Preconditioning for long-duration transportation stress on beef cattle with rumen-protected methionine supplementation: a nutrigenetics study

by

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Abstract

Different participants generally located distantly among them compose the U.S. beef production chain; therefore, shipping is required for cattle at least once in their lifespan. The aim of this study was to determine the effect of Rumen-Protected Methionine (RPM) supplementation on muscle fatigue gene network, creatine synthesis (CKM), and Reactive Oxygen Species (ROS) related-gene expression after 900 miles transportation simulation. Angus heifers (n = 18) were stratified by body weight (408 ± 64 kg; BW) and randomly assigned to dietary treatments: 1) control diet (CTRL), and 2) control diet + 8 gr/hd/day of rumen-protected methionine (RPM). After a successful adaptation period to Calan gates, animals received a common diet of Bermudagrass hay *ad libitum* and a soyhulls and corn gluten feed-based supplement. After 45 days under supplementation, animals were loaded onto a 32 × 7 ft. trailer and transported for 22 hours. Skeletal muscle biopsies, BW and blood samples were obtained on day 0 (Baseline; "BASE"), 43 (Pre-transport, "PRET"), and 45 (Post-transport, "POST"). Heifers' average daily gain did not differ between BASE and PRET ($P = 0.41$). Control heifers' shrink was 10% of BW whereas RPM heifers shrink was 8% ($P = 0.82$). Cortisol level decreased after transportation, but no differences were observed between treatments ($P = 0.94$). Circulating glucose and creatine kinase increased during transportation, but no difference was observed between treatments ($P > 0.10$). Messenger RNA was extracted from skeletal muscle tissue, and gene expression analysis was performed by RT-qPCR. Results showed that *AHCY* (Creatine synthesis pathway), *SSPN* (Sarcoglycan

complex), *DNMT3A* (DNA Methylation), and *SOD2* (Oxidative Stress-ROS) were upregulated ($P < 0.05$) in CTRL between BASE and PRET and decreased between PRET and POST ($P < 0.05$) while they remained unchanged for RPM. Furthermore, *CKM* (Creatine Kinase) was not affected by treatments ($P = 0.11$). In conclusion, muscle fatigue related genes were not affected by RPM. However, RPM effect on *SOD2* gene suggests that it could affect ROS production after long-duration transportation.

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List of abbreviations

ADG	Average Daily Gain
AHCY	Adenosylhomocysteinase
ATP2A1	ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 1
BASE	Baseline
CASQ1	Calsequestrin 1
CKM	Creatine Kinase
CTRL	Control Treatment
DGC	Dystrophin-Glycoprotein complex
DNMT1	DNA Methyltransferase 1
DNMT3A	DNA Methyltransferase 3 Alpha
DM	Dry Matter
GAMT	Guanidinoacetate N-Methyltransferase
GATM	Glycine Amidinotransferase
MTG1	Mitochondrial Ribosome-Associated GTPase 1
NFKB1	Nuclear Factor Kappa B Subunit 1
NO	Nitric Oxide
NOS3	Nitric Oxide Synthase 3
NQO1	NAD(P)H Quinone Dehydrogenase 1
O_2^-	Superoxide
PCr	Creatine Phosphate
PGC1a	PPARG Coactivator 1 Alpha
PRET	Pre-Transportation
POST	Post-Transportation
RPM	Rumen-Protected Methionine Treatment

ROS	Reactive Oxygen Species
RPS15A	Ribosomal Protein S15a
SAM	S-Adenosyl Methionine
SGCB	Sarcoglycan Beta
SLC6A8	Solute Carrier Family 6 Member 8
SNTA1	Syntrophin Alpha 1
SNTB1	Syntrophin Beta 1
SOD1	Superoxide Dismutase 1
SOD2	Superoxide Dismutase 2
SSPN	Sarcospan
SYPL2	Synaptophysin Like 2
UXT	Ubiquitously Expressed Prefoldin Like Chaperone

Chapter I

Literature review

Background

Throughout history, agricultural science has developed numerous achievements by exploring new techniques in order to increase food production in an apparently unalterable climate. Although, nowadays we are situated in a scenario without precedence: competition for natural finite resources and climate change (Godfray *et al.*, 2010). Thus, the outlook has changed to a sustainable and more efficient food production (Eshel *et al.*, 2018). Fortunately, science has on-hand plenty of disciplines to overcome these challenges. Among all of them, the study of gene expression has been increased in the recent years as a resource to progress in a profound understanding of animal physiology and nutrition (Loor et al., 2015).

It is well known that gene expression is affected by both genetic and environmental factors (Cassar-Malek *et al.*, 2008). Nutrigenomic studies are related to the effect of nutrients on gene expression that could be associated with different biological processes (Dawson, 2006). According to these statements, it is possible to regulate the gene expression through nutritional and environmental conditions to achieve specific goals (Kaput & Rodriguez, 2004). In general, these modifications are oriented to achieve profitable characteristics or to develop resistance to specific health issues (Spurlock *et al.*, 2000; Taha-Abdelaziz *et al.*, 2016). Sometimes, a desired characteristic can be achieved by activating the expression of specific genes. Gene activation or inhibition take place at the level of the histone tails or in the DNA, and it is regulated by different processes such as acetylation, phosphorylation, methylation, and others (Biel *et al.*, 2005; Ng & Adrian, 1999). The major challenge is to discover factors, such as environmental or nutritional-related ones, that can be useful for the activation of the expression of desired genes. In our study,

we will assess the effects of the addition of rumen-protected methionine (RPM) in the diet on the expression of genes that encode for proteins related to DNA methylation.

In most of the cases, DNA methylation inhibits gene expression by two main mechanisms: modification of cytosine bases and the inhibition of some DNA binding factors. Furthermore, proteins capable of detecting the location of methyl-CpG groups on the DNA chain can bring out the repressive potential of methylated DNA (Klose & Bird, 2006)

Transportation

Cattle production is one of the most important industries in United States (US)(USDA, 2019). In the Southeast, most of the production systems are cow-calf operations that keep the male calves until weaning or after weaning. Then, weaned calves have to be shipped to the central region of US where their fattening or finishing period takes place. Furthermore, the central area of US is where most of the slaughterhouses are located, diminishing the stress of long-term transportation before slaughter. However, all beef cattle have to be transported for long periods of time, at least once in their life.

Livestock transportation in US has evolved seeking a reduction of cost, shipping time, or better animal welfare conditions. First, in 1607, the "Susan Constant", an English ship, arrived at Jamestown, Virginia with the first cattle shipment; then, in 1700, the port of Philadelphia was an important export trade center, shipping live cattle and packed meat to the West Indias by sea trade (Skaggs, 1986). One and a half century later, the country experimented the first connection by rail between two cities separated by more than six hundred miles: a group of Texas Longhorn cattle traveled from Abilene, KS to Chicago, IL (Kansas Pacific Railway Company, 1874). Afterwards, at the beginning of twentieth century, road transportation became the preferred mode of transport because of economic and logistical reasons to move animals to auctions, feeders, and

abattoirs (Swanson & Morrow-Tesch, 2001). Finally, aircraft transportation can also be used to carry cattle (Moss, 1981). Nowadays, due to the economic impact and volume transported, road transportation is the major mode of transport used in US.

Several research studies have been developed in the last decades in order to achieve a better understanding of the characteristics of road transportation and its effects on cattle (Cernicchiaro *et al.*, 2012a; Cernicchiaro *et al.*, 2012b; Cole *et al.*, 1988; Gonzalez *et al.*, 2012b). It is important to consider that road transportation includes more than the shipping itself; it takes into account the assembly and loading of animals at their place of origin, confinement on a moving or stationary vehicle, unloading, and penning at their final destination (Tarrant, 1990). The analysis of these steps are key to improving animal welfare, implying reductions in economic losses (Losada-Espinosa *et al.*, 2018).

Management pre and during transportation

How an animal is handled early in life will have an effect on its physiological response to stressors later in life, such as during transportation (Grandin, 1997). Therefore, a gentle management allows a quiet and non-stressful behavior, which is important to reduce economic losses, especially with young cattle. Calf training reduces stress and facilitates loading in future shipping (Fukasawa, 2012). At loading time, cattle can be distressed because of inappropriate management practices; as a consequence, they show signs like vocalization, kicking, or struggling. These type of actions can cause economical losses pre-transportation (i.e., bruises) and reduce carcass quality (Ferguson *et al.*, 2001; Grandin, 1997).

Shrink

The body weight (BW) loss due to transportation is known as shrink (Harman *et al.*, 1989). This condition becomes relevant, for example, due to its effect on carcass yield, and its economic yield diminution (Grandin, 2007). Body weight changes during transportation are affected by several factors such as trip distance, time of loading, rest-stop frequency, environmental conditions, breed, sex, age, body condition of the animals and diet composition prior to the trip, among other factors (Gonzalez et al., 2012a; Warriss, 1990).

Different cattle categories (i.e., steers, heifers, cows, etc.) are transported, and each of them present particular characteristics in terms of BW, trip length, stocking density on trucks, place of origin, transportation and handling experience, among other factors (Gonzalez *et al.*, 2012a). For example, feeder cattle are loaded at auctions or farms and have a long transportation period to feed yards; on the contrary, fat or finished cattle are almost exclusively loaded at feed yards and take a short trip to the slaughterhouse. Moreover, fat cattle with destination to the abattoir suffer less shrink during transportation than yearling cattle, mainly because of their body condition and diet quality (Gonzalez et al., 2012a)

Animals during transportation undergo two different types of shrink: “fill shrink” and “tissue shrink”. Fill shrink is caused by losses from the gut contents as manure, approximating 12-25% of the body weight (Grandin, 2007). Furthermore, an additional fill shrink is where animals lose urine from the bladder. On the other hand, “tissue shrink” is caused by the losses of reserves of nutrients and energy through different mechanisms like sweating, oxidation and respiration. Tissue shrink may account for more than 50% of body weight losses (Gonzalez *et al.*, 2012a). Fill shrink depends directly on the time of shipping and the quantity and quality of feed ingested before transportation (Hogan *et al.*, 2007). Gonzalez et al. (2012) reported that cattle loaded at

auction markets shrank less than loaded at feed yards or farms, partially because cattle transported to the sale already had fill shrink, and also the stressful environment of an auction did not encourage animals to eat (Gonzalez *et al.*, 2012a). Furthermore, the quality of the diet ingested is a determining factor in gut fill. For example, diets with high content of forage increase the volume of gut contents, and increase "fill shrink". In contrast, cattle fed with a high concentrate diet have less gut-fill and a lower shrink (Warriss, 1990). Finally, RPM escapes the rumen breakdown and is absorbed in the intestine. Furthermore, no effect on fecal excretion (g/d) is observed due to the addition of RPM in the diet (Torrentera *et al.*, 2017).

In 1997, USDA established the "Twenty-Eight Hour Law" which regulates the maximum time of transportation for animals to 28 consecutive hours. If the trip exceeds the specified time, cattle must be off-loaded for at least 5 consecutive hours to rest. Furthermore, water and feed must to be available for them (USDA, 1997). The "Twenty-Eight Hour Law" is supported by several studies (Gallo *et al.*, 2000; Jones *et al.*, 1990) reporting that the body weight loss is positively correlated to the distance of transportation mainly, because of starvation. However, Knowels et al. (1999) points out that trips longer than 24 hours should not be recommended because the animals change their behavior (i.e., lying down), and increase protein breakdown and dehydration (Knowles *et al.*, 1999). Furthermore, Marti et al. (2017) analyzed the effects of resting stop in a 15-hours-long transportation, concluding that resting stops do not affect BW losses, but stops at 5 hours of transportation prevent short and long-term stress in comparison with stops at 10 or 15 hours of transport (Marti *et al.*, 2017). Not only do the time and distance affect animal welfare, but another crucial condition is high ambient temperature, which increases BW losses as the temperature rises. Greater shrink is observed when animals have to cope with

high environmental temperatures in long journeys (Gonzalez *et al.*, 2012a). Finally, temperature and humidity during transportation have a negative effect on cattle (Randall, 1993).

Serum cortisol, glucose, and creatine kinase

Stress arises when an animal's situation changes the normal physiological activity, and consequently it stimulates a response to restore the previous homeostatic condition (Mumma *et al.*, 2006). Transportation is a stressful event that causes physiological and endocrine changes in animal metabolism. One indicator of these changes is cortisol, a glucocorticoid hormone released from the adrenal gland as a response to stress (Ishizaki & Kariya, 2010). Cortisol acts in all the cells of the body where it regulates translation to proteins which cope against stress (Grandin, 2007). It is widely used as a stress biomarker, and it can be measured from different fluids or excreta; e.g., in saliva (Fukasawa, 2012; Van De Water *et al.*, 2003), in feces (Morrow *et al.*, 2002; Palme *et al.*, 2000), in milk (Verkerk *et al.*, 1998), and in blood (Chulayo *et al.*, 2016). There is a positive correlation between stress and cortisol production (Warriss, 1990). Several studies have shown an increase in cortisol levels due to transportation in cattle, especially in the first hours of the trip (Buckham Sporer *et al.*, 2007; Ishizaki & Kariya, 2010). Cortisol production can be affected by previous experience; for example, animals which had been handled by humans in the past show lower levels of cortisol during handling and transportation because of their experience with stressful practices compared with animals that never had been exposed to those kind of scenarios (Grandin, 1997). Fukasawa et al. (2012) has demonstrated that trained calves showed lower production of cortisol than non-trained calves (Fukasawa, 2012). Having this knowledge can be crucial in order to increase animal welfare and reduce stressful transportation managements. Breed should be also considered when evaluating an animal's stress response (Zavy *et al.*, 1992). Temperament is correlated with handling responses;

among them, stressor situations. In effect, cortisol level is affected by temperament, which is a heritable condition as a result of the breed. *Bos taurus* cattle present a quieter response to handling compared with *Bos indicus* cattle (Grandin, 1997; Hearnshaw & Morris, 1984).

Another metabolite measured in blood circulation was glucose, a monosaccharide which is the most important source of energy for animals. It is formed by hydrolysis of carbohydrates or glycogen, known as glycogenolysis; or by gluconeogenesis, which is the synthesis of glucose from other non-carbohydrates substrates, mainly in the liver. (Ostrowska *et al.*, 2015). The homeostasis of glucose levels is regulated by the action of insulin and glucagon, which promote glucose storage or mobilization, respectively (Baumgard *et al.*, 2017). During muscle contractions, glucose is utilized as energy source by anaerobic and aerobic pathways. Muscle cells during exercise break down glycogen through glycogenolysis in order to obtain glucose (Shearer *et al.*, 2001). During transportation, an increase in glucose levels in blood is observed (Knowles, 1999; Tarrant *et al.*, 1992).

Finally, the last metabolite analyzed in blood was the enzyme creatine kinase. It catalyzes the reversible conversion of creatine and adenosine triphosphate (ATP) to phosphocreatine and adenosine diphosphate (ADP) (Wallimann *et al.*, 1992). After muscular activity, the presence of creatine kinase in blood is greater due to the higher utilization of energy (Volfinger *et al.*, 1994). Also, the increase of creatine kinase shows an increase of the permeability of the skeletal muscle membrane and it is used also as an indicator of stress (Mitchell *et al.*, 1988; Warriss *et al.*, 1984). There are different creatine kinase isoenzymes: creatine kinase-b type, in brain (CKB); creatine kinase m-type, in muscle (CKM); creatine kinase mb-type, (CK-MB), in heart; and mitochondrial creatine kinase (mtCK), in mitochondria (Yamashita & Yoshioka, 1991). In this study, we analyzed the expression of Creatine Kinase M-type (*CKM*).

