

Central Role of the PPAR γ Gene Network in Coordinating Beef Cattle Intramuscular Adipogenesis in Response to Weaning Age and Nutrition

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ABSTRACT: Adipogenic/lipogenic transcriptional networks regulating intramuscular fat deposition (IMF) in response to 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

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Background

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.¹ 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 that is both necessary and sufficient for adipogenesis.² 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 *CEBPA* and *CEBPD* leading to induction of *CEBPA*. *CEBPA* is a transactivator of *PPARG*, and both transcription regulators act together to promote adipogenesis.³ 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, ie, cells that can store triacylglycerol (TAG).^{4,5}



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 (ie, 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.⁶

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; it has been hypothesized that this would allow them to reach their genetic potential to accrete intramuscular fat (“marble”),⁷ and thus achieve a greater meat quality grade at slaughter.⁸ 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 (Fig. 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.

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.⁹ The steers were randomly divided in two groups (EWS and CFS). Seven animals were early-weaned at an age of 141 ± 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,

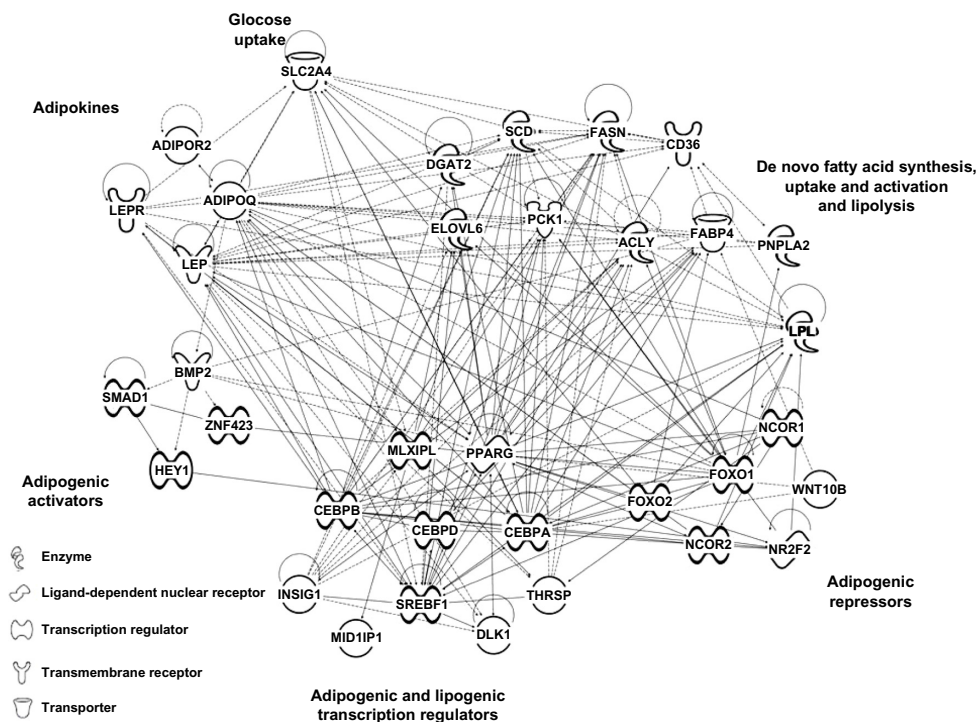


Figure 1. Currently-known relationships among genes analyzed based on manually-curated examination of the published literature within the Ingenuity Pathway Analysis (www.ingenuity.com) knowledge base.

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.

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; Additional Table 1) during the finishing phase.

Ultrasound measurements of back fat thickness, muscle depth and intramuscular fat, using a 500 V Aloka ultrasound with 3.5-MHz transducer (Hitachi Aloka), 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.¹⁰ 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 12th and 13th 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-N₂ 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.^{11,12} 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.¹³ 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 (ie, 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 (ie, genes, production, carcass and blood metabolite) included treatment, breed, time on experiment, treatment \times breed, treatment \times time on experiment and breed \times treatment \times time on experiment interactions when appropriate (eg, 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; μ 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 ϵ_{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 was used for gene expression analysis.⁹ 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 (Additional Tables 6, 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 (Additional Table 1). However, compared with CFS steers, the EWS steers consumed more feed in the first two weeks after normal weaning (Additional Fig. 1). 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.¹⁴ Moreover, there were significant differences in ADG in response to treatments between birth and EW day (Additional Table 1).

Least means squares for ultrasound data are reported in Figure 2. Ultrasound marbling score (Fig. 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 (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 (Fig. 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 (Fig. 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

