine Insulin ELISA kit, Cat No. 10–1201–01, Mercodia AB, Uppsala, Sweden), glucose (Diagnostics Laboratory, College of Veterinary Medicine, University of Illinois) and

adiponectin. The latter was determined using a liquid RIA (Millipore, LA, USA) following a protocol previously described<sup>54</sup>. The sensitivity of the adiponectin assay was 1.6 ng/mL. The intra-assay CV for low (9.9 ng/mL) and high (55.2 ng/mL) concentrations were 10.2% and 13.4%, respectively.

### ***Feed intake and growth***

Individual intakes were obtained utilizing the Grow-Safe® feed monitoring system after normal weaning. Animals were weighed at birth, early weaning, midpoint between early and normal weaning, normal weaning, and every 2 weeks during the finishing phase. Ultrasonic measurements were taken for intramuscular fat deposition score and 12<sup>th</sup> rib fat thickness using ultrasound one day prior to early weaning and normal weaning, and during late finishing phase to sort the cattle into harvest groups with a target harvest backfat thickness of 1.1 cm.

### ***Total and microRNA analysis***

Total and miRNA were extracted using a mir-Vana™ miRNA isolation kit (Ambion, USA). Two hundred mg of *Longissimus* muscle tissue was homogenized in 10 vol of mirVana Lysis/Binding Solution using a Tissue-Tearor™ (BioSpec Products, Inc.) homogenizer at maximum speed. Then 1/10 vol of miRNA homogenate additive was added, mixed thoroughly via vortex for 30 s and incubated on ice for 10 min. An equal vol of Acid-Phenol:Chloroform (Ambion) was then added to each aliquot. The resulting solution were vortexed for 1 min and centrifuged for 5 min at  $10,000 \times g$  at room temperature. The aqueous phase was carefully

removed and then mixed thoroughly with 1.25 vol of 100% ethanol and passed through a mirVana Filter Cartridge in sequential 700  $\mu$ L aliquots. The Filter Cartridge was washed according to the manufacturer's protocol, and RNA was eluted in nuclease-free water at 95°C. Purity of RNA was assessed by ratio of optical density OD<sub>260nm</sub> (oligonucleotides absorbance) / OD<sub>280nm</sub> (protein absorbance) using a NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE).

Quantitative RT-PCR of miRNA expression was performed using the NCode™ miRNA First-Strand Synthesis and qRT-PCR kits (Invitrogen, USA), according to the manufacturer's protocol. For cDNA synthesis, 500 ng of purified RNA sample was added to MIX1, consisting of 5.0  $\mu$ L 5X miRNA Reaction Buffer, 2.5  $\mu$ L 25 mM MnCl<sub>2</sub>, 1  $\mu$ L diluted ATP, 0.5  $\mu$ L Poly A Polymerase and 11  $\mu$ L of DNase/RNase free water was then added. The mixture was incubated at 37°C for 15 min. Four  $\mu$ L polyadenylated RNA (from the above) was added to MIX2, consisting of 1  $\mu$ L of Annealing Buffer and 3  $\mu$ L of Universal RT Primer. The mixture was incubated at 65 °C for 5 min, and then placed on ice for 1 min. MIX3 was added to the above mixture, consisting of 10  $\mu$ L of 2 X First-Strand Reaction Mix and 2  $\mu$ L of SuperScript™ III RT/RNaseOUT™ Enzyme Mix. The mixture was incubated at 85°C for 5 min to stop the reaction.

The cDNA was then diluted 1:10 with DNase/RNase-free water and from this solution a pool of cDNA was made for standard curve preparation using a 1:5 dilution. A combination of 1  $\mu$ L of diluted cDNA with 9  $\mu$ L of the mix of 5  $\mu$ L of Perfecta SYBR Green Super Mix (Cat#95054-02K), 0.2  $\mu$ L each of 10  $\mu$ M sequence-specific forward primer and Universal qPCR Primer, and 3.6  $\mu$ L of DNase/RNase free water was added to each well of a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems). The reactions were performed in an ABI Prism

7900 HT SDS instrument (Applied Biosystems) under the following thermocycler conditions: 95°C for 5 min (Stage 1), and 40 cycles of 95°C for 10 s and 60°C for 30 s (Stage 2). Stage 3 consisted on 95°C for 15 s, 60°C for 15 s and 95°C for 15 s that allows seeing the dissociation curve of each reaction of the qPCR plate. MiRNA forward primers sequences for Bos Taurus (Table 18) were obtained from the microRNA database (<http://www.mirbase.org>). For better interpretation of the miRNA results, transcriptome results from a microarray analysis<sup>45</sup> using the same LM samples of the present study were used to evaluate other significant miRNA targets that were not considered at the beginning of this study (Figure 20).

### ***Statistical analysis***

Quantitative PCR data was analyzed using the MIXED procedure of SAS (SAS 9.1 Institute, Cary, NC, USA). Before statistical analysis, qPCR data was normalized using the geometric mean of miR-16b, miR-let-7a and miR-181a as internal control genes for miRNAs and the geometric mean of *UXT*, *MTG1* and *RPS15A* as internal control genes for the chosen microRNA targets. Fixed effects in the statistical model for each variable analyzed (i.e.: miRNAs and target genes) included plane of nutrition (diet), weaning time (wean), time on experiment (time), diet × wean, diet × time, wean × time, and wean × diet × time interactions. Gene expression data analysis included a repeated-measures statement with an autoregressive covariate structure. Animal performance, carcass quality parameters, blood biomarkers and ultrasound data also were analyzed using the MIXED procedure of SAS with diet as the fixed effect in the statistical model. The random effect in all models was steer.

The statistical model used was:  $Y_{ijklm} = \mu + C_i + T_j + B_k + S_l + (T \times D)_{ij} + (D \times W)_{jk} + (T \times W)_{ik} + (D \times T \times W)_{ijk} + \varepsilon_{ijklm}$ ; where,  $Y_{ijklm}$  is the background-adjusted normalized gene

expression data, etc.;  $\mu$  is the overall mean;  $T_i$  is the fixed effect of time (3 levels);  $D_j$  is the fixed effect of diet (3 levels);  $W_k$  is the fixed effect of wean (2 levels);  $S_l$  is the random effect of steer nested within weaning time;  $T \times D$ ,  $D \times W$ ,  $T \times W$  are the interactions of time by diet, diet by wean and time by wean, respectively;  $D \times T \times W$  is the interaction or third order for the main effects; and  $\varepsilon_{ijklm}$  is the random error ( $0, \sigma_e^2$ ) associated with  $Y_{ijklm}$ .

## Results

### *Animal Performance*

Performance data for the entire group of animals on study are reported elsewhere<sup>61</sup>. Ultrasound measurements performed at EW did not reveal differences ( $P > 0.05$ ) on marbling (intramuscular fat) and back fat thickness (Table 16). The EW steers had greater body weight as compared with NW steers during all the time they were under a finishing diet (Table 17). There were no significant differences ( $P > 0.05$ ) on BW at early weaning between treatments, i.e. all possible combinations between weaning age and cow plane of nutrition (Table 17). The EW steers had greater hip height ( $P = 0.05$ ) compared with NW steers (Table 17). Between 0 and 29 days on feedlot ( $P = 0.03$ ) average daily gain (ADG) was greater in HPN steers compared with other treatments, and between 29 and 57 days on feedlot ( $P = 0.05$ ) MPN steers had greater ADG compared with other treatments. An overall weaning age effect was detected at the beginning (0 – 29 d) of the finishing phase for ADG because of higher values for EW steers ( $P = 0.02$ ), and at the end (84 - 113d) of the finishing phase because of higher values for NW steers ( $P = 0.04$ ) (Table 17). Dry matter intake (DMI) during the finishing phase was lower (diet  $P = 0.04$ ) in LPN as compared with MPN and HPN steers (Table 17).



Age at harvest had a significant weaning effect due mainly to a delay in the harvest time for NW-MPN steers as compared with other treatments ( $P = 0.03$ ). The NW steers required longer to reach the target back fat thickness end point. In contrast, none of the carcass quality parameters presented significant differences between treatments (Table 17).

### ***Blood Biomarkers***

Serum glucose concentration had a significant time effect ( $P < 0.05$ ) with lower values at 296 days of age. Insulin concentration was affected by weaning age, time and weaning  $\times$  time interaction ( $P < 0.05$ ) with a noticeable increase in concentration between 187 and 296 days of age for all treatments (Figure 14). Adiponectin concentration had a significant cow plane of nutrition (diet), time effect ( $P < 0.05$ ) and diet  $\times$  time interaction ( $P < 0.05$ ). The HPN steers had a markedly lower adiponectin concentration as compared with MPN and LPN steers. The general pattern of serum adiponectin concentration was an increase between 78 and 187 days of age followed by a decrease in concentration after 187 days of age for all treatments. It was noteworthy that in EW-HPN the concentration increased between 187 and 296 days of age (Figure 14).