Skeletal muscle structure

Skeletal muscle is one of the three major muscle types present in mammals' body, and it represents up to 50% of the body mass (Hoppeler & Fluck, 2002). Skeletal muscle comprises a group of heterogeneous long multinucleated cells called myofibers. Their highly specialized contractile proteins allow the skeletal muscle to accomplish its role of force generation and movement (Cardinet, 1997; Valberg, 2008). A group of homogenous myofibers and a motor neuron form the motor unit of the muscle (Edstrom & Kugelberg, 1968). Normally, the assembly of several motor units constitutes the skeletal muscle. The variety of myofibers that compose the muscle provides the opportunity of being flexible enough to perform different tasks such as continuous maximal or low-intensity, repeated short tetani, among others (Schiaffino & Reggiani, 2011).

Nowadays, the most common classification for mammalian skeletal muscle fiber types is based on the myosin heavy chain composition (MHC). Type I fibers, also called "red" because of their high myoglobin content, have slow-twitch oxidative fibers and respond to low-intensity long-lasting contraction, producing high resistance to fatigue. Furthermore, they are adapted to oxidative metabolism because of their high mitochondrial density. The other group is called Type II fibers, or "white", due to the lower myoglobin concentration compared with Type I fibers. Type II fibers respond to high-intensity short-duration contractions, which causes lower resistance to fatigue. In contrast to red fibers, white fibers have lower mitochondrial density (Picard *et al.*, 2012). Type II fibers can be subdivided in three groups: IIa, IIx, IIb. Fibers Type IIa are fast oxidative with significant resistance to fatigue, while Type IIb fibers are fast glycolytic with very low fatigue resistance. Type IIx presents intermediate functional

characteristics between IIa and IIb, with a moderate resistance to fatigue (Schiaffino *et al.*, 1989; Schiaffino & Reggiani, 2011).

The concentration of MHC varies among species. For example, in food animals, MHC in cattle have higher percentage of Type I than pigs or poultry animals (Choi & Oh, 2016; Ismail & Joo, 2017; Picard *et al.*, 1998). As a result, the color of the beef is reddish and more resistant to fatigue in comparison to pork (Park *et al.*, 2009; Wright *et al.*, 2018). However, MHC also varies among breeds within the specie. For example, in *Bos taurus*, MHC Type IIx is lower compared with *Bos indicus* breeds. This condition affects tenderness in *Bos indicus*-related meat, nevertheless, other factors like calpastatin activity, connective tissue content and proteolysis also affect the quality of their meat (Wright *et al.*, 2018).

Muscle fatigue and creatine synthesis

Skeletal muscle fatigue can be defined as the decrease of force generation after an intense and repetitive use of muscle. This occurrence is reversible, and can be recovered after a period of rest (Westerblad *et al.*, 1991). Muscle resistance to fatigue depends on the percentage of slow and fast fiber types, which varies among species and breeds.

In order to produce force generation, skeletal muscle has different energy sources available, and the utilization of them varies on the type of muscle fiber and the efficiency of use of that available energy. ATP is the immediate energy source in muscle contraction, and the enzyme responsible to catalyze the formation of ATP (2 ADP ↔ AMP + ATP) is called adenylate kinase. Because muscle consumes ATP rapidly, different pathways are activated in order to supply energy needs through anaerobic and aerobic metabolism. Anaerobic metabolism is important in short-term contractions, whereas aerobic processes are involved in long-term muscle utilization (Sahlin *et al.*, 1998).

In anaerobic pathways, fast muscle cells regenerate ATP from the breakdown of creatine phosphate (PCr), and glycogen. PCr is degraded by creatine kinase, producing ADP and Pi (PCr + ADP ↔ Cr + ATP) (Westerblad *et al.*, 2010). Creatine kinase is in near-equilibrium with adenylate kinase. Interestingly, fast muscle can consume ATP at higher rate than its regeneration rate (Allen *et al.*, 2002). At the beginning of muscle contraction, ATP concentration remains stable, whereas PCr concentration decreases and Cr and Pi concentrations increase. Later, PCr concentration starts its depletion period; consequently, ATP concentration starts to decrease and ADP and AMP concentrations increase significantly (Allen *et al.*, 2002; Westerblad *et al.*, 2010; Zhang *et al.*, 2008). Furthermore, glycogen degradation is catalyzed by the enzyme phosphorylase, which it is active when phosphorylated. This enzyme breaks down glycogen, releasing glucose residues that later enter glycolysis and subsequently are converted to pyruvate. Lactate dehydrogenase is an enzyme that catalyzes the reaction that regenerates NAD^+ ($NADH^+ + H^+ + \text{pyruvate} \rightarrow \text{lactate} + NAD^+$) (Zhang *et al.*, 2008). Generally, the concentration of lactate increases after intensive exercise when ATP consumption is high (Westerblad *et al.*, 2010). The amount of energy produced through PCr depends on the intramuscular concentration and the fiber type (i.e., fast-twitch fibers contain 15-20% more PCr than slow-twitch fibers in humans) (Soderlund *et al.*, 1992).

In the case of the aerobic pathway, it utilizes carbohydrate and fat as metabolic substrates (Spriet & Watt, 2003). The main carbohydrate used is muscle glycogen; however, some studies points out that extracellular glucose is involved in ATP production during exercise (Katz *et al.*, 1991; Katz *et al.*, 1986). The final product of glycogen metabolism is lactate, which is involved in ATP production (Spriet *et al.*, 2000). Triglycerides stored in muscle or in adipose tissue are broken

down to produce free fatty acids. Consequently, fatty acids are oxidized to produce ATP aerobically (Westerblad *et al.*, 2010).

Dystrophin-Glycoprotein complex

The sarcolemma of skeletal and cardiac muscle is provided with a protein assembly called Dystrophin-Glycoprotein complex (DGC). It is composed of a multi-subunit of peripheral and membrane-associated proteins that links the cytoskeleton to the extracellular matrix. This is a physical linkage that stabilizes the sarcolemma during contractions (Holt & Campbell, 1998). The DGC is formed by the dystroglycan complex, composed by dystrophin, the syntrophins, α- and β-dystroglycan, and sarcoglycan complex (SG), which is formed by four different transmembrane glycoproteins called α-, ß-, γ- , and δ -sarcoglycan (Gumerson & Michele, 2011; Tarakci & Berger, 2016). In addition, sarcospan, a transmembrane protein, is recognized as a DGC member (Crosbie *et al.*, 1997; Marshall & Crosbie-Watson, 2013). Furthermore, sarcospan can be included in sarcoglycan complex (Michele & Campbell, 2003).

Dystroglycan complex is present in the sarcolemma, and it is responsible for binding the basal lamina through α-dystroglycan and laminin (Han *et al.*, 2009). Furthermore, it attaches dystrophin, a cytoskeletal actin-binding protein, at its C-terminal domain via ß-dystroglycan (Gumerson & Michele, 2011). At the same time, dystrophin N-terminal binds actin, generating a link between the cytoskeleton and the extracellular matrix (Stone *et al.*, 2005).

The extracellular protein α-Dystroglycan (α-DG) undergoes posttranslational glycosylation in order to bind extracellular binding proteins such as laminin (Rader *et al.*, 2016). In contrast, ß-Dystroglycan (ß-DG) plays a key role in the Dystroglycan complex because it is associated with dystrophin intracellularly, and with α-DG extracellularly (Rader *et al.*, 2016). The SG complex is formed by an assemblage of transmembrane glycoproteins which its main function is to

strengthen the linkage between dystroglycan complex and dystrophin (Tarakci & Berger, 2016). Both ß- and δ-SG are strongly associated, forming a core during assembly due to their affinity. In addition, the SG complex finishes its formation by the attachment of γ-SG to δ-SG, whereas α-SG binds to γ-SG (Noguchi *et al.*, 2000; Shi *et al.*, 2004).

Sarcoplasmic reticulum

Before the generation of fatigue, the muscle must be on a contraction and relaxation period. Calcium cation (Ca^{++}) levels in muscle determine either contracted or relaxed condition. The structure that regulates Ca^{++} levels in muscle milieu is called sarcoplasmic reticulum, and its main function is to store or release Ca^{++} to the tissue. At high Ca^{++} levels within the sarcoplasmic reticulum, the muscle remains relaxed. In contrast, when Ca^{++} is released, contraction takes place (MacLennan & Wong, 1971). Sarco/endoplasmic reticulum Ca^{++}-ATPase (SERCA) is a membrane protein that mediates Ca^{++} transportation from cytosol to the sarcoplasmic reticulum (Pan *et al.*, 2003). Four SERCA isoforms are present in the sarcoplasmic reticulum: SERCA1, SERCA2a, SERCA2b, and SERCA3. SERCA1 is expressed in skeletal muscle, especially in Type II fibers. In contrast, SERCA2a isoform is present in cardiac muscle and Type I skeletal muscle fibers; and both SERCA2b and SERCA3 are present in smooth muscle (MacLennan *et al.*, 1997; Sacchetto *et al.*, 2009). The gene that encodes for SERCA1 is also called ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 1 (*ATP2A1)* (Sacchetto *et al.*, 2009). In addition, Calsequestrin is another important protein involved in Ca^{++} regulation, as its main function is to bind and release Ca^{++} in the lumen of the sarcoplasmic reticulum (Tomasi *et al.*, 2012). Calsequestrin presents high capacity, but low affinity with Ca^{++}, conditions that provide a quick response when Ca^{++} has to be mobilized (Park *et al.*, 2003). Two Calsequestrin isoforms can be expressed depending on the tissue. First, Calsequestrin 1 is presented in both Type I and

Type II fibers of skeletal muscle, and it is encoded by the gene *CASQ1*. In addition, Calsequestrin 2 is present in heart, Type I fibers, and Type II fibers only in early development, and it is encoded by the gene *CASQ2* (Lewis *et al.*, 2016; Tomasi *et al.*, 2012).

Finally, the assembly between sarcoplasmic reticulum and T-tubule, called the triad junction, include a transmembrane protein called mitsugumin29 (MG29), which is encoded by the gen Synaptophysin Like 2 (*SYPL2)* (Takeshima *et al.*, 1998). Because of its similarity in composition to synaptophysin family, MG29 was considered a neurotransmitter releaser (Chen *et al.*, 1998). However, later in time, the function of MG29 was discovered. It performs a key role in triad junction structure and maintenance, and interacts with Ryanodine receptor (RyR) in the Ca^{++} signaling (Komazaki *et al.*, 2001; Zhao *et al.*, 2011). It has been proven that mice lacking MG29 had higher susceptibility to fatigue in both, Type I and Type II muscle fibers (Nagaraj *et al.*, 2000).

Oxidative stress

The generation of free radical elements as Nitric Oxide (NO^-) and superoxide (O_2^-) is a result of the normal cellular metabolism in skeletal muscle (Jackson *et al.*, 2007). Oxygen suffers reductions generating Reactive Oxygen Species (ROS) (i.e., oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-)). Nitric oxide originates Reactive Nitrogen Species (RNS) like peroxynitrous acid (HNO_3^-), proxynitrite ($ONOO^-$), and other nitrogen-derived oxidants (Ferreira & Reid, 2008). These products are released in low concentration during resting, and they are necessary for the normal cellular function (Fukai & Ushio-Fukai, 2011). For example, these compounds are involved in muscle regeneration, acting with other elements like growth factors and chemokines; also, ROS promote mitochondriogenesis during exercise, that generates higher ATP levels for cell utilization (Vina *et al.*, 2009; Wright *et al.*, 2007). Furthermore, mitochondrial synthesis is stimulated by other

factors involved in oxidative stress regulation: the PGC-1α–NRF1–TFAM pathway. PGC-1α is the first stimulator of mitochondrial biogenesis (Vina *et al.*, 2009). However, skeletal muscle generates oxidants at high rates during muscle contraction, and they became blockers of myogenic differentiation, or they can cause injuries by oxidative damage (Barbieri & Sestili, 2012).

Nitric oxide is formed by the conversion of L-arginine to L-citruline by enzymes called NO synthases (NOS) (Ishii *et al.*, 1998). The three isoenzymes that produce NO are located in the muscle fibers. Two of them are expressed constantly: Type I, also called "neuronal" (nNOS), which is located in the sarcolemma of fast fibers; and type III, "endothelial" (eNOS), which is generated in mitochondria (Reid, 1998). The third type, "inducible" (iNOS), is mostly expressed in the course of an inflammation process (Tengan *et al.*, 2012). Among the roles of NO in skeletal muscle, it acts as a signaling molecule (Stamler & Meissner, 2001), reduces force production by inhibiting sarco/endoplasmic reticulum Ca_2^+-ATPase activity (Ishii *et al.*, 1998), increases cGMP establishing mechanism of redox regulation (Kobzik *et al.*, 1994), and inhibits actin-myosin cross-bridge cycling by modulating critical thiols on the myosin head (Perkins *et al.*, 1997).

ROS are produced in different sites in skeletal muscle. Complex I and II of the mitochondrial electron transport chain (Jackson *et al.*, 2007), phospholipase A2 , and metabolism of arachidonic acid by the lipoxygenase pathway apparently are the main ROS producers during resting at low rates (Ferreira & Reid, 2008; Zuo *et al.*, 2004). However, during exercise, both NADPH and phospholipase A2 contributes to increased production of O_2^- (Ferreira & Reid, 2008). Nitric oxide can be inactivated by coupling with O_2^-, producing the oxidant $ONOO^-$ as a final product for the reaction (Fukai & Ushio-Fukai, 2011).

Exercise and fatigue increase muscle $ONOO^-$ and O_2^- producing an excess of oxidants in the cellular milieu. Nevertheless, cellular defense mechanism against oxidative damage can degrade effectively these products (Nikolaidis *et al.*, 2008). Superoxide dismutases (SODs) are oxidoreductases generated by cells as a protection against oxidants. They can perform spontaneously dismutation of O_2^- into H_2O_2. Consequently, they also help reduce the production of $ONOO^-$. Nonetheless, H_2O_2 is another damaging agent, thus, the cell generates thiol-based antioxidants as glutathione peroxidase (GPx) that converts H2O2 into H2O + O2 (Ferreira & Reid, 2008).

Mammals possess three isoforms of SOD, and they are differentiated by their composition and site of action. SOD1 (Cytosolic Cu/ZnSOD) is the main intracellular dismutase, and it is localized in the cytosol and, to a lesser extent, in the intermembrane space of mitochondria. Enzymatic activity of SOD1 depends on the presence of copper and zinc. SOD2 (MnSOD) is a mitochondrial manganese enzyme located in the mitochondrial matrix. Manganese plays a catalytic role in the dismutation of O_2^- to H_2O_2 similarly than SOD1 (Fukai & Ushio-Fukai, 2011). The major site of O_2^- generation is the mitochondrial respiratory chain; consequently, SOD2 is a very important regulator of ROS equilibrium in the cell (Hu *et al.*, 2005). Finally, an extracellular dismutase called SOD3 (ecSOD) catalyzes the ROS in the extracellular matrix. It is mostly expressed in internal organs and blood vessels. Skeletal muscle does not present high expression of SOD3, thus, it is not subject to analysis in our study (Folz & Crapo, 1994; Fukai & Ushio-Fukai, 2011).