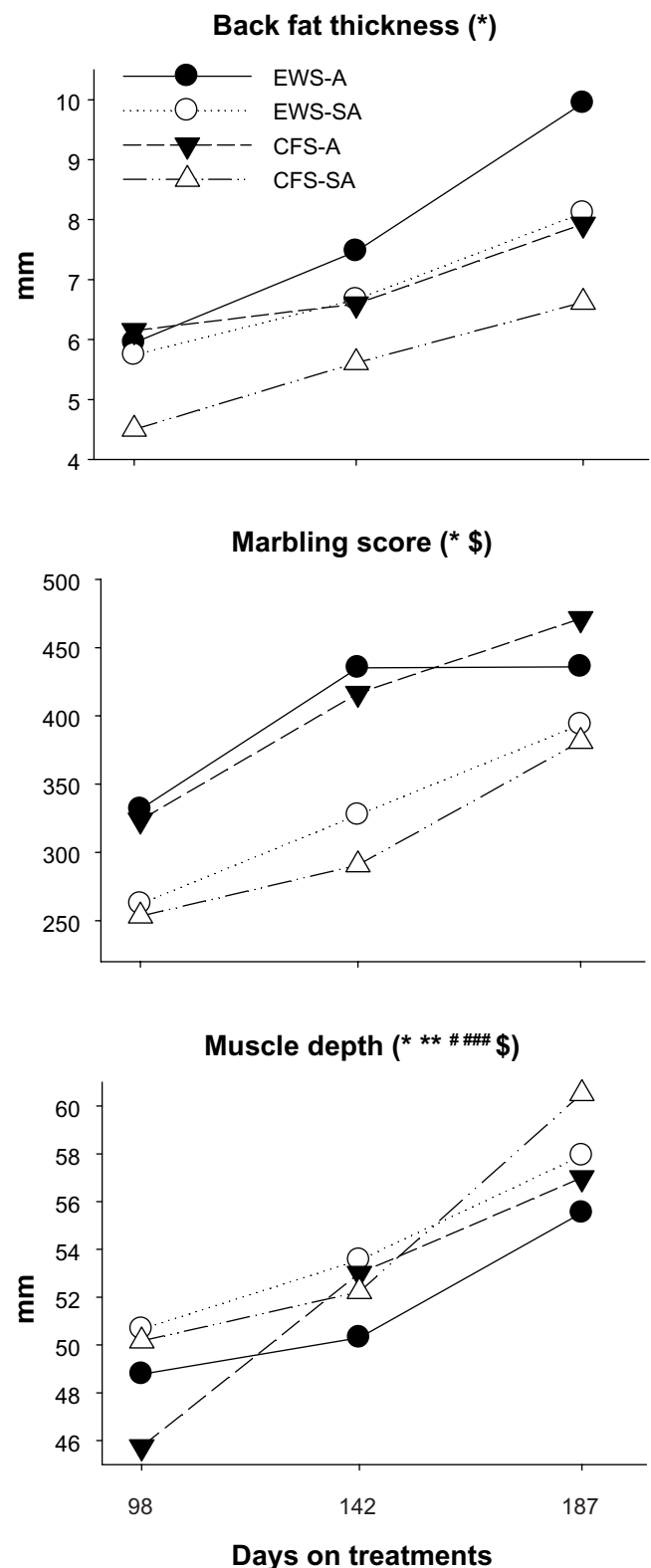


Figure 2. Assessment of muscle depth, subcutaneous and intramuscular fat deposition in early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus \times Simmental (EWS-SA, CFS-SA) steers ($n = 7/\text{treatment}$). All steers were fed a common diet from 96 to 222 days. **Notes:** Marbling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

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.¹⁵

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 (eg, 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 (Additional Fig. 1), 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, ie, 371 ± 8 and 363 ± 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.¹² 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.¹⁶

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.¹⁷ It is well-known that the IGF-1 response to GH decreases when nutrient status or insulin levels are low,¹⁸ 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 (Fig. 3). That response agrees with the gradual increase in fat deposition as the animal ages, ie, more adipocytes able to produce and secrete leptin or adipocytes growing in size.

Expression of adipogenic activators. Details of qPCR performance for all targets measured, primer sequences, and primer sequencing results can be found in Additional Tables 2–5. Results of adipogenic activators indicated that *ZFP423* was

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

ITEM	GROUP				SEM	P VALUE ^A		
	EWS-A	EWS-SA	CFS-A	CFS-SA		T	B	T \times B
Carcass weight (kg)	293	332	339	345	16.0	0.09	0.18	0.32
Marbling ^b	553	567	520	408	55.7	0.10	0.38	0.27
Back fat (cm) ^c	1.30	0.98	1.43	1.14	0.1	0.09	<0.01	0.80
REA (cm ²)	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)								
\geq Low Prime (%)	33	0	0	0	–	0.20	0.20	0.20
\geq High Choice (%)	33	33	0	0	–	0.08	1.00	1.00
\geq Ave Choice (%)	33	67	67	0	–	0.45	0.45	0.04
\geq Low Choice (%)	100	100	100	50	–	0.12	0.12	0.12

Notes: ^aStatistical values for the effect of treatment (EWS or CFS; T), breed (A or SA; B), or interaction of treatment and breed (T \times B). ^bMarbling 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). ^cTarget back fat thickness was 1.2 cm. USDA YG = $2.5 + (2.5 \times 12\text{th rib fat thickness in inches}) + (0.0038 \times \text{hot carcass weight in pounds}) + (0.2 \times \%KPH) - (0.32 \times \text{rib eye area in square inches})$; REA = Rib eye area in centimeters square; KPH = Percent kidney, pelvic and heart fat.