### ***Proadipogenic microRNAs***

#### ***Mir-103***

In our study, miR-103 had a time  $\times$  weaning interaction ( $P = 0.04$ ), a time  $\times$  diet interaction ( $P = 0.07$ ) and a time effect ( $P < 0.01$ ). MiR-103 expression increased between 78 and 187 days of age and decreased between 187 and 354 days of age in NW-MPN steers (Figure 15). In addition, EW-LPN and EW-HPN steers had a decrease in miR-103 expression between

78 and 187 days of age. There were no significant changes due to time for other treatments (Figure 15).

The adipogenic nuclear receptor *PPARG*, a target gene for miR-103 (Table 15), had a significant diet  $\times$  weaning  $\times$  time interaction, a time  $\times$  diet interaction, a weaning  $\times$  time interaction and a time effect (Figure 17). *PPARG* expression increased to the greatest extent in EW-HPN steers between 78 and 187 days of age. Other treatments also had an up-regulation of *PPARG* but to a lesser extent. In contrast, NW-HPN steers had a decrease in *PPARG* expression during this time. Between 187 and 354 days of age, *PPARG* expression decreased significantly for EW-HPN and EW-MPN steers but remained relatively constant for other treatments (Figure 17).

### ***Mir-143***

MiR-143 had a similar miRNA relative abundance compared with miR-103 (Figure 18). MiR-143 had a significant weaning  $\times$  time ( $P < 0.01$ ), a diet  $\times$  weaning interaction ( $P < 0.01$ ) and time effect ( $P < 0.01$ ) (Figure 15). MiR-143 had a slight increase in its expression between 78 and 187 days of age in NW-MPN and NW-LPN steers, and between 187 and 354 days of age it decreased while in all other treatments had an opposite pattern of expression (Figure 15). NW-HPN steers had an unchanged expression level of miR-143 throughout the study. After normal weaning time (187 days of age), the period when NW steers began to receive the high-starch diet, steers had a decrease in miR-143 expression, especially the NW-MPN steers.

The expression of *PPARG*, *CEBPA* and *FABP4* (all targets of miR-143; Table 15) had a significant diet  $\times$  weaning  $\times$  time interaction, a time  $\times$  weaning interaction, time  $\times$  diet interaction and a time effect (Figure 17). These three miRNA targets had a similar pattern of

expression with an increased activation in EW steers between 78 and 187 days of age followed by a marked decrease afterwards only in EW-HPN and EW-MPN steers (Figure 17).

### ***MiR-21-5p***

MiR-21-5p (5p denotes from the 5' arm) was the pro-adipogenic miRNA with lower relative miRNA abundance (Figure 18). MiR-21-5p had a significant diet  $\times$  time interaction ( $P = 0.05$ ). The difference in miR-21-5p expression for NW-HPN steers at 78 days of age as compared to other treatments (Figure 15) was not statistically significant (Figure 19), but leads to a more pronounced downregulation as compared to other treatments between 78 and 187 days of age (Figure 15). In contrast, miR-21-5p expression increased in NW-MPN and EW-LPN while all other treatments had a decrease in expression during this period of time. Between 187 and 354 days of age, miR-21-5p expression decreased in NW-MPN and NW-LPN, increased in EW-HPN and EW-LPN and remained relatively stable for NW-HPN and EW-MPN (Figure 15).

*FABP4* was the target gene measured for miR-21-5p (Table 15). *FABP4* had a significant time  $\times$  weaning  $\times$  diet interaction and it had similar expression pattern for NW-HPN when compared with miR-21-5p expression (Figure 17). Even though, in EW-HPN and EW-MPN steers, miR-21-5p had opposite expression pattern when it is compared with *FABP4* (Figure 17).

### ***MiR-378***

MiR-378 had a significant time  $\times$  diet  $\times$  weaning interaction ( $P = 0.05$ ) and a significant time effect ( $P < 0.01$ ) and it also was the pro-adipogenic miRNA with higher miRNA relative abundance (Figure 18). MiR-378 had a different effect on NW-MPN steers as compared to all

other treatments (Figure 16). For NW-MPN steers, miR-378 had an increase in its expression level between 78 and 187 days of age and a decrease between 187 and 354 days of age.

*PPARG* was the selected target gene of miR-378 (Table 15). MiR-378 had much higher relative miRNA abundance as compared to *PPARG* (Figure 18). At 187 days of age for EW-HPN steers, *PPARG* had the highest expression level while; it was the lowest for miR-378. Furthermore, miR-378 expression had an opposite behavior in NW-MPN steers as compared to other treatments.

### ***Antiadipogenic microRNAs***

#### ***MiR-27 a/b***

Our results revealed that miR-27 a/b was affected by cow plane of nutrition and weaning time (Figure 16). MiR-27a/b had a significant time  $\times$  diet  $\times$  weaning interaction ( $P < 0.01$ ), a time  $\times$  weaning interaction ( $P < 0.01$ ), a diet  $\times$  weaning interaction ( $P = 0.01$ ) and it was the anti-adipogenic miRNA with higher relative miRNA abundance (Figure 18). MiR-27a/b had an increased level of expression between 78 and 187 days of age for EW-LPN, NW-LPN and NW-MPN steers and a decrease for EW-HPN and EW-MPN steers, and no changes for NW-HPN steers at this time (Figure 16). Between 187 and 354 days of age, EW-MPN steers were the only ones that increased its miR-27a/b expression level while in other treatments, miR-27a/b expression decreased at different levels.

*CEBPA* and *PPARG* were the target genes measured for miR-27a/b (Table 15). They had similar expression pattern with a significant time  $\times$  weaning  $\times$  diet interaction ( $P < 0.01$ ), a weaning  $\times$  time interaction ( $P < 0.01$ ), a diet  $\times$  time interaction ( $P < 0.01$ ) and a time effect ( $P <$

0.01) (Figure 17). *PPARG* and *CEBPA* had higher expression level at 187 days of age for EW-HPN steers while miR-27a/b had the lowest expression at that time.

### ***MiR-130a***

Our results showed that miR-130a had a significant time  $\times$  diet  $\times$  weaning interaction ( $P = 0.03$ ), a significant time effect ( $P = 0.03$ ) and a diet effect ( $P < 0.01$ ) (Figure 16) MiR-130a relative miRNA abundance was negligible compared to other miRNAs and miRNA targets analyzed (Figure 18). Steers born from cows that received MPN had an increased level of expression for miR-130a between 78 and 187 days of age, while other treatments had undetectable changes (Figure 16). In contrast, between 187 and 354 days of age, EW-MPN, NW-MPN and EW-HPN steers had a significant decrease in miR-130a expression, while in NW-LPN, NW-HPN and EW-LPN steers remains constant (Figure 16).

*PPARG* was the miR-130a target gene selected (Table 15). *PPARG* was significantly activated between 78 and 187 days of age, while miR-130a remains with no changes during that time. NW-LPN steers had a decrease in miR-130a expression while *PPARG* had a slight increase between 187 and 354 days of age. Furthermore, EW-HPN steers decrease its expression levels for miR-130a and *PPARG* between 187 and 354 days of age (Figure 16 and 17).

### ***Mir-34a***

MiR-34a did not have any significant effect due to time, weaning age or cow plane of nutrition (Figure 16) but if we only consider the early weaning time (78 days of age), miR-34a had a significant cow plane of nutrition effect ( $P < 0.01$ ) with higher expression in HPN and LPN steers (Figure 19). Furthermore, miR-34a was the second anti-adipogenic miRNA with higher relative miRNA abundance within the studied anti-adipogenic miRNAs (Figure 18). In

general, the anti-adipogenic miRNAs measured had lower miRNA relative abundance as compared to the pro-adipogenic miRNAs (Figure 18).

Silent information regulator 1 (*SIRT1*) is the target gene selected for miR-34a (Table 15). *SIRT1* expression decreased between 78 and 187 days of age for NW steers and between 187 and 354 days of age for EW steers.

### ***MiR-369-5p***

MiR-369-5p had a significant weaning effect ( $P = 0.02$ ) and its miRNA relative abundance was negligible as compared with other miRNAs and its targets (Figure 14). MiR-369-5p was only affected by weaning age, presenting an important numerical increase in its expression level for NW-MPN steers which was only a statistical tendency ( $P = 0.06$ ) to be different from other treatments (Figure 16).

*ADIPOQ* and *FABP4* were the target genes selected for miR-369 (Table 15). *ADIPOQ* expression was higher between 78 and 187 days of age especially in EW steers (Figure 17). *ADIPOQ* had a significant time  $\times$  diet  $\times$  weaning interaction ( $P < 0.01$ ), a weaning  $\times$  time interaction ( $P < 0.01$ ), a diet  $\times$  time interaction ( $P < 0.01$ ), a time effect ( $P < 0.01$ ) and a weaning effect ( $P = 0.03$ ). *ADIPOQ* expression did not change for NW steers, it had an increase expression level for NW-LPN steers and it decreased for EW-HPN and EW-MPN steers between 187 and 354 days of age.

### ***MiR-448***

Our results showed that miR-448 had a significant time  $\times$  weaning interaction ( $P < 0.01$ ), a significant time  $\times$  diet interaction ( $P < 0.01$ ) and a significant time effect ( $P = 0.01$ ) (Figure 16). MiR-448 was the miRNA studied with lower relative miRNA abundance compared to all others miRNA and miRNA targets analyzed in this study (Figure 18). EW-MPN steers were the only ones with a differentiated pattern of miR-448 expression (Figure 16). EW-MPN steers had an increment in miR-448 expression between 78 and 187 days of age, followed by downregulation between 187 and 354 days of age, while all remaining treatments had a relatively constant expression level. There were no target genes measured for miR-448 in this study (Table 15).