DNA methylation

The process of methylation in DNA involves the addition of a methyl group to the fifth carbon in cytosine of DNA by enzymes called DNA Methyltransferases (DNMTs). In mammals,

methylation occurs in almost the whole genome, approximately at 75% of CG dinucleotides (Law & Jacobsen, 2010). However, the remaining unmethylated CG dinucleotides are located in CpG islands (Cedar & Bergman, 2009). The main functions of DNA methylation are control of cellular differentiation and development, gene repression, chromosomal integrity, among others (Hermann *et al.*, 2004). The family of DNMTs is composed by DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Jin *et al.*, 2011). DNMT1, DNMT3A, and DNMT3B perform the methylation in cytosines of the genome (Xu *et al.*, 2018). DNMT1, an enzyme codified by the gene *DNMT1*, is involved in maintenance of methylation, whereas DNMT3A and DNMT3B, enzymes codified by the genes *DNMT3A* and *DNMT3B* respectively, are involved in *de novo* methylation (Law & Jacobsen, 2010; Li & Ibeagha-Awemu, 2017).

Chapter II

Gene Expression Response of the Skeletal Muscle of Angus-Simmental Heifers to Transportation under Rumen-Protected Methionine Supplementation

Introduction

Transportation by road is an event that cattle are forced to experience at least once in their lifetime. One of the negative effects of transportation is muscle fatigue, which can be defined as the reversible decrease of force generation after an intense and repetitive use of the muscle (Westerblad *et al.*, 1991). Fatigue occurs once the energy from aerobic and anaerobic metabolism is limited (Sahlin *et al.*, 1998). When transportation takes place, the animals have to be standing; thus, skeletal muscles are generating force constantly. In addition, the case of transportation trips in United States is special because of their long duration, as the distances between cow-calf operations and feedlots are considerable. Consequently, longer trip duration will cause a more repetitive use of the muscle in compared with short-duration trips. Our hypothesis is that Smartamine® (RPM) supplementation could produce a potential increase in creatine synthesis through the upregulation of Creatine Kinase M-type (CKM) that helps skeletal muscle during conditions that cause muscle fatigue.

The objective of this study was to analyze the effect of administration of RPM on the muscle fatigue gene network during pre- and post- exposure to a long-duration transportation simulation; also to determine if RPM induces changes in expression of genes related to oxidative stress, and finally to identify biomarkers of beef muscle fatigue to be used in the future for selection of animals less susceptible to transportation stress.

Materials and methods

Angus heifers (n = 18) were assigned to two groups of 9 animals in order to achieve uniformity of average body weight (BW; 408 ± 64 kg) and age (375 ± 44 days old) within treatment groups. All animals were located at Beef Evaluation Center, Auburn University, Auburn, Alabama. All procedures were approved by the Auburn University Animal Care and Use Committee (IACUC; PRN# 2017-3129). After a successful adaptation to Calan Gate System (Northwood, NH), use for better control of individual dry matter intake (DMI), both groups received once per day 2 pounds of soyhulls (50%) and corn gluten feed (50%) in combination with Bermudagrass Hay *ad libitum*. Additionally, 9 heifers received supplement containing 8 gr/hd/day of top-dressed RPM (0.07% of DMI), whereas other 9 heifers received supplement without the addition of top-dressed RPM. Heifers' health status, DMI and RPM intake were individually monitored on a daily basis.

After forty-five days (from 10/31/2017 to 12/14/2017) on test diets, both cohorts were loaded by reference to an optimal animal density of 10.40 sq. ft. for an 800-lb steer onto a 32' × 7' steel gooseneck trailer (Circle W trailers, McKenzie, AL 36456) hooked up to a pickup truck. Subsequently, animals were transported from the Beef Evaluation Center located at 405 Shug Jordan Parkway, Auburn, AL 36832 to the National Center for Asphalt Technology Test Track (NCAT) located at 1600 Lee Road 151, Opelika, AL 36804 (~18 miles apart) (Tucker *et al.*, 2015). The "transportation simulation" took place on a 1.7-mile oval test track at NCAT, where animals remained loaded and transported for 22 hours at 45 mph. Truck drivers (three in total) followed a modified version of the NCAT driver safety schedule which consisted of stopping once per hour (5 minutes per stop) to rest, and every three hours to load 28 gallons of gas until full tank (~10 minutes per load). Every time the truck was stopped, visual evaluations of the

animals were performed in order to make sure about their well-being status. Animals did not have access to water or feed on the truck during the transportation neither the resting stops. The average ambient temperature and relative humidity during the pre-conditioning period was 11°C and 72%, respectively. During transportation, the average ambient temperature was 4°C and the relative humidity was 48%. No precipitation was detected during any biopsy timepoints neither transportation simulation. The weather information was obtained at the AWIS (Agricultural Weather Information Service, Inc.) website from the Auburn Mesonet weather station (Auburn_CR10_92-18, Lee County). Since the temperature was close to 0°C, the Temperature and Humidity Index was the minimum, which is equal to 50 (Lees *et al.*, 2019; Mader, 2003) Animals were weighed at the beginning of the adaptation period to Calan Gate System, at the beginning of the study (Day 0), 3 days before transportation (Day 43, pre-transportation), and immediately after transportation (Day 45, post-transportation).

The first skeletal muscle biopsy was performed at the beginning of the administration of RPM (Day 0). The second biopsy was performed 3 days prior to transportation simulation (PRET) and, a final biopsy was performed immediately after unloading the truck (POST). These biopsies were taken in order to assess gene expression changes related to muscle fatigue due to RPM supplementation throughout the preconditioning period, during and after transportation. Animals IDs were recorded so that the same animals were biopsied at each time point to provide repeated sampling.

Skeletal muscle biopsies of *Longissimus dorsi* muscle were performed for gene expression analysis (RT-qPCR). Each muscle sample was obtained from the left side with incision site positioned progressively more caudal (longitudinally) with each sequential biopsy. Anesthesia injections of 5 mL of Lidocaine 2% (VetOne®, Boise, ID) were placed before each biopsy

incision. A biopsy core of muscle (500 - 600 mg) was removed using a biopsy needle (12-gauge × 16 cm) inserted in a biopsy gun instrument (Bard Magnum MG#1522, Tempe, AZ). The first biopsy area coincided with the *Longissimus dorsi* muscle at the level of the last rib. The second biopsy was performed 10 cm towards the head from the first biopsy, and the last biopsy, 10 cm towards the head from the second biopsy. Incision sites for all the biopsies were located 5 cm down the vertebral column with the aim to take samples from the center of the *Longissimus dorsi* muscle (Figure 3). Ten mL of blood were collected via jugular venipuncture at day 0, before and immediately following transportation simulation to determine circulating serum cortisol, glucose and creatine kinase levels.

No signs of infection, swelling or external bleeding were detected on the biopsy site after each of the procedures, and in the following days. All animals ate normally after each biopsy.

Serum cortisol, glucose, and creatine kinase analysis

In order to asses cortisol, glucose, and creatine kinase levels in circulating blood, 10 mL of blood were collected via jugular venipuncture. Serum were separated by centrifugation at 1,500 × g for 15 min, and an aliquot was stored frozen at -20 °C until analyzed for cortisol, glucose and creatine kinase. Serum glucose, creatine kinase and cortisol concentration were measured at the Auburn University Endocrine Diagnostics Lab. Cortisol levels were measured with IMMULITE 2000 analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA), which uses a solid-phase competitive enzyme-amplified chemiluminescent immunoassay. Glucose concentration was measured using a Roche/Hitachi Cobas C analyzer for clinical chemistry using Reagent 1 (MES buffer: 5.0 mmol/L, pH 6.0; Mg^{2+}: 24 mmol/L; ATP: $\geq$ 4.5 mmol/L; NADP: $\geq$ 7.0 mmol/L; preservative), and Reagent 2 (HEPES buffer: 200 mmol/L, pH 8.0; Mg^{2+}: 4 mmol/L; HK (yeast): $\geq$ 300 µkat/L; G6PDH (E. Coli): $\geq$ 300 µkat/L; preservative).

Creatine Kinase concentration was measured using a Roche/Hitachi Cobas C analyzer using Reagent 1 (Imidazole buffer: 123 mmol/L, pH 6.5 (37°C); EDTA: 2.46 mmol/L; Mg^{2+}: 12.3 mmol/L; ADP: 2.46 mmol/L; AMPR: 6.14 mmol/L; diadenosine pentaphosphate: 19 µmol/L; $NADP^{+}$ (yeast): 2.46 mmol/L; N-acetylcysteine: 24.6 mmol/L; HK (yeast): $\geq$ 36.7 µkat/L; G6PDH (E. Coli): $\geq$ 23.4 µkat/L; preservative; stabilizers; additives), and Reagent 2 (CAPSO* buffer: 20 mmol/L, pH 8.8 (37 ºC); glucose: 120 mmol/L; EDTA: 2.46 mmol/L; creatine phosphate : 184 mmol/L; preservative; stabilizers).

RNA extraction

In order to extract RNA from skeletal muscle, ~100 mg. of tissue from each animal at each biopsy date was immersed in 1 mL of QIAzol Lysis Reagent (Qiagen, Hilden, Germany; Cat. #: 79306) and homogenized for 1 minute, cooled on ice for 1 minute, and finally homogenized again for 1 additional minute. Then, after 5 minutes of incubation on ice, each sample received 0.2 mL of chloroform and were shaken by hand for 15 seconds. After 2 minutes of incubation, samples were centrifuged for 10 minutes at 12,000 × g at 4ºC. After centrifugation, each tube presented a lower red phenol-chloroform layer, an interphase, and a colorless upper aqueous phase, which contains the RNA. The upper phase was transferred to a new tube, and 0.5 mL of isopropanol was added and then incubated for 10 minutes. The following step consisted of centrifugation for 10 minutes at 12,000 × g in which total RNA is precipitated as a white pellet at the bottom of the tube. The supernatant was discarded, and the pellet was re-suspended in 1 mL of 75% ethanol and centrifuged for 5 minutes at 7,500 × g at 4ºC. Afterward, the tubes containing RNA pellets were opened for 10 minutes in the hood in order to be exposed to air drying. Finally, 20 µL of RNase-free water was added directly to resuspend the RNA pellet. The RNA concentration was obtained using Nanodrop OneC. Samples were cleaned with RNA Clean

& Concentrator kit according to manufacturer's protocol (Zymo Research Catalog Nos. R1013 & R1014) if they had lower 260/280 (>1.90) and 260/230 (>1.70) ratios. RNA quality integrity was assessed by electrophoresis gel, and the samples that showed two clear ribosomic bands were accepted.

Primer design

The cDNA sequences for genes used were found at National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) or at University of California-Santa Cruz's Genome Browser (https://www.genome.ucsc.edu/). The sequences obtained were entered into Primer Express 3.0.1 software (ABI). The default settings (TaqMan® MGB quantification) were used; however, amplicon size was modified to 100 base pairs. The designed primer sequences were uploaded in blast tool of NCBI Nucleotide Blast and ordered from Integrated DNA Technologies (https://www.idtdna.com).

cDNA synthesis

In order to prepare the complementary DNA (cDNA), the RNA obtained after cleaning was diluted to a concentration of 100 ng/µL. First, Master Mix 1 (MM1) was prepared by mixing 9 µL of RNase-free water to 1 µL of Random Primers (Roche Diagnostics, Indianapolis, IN). Later, 1 µL of 100 ng total RNA was added. The mixture was incubated at 65ºC for 5 minutes. Then, the samples were incubated on ice for 3 minutes. For each sample, Master Mix 2 (MM2) was prepared by mixing 1.625 µL RNase-free water, 4 µL 5X First-Strand Buffer, 1 µL Oligo dT18, 2 µL 10 mM dNTP mix (10 mM), 0.25 µL of Revert aid (200 U/µL), and 0.125 µL of RNase inhibitor (20U/µL). Then, MM2 was mixed to MM1 + RNA (final volume of 20 µL and multiplied by total genes analyzed). The incubation protocol was as follows: 25ºC for 5 minutes, 42ºC for 60 minutes, and 70ºC for 5 minutes followed by 4ºC. A pooled sample was obtained

from all samples to design the Standard Curve. Then, the pooled sample was diluted to a 1:2 ratio with RNase-free water for the first standard curve point. The subsequent standard curve points were diluted to a 1:4 ratio.

Preliminary primer testing

In order to test each primer, the following products were mixed in a PCR tube: 1 µL of Forward primer, 1 µL Reverse primer, 8 µL of pooled cDNA, and 10 µL of Perfecta SYBR Green. Samples were placed in an Eppendorf nexus gradient thermocycler for 2 minutes at 50ºC, 10 minutes at 95ºC, 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC for denaturation. Five µL of the PCR product were transferred to a new 0.2-mL PCR tube for agarose gel electrophoresis and mixed with 2 µL of loading dye. The ladder was prepared by mixing 0.6 µL of ladder (25 bp, from Invitrogen, Carlsbad, CA) with 2 µL of loading dye.

In addition, 3 g of OmniPur® agarose (Calbiochem, San Diego, CA) were dissolved in 150 mL of TAE Buffer (Invitrogen, Carlsbad, CA; Cat# 15558-026). The agarose mix was heated for 1 minute in a microwave. Two µL of SYBR Safe were added to the agarose mix before cooling, and then placed in the agarose gel apparatus. Ladders were added to the first well of each row, and the samples were added to the remaining empty wells. The gel ran at 80 mV until samples and ladder reached ¾ of the gel.

Bio-Rad Chemi Doc apparatus was used to analyze the gel, utilizing Image Lab software. The accepted primers had a clear and single band at 100 bp. Finally, the accepted PCR products were cleaned with QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany; Cat. #28106), before sending them for Sanger sequencing analysis to the University of Illinois Core Sequencing facility. Sequencing results were blast in NCBI website. The sequencing results that matched the primers blast were utilized. The following genes were selected as internal controls:

Mitochondrial Ribosome-Associated GTPase 1 (*MTG1)*, Ribosomal Protein S15a (*RPS15A*), and Ubiquitously Expressed Prefoldin Like Chaperone (*UXT*). Furthermore, for the Dystroglycan complex, we selected Sarcoglycan Beta (*SGCB*), Syntrophin alpha 1 (*SNTA1*), Syntrophin beta 1 (*SNTB1*), Sarcospan (*SSPN*). For genes present in the sarcoplasmic reticulum, we selected ATPase Sarcoplasmic/Endoplasmic Reticulum Ca^{++} Transporting 1 (*ATP2A1*), Calsequestrin (*CASQ1*), Synaptophysin Like 2/MG29 (*SYPL2*). For the creatine synthesis pathway, we considered Adenosylhomocysteinase (*AHCY*), Creatine Kinase M-type (*CKM*), Guanidinoacetate N-methyltransferase (*GAMT*), Glycine amidinotransferase (*GATM*), and Solute carrier family 6 member 8 (*SLC6A8*). Genes related to DNA methylation were DNA Methyltransferase 1 (*DNMT1*) and DNA Methyltransferase 3 alpha (*DNMT3A*). Finally, for Oxidative Stress, we selected the genes: Superoxide dismutase 1 (*SOD1*), Superoxide dismutase 2 (*SOD2*), Nitric Oxide synthase 3 (*NOS3*), Nuclear factor kappa B subunit 1 (*NFKB1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), and PPARg coactivator alpha (*PGC1a*).