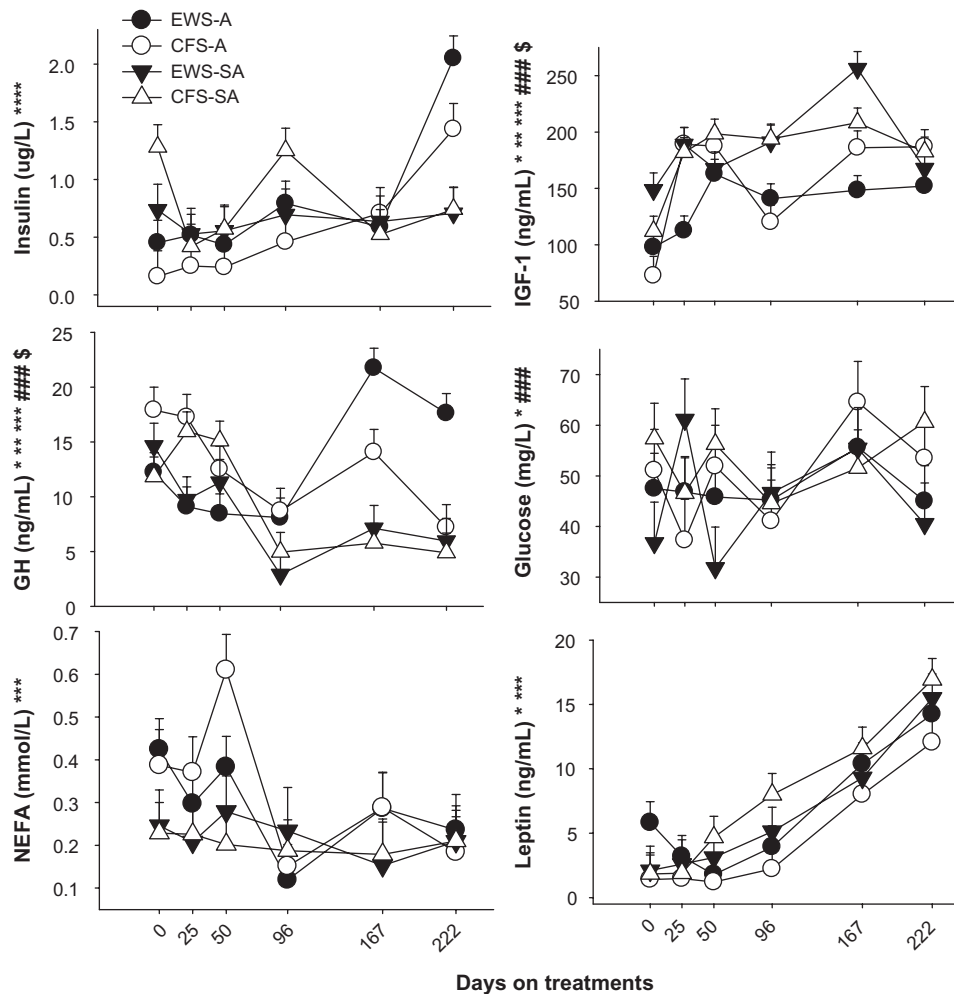


Figure 3. Blood serum concentration of insulin, insulin-like growth factor-1 (IGF-1) and growth hormone (GH), non-esterified fatty acids (NEFA), glucose and leptin in early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus \times Simmental (EWS-SA, CFS-SA) steers.

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.

the only gene that did not have a significant breed \times treatment \times time interaction (Fig. 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 *SMAD1*). The correlation between marbling and *SMAD1* 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*¹⁹ and *CEBPA*²⁰ 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 compared 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.²¹ In contrast, this protein might serve to revamp this process in CFS steers during the finishing phase, representing a sort of compensatory mechanism in response to the higher-energy feeding prior to slaughter.²²