## **Discussion**

### ***Animal Performance***

Overall, steer feedlot performance in terms of ADG was positively influenced by the plane of nutrition of the dam, and coincided with results from a previous study of similar design<sup>12</sup>. However, unlike another study where cow precalving BCS was manipulated through diet<sup>4</sup>, the MPN offspring had better performance during the first half and lower performance during the second half of the growing phase. More specifically, between 78 and 187 days of age, access to a high-starch diet was advantageous to EW-MPN steers which had a higher growth performance compared with NW-MPN steers which were grazing and nursing their dams during the same time-frame. This difference in nutrition during the growing phase resulted in more efficient growth in EW-MPN steers; in contrast, NW-MPN remained longer in the feedlot due to a lower efficiency of growth during the growing phase, i.e. nutritionally grazing and nursing “delayed” their target back fat thickness end point during finishing phase.

### ***Blood Biomarkers***

The decrease in glucose concentration between 187 and 296 days of age might reflect a greater rate of glucose uptake from the bloodstream. Higher plasma insulin levels between 187 and 296 days of age are common in animals fed diets that result in greater production of propionate (i.e. high-starch diets). Similar to a previous study<sup>46</sup>, serum glucose and insulin concentrations were not affected by weaning time. Adiponectin is exclusively secreted from white adipose tissue<sup>16</sup>. Levels of this adipokine are usually inversely correlated with body fat percentage and positively correlated with body size in humans<sup>62</sup>. Alterations in plasma adiponectin levels are inversely correlated with biomarkers of adipose tissue mobilization (i.e. NEFA), some of which might be caused by a local inflammatory response<sup>25</sup>. The lower plasma adiponectin levels measured in HPN steers could be related to the presence of an inflammatory response produced via macrophage infiltration in visceral or subcutaneous adipose tissue<sup>20; 19</sup>. Adiponectin also increases tissue sensitivity to insulin in non-ruminants, hence, greater adiponectin concentrations may facilitate a biological response to lower concentrations of insulin<sup>1</sup>. The low serum adiponectin concentrations in HPN steers could be taken as an indication of reduced insulin sensitivity<sup>48</sup>.

### ***Proadipogenic microRNAs***

#### ***Mir-103***

In a mouse study with two different obese models (diet-induced obesity and leptin-deficient ob/ob) treatment of animals with a miR-103 antagonist (i.e. *TNF- $\alpha$* ) the in vivo induction of adipogenesis by miR-103 was confirmed because triglyceride accumulation increased during early stages of adipogenesis. In contrast, in vitro, in both obesity models *TNF- $\alpha$*



treatment of differentiated adipocytes led to miR-103 downregulation<sup>68</sup>. In another study with guinea pig offspring with intrauterine growth restriction, miR-103 was greater in low birth weight male offspring with greater visceral adiposity<sup>60</sup>.

MiR-103 is an intronic miRNA that resides in the sense orientation (5' to 3') in intron 5 of pantothenate kinase family member genes (*PANK*), hence, miR-103 is associated with *PANK*<sup>51</sup> which has a role in the rate-limiting step of CoA generation. Phosphorylation of pantothenate (Vitamin B5)<sup>66</sup> is governed by *PANK*, and constitutes an important step in the overall metabolism of pyruvate, lactate, glucose and the citric acid cycle, or its incorporation into the acyl-carrier-protein needed for fatty acids synthesis<sup>55</sup>. In a previous study, miR-103 was co-regulated with *PANK* during 3T3-L1 adipogenesis<sup>68</sup>. *PANK* is located in the mitochondria and it senses the levels of palmitoyl-carnitine and increases CoA biosynthesis in response to an increased mitochondrial demand for the cofactor to support beta-oxidation<sup>37</sup>.

More data supporting a pro-adipogenic role for miR-103 (after ectopic expression) during adipocyte development in 3T3-L1 cells include an increase in the rate of triglyceride accumulation in adipocytes and also upregulation of the expression of important transcription factors such as *PPARG*, key cell cycle regulators such as G0/G1 switch 2 (*G0S2*), and molecules associated with lipid metabolism (*FABP4*), glucose homeostasis (*SLC2A4*) and endocrine functions (*ADIPOQ*) of adipocytes<sup>68</sup>. The upregulation between 78 and 187 days of age of *SLC2A4* coupled with downregulation of *G0S2* (Figure 20) argues against an effect of miR-103 on cell cycle through *G0S2*. It suggests that miR-103 helps regulate glucose homeostasis through *SLC2A4* in NW-MPN steers.

MiR-103 also was associated with insulin and glucose levels through inhibition of caveolin 1 (*CAVI*) which influences lipid raft signaling, through destabilization of the insulin receptor (*INSR*), leading to increased insulin resistance in the adipose tissue<sup>64; 58</sup>. *CAVI* inhibition supports this mechanism for NW-MPN steers between 78 and 187 days of age (Figure 20). Taken together, data suggest that miR-103 and *PANK1* could activate adipogenesis in LM of NW-MPN steers but, alternatively, they could have increased insulin resistance in the adipose tissue of NW-MPN steers through downregulation of *INSR* due to *CAVI* inhibition.

### ***Mir-143***

In cultured preadipocytes, miR-143 was upregulated on day 4 and increased further on day 8 after induction of differentiation, indicating that miRNA-143 normally promotes intramuscular preadipocyte differentiation; it was also reported that miR-143 is highly-expressed in bovine intramuscular fat<sup>65</sup>. Furthermore, in cells transfected with an miR-143 inhibitor, *CEBPA* and *FABP4* expression was downregulated<sup>38</sup>. The transfection of the fibroblast-like preadipocytes with miRNA-143 antisense inhibitor induced a significant suppression of differentiation, indicated by a decrease in storage of lipid droplets<sup>38</sup>. In contrast, cell proliferation was increased with the transfection of a miRNA-143 inhibitor. Taken together, the above results and those of the present study confirm the importance of miR-143 in bovine intramuscular fat development.

Greater miRNA-143 expression in NW compared with EW steers before weaning (187 days of age) suggests that in those steers epigenetic control of adipogenesis via miR-143 was more pronounced. In human adipocytes, free fatty acids downregulate miR-143<sup>72</sup>. At late terminal differentiation stage of adipogenesis, miR-143 leads to downregulation of delta-like 1

(*DLK1*), an inhibitor of adipocyte differentiation through *ERK1/2* activation<sup>34</sup>. Furthermore, hyperinsulinemia significantly suppressed *DLK1*<sup>29</sup>. Before 187 days of age, the fact that NW were nursing their dam, consuming pasture, and receiving some grain meant that insulin secretion was less pronounced compared with EW steers fed the high-starch diet (Figure 14). The expression of *DLK1* was downregulated between 78 and 187 days of age (Figure 20), hence, it could be possible that miR-143 activation in NW steers and the higher serum insulin concentration in EW steers were mechanisms that ensured downregulation of *DLK1*. In contrast to what was expected, miR-143 target genes (*PPARG*, *CEBPA* and *FABP4*) were downregulated when miR-143 was upregulated suggesting that miR-143 was not influencing these targets genes. Taken together, the main mechanism of action of miR-143 for promoting adipogenesis in LM was by blocking adipogenic repressors (*DLK1*) more than promoting proadipogenic gene activation.

### ***MiR-21-5p***

MiR-21 is upregulated in white adipose tissue (WAT) of obese humans as compared with lean controls<sup>30</sup>. MiR-21 also controls adipogenesis by modulating its target transforming growth factor beta receptor 2 (*TGFBR2*)<sup>33</sup>. In human adipose tissue-derived mesenchymal stem cells (hASCs) induced to overexpress miR-21, TGF- $\beta$ -induced inhibition of adipocyte differentiation was significantly inhibited. In our study, *TGFBR2*, *TGF- $\beta$ 2* and *TGF- $\beta$ 3* were affected by time (Figure 20) with activation of the receptor between 187 and 354 days and inhibition of TGF- $\beta$ 3 before 187 days and TGF- $\beta$ 2 between 187 and 354 days. Judging by their expression pattern, *TGFBR2* seems to be more susceptible to miR-21 control because its expression did not change when miR-21 was upregulated (i.e. NW-MPN steers).

In a study using human adipose tissue-derived mesenchymal stem cells (hASCs), *STAT3* was detected as the most likely target gene for miR-21. MiR-21 reduced proliferation of hASCs by impairing total and phospho-STAT3 expression which inhibits self-renewal of stem cells<sup>32</sup>. In contrast, it has been reported that miR-21 increases proliferation in cancer cells through inhibition of programmed cell death protein 4 (*PDCD4*) and phosphatase and tensin homolog (*PTEN*)<sup>11</sup>. By means of its interaction with *STAT3*, in the present study, miR-21 inhibition between 78 and 187 days of age for NW-HPN and EW-HPN might have generated an increment in cell proliferation during the clonal stage of adipogenic expansion; between 187 and 354 days of age, when the rate of fat accumulation was likely greater in the steers (especially EW), miR-21 could have interacted with *PDCD4* leading to an anti-apoptotic effect. As such, the adipocyte differentiation process would have continued undisrupted.