RT- PCR

The last step in the gene expression analysis was RT-PCR. Eight µL of diluted cDNA sample, negative control, and standard curve were pipetted into their respective wells of MicroAmp™ Optical 96-well reaction plate in duplicates. Later, 12 µL of SYBR Green Master Mix (8 µL of SYBR Green, 0.6 µL Forward Primer, 0.6 µL Reverse Primer, and 0.4 µL of water) was pipetted into each well. The PCR reaction was executed in an ABI Prism 7500 HT SDS machine set to 2 minutes at 50ºC, 10 minutes at 95ºC for holding stage; 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC for cycling stage; and 15 seconds at 95 ºC, 1 minute at 60 ºC, 30 seconds at 95 ºC, and 15 seconds at 60 ºC for melt curve stage. The data obtained were analyzed using the 7500 HT Sequence Detection Systems Software (version 2.3, Applied Biosystems, Foster City, CA).

Statistical analysis

Quantitative PCR data were analyzed using the MIXED procedure of SAS (SAS 9.4 Institute, Cary, NC, USA). Prior to 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. beginning of study). To estimate standard errors at day 0 and minimized 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, cortisol) included treatment (RPM or CTRL), time (BASE, PRET and POST) on experiment and treatment × 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. Significant differences were declared at $P < 0.05$ and tendencies between $0.06 < P < 0.1$. Serum cortisol, glucose, and creatine kinase cortisol levels, and shrink 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 heifer within treatment. Average Daily Gain data was analyzed using PROC GLM procedure of SAS.

The statistical model used was: $Y_{ijl} = \mu + C_i + T_j + S_l + (C \times T)_{ij} + \varepsilon_{ijl}$; where, Y_{ijl} is the background-adjusted normalized fold change or blood data value; μ is the overall mean; C_i is the fixed effect of time (3 levels); T_j is the fixed effect of treatment (2 levels); S_l is the random effect of heifer nested within treatment; C × T is the interactions of time by treatment and ε_{ijl} is the random error $(0, \sigma_e^2)$ associated with Y_{ijl}.

Results

Animal performance

Body weight (BW) at BASE differed ($P < 0.05$) from BW at PRET (Figure 4). Animals in RPM group had a BW of 365 kg at BASE and, their BW was increased to 407 kg at PRET. In the case of CTRL group animals, their BW at BASE was 360 kg and, their BW increased to 409 kg at PRET. Later, BW at PRET differed ($P < 0.05$) from BW at POST. Animals in RPM group had a BW of 407 kg at PRET, but after transportation they had a BW of 373 kg. Similarly, heifers in the CTRL group had a BW of 408 kg. at PRET, and BW decreased to 366 kg at POST.

The treatment × time interaction for BW was not significant ($P < 0.37$). RPM shrank 8.13 kg less than CTRL group (8.40% vs 10.47% of BW respectively). Furthermore, average daily gain (ADG) did not differ between treatments ($P < 0.41$) during the supplementation period (i.e., between BASE and PRET). Heifers in RPM group had an ADG of 0.98 kg/day, whereas heifers in CTRL an ADG of 1.12 kg/day.

Serum cortisol, glucose, and creatine kinase

Cortisol levels did not show a statistical difference in treatment × time interaction ($P < 0.67$) even though, there was an important reduction in cortisol levels after transportation. Heifers in CTRL group had 141.08 nMol/L at PRET, which decreased to 100.64 nMol/L at POST; whereas animals in RPM group had 150.08 nMol/L at PRET, and followed by reduction to 85.71 nMol/L at POST.

Glucose levels showed a treatment × time interaction ($P < 0.05$) and a time effect ($P < 0.05$). There was not significant difference in glucose concentration in both treatments between BASE and PRET. Heifers in RPM group had a significant increase in glucose levels between PRET

(83.9 mg/dL) and POST (98.8 mg/dL); and heifers in CTRL did not show a significant difference between PRET (91.7 mg/dL), and POST (96.8 mg/dL) (Figure 4).

Circulating creatine kinase levels did not show a treatment × time interaction ($P = 0.16$). However, creatine kinase concentration showed a time effect ($P < 0.05$). Both groups showed a significant increase between PRET and POST. Heifers in CTRL group had 227.4 U/L at PRET, and 406 U/L at POST; similarly, animals in RPM showed 266.7 U/L at PRET, and 424.8 U/L at POST (Figure 4).

Dystrophin-Glycoprotein complex

First, there was a treatment × time interaction ($P < 0.05$) and time effect ($P < 0.05$) for *SGCB*, *SNTA1*and *SSPN* (Figure 6). The mRNA expression of *SGCB* in CTRL group was upregulated between BASE and PRET ($P < 0.05$), and then it was decreased between PRET and POST ($P < 0.05$). In RPM treatment, *SNTA1* mRNA expression was downregulated between BASE and PRET ($P < 0.05$) and downregulated in CTRL between PRET and POST ($P < 0.05$). Expression of *SSPN* had a treatment × time interaction ($P < 0.01$), treatment effect ($P < 0.05$), and time effect ($P < 0.01$). Its expression was upregulated in CTRL between BASE and PRET ($P < 0.05$), and then downregulated between PRET and POST ($P < 0.05$). However, *SSPN* expression decreased in RPM between BASE and PRET ($P < 0.05$). The mRNA expression of *SGCB*, SSPN, and STNA1 in CTRL were greater than RPM ($P < 0.05$) at PRET.

Finally, *SNTB1* did not showed a significant treatment × time interaction ($P = 0.34$).

Sarcoplasmic reticulum

There was a treatment × time interaction ($P < 0.01$) and a time effect ($P < 0.01$) for both *CASQ1* and *SYPL2* (Figure 7). Furthermore, *ATP2A1* showed a treatment × time interaction ($P < 0.01$). In all cases, *ATP2A1*, *CASQ1* and *SYPL2* mRNA expression for CTRL was significantly greater ($P < 0.05$) than RPM at PRET. Furthermore, *ATP2A1*, *CASQ1* and *SYPL2* expressions for CTRL increased ($P < 0.05$) between BASE and PRET; however, their expressions decreased ($P < 0.05$) between PRET and POST.

In addition, *SYPL2* had significant upregulation ($P < 0.05$) of CTRL between BASE and PRET. Furthermore, RPM had a significant downregulation ($P < 0.05$) between BASE and PRET. For only the CTRL group did *SYPL2* expression decrease ($P < 0.05$) between PRET and POST.

Creatine synthesis pathway

Expression of *CKM* showed a significant treatment × time interaction ($P < 0.01$) (Figure 8). Furthermore, there was a significant treatment × time interaction ($P < 0.01$), treatment effect ($P < 0.05$), and time effect ($P < 0.01$), for *AHCY* mRNA expression. The mRNA expression of *GAMT* and *GATM* showed a treatment × time interaction ($P < 0.05$) and time effect ($P < 0.01$). The mRNA expression of *SLC6A8* showed a treatment × time interaction ($P < 0.01$) and a tendency for a time effect ($P < 0.08$). In addition, *AHCY*, *CKM* and *SLC6A8* mRNA expression on CTRL was greater ($P < 0.05$) than RPM at PRET. Finally, CKM expression was increased ($P < 0.05$) between BASE and PRET in CTRL, but its expression was decreased ($P < 0.05$) between PRET and POST. There was a significant downregulation of *AHCY* ($P < 0.05$) on RPM between BASE and PRET; however, it was upregulated ($P < 0.05$) between PRET and POST. In CTRL animals, *GATM* and *GAMT* expressions were downregulated ($P < 0.05$) between PRET and POST. In RPM heifers, *SLC6A8* expression was decreased ($P < 0.05$) between BASE and PRET,

and CTRL increased ($P < 0.05$) between PRET and POST. Finally, *SLC6A8* had a tendency for a time effect (P = 0.07).

Oxidative stress

There was a treatment × time interaction ($P < 0.05$) and time effect ($P < 0.01$) for *SOD1, NQO1,* and *NOS3* mRNA expression (Figure 9). Furthermore, *SOD2* showed a treatment × time interaction ($P < 0.01$) and treatment effect ($P < 0.05$). There was no treatment × time interaction ($P > 0.05$) for either *PGC1a* and *NFKB1.* At PRET, CTRL had a greater expression ($P < 0.05$) for *SOD2,* and *NQO1* expressions than RPM. There was an increase ($P < 0.05$) in *SOD1, NOS3, NFKB1,* and *PGC1a* mRNA expressions in CTRL between BASE and PRET. In CTRL heifers, *SOD2* mRNA expression showed an upregulation ($P < 0.05$) between BASE and PRET; however, its expression was downregulated between PRET and POST. In the case of *SOD2* expression in RPM, a decrease ($P < 0.05$) in expression was observed between BASE and PRET. Finally, *NQO1* mRNA expression increased ($P < 0.05$) in CTRL between BASE and PRET; however, a decrease (P < 0.05) was observed between PRET and POST.

DNA methylation

There was a treatment × time interaction ($P = 0.05$), and a time effect ($P < 0.01$) for *DNMT1* mRNA expression (Figure 10). Furthermore, *DNMT3A* showed a treatment × time interaction ($P < 0.01$), a treatment effect ($P = 0.05$), and a time effect ($P < 0.01$). For *DNMT3A,* CTRL had a greater expression (P < 0.05) at PRET than RPM.

There was an increase of *DNMT1* mRNA expression in CTRL between PRET and POST. Finally, *DNMT3A* mRNA expression decreased ($P < 0.05$) in RPM between BASE and PRET. Similarly, *DNMT3A* expression was downregulated ($P < 0.05$) in CTRL between PRET and POST.

Discussion

Animal performance

In terms of animal performance, CTRL group showed no difference in ADG compared with RPM (1.12 kg/d vs. 0.98 kg/d). There are no previous studies that report the performance of beef heifers under RPM supplementation; however, our results support Chen et al. (2011), who evaluated RPM supplementation (Smartamine) in dairy cows, and reported no difference with a control group (Chen *et al.*, 2011). In addition, Torrentera et al. (2017) tested the performance of Holstein steers (127±4.9 kg) under the inclusion of different levels of RPM. They obtained superior results with RPM supplementation when Smartamine was included at level of 0.096% of dietary dry matter (DM) (Torrentera *et al.*, 2017). In our study, the RPM supplementation was 0.07% of DM; inclusion up to 0.09% might possibly have improved animals' performance. However, Gomez et al. (2011) reported an increase in ADG ($P < 0.05$) in grazing Holstein heifers (227 ± 33 kg BW) supplemented with urea, blood meal, and RPM (Gomez *et al.*, 2011). Furthermore, unpublished data from a calving season study completed in our lab showed that ADG in calves born in Fall calving season under RPM supplementation tended to improve ADG, but no difference was observed in calves born in Winter season under RPM supplementation. Numerous factors affect shrink in livestock transportation. First, transportation duration and distance travelled are especially critical. In our study, animals were loaded on the trailer for 22 hours; however, because of the stops to rest, the net transportation time was 20 hours. The truck travelled to a velocity of 45 mph during the entire duration of the simulation, following NCAT safety rules. Even though animals were transported around the test track and no destination point was assigned, it is possible to calculate the estimated distance travelled (i.e., ~900 miles). The distance travelled by the animals could be compared with a typical commercial beef cattle

transportation from Auburn, AL to Kansas City, KS (838 miles); Lincoln, NE (1,033 miles); or Amarillo, TX (1,061 miles). Interestingly, Lambooy & Hulsegge (1988) studied the effect of 24 hours transportation in pregnant heifers, and reported body weight losses of 8% on average (Lambooy & Hulsegge, 1988). This result is similar to the shrink loss we obtained for RPM heifers. Another important factor that affects body weight loss due to transportation is the cattle category (i.e., steer, heifer, etc.). Gonzalez et al (2012a) assessed the differences in shrink depending on the animal category. Even though there was not a specific category for heifer, it is possible to compare with feeder cattle (388 ± 4.8 kg), for which BW is very similar to heifers' BW in our study (Gonzalez *et al.*, 2012a). For feeder cattle, transportation for more than 400 km produced 7.94% ± 0.15 BW losses. Similarly, 24 hours of transportation in heifers and steers (217 ± 3 kg BW) produced 9.6% BW losses (Marques *et al.*, 2012).

Regarding environmental conditions during the transportation day, according to AWIS (Agricultural Weather Information Service, Inc.) at Auburn Mesonet weather station (Auburn_CR10_92-18, Lee County) which is located at 5.2 miles from the National Center for Asphalt Technology Test Track (NCAT), the temperature was 5 ºC on average, relative humidity was 48.5%, and no rain was recorded during the 22 hours of transportation nor on the previous or subsequent days. According to Gonzalez et al. (2012), 20-25 hours transportation with an ambient temperature of ~10 ºC will produce a ~8% of BW losses on feeder cattle, and ~10% of BW losses on cull cattle (Gonzalez *et al.*, 2012a).

To the best of our knowledge, there are no reports on the effects of a rumen-protected amino acid on body weight losses due to transportation. Even though there was no difference between RPM and CTRL groups' shrink, it would be possible to consider that the numerical difference observed in shrink was produced by both: "fill shrink", and "tissue shrink" (i.e., oxidation,

respiration). However, we did not measure fecal and urine excretion nor individual DMI; consequently, it is not possible to estimate the percentage of shrink that belongs to fill shrink or tissue shrink.

Serum cortisol, glucose, and creatine kinase

The release of blood cortisol increases when the transportation starts, reaching its highest point at ~4 hours, and then it starts to decline until lower levels compared with the initial cortisol levels at the beginning of transportation. This reduced cortisol level is maintained for the next 24 hours post-transportation (Schmidt *et al.*, 2013; Sporer *et al.*, 2008). However, these studies assessed the effect of shorter transportation (9 hours maximum), compared with a longer transportation period used in this study.

Nevertheless, evidence of the reduction of blood cortisol level due to a long transportation could be associated with a familiarization of the animal with the truck environment (Van Engen *et al.*, 2014). In addition, in mammals, cortisol levels have pulsatility variation throughout the course of the day (Veldhuis *et al.*, 1989; Windle *et al.*, 1998; Young *et al.*, 2004). Van Engen *et al* (2014) assessed cortisol levels at 24 hours of the initiation of transportation, decreasing the pulsatility effect, and being representative to our study, where we took blood samples at pre-transportation and immediately after a 22 hours of transportation simulation (Van Engen *et al.*, 2014).

In total, our truck was stopped 160 minutes during the 22 hours of the trip, either to rest or to load gas, and visual evaluations of the animals' status was made during stops. The amount of time that the truck was stopped could be beneficial for both truck drivers and animals. Grandin (1997; 2007) points out that frequent rest stops with loading and unloading may be more stressful than being in the vehicle in movement. Consequently, this practice is detrimental for the well-being of the animals (Grandin, 1997; Grandin, 2007). Stops, in addition to visual

evaluation, during the first 24 hours without unloading animals, could be considered a good management practice to reduce stress conditions.

Glucose level in blood is used as a metabolic indicator of energy status in cattle. Our results suggest that glucose levels in blood during the preconditioning period are comparable to previous studies that reported 80.53 mg/100 mL of glucose on growing steers consuming high forage diet (Vasconcelos *et al.*, 2009). Tarrant et al (1992) assessed the metabolic changes on Friesian steers after 24-hour transportation (Tarrant *et al.*, 1992). They showed that glucose level at pre-transportation was 4.95 mmol/L (89.2 mg/dL), and at post-transportation was 5.9 mmol/L (106.30); these values are comparable to our results. In addition, Earley *et al* (2010) reported glucose levels in blood of growing Charolais bulls of 4.3 mmol/L (77.5 mg/dL) at pre-transportation, and 5.8 mmol/L (104.5 mg/dL) immediately after 24-hour transportation was completed (Earley *et al.*, 2010). However, the increase in glucose in RPM during transportation could be related to greater glycogenolysis (Brockman & Laarveld, 1986).