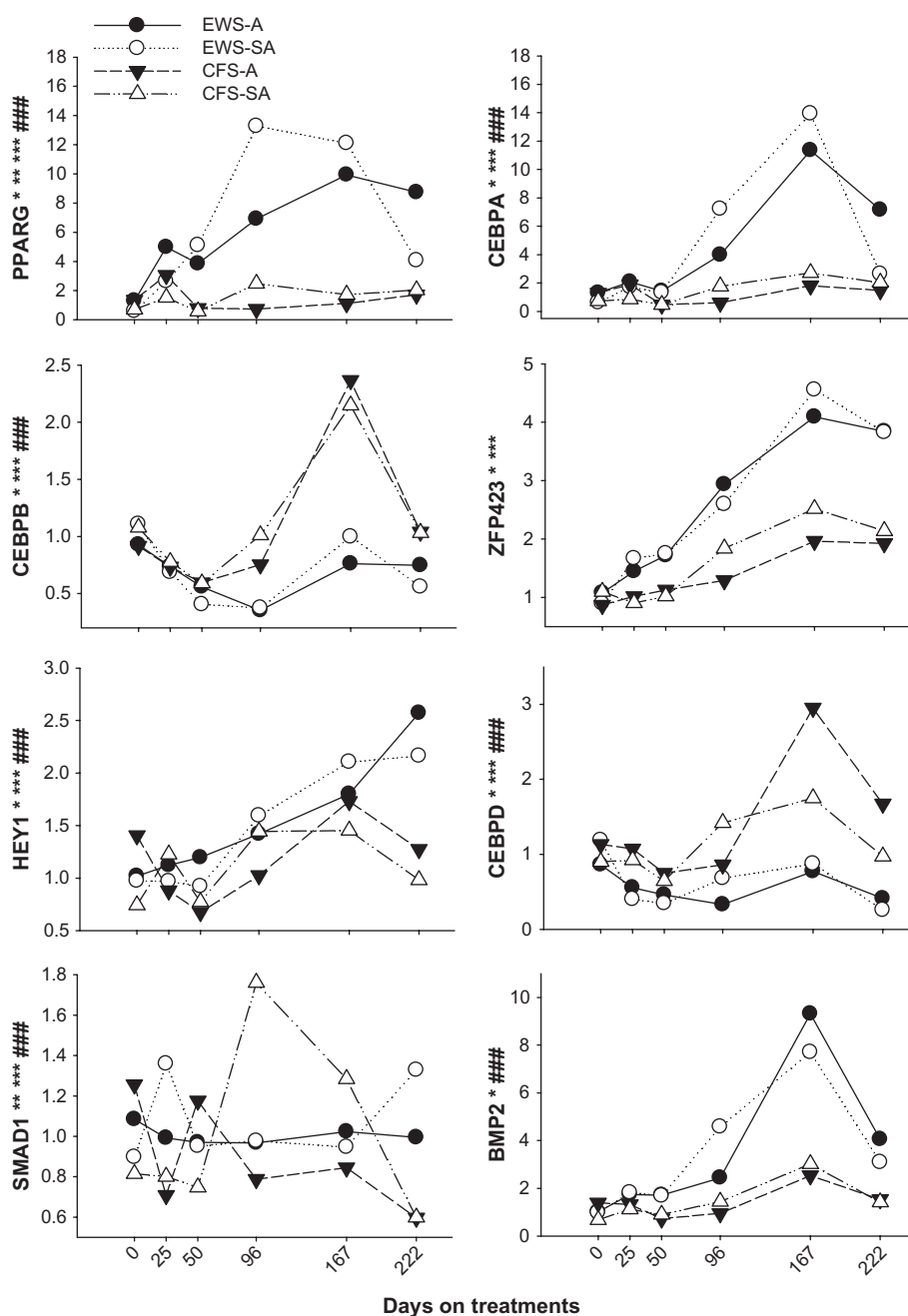


Figure 4. Patterns of mRNA expression (fold-change) of adipogenic activators in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus \times Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

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.

Initial studies suggested that *BMP2* induced adipocyte differentiation in a Smad-1 independent fashion via the induction of *PPARG*.²³ However, it is now clear that BMPs activate *PPARG* during adipogenesis through the interaction of *ZFP423* and *SMAD1*.²⁴ SMAD family member 1 (*SMAD1*) is a signal transducer that strengthens the association between the transcription regulator with DNA, furnishing transcriptional activation capacity.²⁵ Zinc finger protein 423 (*ZFP423*) is the DNA binding protein that associates with *SMAD1*.²⁶ 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.²⁷ 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.²⁷ 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 (ie, *SMAD6*),²⁸ 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*.²⁹ The response to nutrition and weaning age for *CEBPB* and *CEBPD* was exactly the opposite of other adipogenic transcription activators. 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.^{30,31} 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 (eg, 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.³² 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, in adipogenesis.

The dynamics of the *PPARG* and *CEBPA* response, along with those 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.³³

Expression of adipogenic repressors. In the case of the adipogenic repressors (Fig. 5), there was a significant breed × treatment × 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.³⁴ In the absence of ligand binding, *PPARG* forms a protein complex with its corepressors leading to reduced transcriptional activation of target genes.³⁵ 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.³⁶

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.³⁷

These corepressors could be nutrient-independent-like sensors that 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 (ie, 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.³⁸ 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* (Fig. 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.³⁹ *NR2F2* positively regulates adipogenesis at an early expansion phase, promoting formation of small adipocytes; whereas, the persistent expression of *NR2F2* during differentiation may