Subedi and coworkers<sup>63</sup> suggested that miR-21/*PDCD4* axis may be a novel target mediating adiponectin-induced biological responses because miR-21 increase adiponectin mRNA and protein expression levels<sup>28</sup>. In that context, the fact that EW-HPN and NW-HPN steers had a lower level of serum adiponectin and a simultaneous decrease in miR-21 expression suggest the existence of a similar effect.

### ***MiR-378***

In beef cattle, miR-378 is located on intron 1 of peroxisome proliferator-activated receptor gamma coactivator 1 beta (*PGC-1 $\beta$* )<sup>7; 57</sup> and is strongly associated with back fat thickness<sup>22</sup>. Mitogen-activated protein kinase 1 (*MAPK1*) is a target of miR-378<sup>13</sup>, and it can mediate phosphorylation of *PPARG* leading to a reduction in transcriptional activity<sup>22</sup>. This suggests that miR-378 may induce both *PPARG* and *PGC-1 $\beta$*  to allow both to function

synergistically in the regulation of lipid metabolism. Importantly, this induction is not limited to mouse 3T3-L1 cells as miR-378 was recently identified as the most highly up-regulated miRNA during the differentiation of both mouse and human primary adipocytes. In general, we observed that when *PPARG* was activated miR-378 was inhibited, hence, casting doubt of a positive effect of miR-378 on *PPARG* activation. MiR-378 activation leads to inhibition of *ADIPOQ*<sup>18</sup>, but only in the case of NW-MPN steers there was a similar expression profile of miR-378 and both *PPARG* and *ADIPOQ* (Figure 15 and 17). The data suggest that miR-378 activation in NW-MPN steers before 187 days of age might have been influenced by a higher proportion of preadipocytes in the LM or by a higher intracellular concentration of free fatty acids in LM which were previously reported to activate both miR-378 and adipocyte differentiation<sup>14</sup>.

MiR-378 is considered a novel target for controlling adipose tissue inflammation because it has been established that secretion of adipokines and cytokines (i.e. IL-6, TNF- $\alpha$ ) due to onset of obesity upregulate miR-378 expression through *SREBP* and *C/EBP* binding sites<sup>21</sup>. In the microarray analysis of LM from these animals<sup>45</sup>, data revealed a clear innate immune response in EW steers between 78 and 187 days of age, hence, it is unlikely that the marked upregulation of miR-378 through 187 days of age in NW-MPN steers was related to IMF inflammation. Furthermore, *SREBF1* activation was not significant ( $P = 0.08$ ) (Figure 20) while *CEBPA* was upregulated (Figure 17) when miR-378 was downregulated (Figure 15). Thus, the data could suggest that secretion of cytokines within the LM might control miR-378 expression more likely through its C/EBP binding sites.

In conclusion, at 187 days NW-MPN steers had the highest level of activation of all proadipogenic miRNAs analyzed. Because the NW-MPN steers remained in the feedlot for a longer period of time to achieve the desired threshold of backfat for slaughter (Table 16), it is

possible that in these animals adipogenic corepressors in LM hampered the activity of the proadipogenic microRNAs. Hence, the marked upregulation of these miRNAs.

### ***Antiadipogenic microRNAs***

#### ***MiR-27 a/b***

MiR-27 inhibits adipogenesis partially by repressing the early adipogenic transcription factor cAMP response element-binding protein (*CREB*) by directly targeting its 3' UTR<sup>73</sup>. Tumor necrosis factor alpha (*TNF-α*) treatment upregulates miR-27 through the *NF-κB* pathway<sup>73</sup>. The utilization of an anti-miR-27 reduces the *TNF-α*-induced inhibition of adipogenesis. The levels of miR-27 expression decrease in mature adipocytes of obese mice when compared with lean mice<sup>73</sup>. In the present study, the downregulation of miR-27 a/b when *TNF* was activated (Figure 16 and Figure 20) suggests that control of adipogenesis through miR-27 after 187 days of age could have been impaired by the cytokine, especially in NW-MPN and NW-LPN steers. This hypothesis is supported not by the degree of change in miR-27a/b expression but because miR-27 a/b was very abundant as compared to other anti-adipogenic miRNAs. As such, it can be surmised that a slight upregulation of miR-27a/b could be enough to produce the inhibitory effect in adipogenesis.

The negative effect of miR-27 on adipogenesis is counterbalanced by activation of Wnt/ $\beta$ -catenin signaling<sup>53</sup> leading to myocyte differentiation<sup>71</sup>. In a previous study, marked upregulation of miR-27b partly explained muscle hypertrophy in Piedmontese cattle due to its linkage with Myostatin (*MSTN*)<sup>44</sup>. MiR-27a over expression resulted in reduced *MSTN* expression, skeletal muscle hypertrophy and increase in the number of activated satellite cells<sup>41</sup>. In the present study, the upregulation of miR-27a/b could have affected *MSTN* expression but

*MSTN* downregulation was not significant ( $P = 0.41$ ) between 78 and 187 days of age (Figure 20). Taken together, there was no consistency in miR-27a/b response to treatments, and only in the case of NW steers between 187 and 354 days of age (especially for NW-MPN steers) miR-27a/b expression decreased in general but it did not seem to correlate with lower intramuscular fat in those steers.

### ***MiR-130a***

A previous study provided evidence of miR-130 as a potential negative regulator of adipogenesis by inhibiting *PPARG*<sup>15</sup>. In another study with adipocytes<sup>31</sup>, pro-inflammatory responses mediated by TNF $\alpha$  treatment resulted in adipocyte dysfunction by down-regulating *PPARG* expression. Inhibition of miR-130a and miR-130b using specific miRNA inhibitors significantly restored *PPARG* expression upon TNF $\alpha$  stimulation. These results indicate that miR-130 induced by the TNF $\alpha$ -mediated inflammatory response could downregulate *PPARG* expression. Taken together, miR-130a action in MPN steers could have impaired normal *PPARG* activation between 78 and 187 days of age, and subsequently between 187 and 354 days of age and through *TNF* stimulation, maintain *PPARG* expression at a constant level (i.e. impairing its activation) in steers that received less dietary starch in utero and postpartum (Figure 16, 17 and Figure 20).

### ***Mir-34a***

The different pattern of expression of miR-34a in response to maternal plane of nutrition at 78 days of age provides the closest evidence for epigenetic regulation of LM in offspring steers. In human subcutaneous adipose tissue, miR-34a was positively upregulated during adipogenesis, and correlated with body mass index (BMI)<sup>50</sup>. MiR-34a inhibits *SIRT1* expression

through a miR-34a-binding site within the 3'UTR of *SIRT1*<sup>6</sup>. *TP53* induces expression of miR-34a which suppresses *SIRT1*<sup>70</sup>. *SIRT1* also regulates *TP53*-dependent apoptosis through deacetylation and stabilization of *TP53*. Furthermore, *TP53* and miR-34 function in parallel to activate *p21* to induce cell cycle arrest<sup>69</sup>. Between 78 and 354 days of age, miR-34a expression was not affected by treatment (Figure 16), but it was significantly different at 78 days of age (Figure 19). In contrast, *SIRT1* remained unchanged in EW steers while *TP53* was upregulated at this time (Figure 20). This situation suggests that miR-34a has a role on the activation of cell cycle arrest in LM by suppressing *SIRT1* activation, which leads to *TP53* activation between 78 and 187 days of age.

### ***MiR-369-5p***

The relative miRNA abundance of miR-369-5p was the second lowest in the present study, thus, its relevance to intramuscular adipogenesis appears doubtful. It was previously determined that miR-369-5p directly targets *FABP4* in human mesenchymal stem cells (MSC)<sup>5</sup>. MiR-369-5p binds directly to the 3'UTR of *FABP4* to decrease gene expression and thereby inhibit adipogenesis. This miRNA inhibits the adipogenic factors *ADIPOQ* and *FABP4* and causes an overall reduction in lipid droplet formation<sup>5</sup>. In addition to its low miRNA abundance, the fact that expression of *CEBPA* and *PPARG* did not correlate with that of miR-369-5p is further support that this miRNA is not relevant for intramuscular fat deposition in steers.

### ***MiR-448***

MiR-448-mediated repression of Krüppel-like factor 5 (*KLF5*) at its 3' UTR contributes to inhibition of adipocyte differentiation<sup>49</sup>, which was confirmed by the lower expression of adipogenic genes and intracellular triglyceride<sup>35</sup>. *KLF5* is activated by *C/EBPB* and *C/EBPD*



and, in concert with these C/EBPs<sup>10</sup>, contributes to *PPARG* induction which regulates adipocyte differentiation<sup>49</sup>. The expression of *CEBPA* and *PPARG* was decreased significantly in cells transfected with miR-448. In contrast, *C/EBPB* and *C/EBPD* were not affected significantly by miR-448<sup>35</sup>. From the microarray data it can be surmised that *KLF5* upregulation between 187 and 354 days of age in EW-MPN (Figure 20) was partly due to the downregulation of miR-448 steers. It is noteworthy that miR-448 relative expression was quite low as compared with other miRNAs, but its expression in EW-MPN between 78 and 187 days increased substantially. Clearly, additional research needs to be performed to better understand the functional cause of miR-448 activation in LM of EW-MPN steers.