Finally, creatine kinase in blood is commonly used in studies related to muscle fatigue for assessing the activity of the muscle (Edwards, 1986; Newham *et al.*, 1983). Previous studies reported creatine kinase activity due to transportation in cattle. Tarrant *et al* (1992) reported 234 U/L of increase in creatine kinase after a 24-hours transportation with a stocking density (1.13 m^2/hd) similar to the one used in our study (1.15 m^2/hd). However, we reported a lower increase in circulating creatine kinase during transportation; RPM increased 158.1 U/L, and CTRL 178 U/L ($P = 0.16$). The lower increase in our study may be related with the BW of the animals. Tarrant *et al* (1992) used Friesian bulls with BW of 618 kg (Min, 537 kg; Max 900 kg) at pre-transportation; and in our study, heifers had an average BW of 408 kg (Min, 351 kg; Max 472 kg) at PRET. The lower BW of heifers may cause a decrease in units of creatine kinase per liter

of blood compared with steers with greater BW (Tarrant *et al.*, 1992). In addition, Earley *et al* (2010) reported an increase in circulating creatine kinase levels after 24-hours of transportation in Charolais bulls with BW of 367 kg (+/- 35 kg). In that study, before transportation, creatine kinase level was 162.3 U/L, which is lower compared with our study at PRET (CTRL, 227.4 U/L; RPM, 266.7 U/L). However, circulating creatine kinase after 24-hours transportation was 496 U/L, which may be consider similar to heifers' creatine kinase levels at POST showed in this study (CTRL, 406 U/L; RPM, 424.8 U/L) (Earley *et al.*, 2010). Another possible explanation for the greater creatine kinase in blood in the previous studies mentioned is that male animals show greater levels than females, as reported by (Mpakama *et al.*, 2014). In conclusion, beyond the clear increase in creatine kinase levels in blood after transportation, no significant effect was observed due to the addition of RPM in heifers' diet. Contrarily to our hypothesis, there was no a greater concentration of creatine kinase, which could mitigate the effect of muscle fatigue due to a greater creatine phosphate synthesis.

Dystrophin-Glycoprotein complex

Dystrophin-Glycoprotein complex (DGC) is a key element for integrity of the sarcolemma, composed primarily of dystrophin and sarcoglycan complexes. The function of DGC is associated with the anchoring of sarcolemma to the actin cytoskeleton (Campbell, 1995; Michele & Campbell, 2003).

High levels of sarcospan provide stabilization to the sarcolemma in individuals under Duchenne muscular dystrophy (DMD), a muscular dystrophy caused by a genetic disorder (Gibbs *et al.*, 2016; McGreevy *et al.*, 2015). In addition, to the best of our knowledge, there is no literature reporting the effects of fatigue on *SSPN* expression. In the light of this, it is conceivable to think that CTRL group achieved greater stabilization in the sarcolemma by *SSPN* upregulation

between BASE and PRET. However, Lebakken et al. (2012) reported that *SSPN*-deficient mice had normal creatine kinase levels, sarcolemma integrity, and force generation capacity (Lebakken *et al.*, 2000).

The gene expression regulation of Syntrophin isoforms is an important factor for sarcolemma integrity and binding of signaling proteins. If an isoform is missing or deleted, other isoforms can replace their function and no effects are observed (Kim *et al.*, 2018).

The absence of α-Syntrophin 1 (*SNTA1*) gene expression is a characteristic of DMD as previously described (Adams *et al.*, 1995). However, α1syn$^{-/-}$ mutant mice did not show reduced force recovery in a fatigue stimulation trial (Yokota *et al.*, 2014). Kim et al. (2018) showed that mice lacking α- and ß-syntrophins (*SNTA1* and *SNTB1*, respectively) had decreased in skeletal muscle function (Kim *et al.*, 2018). It is possible that sarcolemma integrity might not be affected by RPM supplementation nor fatigue due to transportation, because both *SNTA1* and *SNTB1* expression did not differ between treatments. Also, there would be a compensatory relationship between *SNTA1* and *SNTB1*, because both treatments decreased numerically in expression between PRET and POST in *SNTA1*, and increased numerically *SNTB1*, respectively. However, further studies are needed in order to confirm this numerical difference. Similarly, ß-sarcoglycan (*SGCB*), a gene that encodes for a transmembrane protein in the sarcoglycan complex, showed a similar pattern of expression as to *SNTA1* and *SSPN*, indicating its possible correlation with the entire Dystrophin-Glycoprotein complex.

Because *SSPN* was the unique gene that showed a treatment effect in the Dystrophin-Glycoprotein complex, it would not be accurate to declare that the sarcolemma is more stable because of the unique upregulation of *SSPN*. Consequently, administration of RPM may not alter skeletal muscle membrane integrity in growing heifers. Further analysis of more genes related to

Dystrophin-Glycoprotein complex will help to fully understand the mechanisms behind a muscle fatigue condition.

Sarcoplasmic reticulum

The regulation of Ca^{++} ions in muscle cells was widely studied in different species; however, little is known about the effect of fatigue on beef cattle. Kinnunen & Manttari (2012) reported a downregulation of *CASQ1* protein expression in mice after an endurance trial (Kinnunen & Manttari, 2012). These results are consistent with our study, because there was a downregulation of *CASQ1* in CTRL between PRET and POST. Similarly, reduction in activity of *ATP2A1* in fatigued skeletal muscle was reported in rats (Belcastro *et al.*, 1981; Byrd *et al.*, 1989), and humans (Li *et al.*, 2002).

Even though we did not measure Ca^{++} levels in skeletal muscle, the downregulation of *CASQ1*, *ATP2A1*, and *SYPL2* in CTRL during transportation could be related to Ca^{++} depletion after a repetitive use of muscle as reported by Westerblad *et al*. (1991) (Westerblad *et al.*, 1991). The administration of RPM downregulated only one of the genes that we selected to study within the sarcoplasmic reticulum complex, *SYPL2*, between BASE and PRET. It is possible that methyl groups provided from RPM inhibited *SYPL2* expression. Nagaraj et al (2000) reported that wild-type mice and mice lacking MG29, the protein for which *SYPL2* encodes, exhibited lower force generation after fatigue in the diaphragm, a skeletal mixed-fiber muscle such as *Longissimus Dorsi* (Nagaraj *et al.*, 2000). In addition, mice lacking *SYPL2* did not show decreased force generation after fatigue compared with wild-type mice, indicating the effect of MG29 would be compensated in a muscle fatigue scenario for that specie. In RPM, *ATP2A1* and *CASQ1* remained unchanged among time points, suggesting that the greater availability of methyl groups does not modify Ca^{++} regulation in skeletal muscle or inhibit the expression of genes related to

sarcoplasmic reticulum. Skeletal muscle fatigue impairs Ca^{2+} uptake as described by Allen *et al* (2008) and Tupling (2004). Down-regulation in the expression of all the genes analyzed related to sarcoplasmic reticulum between PRET and POST in CTRL may suggest that Ca^{2+} metabolism could be impaired under fatigue conditions. In contrast, RPM did not show a significant difference between PRET and POST. Thus, the stabilization of the expression of genes related to sarcoplasmic reticulum could potentially explain a better Ca^{2+} uptake by the skeletal muscle (Allen *et al.*, 2008; Tupling, 2004).

Creatine synthesis pathway

To the best of our knowledge, there are no published nutrigenomics studies of muscle fatigue during transportation stress in cattle. Because of this, we utilized information from other species for our discussion, especially non-ruminants like mice, humans, and horses. Even though the utilization of these data would potentially differ from ruminants, it is the best available source of information with which to compare our data. Creatine biosynthesis occurs mainly in the kidney, pancreas and liver, and involves two steps. First, glycine amidinotransferase (*GATM* or *AGAT*) catalyzes the reversible transamidination the of guanidine group from arginine to glycine, producing ornithine and guanidinoacetate (GAA). Second, guanidinoacetate methyl transferase (*GAMT*) catalyzes the addition of a methyl group from S-adenosyl methionine (SAM) to GAA, producing creatine and S-adenosyl homocysteine (SAH). Once creatine is formed, it reaches different tissues via its transporter called solute carrier family 6 member 8 (*SLC6A8*) (Choe *et al.*, 2013; Demant & Rhodes, 1999; Iqbal *et al.*, 2017).

The difference in *GATM* expression can be explained by the maintenance of the expression in RPM in POST compared with CTRL, which was downregulated. In mice, Stockebrand et al (2008) reported that the upregulation of *GATM* in skeletal muscle does not compensate

deficiency in *SLC6A8* (Stockebrand *et al.*, 2018). Thus, it is possible that the *GATM* expression in RPM does not compensate for the decreased expression of *CKM* and *SLC6A8* at POST.

In addition, Kan et al. (2005) reported that *GAMT* deficiency has a negative effect in force maintenance in both high-intensity electrical stimulation and lower electrical stimulation frequency in mice (Kan *et al.*, 2005). The downregulation of *GAMT* expression in CTRL between PRET and POST could explain a decrease in creatine synthesis due to muscle fatigue, which also could be supported by the downregulation of *CKM* expression in CTRL between PRET and POST.

S-Adenosyl Homocysteine is an important inhibitor of the majority of the methytransferases, affecting DNA methylation, RNA, and protein methylation in humans (James *et al.*, 2002). The reversible hydrolysis of S-Adenosyl Homocysteine to Homocysteine is catalyzed by the enzyme called S-Adenosylhomocysteine, which is codified by the gene *AHCY* (Gellekink *et al*., 2004; Vugrek *et al*., 2009). This chemical reaction also plays an important role in regulating the addition of methyl groups to other compounds (i.e., methylation) (Ponnaluri *et al.*, 2018). Therefore, *AHCY* principal role in metabolism is to hydrolyse and efficiently remove S-adenosylhomocysteine, the by-product of transmethylation reactions and one of the most potent methyltransferase inhibitors (Motzek *et al.*, 2016). S-adenosylhomocysteine hydrolase (*AHCY*), the only mammalian enzyme capable of hydrolyzing S-adenosyl-l-homocysteine binds to *DNMT1* during DNA replication. Hypermethylation of the genome can occur due to *DNMT1* overexpression cause by *AHCY* regulation in vitro (Ponnaluri *et al.*, 2018). Furthermore, in isolated guinea pig heart muscles, adrenaline secretion inhibits S-adenosylhomocysteine hydrolase activity by a calcium mediated mechanism (Suarez *et al.*, 1996).

Stress can also directly influence the transcriptional regulation at the epigenetic level. In a previous study, prenatal transportation stress in Brahman bull calves was assessed at the methylome level. Results showed at least 10% more methylation in stressed calves as compared to controls (Littlejohn *et al.*, 2018). In our study, RPM produce the inhibition of *AHCY* during preconditioning. However, under the stress of transportation, *AHCY* had activation in RPM heifers, coincidentally with *DNMT1* upregulation. Furthermore, in a previous study, the transcriptional repression of *DNMT1* by glucocorticoid exposure is considered as a proxy for stress response (Klengel & Binder, 2015). If hypermethylation plays a role in controlling the physiological response to stressors, it maybe by regulating the release of glucocorticoids in response to challenges. Our results allow us to suggest that preconditioning with RPM supplementation produce an activation of the process of DNA methylation during transportation stress. The animal's benefit due to this metabolic response (i.e., hypermethylation) to long-term transportation stress still needs to be elucidated.

Gu *et al.* (2010) reported no difference in *CKM* expression in skeletal muscle of Thoroughbred horses under moderate and high intensity work on treadmill (Gu *et al.*, 2010). Samples were taken before exercise, immediately after exercise, and 4 hours later. It is possible to compare these results with ours because in the present study, the POST samples were taken immediately after transportation. However, even though RPM treatment remained unchanged among time points for CKM expression, it is not possible to explain the upregulation of CTRL between BASE and PRET.

Interestingly, *SLC6A8* showed the same pattern as *CKM* expression for both treatments among all time points. Since *SLC6A8* encodes for the major creatine transporter, the decrease of creatine synthesis produces an inhibition of *SLC6A8* expression such as Russel et al. (2014) and

Stockebrand et al. (2018) reported with creatine transporter deficiency in mice (Russell *et al.*, 2014; Stockebrand *et al.*, 2018).

Because we did not measure creatine levels in blood nor skeletal muscle, but we did measure the expression of genes involved in creatine synthesis, we can conclude that creatine synthesis could not have been affected by the addition of RPM.

Oxidative stress

Expression of *SOD1* and *SOD2* was activated in CTRL heifers between BASE and PRET compared with RPM heifers. This lack of response in RPM heifers could be interpreted as a better controlled oxidants-antioxidant balance in skeletal muscle. We base our hypothesis on the suggestion that regular exercise appears to gradually increase the level of adaptation by the repeated activation of antioxidant genes and proteins (Radak et al., 2013).

As reported in our study, BW losses of 8% in RPM, and 10% in CTRL were caused by the transportation, and up to 50% of that BW loss can belong to tissue partitioning (Gonzalez *et al.*, 2012a). The higher expression of superoxide agents in CTRL may be related to the oxidative stress imbalance due to tissue partitioning in heifers' adipose and skeletal muscle tissues, because of the long-duration transportation without access to water and feed. The occurrence of oxidative stress due to starvation has been reported in different species (McDougald *et al.*, 2002; Morales *et al.*, 2004; Schraag *et al.*, 2007; Sorensen *et al.*, 2006). Wasselin *et al* (2014) reported that fasting rats showed a higher hepatic oxidative stress imbalance due to different processes of tissue degradation such as lipid peroxidation, protein misfolding, and glycolysis (Wasselin *et al.*, 2014). In addition, Qi *et al* (2014) reported an increase in ROS generation in skeletal muscle after 24 of fasting in mice (Qi *et al.*, 2014). We believe that, after a continuous contraction of skeletal muscles in order to maintain equilibrium during the 22 hours on the trailer, and the lower

tissue partitioning in RPM compared with CTRL, expressed in lower shrink, could explain *SOD1* and SOD2 down-regulation.