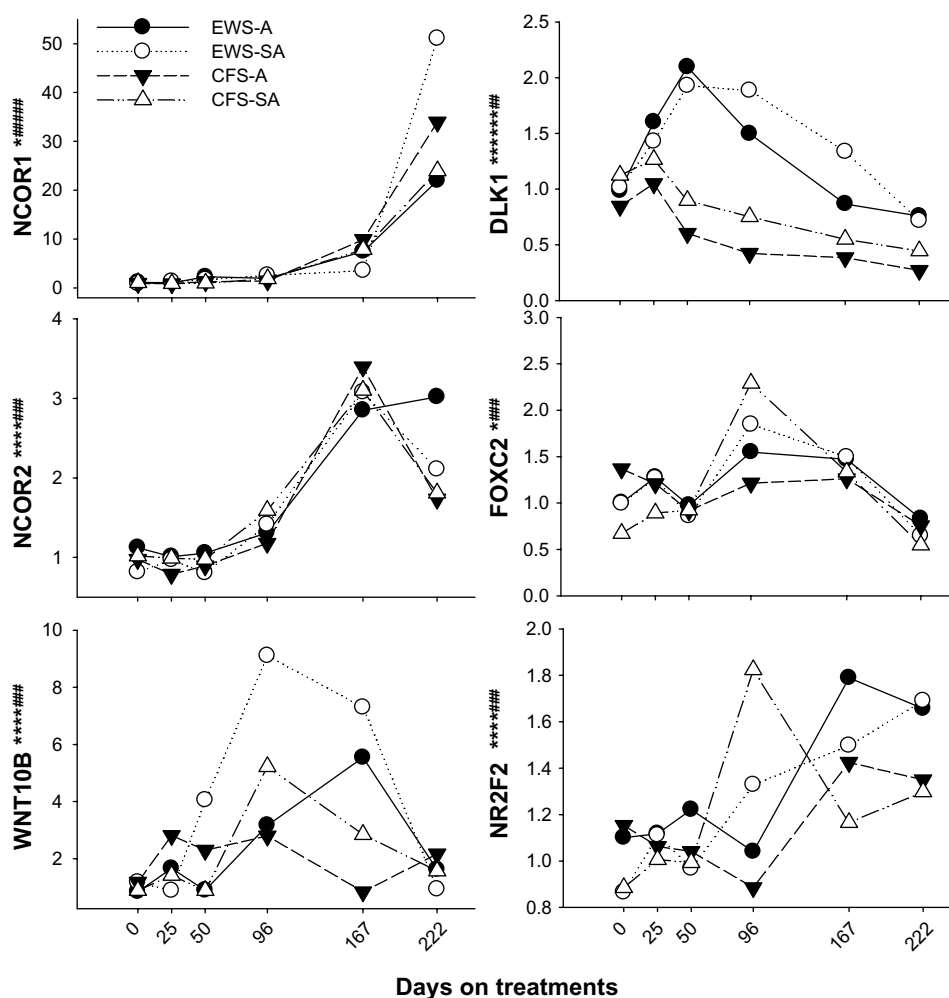


Figure 5. Patterns of mRNA expression (fold-change) of adipogenic repressors in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus \times Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days. **Notes:** Statistical difference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

be inhibitory to the increase in adipose tissue mass.⁴⁰ Similar to a previous study,¹⁰ 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 (Fig. 5) could be a signal of greater amounts of small preadipocytes.

Wnt signaling through β -catenin and TCF maintains preadipocytes in a non-differentiated stage.⁴¹ Wnt/ β -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.⁴² 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 β -adrenergic-cAMP-protein kinase A (PKA) signaling pathway⁴³ and by

inhibiting the capacity of *PPARG* to promote the expression of genes recognized as markers of mature adipocytes (*CEBPA*, *ADIPOQ*, and *FABP4*).⁴⁴ 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, there was a gradual increase in expression until 96 days after EW in all treatments, followed by a 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 (Fig. 6). Of these genes, the response in *LEP* expression in EW steers was the most marked. 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

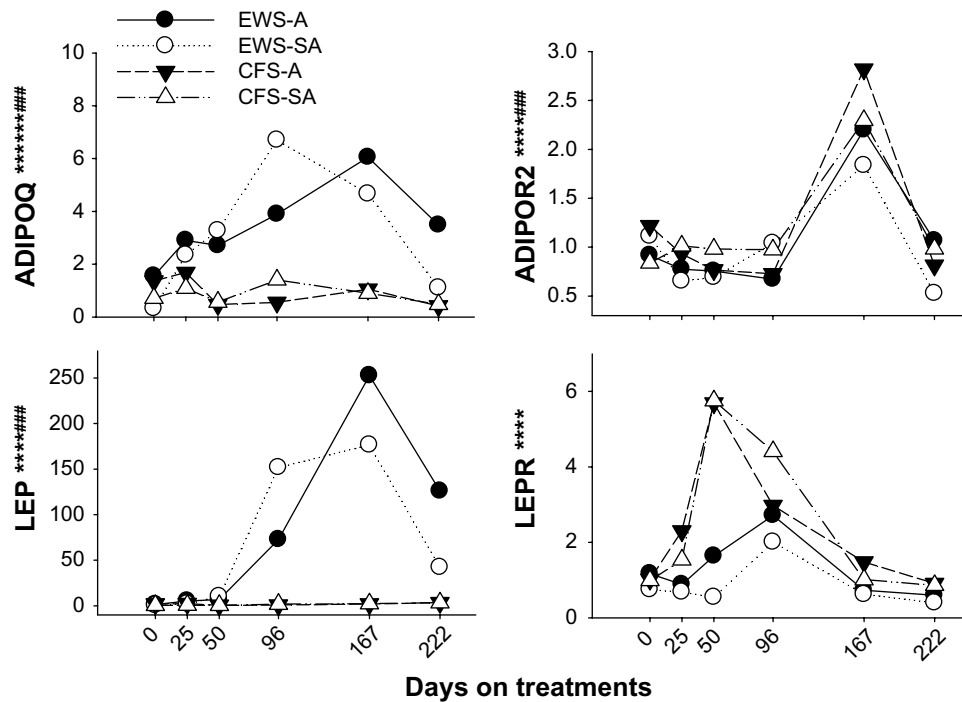


Figure 6. Patterns of mRNA expression (fold-change) of adipokines in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus \times Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days. **Notes:** Statistical difference due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

type of diet leads to an increase in capillary networks.⁴⁵ These capillary networks supply nutrients to fat cells allowing the adipocytes to actively secrete adipokines (eg, leptin) at a rate strongly related with adiposity.⁴⁶ While adipocytes increase in size and mass, concentrations of leptin (*LEP*) in blood also increase.⁴⁷ Moreover, a positive correlation exists between adipocyte diameter and *LEP* mRNA levels in crossbred steers.⁴⁸

A previous study reported a significant effect of breed and fat depot anatomical site,⁴⁹ with greater *LEP* mRNA expression in the intramuscular fat of Angus steers than in subcutaneous fat. The opposite was observed in Limousin steers.⁴⁹ 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.⁵⁰ Upon action of *ADIPOQ*, adipogenesis is accelerated by ensuring a marked and prolonged expression of *PPARG* and *CEBPA*.^{50,51} In fully differentiated adipocytes,^{50,52} 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 (see Additional Fig. 2).