## Conclusions

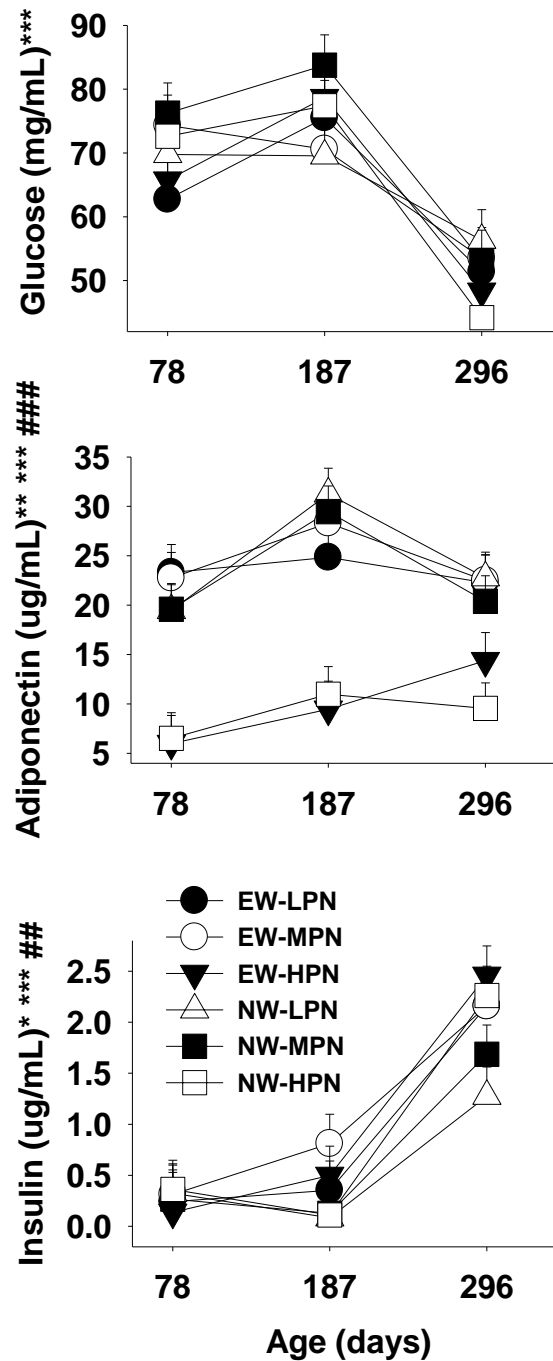
MiR-34a was the sole miRNA studied in offspring LM that had a significant change in expression at 78 days of age due to cow plane of nutrition. Within the miRNAs studied, this fact makes miR-34a, the one with the clearest sign of epigenetic regulation of LM of beef offspring due to cow plane of nutrition during late gestation. MiR-34a has a role on the activation of cell cycle arrest in LM of HPN and LPN steers by suppressing *SIRT1*, which leads to an activation of *TP53* between 78 and 187 days of age. In conclusion, cow plane of nutrition regulates miR-34a expression in offspring LM before weaning. Furthermore, epigenetic control of LM metabolism was positively affected by selected proadipogenic miRNAs between 78 and 187 days of age, especially in animals that had a delay in reaching target back fat thickness like NW-MPN steers during the finishing phase.

## Tables and Figures

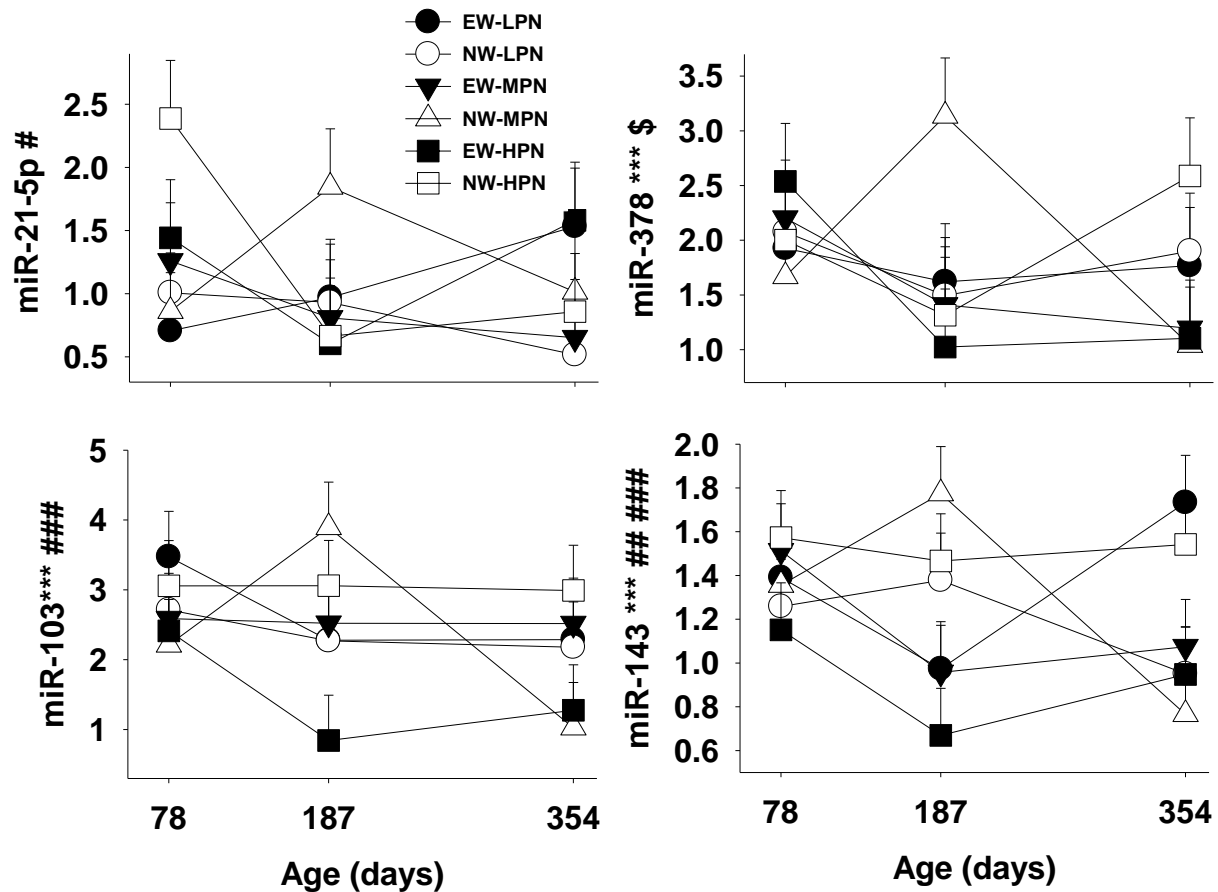
**Table 15:** Literature utilized to select proadipogenic and antiadipogenic miRNAs from studies of Mammalian (Ma), Human (H), Mouse (Mo) and Bos Taurus (B) miRNAs.

miRNAs	Function	Model system	Targets	References
miR-21-5p	Proadipogenic	B	<i>PTEN, PDCD4, FABP4</i>	52; 17;27
miR-27 a/b	Antiadipogenic	Ma	<i>PPARG, CEBPA</i>	39; 42; 56; 59
miR-34a	Antiadipogenic	Ma/H	<i>SIRT1</i>	36; 17; 56
miR-103	Proadipogenic	Mo/Ma	<i>PPARG, PANK, Cav-1, FASN, ADIPOQ, FABP4</i>	56; 65; 24; 40
miR-130a	Antiadipogenic	H	<i>PPARG</i>	17; 56
miR-143	Proadipogenic	Ma/Mo/B/H	<i>ERK5, GLUT4, aP2, HSL, PPARG2, CEBPA, FABP4</i>	9; 26; 69; 22; 38; 56
miR-369-5p	Antiadipogenic	H	<i>ADIPOQ, FABP4</i>	5; 17
miR-378	Proadipogenic	B/Mo	<i>PPARGC1B, PPARG, PGC1B, MAPK1</i>	14; 22; 24; 59
miR-448	Antiadipogenic	Ma	<i>KLF5</i>	35; 56