An increased concentration of H_2O_2 produce modulation of NF-κB pathway as reported by Oliveira-Marques et al (2000), activation of which is translated as adaptation to exercise (Gomez-Cabrera *et al.*, 2005; Oliveira-Marques *et al.*, 2009). At low ROS levels, NF-κB has also been shown to have many antioxidant targets, including manganese superoxide dismutase (SOD2) (Kairisalo *et al.*, 2007). Acute fatiguing resistance exercise decreases NF-κB binding to DNA in skeletal muscle from healthy humans and mice (Durham *et al.*, 2004). Han *et al* (2018) reported an increased in *NFKB1* on dairy cows under RPM supplementation (Han *et al.*, 2018). However, NFKB1 is a transcription factor involved in several pathways, such as innate immune response, cell proliferation and apoptosis, and stress responses to a variety of noxious stimuli (Hayden & Ghosh, 2004). Our results showed that RPM heifers did not have a significant *NFKB1* activation, indicating that ROS levels could be lower, or that its upregulation could not be required. Metabolic stress inherent in endurance exercise has been demonstrated to stimulate mitochondrial biogenesis (Donato *et al.*, 2014; Perry *et al.*, 2010). Mitochondrial synthesis is stimulated by the PGC-1α - NRF1 - TFAM pathway (Steinbacher & Eckl, 2015). Therefore, PGC-1α is the first stimulator of mitochondrial biogenesis (Vina *et al.*, 2009), and its expression increases with exercise training (Mathai *et al.*, 2008; Steiner J. L. *et al.*, 2011). PGC-1α is a key player in the adaptation of muscle cells to exercise (Steinbacher & Eckl, 2015). Oxidative stress increases expression of *PGC-1α* (Kang *et al.*, 2009), and PGC-1α controls cellular antioxidant homeostasis by stimulating the gene expression of superoxide dismutase-2 (*SOD2*), catalase, glutathione peroxidase 1 (*GPx1*), and uncoupling protein (*UCP*) (Kang & Li Ji, 2012). In our study, administration of RPM or transportation stress did not affect *PGC-1α* expression, although

the peak in PGC-1α expression at PRET in CTRL heifers leads us to suggest that RPM could be producing an inhibition in the expression of this gene. Furthermore, the presence of additional methyl groups could be producing a more balanced environment in the RPM heifer's skeletal muscle, where the activation of the oxidative stress-related gene network was not required. In contrast to our results, Herzig *et al* (2001) and Yoon *et al* (2001) reported an upregulation in mRNA expression of *PGC-1α* in liver of fasting mice (Herzig *et al.*, 2001; Yoon *et al.*, 2001). However, *PGC-1α* stabilization in RPM during transportation could mean that tissue partitioning caused by fasting metabolism was not related to the expression of this gen. The role of *PGC-1α* expression in skeletal muscle during the transition from fed to fasted status could not be relevant, supporting the previous results reported by Gudiksen & Pilegaard (2017), whom assessed the effect of mice lacking *PGC-1α* on substrate utilization from fed to fasted state (Gudiksen & Pilegaard, 2017). This could explain the lack of significant treatment effect or treatment × time interaction in *PGC-1α*.

The generation of Nitric Oxide (NO) in endothelial cells is produced by Nitric oxide synthetase Type III or eNOS, which is encoded by *NOS3*. The presence of O_2^- can stimulate the development of fatigue. Thus, the inactivation of O_2^- by NO may be protective for the muscle cell (Kobzik *et al.*, 1995; Reid *et al.*, 1992). Our results showed that the addition of RPM did not modify the expression of *NOS3*, suggesting that O_2^- remained unchanged among time points and an upregulation of the gene expression might not have been required. There could be a possible relationship between mithocondrogenesis, PGC-1α and NOS3. Nisoli *et al* (2014) study supports our results, showing that eNOS $^{-/-}$ muscle cells have lower mitochondriogenesis activity, and lower expression of PGC-1α (Nisoli et al., 2004).

NQO1 is a member of an antioxidant defense system. An acute bout of exercise at sufficient intensity activates this antioxidant enzyme among others (Ji, 1995). In contrast, in patients with chronic fatigue syndrome, *NQO1* was downregulated (Pietrangelo *et al.*, 2009). Similarly, the other antioxidant assessed in this study, *NQO1* expression remained unchanged among time points in RPM heifers. The higher concentration of available methyl groups provided by the addition of RPM could decrease ROS production. Consequently, the activation of *NQO1* would not be necessary for the muscle cells after the transportation was performed.

DNA methylation

DNA methylation is the major regulator of epigenetics in the genome of mammalians by the covalent addition of a methyl group (CH_3) to cytosine in combination of CpG dinucleotide called "CpG" islands (Laker & Ryall, 2016; Robertson & Jones, 2000; Xu *et al.*, 2018). Enzymes capable of adding methyl groups to the hemimethylated and unmethylated DNA are called DNA methyltransferase (DNMT), like DNMT1 and DNMT3A, respectively (Fatemi *et al.*, 2002). Thus, *DNMT1* and *DNMT3A* gene expressions in skeletal muscle would potentially explain epigenetic changes due to muscle fatigue. Interestingly, *DNMT1* and *DNMT3A* cooperate in methylation processes during early developmental stages in embryonic stem cells in mice (Liang *et al.*, 2002). Furthermore, a cooperation between DNMT1 and DNMT3B was observed in humans; when disrupted, DNA methylation was decreased by 95% in colon rectal cancer cell (Rhee *et al.*, 2002).

The addition of RPM to the diet increases Methionine availability. Consequently, it can be converted to *S*-adenosylmethionine (SAM), which is a molecule considered as the universal methyl donor (Graulet *et al.*, 2005; Martinov *et al.*, 2010). Similar to our results, Osorio et al. (2014) did not obtain difference in hepatic *DNMT1* expression in periparturient dairy cows under

Smartamine supplementation. Furthermore, in contrast to our results, hepatic *DNMT3A* showed a greater expression after 11 days under Smartamine supplementation during pre-partum period (Osorio *et al.*, 2014). However, the difference was mitigated after calving, and no difference was obtained from 11 to 31 days of supplementation.

In addition, there was a similar pattern of expression of *DNMT1* and *DNMT3A* in CTRL among time points, similar to what Rhee et al (2002) reported, but this pattern was not observed in RPM (Rhee *et al.*, 2002).

Summary and conclusions

The addition of RPM had been tested in several research studies in bovine species, especially in dairy cattle, because of two main reasons: first, addition of methionine would be beneficial since it is the most limiting amino acid in ruminants' diets; and second, it would increase the availability of methyl groups for methylation processes in DNA. Our results indicate that RPM could promote a better response to transportation stress in heifer's skeletal muscle due to a possible more stable redox balance after a long-duration transportation.

The most important parameter to assess, from the production standpoint, is the body weight loss or shrink. A numerical difference is not sufficient to indicate that RPM reduce shrink; however, the utilization of more animals in further studies, could make this difference significant. In addition, cortisol level was not affected by the administration of RPM, suggesting that the control of stress level could not be enhanced by a greater methionine availability.

Finally, gene expression results were consistent because CTRL heifers had a greater gene expression response throughout the study compared with RPM heifers. In the light of this thought, it is not possible to confirm that the integrity of sarcolemma after a fatigue condition is positively affected by RPM supplementation. However, further studies are required to

understand why the majority of the genes in CTRL had an upregulation in their expression between BASE and PRET. Similar to the results obtained from previous studies in other animals, the majority of the genes were downregulated in CTRL between PRET and POST because of a potential fatigue condition. The regulation of gene expression in muscle fatigue and oxidative stress metabolic pathways due to an increase in DNA methylation levels through RPM could be related to the maintenance of stable levels of genes expression observed in our study.

In conclusion, in contrast to our hypothesis, RPM administration does not upregulate *CKM*, or serum creatine kinase levels. Furthermore, muscle fatigue-related genes during the preconditioning period or the long-term-transportation simulation did not affect the integrity of the muscle membrane. Even though, administration of RPM in growing Angus × Simmental heifers may have an effect on oxidative stress (*SOD2*), and hypermethylation (*AHCY).*

References

Adams ME, Dwyer TM, Dowler LL, White RA, & Froehner SC (1995). Mouse α1- and β2-Syntrophin gene structure, chromosome localization, and homology with a discs large domain. Journal of Biological Chemistry 270**:** 25859-25865.

Allen DG, Kabbara AA, & Westerblad H (2002). Muscle fatigue: The role of intracellular calcium stores. Can J Appl Physiol 27**:** 83-96.

Allen DG, Lamb GD, & Westerblad H (2008). Skeletal muscle fatigue: Cellular mechanisms. Physiological Reviews 88**:** 287-332.

Barbieri E, & Sestili P (2012). Reactive oxygen species in skeletal muscle signaling. J Signal Transduct 2012**:** 982794.

Baumgard LH, Collier RJ, & Bauman DE (2017). A 100-Year review: Regulation of nutrient partitioning to support lactation. Journal of Dairy Science 100**:** 10353-10366.

Belcastro AN, Rossiter M, Low MP, & Sopper MM (1981). Calcium activation of sarcoplasmic reticulum ATPase following strenuous activity. Canadian Journal of Physiology and Pharmacology 59**:** 1214-1218.

Bellinger AM, Reiken S, Carlson C, Mongillo M, Liu X, Rothman L, *et al.* (2009). Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. Nat Med 15**:** 325-330.

Biel M, Wascholowski V, & Giannis A (2005). Epigenetics—an epicenter of gene regulation: histones and histone-modifying enzymes. Angewandte Chemie International Edition 44**:** 3186-3216.

Brockman RP, & Laarveld B (1986). Hormonal regulation of metabolism in ruminants; a review. Livestock Production Science 14**:** 313-334.

Buckham Sporer KR, Burton JL, Earley B, & Crowe MA (2007). Transportation stress in young bulls alters expression of neutrophil genes important for the regulation of apoptosis, tissue remodeling, margination, and anti-bacterial function. Vet Immunol Immunopathol 118**:** 19-29.

Byrd SK, Bode AK, & Klug GA (1989). Effects of exercise of varying duration on sarcoplasmic reticulum function. Journal of Applied Physiology 66**:** 1383-1389.

Campbell KP (1995). Three muscular dystrophies: Loss of cytoskeleton-extracellular matrix linkage. Cell 80**:** 675-679.

Cardinet GH (1997). Chapter 16 - Skeletal muscle function. In clinical biochemistry of domestic animals (fifth edition). eds Kaneko J.J., Harvey J.W., & Bruss M.L. Academic Press: San Diego, pp 407-440.

Cassar-Malek I, Picard B, Bernard C, & Hocquette J-F (2008). Application of gene expression studies in livestock production systems: a European perspective. Australian Journal of Experimental Agriculture 48**:** 701-710.

Cedar H, & Bergman Y (2009). Linking DNA methylation and histone modification: Patterns and paradigms. Nature Reviews Genetics 10**:** 295.

Cernicchiaro N, White BJ, Renter DG, Babcock AH, Kelly L, & Slattery R (2012a). Associations between the distance traveled from sale barns to commercial feedlots in the United States and overall performance, risk of respiratory disease, and cumulative mortality in feeder cattle during 1997 to 2009. J Anim Sci 90**:** 1929-1939.

Cernicchiaro N, White BJ, Renter DG, Babcock AH, Kelly L, & Slattery R (2012b). Effects of body weight loss during transit from sale barns to commercial feedlots on health and performance in feeder cattle cohorts arriving to feedlots from 2000 to 2008. J Anim Sci 90**:** 1940-1947.

Chen D, Zhao CM, Andersson K, Meister B, Panula P, & Håkanson R (1998). ECL cell morphology. The Yale journal of biology and medicine 71**:** 217-231.

Chen ZH, Broderick GA, Luchini ND, Sloan BK, & Devillard E (2011). Effect of feeding different sources of rumen-protected methionine on milk production and N-utilization in lactating dairy cows. J Dairy Sci 94**:** 1978-1988.

Choe CU, Nabuurs C, Stockebrand MC, Neu A, Nunes P, Morellini F, *et al.* (2013). L-arginine:glycine amidinotransferase deficiency protects from metabolic syndrome. Hum Mol Genet 22**:** 110-123.

Choi YM, & Oh HK (2016). Carcass performance, muscle fiber, meat quality, and sensory quality characteristics of crossbred pigs with different live weights. 36.

Chulayo AY, Bradley G, & Muchenje V (2016). Effects of transport distance, lairage time and stunning efficiency on cortisol, glucose, HSPA1A and how they relate with meat quality in cattle. Meat Sci 117**:** 89-96.

Cole NA, Camp TH, Rowe LD, Jr., Stevens DG, & Hutcheson DP (1988). Effect of transport on feeder calves. Am J Vet Res 49**:** 178-183.

Crosbie RH, Heighway J, Venzke DP, Lee JC, & Campbell KP (1997). Sarcospan, the 25-kDa transmembrane component of the dystrophin-glycoprotein complex. J Biol Chem 272**:** 31221-31224.

Dawson KA (2006). Nutrigenomics: Feeding the genes for improved fertility. Anim Reprod Sci 96**:** 312-322.

Demant TW, & Rhodes EC (1999). Effects of creatine supplementation on exercise performance. Sports Med 28**:** 49-60.

Donato DMD, West DWD, Churchward-Venne TA, Breen L, Baker SK, & Phillips SM (2014). Influence of aerobic exercise intensity on myofibrillar and mitochondrial protein synthesis in young men during early and late postexercise recovery. American Journal of Physiology-Endocrinology and Metabolism 306**:** E1025-E1032.

Durham WJ, Li YP, Gerken E, Farid M, Arbogast S, Wolfe RR, *et al.* (2004). Fatiguing exercise reduces DNA binding activity of NF-kappaB in skeletal muscle nuclei. J Appl Physiol (1985) 97**:** 1740-1745.

Earley B, Murray M, & Prendiville DJ (2010). Effect of road transport for up to 24 hours followed by twenty-four hour recovery on live weight and physiological responses of bulls. BMC Veterinary Research 6**:** 38.

Edstrom L, & Kugelberg E (1968). Histochemical composition, distribution of fibres and fatiguability of single motor units. Anterior tibial muscle of the rat. J Neurol Neurosurg Psychiatry 31**:** 424-433.

Edwards RH (1986). Muscle fatigue and pain. Acta Med Scand Suppl 711**:** 179-188.

Eshel G, Shepon A, Shaket T, Cotler BD, Gilutz S, Giddings D, *et al.* (2018). A model for 'sustainable' US beef production. Nat Ecol Evol 2**:** 81-85.

Fatemi M, Hermann A, Gowher H, & Jeltsch A (2002). Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. European Journal of Biochemistry 269**:** 4981-4984.

Ferguson DM, Bruce HL, Thompson JM, Egan AF, Perry D, & Shorthose WR (2001). Factors affecting beef palatability — farmgate to chilled carcass. Australian Journal of Experimental Agriculture 41**:** 879-891.

Ferreira LF, & Reid MB (2008). Muscle-derived ROS and thiol regulation in muscle fatigue. Journal of Applied Physiology 104**:** 853-860.

Folz RJ, & Crapo JD (1994). Extracellular superoxide dismutase (SOD3): tissue-specific expression, genomic characterization, and computer-assisted sequence analysis of the human EC SOD gene. Genomics 22**:** 162-171.

Fukai T, & Ushio-Fukai M (2011). Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid Redox Signal 15**:** 1583-1606.

Fukasawa M (2012). Calf training for loading onto vehicle at weaning. Anim Sci J 83**:** 759-766.

Gallo C, Perez S, Sanhueza C, & Gasic J (2000). [Efectos del tiempo de transporte de novillos previo al faenamiento sobre el comportamiento, las pérdidas de peso y algunas características de la canal]. Archivos de medicina veterinaria 32**:** 157-170.

Gellekink H, Heijer Md, Kluijtmans LAJ, & Blom HJ (2004). Effect of genetic variation in the human S-adenosylhomocysteine hydrolase gene on total homocysteine concentrations and risk of recurrent venous thrombosis. European Journal of Human Genetics 12**:** 942-948.

Gibbs EM, Marshall JL, Ma E, Nguyen TM, Hong G, Lam JS, *et al.* (2016). High levels of sarcospan are well tolerated and act as a sarcolemmal stabilizer to address skeletal muscle and pulmonary dysfunction in DMD. Hum Mol Genet 25**:** 5395-5406.

Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, *et al.* (2010). Food security: The challenge of feeding 9 billion people. Science 327**:** 812-818.

Gomez-Cabrera MC, Borras C, Pallardo FV, Sastre J, Ji LL, & Vina J (2005). Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. J Physiol 567**:** 113-120.

Gomez A, Mendoza GD, Garcia-Bojalil C, Barcena R, Ramos JA, Crosby MM, *et al.* (2011). Effect of supplementation with urea, blood meal, and rumen-protected methionine on growth performance of Holstein heifers grazing kikuyu pasture. Trop Anim Health Prod 43**:** 721-724.