In contrast to *LEP*, smaller adipocytes secrete more *ADIPOQ*, resulting in enhanced adipocyte differentiation and lipid accumulation.⁵⁰ Coinciding with previous results,¹⁰ 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.⁵³ The *ADIPOR2* response, however, might have been linked with the marked *ADIPOQ* expression because *ADIPOQ* overexpression in adipocytes was reported to decrease lipid accumulation.⁵⁰

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 (Fig. 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 (eg, *NCOR2*).

Expression of lipogenic regulators. With the exception of *SREBF1*, all the genes classified as lipogenic transcription regulators (Fig. 7) had a breed \times treatment \times time

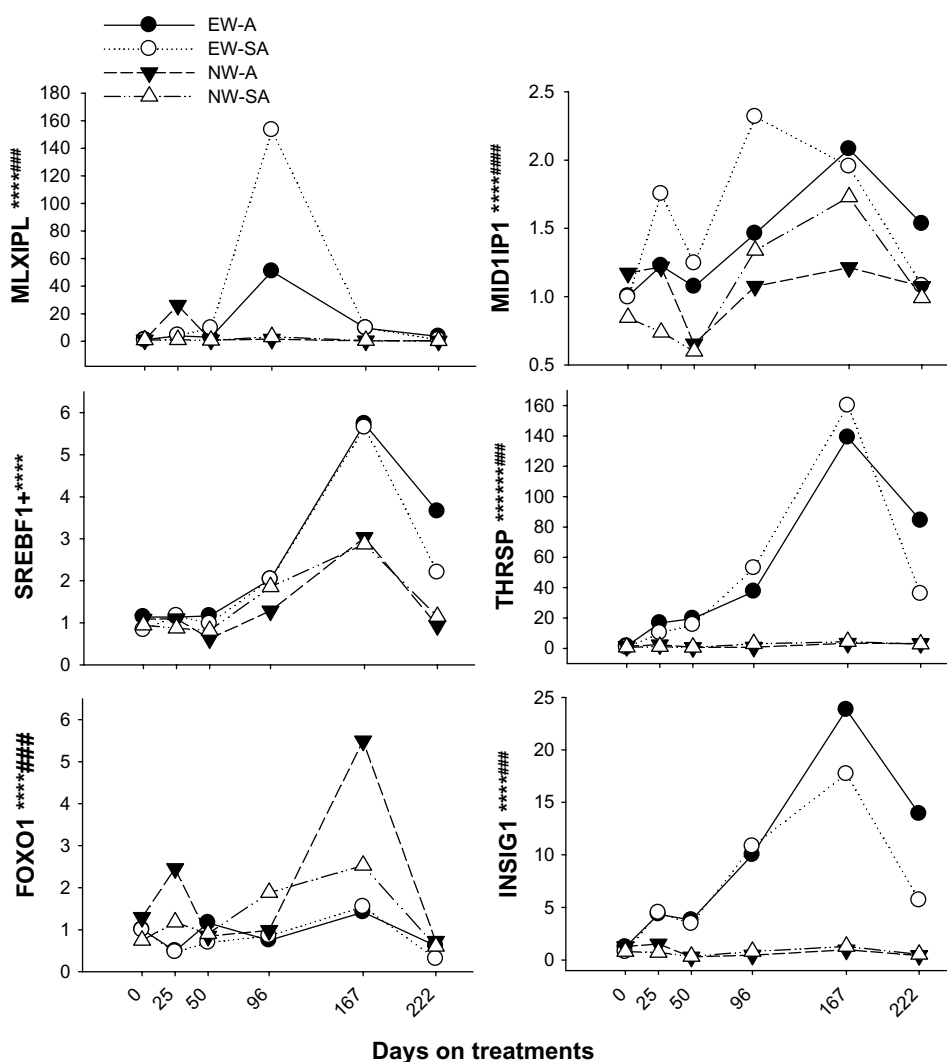


Figure 7. Patterns of mRNA expression (fold-change) of lipogenic regulators in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus \times Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

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 *MLXIPL*, *MID1P1*, *SREBF1*, *THRSP*, *FOXO1*, and *INSIG1* was 21, 0.3, 0.6, 19, 0.5, and 3, respectively.