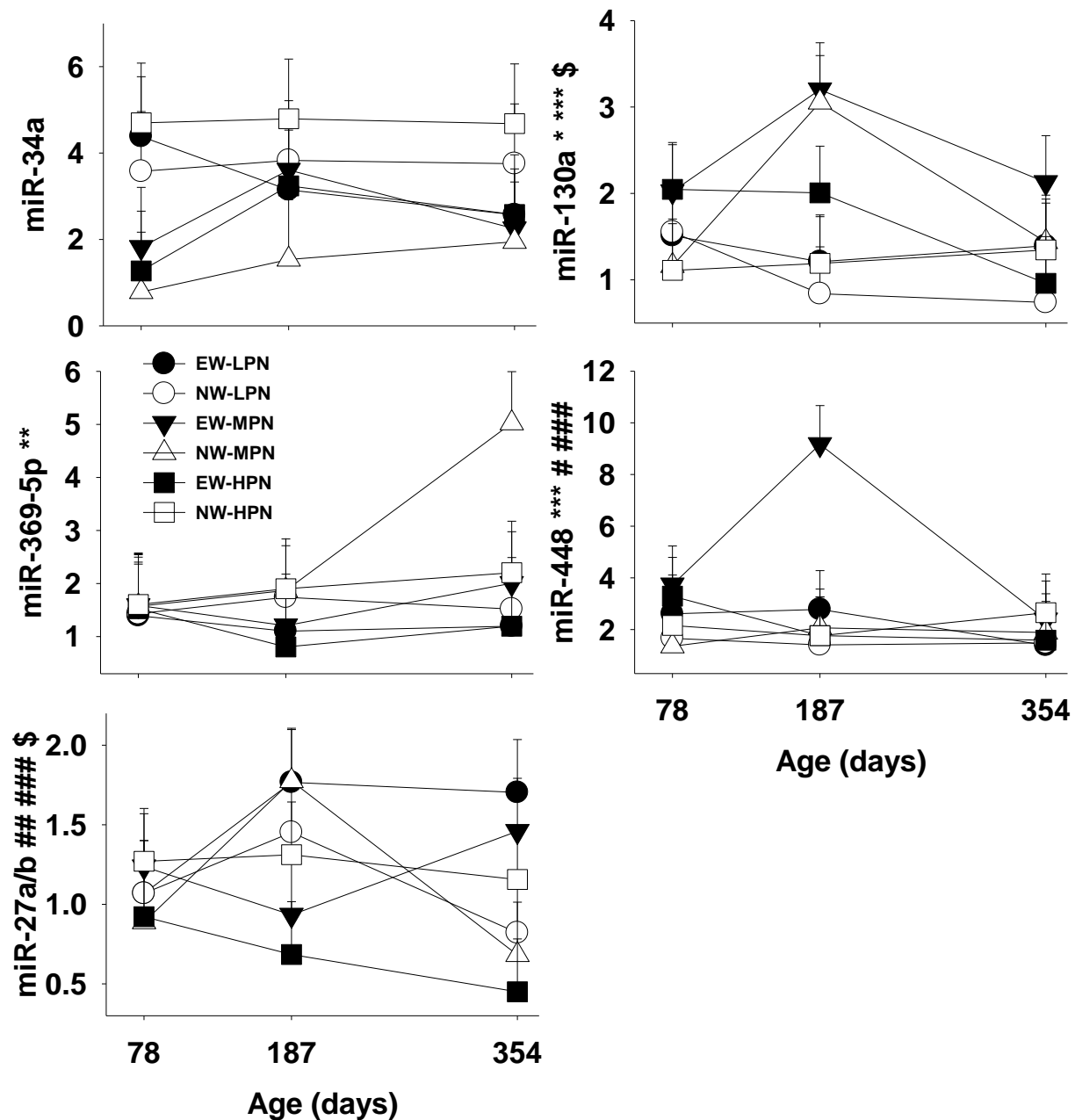
**Figure 14.** Glucose, adiponectin and insulin serum levels for Angus  $\times$  Simmental steers from cows that received a low (LPN), medium (MPN) or a high (HPN) plane of nutrition during the late gestation period. Weaning times are early weaning (EW) and normal weaning (NW). \* Weaning, \*\* Diet, \*\*\* Time, # weaning  $\times$  time, ## diet  $\times$  weaning, ### diet  $\times$  time and \$ time  $\times$  weaning  $\times$  diet interaction. Significant differences are declared at  $P < 0.05$ .



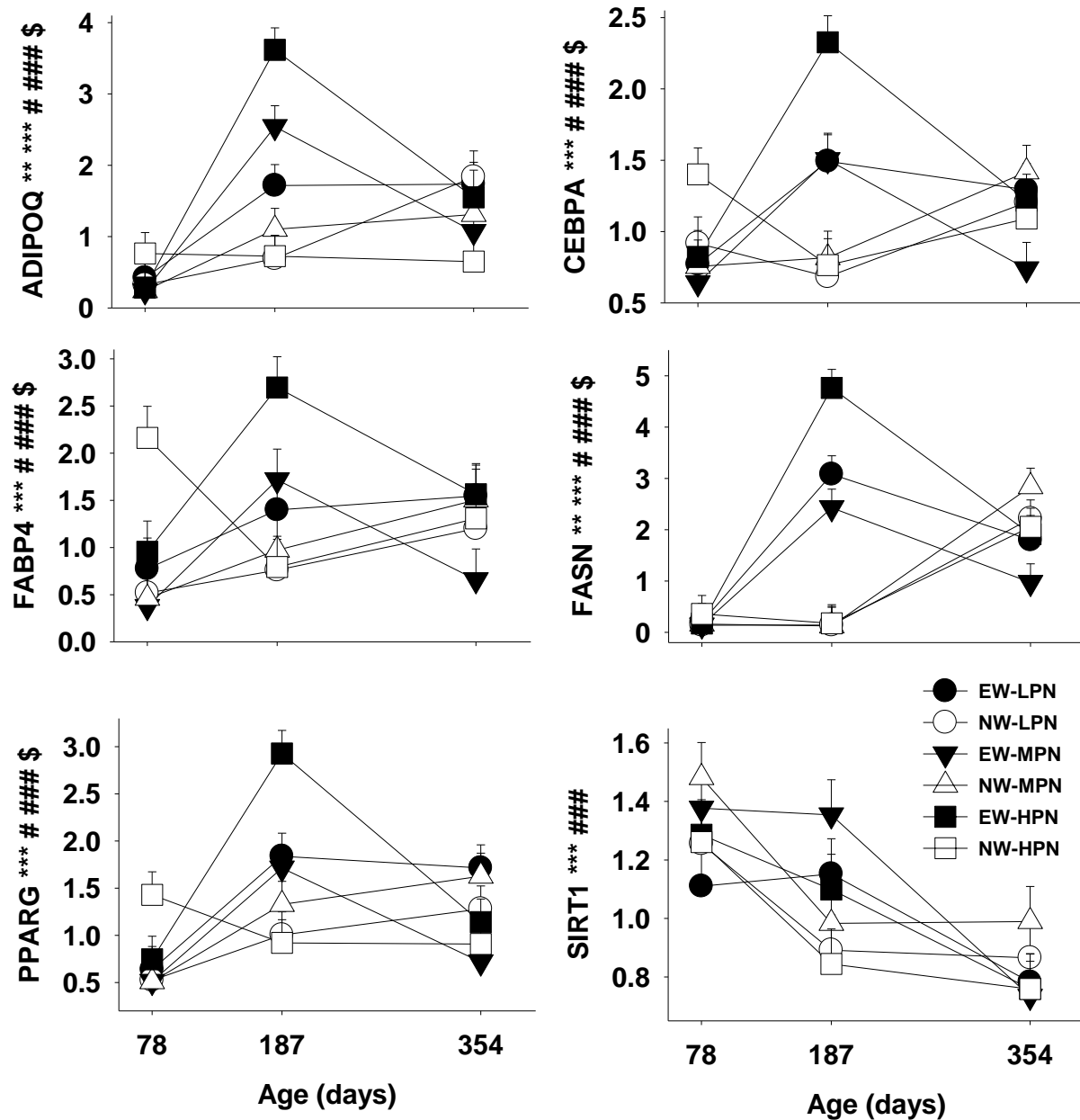
**Figure 15.** Pro-adipogenic microRNA expression of *Longissimus* muscle for Angus × Simmental steers from cows that received a low (LPN), medium (MPN) or a high (HPN) plane of nutrition during the late gestation period. Weaning times are early weaning (EW) and normal weaning (NW). \* Diet, \*\* Weaning, \*\*\* Time, # diet × time, ## diet × weaning, ### time × weaning and \$ time × weaning × diet interaction. Significant differences are declared at  $P < 0.05$ .