Gonzalez LA, Schwartzkopf-Genswein KS, Bryan M, Silasi R, & Brown F (2012a). Factors affecting body weight loss during commercial long haul transport of cattle in North America. J Anim Sci 90**:** 3630-3639.

Gonzalez LA, Schwartzkopf-Genswein KS, Bryan M, Silasi R, & Brown F (2012b). Relationships between transport conditions and welfare outcomes during commercial long haul transport of cattle in North America. J Anim Sci 90**:** 3640-3651.

Grandin T (1997). Assessment of stress during handling and transport. J Anim Sci 75**:** 249-257.

Grandin T (2007) Livestock handling and transport. 3 edn.

Graulet B, Richard C, & Robert JC (2005). Methionine availability in plasma of dairy cows supplemented with methionine hydroxy analog isopropyl ester. Journal of Dairy Science 88**:** 3640-3649.

Gu J, MacHugh DE, McGivney BA, Park SD, Katz LM, & Hill EW (2010). Association of sequence variants in CKM (creatine kinase, muscle) and COX4I2 (cytochrome c oxidase, subunit 4, isoform 2) genes with racing performance in Thoroughbred horses. Equine Vet J Suppl**:** 569-575.

Gudiksen A & Pilegaard H (2017). PGC-1α and fasting-induced PDH regulation in mouse skeletal muscle. Physiol Rep 5**:** e13222.

Gumerson JD, & Michele DE (2011). The dystrophin-glycoprotein complex in the prevention of muscle damage. J Biomed Biotechnol 2011**:** 210797.

Han L, Batistel F, Ma Y, Alharthi ASM, Parys C, & Loor JJ (2018). Methionine supply alters mammary gland antioxidant gene networks via phosphorylation of nuclear factor erythroid 2-like 2 (NFE2L2) protein in dairy cows during the periparturient period. J Dairy Sci 101**:** 8505-8512.

Han R, Kanagawa M, Yoshida-Moriguchi T, Rader EP, Ng RA, Michele DE, *et al.* (2009). Basal lamina strengthens cell membrane integrity via the laminin G domain-binding motif of alpha-dystroglycan. Proc Natl Acad Sci U S A 106**:** 12573-12579.

Harman BR, Brinkman MH, Hoffman MP, & Self HL (1989). Factors affecting in-transit shrink and liver abscesses in fed steers. Journal of Animal Science 67**:** 311-317.

Hayden MS, & Ghosh S (2004). Signaling to NF-kappaB. Genes Dev 18**:** 2195-2224.

Hearnshaw H, & Morris C (1984). Genetic and environmental effects on a temperament score in beef cattle. Australian Journal of Agricultural Research 35**:** 723-733.

Hermann A, Gowher H, & Jeltsch A (2004). Biochemistry and biology of mammalian DNA methyltransferases. Cellular and Molecular Life Sciences CMLS 61**:** 2571-2587.

Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, *et al.* (2001). CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature 413**:** 179-183.

Hogan JP, Petherick JC, & Phillips CJ (2007). The physiological and metabolic impacts on sheep and cattle of feed and water deprivation before and during transport. Nutr Res Rev 20**:** 17-28.

Holt KH, & Campbell KP (1998). Assembly of the sacroglycan complex. The journal of biological chemistry 273**:** 34667-34670.

Hoppeler H, & Fluck M (2002). Normal mammalian skeletal muscle and its phenotypic plasticity. J Exp Biol 205**:** 2143-2152.

Hu Y, Rosen DG, Zhou Y, Feng L, Yang G, Liu J, *et al.* (2005). Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: Role in cell proliferation and response to oxidative stress. J Biol Chem 280**:** 39485-39492.

Iqbal F, Hoeger H, Lubec G, & Bodamer O (2017). Biochemical and behavioral phenotype of AGAT and GAMT deficient mice following long-term Creatine monohydrate supplementation. Metabolic Brain Disease 32**:** 1951-1961.

Ishii T, Sunami O, Saitoh N, Nishio H, Takeuchi T, & Hata F (1998). Inhibition of skeletal muscle sarcoplasmic reticulum Ca2+-ATPase by nitric oxide. FEBS Letters 440**:** 218-222.

Ishizaki H, & Kariya Y (2010). Road transportation stress promptly increases bovine peripheral blood absolute NK cell counts and cortisol levels. J Vet Med Sci 72**:** 747-753.

Ismail I, & Joo ST (2017). Poultry meat quality in relation to muscle growth and muscle fiber characteristics. Korean J Food Sci Anim Resour 37**:** 873-883.

Jackson MJ, Pye D, & Palomero J (2007). The production of reactive oxygen and nitrogen species by skeletal muscle. Journal of Applied Physiology 102**:** 1664-1670.

James SJ, Melnyk S, Pogribna M, Pogribny IP, & Caudill MA (2002). Elevation in S-Adenosylhomocysteine and DNA hypomethylation: Potential epigenetic mechanism for homocysteine-related pathology. The Journal of Nutrition 132**:** 2361S-2366S.

Ji L (1995). Oxidative stress during exercise: Implication of antioxidant nutrients. Free Radical Biology and Medicine 18**:** 1079-1086.

Jin B, Li Y, & Robertson KD (2011). DNA methylation: Superior or subordinate in the epigenetic hierarchy? Genes & cancer 2**:** 607-617.

Jones SDM, Schaefer AL, Robertson WM, & Vincent BC (1990). The effects of withholding feed and water on carcass shrinkage and meat quality in beef cattle. Meat Science 28**:** 131-139.

Kairisalo M, Korhonen L, Blomgren K, & Lindholm D (2007). X-linked inhibitor of apoptosis protein increases mitochondrial antioxidants through NF-κB activation. Biochemical and Biophysical Research Communications 364**:** 138-144.

Kan HE, Buse-Pot TE, Peco R, Isbrandt D, Heerschap A, & de Haan A (2005). Lower force and impaired performance during high-intensity electrical stimulation in skeletal muscle of GAMT-deficient knockout mice. Am J Physiol Cell Physiol 289**:** C113-119.

Kang C, & Li Ji L (2012). Role of PGC-1alpha signaling in skeletal muscle health and disease. Ann N Y Acad Sci 1271**:** 110-117.

Kang C, O'Moore KM, Dickman JR, & Ji LL (2009). Exercise activation of muscle peroxisome proliferator-activated receptor-γ coactivator-1α signaling is redox sensitive. Free Radical Biology and Medicine 47**:** 1394-1400.

Kaput J, & Rodriguez RL (2004). Nutritional genomics: The next frontier in the postgenomic era. Physiol Genomics 16**:** 166-177.

Katz A, Sahlin K, & Broberg S (1991). Regulation of glucose utilization in human skeletal muscle during moderate dynamic exercise. American Journal of Physiology-Endocrinology and Metabolism 260**:** E411-E415.

Katz A, Sahlin K, & Henriksson J (1986). Muscle ATP turnover rate during isometric contraction in humans. Journal of Applied Physiology 60**:** 1839-1842.

Kim MJ, Whitehead NP, Bible KL, Adams ME, & Froehner SC (2018). Mice lacking α-, β1- and β2-syntrophins exhibit diminished function and reduced dystrophin expression in both cardiac and skeletal muscle. Human Molecular Genetics 28**:** 386-395.

Kinnunen S, & Manttari S (2012). Specific effects of endurance and sprint training on protein expression of calsequestrin and SERCA in mouse skeletal muscle. J Muscle Res Cell Motil 33**:** 123-130.

Klengel T, & Binder EB (2015). Epigenetics of stress-related psychiatric disorders and gene x environment interactions. Neuron 86**:** 1343-1357.

Klose RJ, & Bird AP (2006). Genomic DNA methylation: The mark and its mediators. Trends Biochem Sci 31**:** 89-97.

Knowles G (1999). A review of the road transport of cattle. Veterinary Record 144**:** 197.

Knowles TG, Warriss PD, Brown SN, & Edwards JE (1999). Effects on cattle of transportation by road for up to 31 hours. Vet Rec 145**:** 575-582.

Kobzik L, Reid MB, Bredt DS, & Stamler JS (1994). Nitric oxide in skeletal muscle. Nature 372**:** 546-548.

Kobzik L, Stringer B, Balligand JL, Reid MB, & Stamler JS (1995). Endothelial type nitric oxide synthase in skeletal muscle fibers: Mitochondrial relationships. Biochem Biophys Res Commun 211**:** 375-381.

Komazaki S, Nishi M, Takeshima H, & Nakamura H (2001). Abnormal formation of sarcoplasmic reticulum networks and triads during early development of skeletal muscle cells in mitsugumin29-deficient mice. Dev Growth Differ 43**:** 717-723.

Laker RC, & Ryall JG (2016). DNA methylation in skeletal muscle stem cell specification, proliferation, and differentiation. Stem Cells Int 2016**:** 5725927.

Lambooy E, & Hulsegge B (1988). Long-distance transport of pregnant heifers by truck. Applied Animal Behaviour Science 20**:** 249-258.

Law JA, & Jacobsen SE (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nature Reviews Genetics 11**:** 204.

Lebakken CS, Venzke DP, Hrstka RF, Consolino CM, Faulkner JA, Williamson RA, *et al.* (2000). Sarcospan-deficient mice maintain normal muscle function. Mol Cell Biol 20**:** 1669-1677.

Lees AM, Sejian V, Wallage AL, Steel CC, Mader TL, Lees JC, *et al.* (2019). The impact of heat load on cattle. 9**:** 322.

Lewis KM, Munske GR, Byrd SS, Kang J, Cho HJ, Rios E, *et al.* (2016). Characterization of post-translational modifications to calsequestrins of cardiac and skeletal muscle. Int J Mol Sci 17.

Li JL, Wang XN, Fraser SF, Carey MF, Wrigley TV, & McKenna MJ (2002). Effects of fatigue and training on sarcoplasmic reticulum Ca2+ regulation in human skeletal muscle. Journal of Applied Physiology 92**:** 912-922.

Li R, & Ibeagha-Awemu EM (2017). Altered gene expression of epigenetic modifying enzymes in response to dietary supplementation with linseed oil. J Dairy Res 84**:** 119-123.

Liang G, Chan MF, Tomigahara Y, Tsai YC, Gonzales FA, Li E, *et al.* (2002). Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Molecular and cellular biology 22**:** 480-491.

Littlejohn BP, Price DM, Neuendorff DA, Carroll JA, Vann RC, Riggs PK, *et al.* (2018). Prenatal transportation stress alters genome-wide DNA methylation in suckling Brahman bull calves. J Anim Sci 96**:** 5075-5099.

Loor JJ, Vailati-Riboni M, McCann JC, Zhou Z, & Bionaz M (2015). Triennial lactation symposium: Nutrigenomics in livestock: Systems biology meets nutrition. J Anim Sci 93**:** 5554-5574.

Losada-Espinosa N, Villarroel M, María GA, & Miranda-de la Lama GC (2018). Pre-slaughter cattle welfare indicators for use in commercial abattoirs with voluntary monitoring systems: A systematic review. Meat Science 138**:** 34-48.

MacLennan DH, Rice WJ, & Green NM (1997). The Mechanism of Ca2+ Transport by Sarco(Endo)plasmic Reticulum Ca2+-ATPases. Journal of Biological Chemistry 272**:** 28815-28818.

MacLennan DH, & Wong PT (1971). Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc Natl Acad Sci U S A 68**:** 1231-1235.

Mader TL (2003). Environmental stress in confined beef cattle. Journal of Animal Science 81**:** E110-E119.

Marques RS, Cooke RF, Francisco CL, & Bohnert DW (2012). Effects of twenty-four hour transport or twenty-four hour feed and water deprivation on physiologic and performance responses of feeder cattle. J Anim Sci 90**:** 5040-5046.

Marshall JL, & Crosbie-Watson RH (2013). Sarcospan: a small protein with large potential for Duchenne muscular dystrophy. Skelet Muscle 3**:** 1.

Marti S, Wilde RE, Moya D, Heuston CE, Brown F, & Schwartzkopf-Genswein KS (2017). Effect of rest stop duration during long-distance transport on welfare indicators in recently weaned beef calves. J Anim Sci 95**:** 636-644.

Martinov MV, Vitvitsky VM, Banerjee R, & Ataullakhanov FI (2010). The logic of the hepatic methionine metabolic cycle. Biochim Biophys Acta 1804**:** 89-96.

Mathai AS, Bonen A, Benton CR, Robinson DL, & Graham TE (2008). Rapid exercise-induced changes in PGC-1α mRNA and protein in human skeletal muscle. Journal of Applied Physiology 105**:** 1098-1105.

McDougald D, Gong L, Srinivasan S, Hild E, Thompson L, Takayama K, *et al.* (2002). Defences against oxidative stress during starvation in bacteria. Antonie van Leeuwenhoek 81**:** 3-13.

McGreevy JW, Hakim CH, McIntosh MA, & Duan D (2015). Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. Disease Models & Mechanisms 8**:** 195-213.

Michele DE, & Campbell KP (2003). Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. J Biol Chem 278**:** 15457-15460.

Mitchell G, Hattingh J, & Ganhao M (1988). Stress in cattle assessed after handling, after transport and after slaughter. Veterinary Record 123**:** 201.

Morales AE, Pérez-Jiménez A, Carmen Hidalgo M, Abellán E, & Cardenete G (2004). Oxidative stress and antioxidant defenses after prolonged starvation in Dentex dentex liver. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 139**:** 153-161.

Morrow CJ, Kolver ES, Verkerk GA, & Matthews LR (2002). Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. Gen Comp Endocrinol 126**:** 229-241.

Moss R (1981). Divisional Veterinary Officer, Ministry of Agriculture, Fisheries and Food. In transport of animals intended for breeding, production and slaughter: A seminar in the CEC programme of coordination of research on animal welfare, organised by R Moss, and held in Brussels, 7–8 July, 1981. Springer Science & Business Media, p 147.

Motzek A, Knezevic J, Switzeny OJ, Cooper A, Baric I, Beluzic R, *et al.* (2016). Abnormal hypermethylation at imprinting control regions in patients with S-Adenosylhomocysteine Hydrolase (AHCY) deficiency. Plos One 11.

Mpakama T, Chulayo AY, & Muchenje V (2014). Bruising in slaughter cattle and its relationship with creatine kinase levels and beef quality as affected by animal related factors. Asian-Australasian journal of animal sciences 27**:** 717-725.

Mumma JO, Thaxton JP, Vizzier-Thaxton Y, & Dodson WL (2006). Physiological stress in laying hens. Poult Sci 85**:** 761-769.

Nagaraj RY, Brotto CAP, Nishi M, Takeshima H, Nosek TM, & Ma J (2000). Increased susceptibility to fatigue of slow- and fast-twitch muscles from mice lacking the MG29 gene. Physiological Genomics 4**:** 43-49.

Newham DJ, Jones DA, & Edwards RHT (1983). Large delayed plasma creatine kinase changes after stepping exercise. 6**:** 380-385.

Ng H-H, & Adrian B (1999). DNA methylation and chromatin modification. Current Opinion in Genetics & Development 9**:** 158-163.

Nikolaidis MG, Jamurtas AZ, Paschalis V, Fatouros IG, Koutedakis Y, & Kouretas D (2008). The effect of muscle-damaging exercise on blood and skeletal muscle oxidative stress. Sports Medicine 38**:** 579-606.

Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, *et al.* (2004). Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proceedings of the National Academy of Sciences of the United States of America 101**:** 16507-16512.