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.⁵⁴ Moreover, a recent study confirmed an association of bovine fatty acid composition with a nucleotide polymorphism in *THRSP*.⁵⁵ *THRSP* was highly-correlated with marbling content in breeds widely known to possess extremely high capacity for marbling such as Japanese Black or Wagyu.^{56,57} 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.⁵⁸ 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 (ie, 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.⁵⁹ Our results agree with a previous study,¹⁰ 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 and leading to synthesis of palmitic and oleic acids and formation of triacylglycerol.⁶⁰ 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.⁵⁴

Forkhead box protein O1 (*FOXO1*) can have both a positive and a negative effect on adipogenesis. Insulin-induced phosphorylation inactivates *FOXO1* and holds it in the cytoplasm,⁶¹ thus preventing binding to DNA and reducing the *FOXO1-PPARG* interactions.⁶² 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 p21.⁶³ At this stage of adipogenesis, *FOXO1* is activated by a glucose-derived O-linked β -N-acetylglucosamine (O-GlcNAc),⁶⁴ and cooperates with *PPARG* to trigger a postmitotic 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 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 (*MIDI1P1*) binds and forms a heterodimer with *THRSP*.⁶⁵ In the absence of *THRSP*, *MIDI1P1* forms a homodimer, a process required for efficient fatty acid and lipid biosynthesis,^{66,67} including triacylglycerol, diacylglycerol and phospholipid.⁶⁸ 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 *MIDI1P1* 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 to lipolysis and lipogenesis that did not have a breed \times treatment \times time interaction (Additional Figure 2). 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 (Fig. 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.⁶⁹ For subcutaneous adipose tissue, acetate is the main

source for fatty acid synthesis.⁷⁰ Lactate requires the enzymes ATP-citrate lyase (*ACLY*) and NADP-malate dehydrogenase in order to be converted to fatty acids.⁷¹ 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.⁷² In our study, *FASN* had a clear difference of expression between CFS and EWS steers, with greater levels of expression in EWS steers (Fig. 7). Fatty acid binding protein 4 (*FABP4*) delivers long-chain fatty acids (LCFA) and retinoic acid to their cognate receptors in the adipocyte nucleus.⁷³ *FABP4* followed a similar pattern of expression as *FASN*, with an earlier and stronger response in EWS-SA as compared with EWS-A (Fig. 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 a robust lipogenic process.⁷⁴ Moreover, increased *FABP4* expression is associated with increased marbling in cattle and increased TAG content.^{73,75}

Desaturation. Stearoyl-CoA desaturase (*SCD* or $\Delta 9$ desaturase) converts a portion of stearic acid (18:0) into oleic acid (18:1).⁷⁶ Several studies have indicated that time on a corn-based finishing diet increases muscle and adipose tissue *SCD* gene expression.⁷⁷ 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.⁷⁸ 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*).⁷⁹ 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.⁸⁰ The results underscore 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.

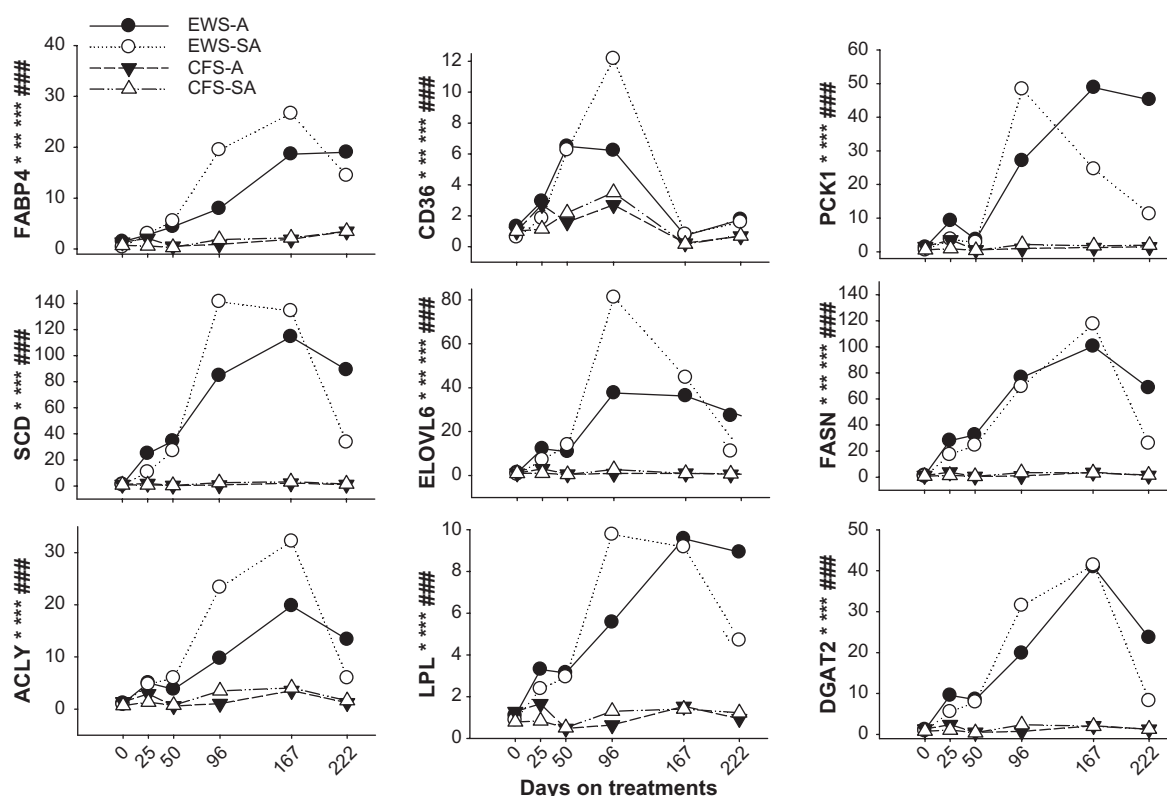


Figure 8. Patterns of mRNA expression (fold-change) of lipogenic enzymes in *Longissimus* tissue from early weaned (EWS) and normal weaned (CFS) Angus (EWS-A, CFS-A) and Angus \times Simmental (EWS-SA, CFS-SA) steers. All steers were fed a common diet from 96 to 222 days.