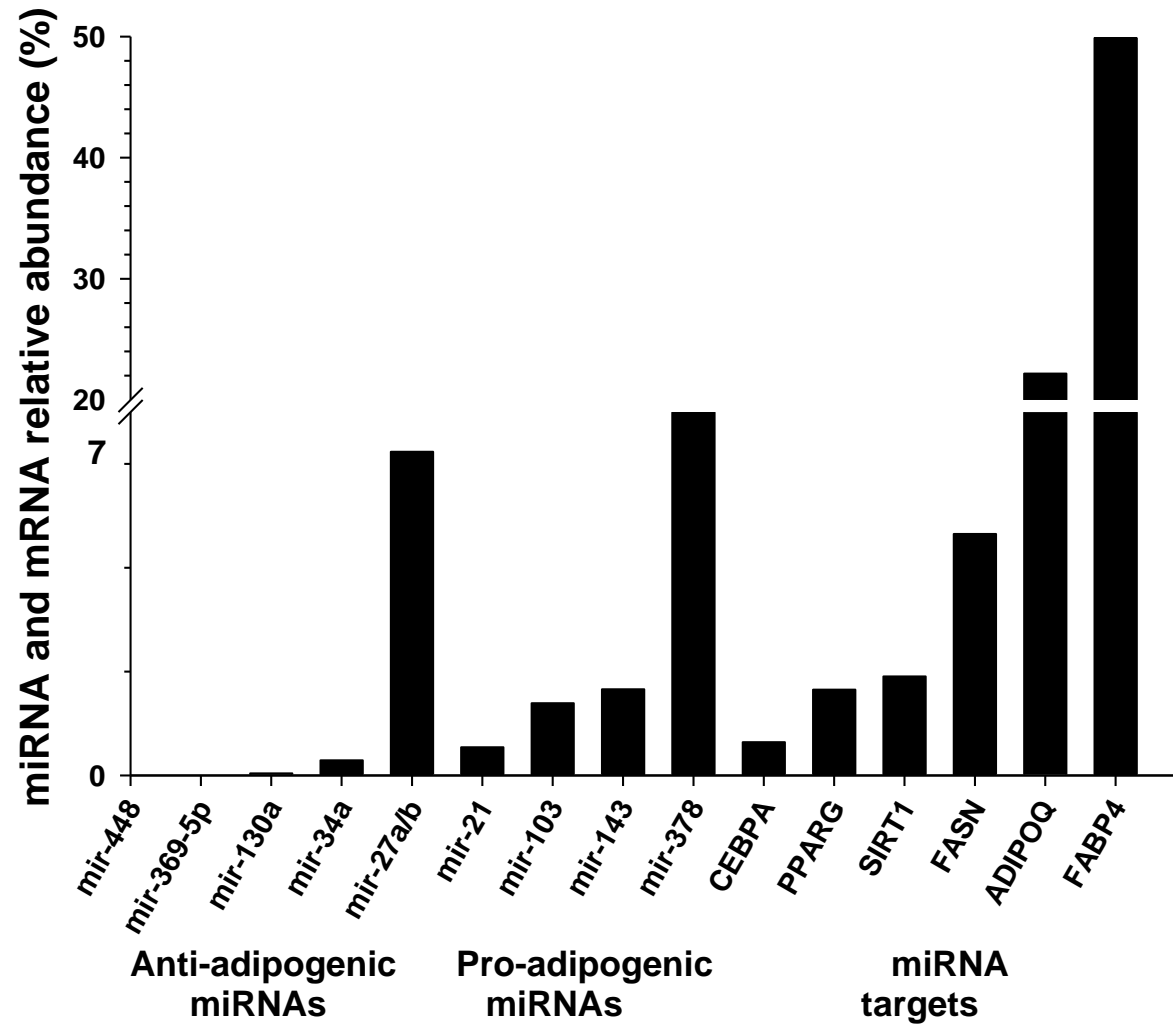
**Figure 16.** Anti-adipogenic microRNA expression of *Longissimus* muscle for Angus × Simmental steers from cows that received a low (LPN), medium (MPN) or a high (HPN) plane of nutrition during the late gestation period. Weaning times are early weaning (EW) and normal weaning (NW). \* Diet, \*\* Weaning, \*\*\* Time, # diet × time, ## diet × weaning, ### time × weaning and \$ time × weaning × diet interaction. Significant differences are declared at  $P < 0.05$ .



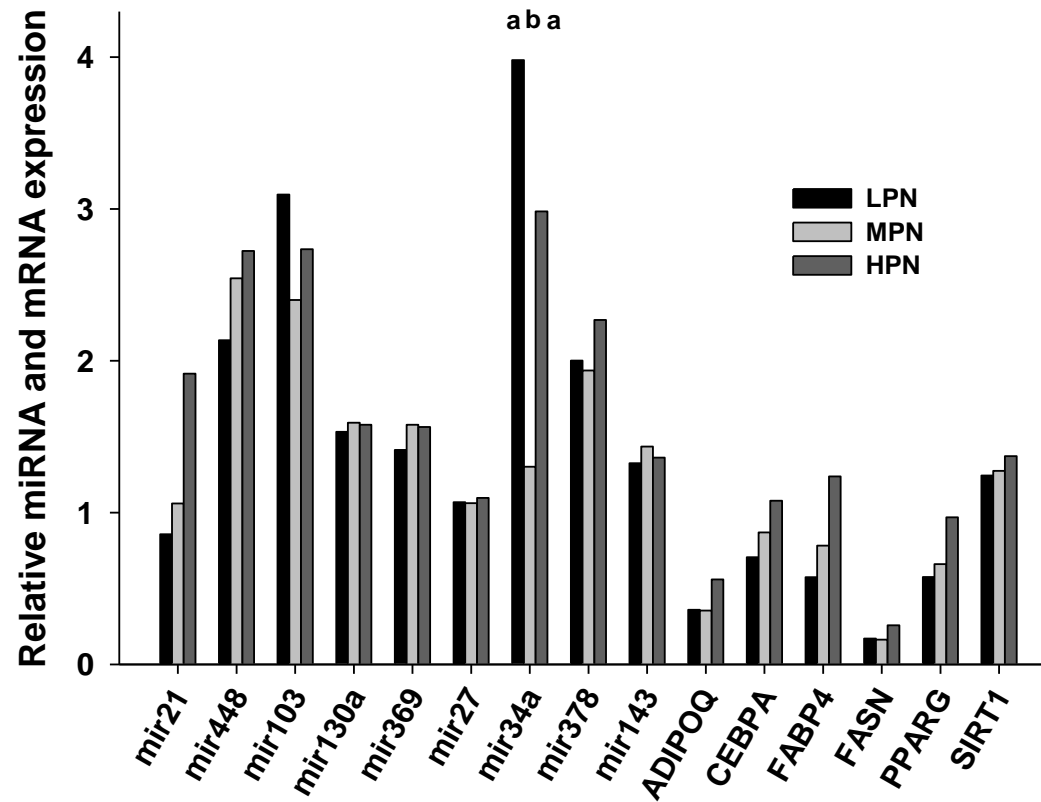
**Figure 17.** microRNA targets expression of *Longissimus* muscle for early weaning biopsy day of Angus  $\times$  Simmental steers from cows that received a low (LPN), medium (MPN) or a high (HPN) plane of nutrition during the late gestation period. Weaning times are early weaning (EW) and normal weaning (NW). \* Diet, \*\* Weaning, \*\*\* Time, # diet  $\times$  time, ## diet  $\times$  weaning, ### time  $\times$  weaning and \$ time  $\times$  weaning  $\times$  diet interaction. Significant differences are declared at  $P < 0.05$ .



**Figure 18.** Pro-adipogenic and anti-adipogenic microRNA and its targets relative mRNA abundance percentage.

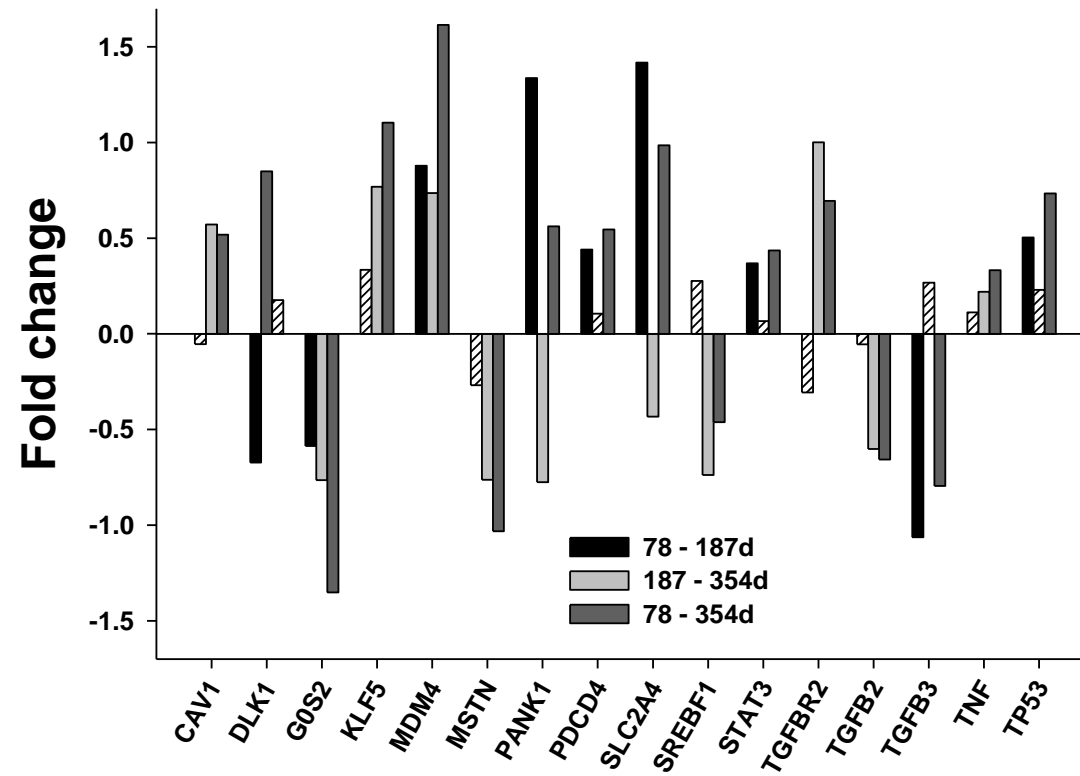


**Figure 19.** Pro-adipogenic and anti-adipogenic microRNA and its selected target genes expression at 78 days of age of *Longissimus* muscle for Angus × Simmental steers from cows that received a low (LPN), medium (MPN) or a high (HPN) plane of nutrition (diet) during the late gestation period. Weaning was not considered in the analysis. Different letters denote differences due to cow plane of nutrition. Significant differences are declared at  $P < 0.05$ .