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.

Glyceroneogenesis. Phosphoenolpyruvate carboxykinase (*PCK1*) is a cytosolic enzyme that along with GTP, catalyzes the formation of phosphoenolpyruvate from oxaloacetate.⁸¹ In monogastrics, *PCK1* expression is regulated by insulin, glucagon and diet, and it is responsible for the synthesis of glycerol-3-phosphate and re-esterification of free fatty acids (FFA) to generate TAG.^{82,83} 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; this suggests 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 (Fig. 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.¹ High-carbohydrate diets increase the *LPL* expression in both adipose tissue and skeletal muscle.²⁹ By increasing *LPL* gene transcription,⁸⁴ 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.⁸⁵ In a previous study using Hereford \times Angus steers, *DGAT2* activity was higher in subcutaneous adipose tissue compared with IMF or muscle tissue.⁸⁶ The difference in *DGAT2* expression between treatments may be associated with differences in the positional distribution of fatty acids in the triacylglycerol molecule.⁸⁷

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.^{73,84} 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

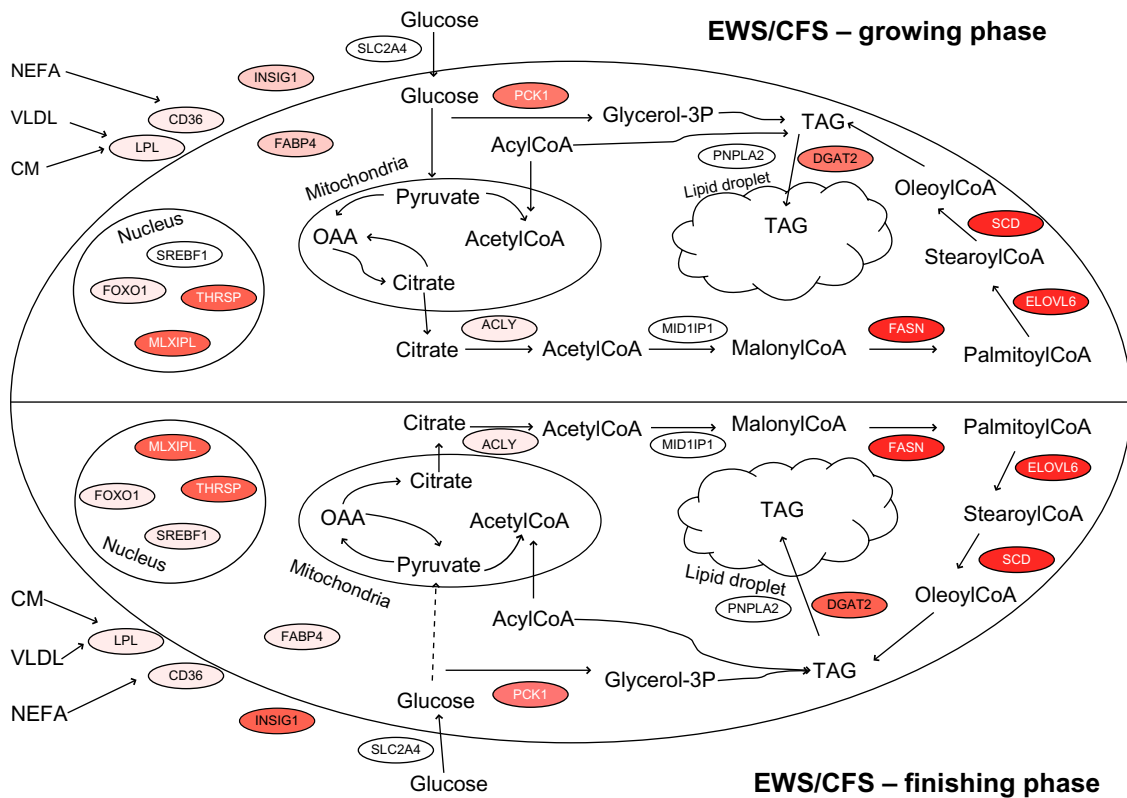


Figure 9. Putative model of transcriptional adaptations during the growing and finishing phase in response to plane of nutrition. **Notes:** Gene names with differences in color intensity denote expression level: darker red color (high expression) and white color (slightly expressed). Non-esterified fatty acids (NEFA), very low density lipoprotein (VLDL) and chylomicron (CM).

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, the 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.

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Author Contributions

Conceived and designed the experiments: DWS, DBF, JLL. Conceived and performed the analyses: SJM, WTM, DK. Wrote the manuscript: SJM, DWS, JLL. Agree with manuscript results and conclusions: SJM, DWS, WTM, DK, DBF, JLL. Jointly developed the structure and arguments for the paper: SJM, JLL. Made critical revisions and approved final version: SJM, DWS, JLL. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.



Supplementary Material

- Additional Tables 1**
- Additional Tables 2**
- Additional Tables 3**
- Additional Tables 4**
- Additional Tables 5**
- Additional Tables 6**
- Additional Tables 7**
- Additional Tables 8**
- Additional Figure 1**
- Additional Figure 2**

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