**Figure 20.** MicroRNA targets fold expression due to time effect from microarray analysis at 78, 187 and 354 days of age of *Longissimus* muscle for Angus  $\times$  Simmental steers. Black bars represent time comparison 78 - 187 days of age, light gray bars for comparison 187 - 354 days of age and dark gray bar for comparison 78 - 354 days of age. White hatched bars denote not significant differences ( $P > 0.05$ ). Significant differences are declared at  $P < 0.05$  and FDR  $< 0.10$ .



**Table 16.** Ultrasound measurements and carcass quality parameters for Angus × Simmental biopsied steers (n = 35) from cows that received a low (LPN), medium (MPN) or high (HPN) plane of nutrition during the late gestation period. Weaning times are early weaning (EW) and normal weaning (NW). Significant differences are declared at  $P < 0.05$ .

	Treatments						SEM	P value		
	EW			NW				W	D	W*D
	LPN	MPN	HPN	LPN	MPN	HPN				
Ultrasound measurements										
BF at EW (cm)	0.31	0.35	0.33	0.31	0.35	0.34	0.02	0.81	0.13	0.92
BF at NW (cm)	0.33	0.34	0.38	0.32	0.32	0.32	0.02	0.14	0.38	0.65
Marbling at EW	427	389	376	428	413	400	28	0.50	0.38	0.89
Marbling at NW	330	400	342	365	427	421	14	0.16	0.26	0.77
Carcass quality parameters										
HCW (Kg)	340	359	373	323	336	351	17	0.11	0.16	0.97
Calculated YG	3.15	3.21	2.94	3.09	2.94	3.03	0.23	0.65	0.83	0.71
LM area (cm2)	77.34	81.9	87.91	78.3	79.64	83.64	3.69	0.54	0.11	0.78
Marbling score	418	586	486	478	500	484	49	0.82	0.17	0.34
Back fat thickness (cm)	1.24	1.38	1.29	1.32	1.17	1.13	0.13	0.39	0.85	0.53
KPH (%)	2.11	2.03	2.07	2.35	2.12	2.11	0.11	0.16	0.30	0.63
Days to harvest	367	371	387	392	407	387	11	0.03	0.67	0.26

**Table 17.** Animal performance at the feedlot of biopsied Angus × Simmental steers (n = 35) from cows that received different diets during the late gestation period. Diets (D) are cow low plane of nutrition (LPN), medium plane of nutrition (MPN) and high plane of nutrition (HPN). Weaning times (W) are early weaning (EW) and normal weaning (NW). BW = body weight, BF = back fat thickness, ADG = average daily gain, 0 days = beginning of administration of finishing diet, overall dry matter intake (DMI) = from 0 to 140 days in the feedlot. Weaning age × diet interaction (W\*D). Significant differences are declared at  $P < 0.05$ .

	Treatments						P Value			
	EW			NW			SEM	W	D	W*D
	LPN	MPN	HPN	LPN	MPN	HPN				
Feedlot Performance										
Hip Height (cm)	112	113	111	106	108	111	2	0.05	0.73	0.47
BW at EW (Kg)	100	104	104	99	106	109	6	0.62	0.45	0.93
BW at NW (Kg)	217	231	245	173	159	192	15	0.01	0.21	0.64
BW at 0 d (Kg)	258	278	290	212	204	233	17	0.01	0.25	0.68
BW at 28 d (Kg)	308	335	354	253	247	288	20	0.01	0.11	0.67
BW at 56 d (Kg)	362	402	410	304	310	350	22	0.01	0.09	0.66
BW at 84 d (Kg)	417	453	459	360	365	410	23	0.01	0.12	0.63
BW at 112 d (Kg)	462	494	496	408	410	457	23	0.01	0.17	0.54
BW at 140 d (Kg)	507	541	557	461	459	507	27	0.01	0.15	0.71
Final BW (Kg)*	548	580	601	521	542	566	27	0.11	0.16	0.97
ADG (0 – 28 d)	1.76	2.03	2.28	1.49	1.56	1.96	0.18	0.02	0.03	0.83
ADG (29 – 56 d)	1.96	2.39	2.01	1.8	2.25	2.22	0.18	0.84	0.05	0.49
ADG (57 – 84 d)	1.95	1.81	1.75	2.01	1.94	2.14	0.14	0.09	0.72	0.44
ADG (85 – 112 d)	1.61	1.5	1.32	1.72	1.61	1.69	0.13	0.04	0.30	0.33
Overall DMI (Kg)	14.7	17.5	16.3	15.3	15.8	18	0.8	0.72	0.04	0.11

\*Based on 62% dressing percentage

**Table 18.** Measured microRNA sequences, functions and corresponding target genes.

miRNAs	Function	Sequence	Target genes
miR-130a	Antiadipogenic	CAGTGCAATGTTAAAAGGGCAT	PPARG
miR-34a	Antiadipogenic	TGGCAGTGTCTTAGCTGGTTGT	SIRT1
miR-369-5p	Antiadipogenic	ATCGACCGTGTTATATTCGC	ADIPOQ, FABP4
miR-448	Antiadipogenic	TTGCATATGTAGGATGTCCCAT	KLF5
miR-181a	Internal Control Gene	AACATTCAACGCTGTCGGTGAGTT	-
miR-let-7a	Internal Control Gene	CGGTGAGGTAGTAGGTTGTATAGTT	-
miR-16b	Internal Control Gene	TAGCAGCACGTAAATATTGGC	-
miR-103	Proadipogenic	AGCAGCATTGTACAGGGCTATGA	PPARG
miR-143	Proadipogenic	TGAGATGAAGCACTGTAGCTCG	PPARG, CEBPA, FABP4
miR-21-5p	Proadipogenic	TAGCTTATCAGACTGATGTTGACT	PTEN, PDCD4
miR-27 a/b	Proadipogenic	TTCACAGTGGCTAAGTTCTGC	PPARG, CEBPA
miR-378	Proadipogenic	ACTGGACTTGGAGTCAGAAGGC	PPARG

**Table 19.** qPCR performance for microRNAs and microRNA target genes.

Gene	Median Ct <sup>1</sup>	Median $\Delta$ Ct <sup>2</sup>	Slope <sup>3</sup>	(R <sup>2</sup> ) <sup>4</sup>	Efficiency <sup>5</sup>	relative mRNA abundance <sup>6</sup>	1/E $\Delta$ Ct <sup>7</sup>
<b>mir-21</b>	25.7046	5.1573	-3.1602	0.9946	2.0722	0.0233	0.0054
<b>mir-448</b>	29.6263	9.0919	-2.5191	0.9873	2.4944	0.0002	0.0001
<b>mir-103</b>	24.5853	4.0853	-3.3439	0.9978	1.9909	0.0600	0.0139
<b>mir-130a</b>	28.0769	7.9321	-2.8475	0.9971	2.2449	0.0016	0.0004
<b>mir-369-5p</b>	30.9458	10.2857	-3.1683	0.9906	2.0684	0.0006	0.0001
<b>mir-27a/b</b>	22.4407	1.9472	-3.4132	0.9916	1.9633	0.2688	0.0624
<b>mir-34a</b>	25.4864	4.7408	-2.4918	0.9902	2.5195	0.0125	0.0029
<b>mir-378</b>	21.7708	1.4421	-3.4186	0.9889	1.9612	0.3786	0.0878
<b>mir-143</b>	24.6768	3.8721	-3.3808	0.9978	1.9760	0.0716	0.0166
<b>ADIPOQ</b>	22.0669	0.0621	-3.1812	0.9963	2.0623	0.9560	0.2217
<b>CEBPA</b>	27.3818	5.3933	-3.4597	0.9847	1.9455	0.0276	0.0064
<b>FABP4</b>	21.0294	-0.9827	-2.9434	0.9940	2.1865	2.1570	0.5002
<b>FASN</b>	24.4363	2.1586	-3.0930	0.9965	2.1053	0.2005	0.0465
<b>PPARG</b>	25.4338	3.3727	-2.9395	0.9912	2.1887	0.0712	0.0165
<b>SIRT1</b>	25.2388	3.2817	-3.0241	0.9954	2.1413	0.0822	0.0191

<sup>1</sup> The median is calculated considering all time points and all steers.

<sup>2</sup> The median of  $\Delta$ Ct is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each steer.

<sup>3</sup> Slope of the standard curve.

<sup>4</sup> R<sup>2</sup> stands for the coefficient of determination of the standard curve.

<sup>5</sup> Efficiency is calculated as  $[10^{(-1 / \text{Slope})}]$ .

<sup>6</sup> relative mRNA abundance =  $1 / \text{Efficiency}^{\text{Median } \Delta\text{Ct}}$

<sup>7</sup>  $1/E\Delta\text{Ct} = \text{relative mRNA abundance} / \sum \text{relative mRNA abundance}$

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