Noguchi S, Wakabayashi E, Imamura M, Yoshida M, & Ozawa E (2000). Formation of sarcoglycan complex with differentiation in cultured myocytes. European Journal of Biochemistry 267**:** 640-648.

Oliveira-Marques V, Marinho HS, Cyrne L, & Antunes F (2009). Role of hydrogen peroxide in NF-kappaB activation: from inducer to modulator. Antioxid Redox Signal 11**:** 2223-2243.

Osorio JS, Ji P, Drackley JK, Luchini D, & Loor JJ (2014). Smartamine M and MetaSmart supplementation during the peripartal period alter hepatic expression of gene networks in 1-carbon metabolism, inflammation, oxidative stress, and the growth hormone-insulin-like growth factor 1 axis pathways. J Dairy Sci 97**:** 7451-7464.

Ostrowska M, Jarczak J, & Zwierzchowski L (2015). Glucose transporters in cattle - a review. Animal Science Papers & Reports 33**:** 191-212.

Palme R, Robia C, Baumgartner W, & Mostl E (2000). Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. Vet Rec 146**:** 108-109.

Pan Y, Zvaritch E, Tupling AR, Rice WJ, de Leon S, Rudnicki M, *et al.* (2003). Targeted disruption of the ATP2A1 gene encoding the sarco(endo)plasmic reticulum Ca2+ ATPase isoform 1 (SERCA1) impairs diaphragm function and is lethal in neonatal mice. J Biol Chem 278**:** 13367-13375.

Park H, Wu S, Dunker AK, & Kang C (2003). Polymerization of calsequestrin: implication for Ca2+ REGULATION. Journal of Biological Chemistry 278**:** 16176-16182.

Park SK, Gunawan AM, Scheffler TL, Grant AL, & Gerrard DE (2009). Myosin heavy chain isoform content and energy metabolism can be uncoupled in pig skeletal muscle. Journal of Animal Science 87**:** 522-531.

Perkins WJ, Han Y-S, & Sieck GC (1997). Skeletal muscle force and actomyosin ATPase activity reduced by nitric oxide donor. Journal of Applied Physiology 83**:** 1326-1332.

Perry CGR, Lally J, Holloway GP, Heigenhauser GJF, Bonen A, & Spriet LL (2010). Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. The Journal of physiology 588**:** 4795-4810.

Picard B, Duris MP, & Jurie CJTHJ (1998). Classification of bovine muscle fibers by different histochemical techniques. 30**:** 473-477.

Picard M, Hepple RT, & Burelle Y (2012). Mitochondrial functional specialization in glycolytic and oxidative muscle fibers: tailoring the organelle for optimal function. Am J Physiol Cell Physiol 302**:** C629-641.

Pietrangelo T, Mancinelli R, Toniolo L, Montanari G, Vecchiet J, Fano G, *et al.* (2009). Transcription profile analysis of vastus lateralis muscle from patients with chronic fatigue syndrome. Int J Immunopathol Pharmacol 22**:** 795-807.

Ponnaluri VKC, Esteve PO, Ruse CI, & Pradhan S (2018). S-adenosylhomocysteine Hydrolase participates in DNA methylation inheritance. J Mol Biol 430**:** 2051-2065.

Qi Z, He Q, Ji L, & Ding S (2014). Antioxidant supplement inhibits skeletal muscle constitutive autophagy rather than fasting-induced autophagy in mice. Oxid Med Cell Longev 2014**:** 315896.

Radak Z, Zhao Z, Koltai E, Ohno H, & Atalay M (2013). Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. Antioxid Redox Signal 18**:** 1208-1246.

Rader EP, Turk R, Willer T, Beltran D, Inamori K, Peterson TA, *et al.* (2016). Role of dystroglycan in limiting contraction-induced injury to the sarcomeric cytoskeleton of mature skeletal muscle. Proc Natl Acad Sci U S A 113**:** 10992-10997.

Randall J (1993). Environmental parameters necessary to define comfort for pigs, cattle and sheep in livestock transporters. Animal Science 57**:** 299-307.

Reid MB (1998). Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. Acta Physiol Scand 162**:** 401-409.

Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L, & West MS (1992). Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. Journal of Applied Physiology 73**:** 1797-1804.

Rhee I, Bachman KE, Park BH, Jair K-W, Yen R-WC, Schuebel KE, *et al.* (2002). DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416**:** 552-556.

Robertson KD, & Jones PA (2000). DNA methylation: past, present and future directions. Carcinogenesis 21**:** 461-467.

Russell AP, Ghobrial L, Wright CR, Lamon S, Brown EL, Kon M, *et al.* (2014). Creatine transporter (SLC6A8) knockout mice display an increased capacity for in vitro creatine biosynthesis in skeletal muscle. Front Physiol 5**:** 314.

Sacchetto R, Testoni S, Gentile A, Damiani E, Rossi M, Liguori R, *et al.* (2009). A defective SERCA1 protein is responsible for congenital pseudomyotonia in Chianina cattle. Am J Pathol 174**:** 565-573.

Sahlin K, Tonkonogi M, & Soderlund K (1998). Energy supply and muscle fatigue in humans. Acta Physiol Scand 162**:** 261-266.

Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, *et al.* (1989). Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J Muscle Res Cell Motil 10**:** 197-205.

Schiaffino S, & Reggiani C (2011). Fiber types in mammalian skeletal muscles. Physiol Rev 91**:** 1447-1531.

Schmidt TB, Broadway PR, Buntyn JO, Elsasser TH, Sartin JL, Keisler DH, *et al.* (2013). Evaluation of the endocrine response of cattle during the relocation process. Livestock Science Volume 151**:** 203-212.

Schraag S, Mandach U, Schweer H, & Beinder E (2007). Metabolic changes, hypothalamo-pituitary-adrenal axis and oxidative stress after short-term starvation in healthy pregnant women. J Perinat Med 35**:** 289-294.

Shearer J, Marchand I, Tarnopolsky MA, Dyck DJ, & Graham TE (2001). Pro- and macroglycogenolysis during repeated exercise: roles of glycogen content and phosphorylase activation. 90**:** 880-888.

Shi W, Chen Z, Schottenfeld J, Stahl RC, Kunkel LM, & Chan YM (2004). Specific assembly pathway of sarcoglycans is dependent on beta- and delta-sarcoglycan. Muscle Nerve 29**:** 409-419.

Skaggs JM (1986) Prime Cut: Livestock Raising and Meatpacking in the United States, *1607-1983* Texas, Texas A&M University Press.

Soderlund K, Greenhaff PL, & Hultman E (1992). Energy metabolism in type I and type II human muscle fibres during short term electrical stimulation at different frequencies. Acta Physiologica Scandinavica 144**:** 15-22.

Sorensen M, Sanz A, Gómez J, Pamplona R, Portero-Otín M, Gredilla R, Barga G (2006). Effects of fasting on oxidative stress in rat liver mitochondria. Free Radical Research 40**:** 339-347.

Sporer KR, Xiao L, Tempelman RJ, Burton JL, Earley B, & Crowe MA (2008). Transportation stress alters the circulating steroid environment and neutrophil gene expression in beef bulls. Vet Immunol Immunopathol 121**:** 300-320.

Spriet LL, Howlett RA, & Heigenhauser GJF (2000). An enzymatic approach to lactate production in human skeletal muscle during exercise. Medicine & Science in Sports & Exercise 32**:** 756-763.

Spriet LL, & Watt MJ (2003). Regulatory mechanisms in the interaction between carbohydrate and lipid oxidation during exercise. Acta Physiologica Scandinavica 178**:** 443-452.

Spurlock ME, Houseknecht KL, Portocarrero CP, Cornelius SG, Willis GM, & Bidwell CA (2000). Regulation of PPARgamma but not obese gene expression by dietary fat supplementation. J Nutr Biochem 11**:** 260-266.

Stamler JS, & Meissner G (2001). Physiology of nitric oxide in skeletal muscle. Physiol Rev 81**:** 209-237.

Steinbacher P, & Eckl P (2015). Impact of oxidative stress on exercising skeletal muscle. Biomolecules 5**:** 356-377.

Steiner J. L., Murphy A.E., McClellan J.L., Carmichael M.D., & J.M. D (2011). Exercise training increases mitochondrial biogenesis in the brain. Journal of Applied Physiology 111**:** 1066-1071.

Stockebrand M, Sasani A, Das D, Hornig S, Hermans-Borgmeyer I, Lake HA, *et al.* (2018). A mouse model of creatine transporter deficiency reveals impaired motor function and muscle energy metabolism. Front Physiol 9**:** 773.

Stone MR, O'Neill A, Catino D, & Bloch RJ (2005). Specific interaction of the actin-binding domain of dystrophin with intermediate filaments containing keratin 19. Mol Biol Cell 16**:** 4280-4293.

Suarez J, HernandezMunoz R, Sanchez L, & deSanchez VC (1996). Adrenaline inhibits S-adenosylhomocysteine hydrolase activity by a calcium mediated mechanism in isolated guinea pig heart muscles. Faseb J 10**:** 2277-2277.

Swanson JC, & Morrow-Tesch J (2001). Cattle transport: Historical, research, and future perspectives. Journal of Animal Science 79**:** E102-E109.

Taha-Abdelaziz K, Wyer L, Berghuis L, Bassel LL, Clark ME, & Caswell JL (2016). Regulation of tracheal antimicrobial peptide gene expression in airway epithelial cells of cattle. Vet Res 47**:** 44.

Takeshima H, Shimuta M, Komazaki S, Ohmi K, Nishi M, Iino M, *et al.* (1998). Mitsugumin29, a novel synaptophysin family member from the triad junction in skeletal muscle. Biochem J 331 317-322.

Tarakci H & Berger J (2016). The sarcoglycan complex in skeletal muscle. Front Biosci (Landmark Ed) 21**:** 744-756.

Tarrant PV (1990). Transportation of cattle by road. Applied Animal Behaviour Science 28**:** 153-170.

Tarrant PV, Kenny FJ, Harrington D, & Murphy M (1992). Long distance transportation of steers to slaughter: effect of stocking density on physiology, behaviour and carcass quality. Livestock Production Science 30**:** 223-238.

Tengan CH, Rodrigues GS, & Godinho RO (2012). Nitric oxide in skeletal muscle: role on mitochondrial biogenesis and function. Int J Mol Sci 13**:** 17160-17184.

Tomasi M, Canato M, Paolini C, Dainese M, Reggiani C, Volpe P, *et al.* (2012). Calsequestrin (CASQ1) rescues function and structure of calcium release units in skeletal muscles of CASQ1-null mice. American Journal of Physiology-Cell Physiology 302**:** C575-C586.

Torrentera N, Carrasco R, Salinas-Chavira J, Plascencia A, & Zinn RA (2017). Influence of methionine supplementation of growing diets enriched with lysine on feedlot performance and characteristics of digestion in Holstein steer calves. Asian-Australas J Anim Sci 30**:** 42-50.

Tucker CB, Coetzee JF, Stookey JM, Thomson DU, Grandin T, & Schwartzkopf-Genswein KS (2015). Beef cattle welfare in the USA: identification of priorities for future research. Anim Health Res Rev 16**:** 107-124.

Tupling AR (2004). The sarcoplasmic reticulum in muscle fatigue and disease: role of the sarco(endo)plasmic reticulum Ca2+-ATPase. Can J Appl Physiol 29**:** 308-329.

USDA (1997). Transportation of animals. In 49. ed. USDA: Government Printing Office., p 1219.

USDA (2019). Livestock, dairy, and poultry outlook. https://downloadsusdalibrarycornelledu/usda-esmis/files/g445cd121/7d2792872/4x51ht669/LDPM299pdf May 2019.

Valberg SJ (2008). Chapter 15 - Skeletal muscle function. In clinical biochemistry of domestic animals (Sixth Edition). eds Kaneko J.J., Harvey J.W., & Bruss M.L. Academic Press: San Diego, pp 459-484.

Van De Water G, Heylen T, Swinnen K, & Geers R (2003). The impact of vertical vibrations on the welfare of calves. Dtsch Tierarztl Wochenschr 110**:** 111-114.

Van Engen NK, Stock ML, Engelken T, Vann RC, Wulf LW, Karriker LA, *et al.* (2014). Impact of oral meloxicam on circulating physiological biomarkers of stress and inflammation in beef steers after long-distance transportation. J Anim Sci 92**:** 498-510.

Vasconcelos JT, Sawyer JE, Tedeschi LO, McCollum FT, & Greene LW (2009). Effects of different growing diets on performance, carcass characteristics, insulin sensitivity, and accretion of intramuscular and subcutaneous adipose tissue of feedlot cattle. Journal of Animal Science 87**:** 1540-1547.

Veldhuis JD, Iranmanesh A, Lizarralde G, & Johnson ML (1989). Amplitude modulation of a burstlike mode of cortisol secretion subserves the circadian glucocorticoid rhythm. 257**:** E6-E14.

Verkerk GA, Phipps AM, Carragher JF, Matthews LR, & Stelwagen K (1998). Characterization of milk cortisol concentrations as a measure of short-term stress responses in lactating dairy cows. Animal Welfare 7 (1)**:** 77-88.

Vina J, Gomez-Cabrera MC, Borras C, Froio T, Sanchis-Gomar F, Martinez-Bello VE, Pallardo FV. (2009). Mitochondrial biogenesis in exercise and in ageing. Adv Drug Deliv Rev 61**:** 1369-1374.

Volfinger L, Lassourd V, Michaux JM, Braun JP, & Toutain PL (1994). Kinetic evaluation of muscle damage during exercise by calculation of amount of creatine kinase released. 266**:** R434-R441.

Vugrek O, Belužić R, Nakić N, & Mudd SH (2009). S-adenosylhomocysteine hydrolase (AHCY) deficiency: Two novel mutations with lethal outcome. 30**:** E555-E565.

Wallimann T, Wyss M, Brdiczka D, Nicolay K, & Eppenberger HM (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and

fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 281 (Pt 1)**:** 21-40.

Warriss PD (1990). The handling of cattle pre-slaughter and its effects on carcass and meat quality. Applied Animal Behaviour Science 28**:** 171-186.

Warriss PD, Kestin SC, Brown SN, & Wilkins LJ (1984). The time required for recovery from mixing stress in young bulls and the prevention of dark cutting beef. Meat Science 10**:** 53-68.

Wasselin T, Zahn S, Maho YL, Dorsselaer AV, Raclot T, & Bertile F (2014). Exacerbated oxidative stress in the fasting liver according to fuel partitioning. Proteomics 14**:** 1905-1921.

Westerblad H, & Allen DG (2011). Emerging roles of ROS/RNS in muscle function and fatigue. Antioxid Redox Signal 15**:** 2487-2499.

Westerblad H, Bruton JD, & Katz A (2010). Skeletal muscle: Energy metabolism, fiber types, fatigue and adaptability. Experimental Cell Research 316**:** 3093-3099.

Westerblad H, Lee JA, Lannergren J, & Allen DG (1991). Cellular mechanisms of fatigue in skeletal muscle. Am J Physiol 261**:** C195-209.

Windle RJ, Wood SA, Shanks N, Lightman SL, & Ingram CD (1998). Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress. Endocrinology 139**:** 443-450.

Wright DC, Han D-H, Garcia-Roves PM, Geiger PC, Jones TE, & Holloszy JO (2007). Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1α expression. Journal of Biological Chemistry 282**:** 194-199.

Wright SA, Ramos P, Johnson DD, Scheffler JM, Elzo MA, Mateescu RG, *et al.* (2018). Brahman genetics influence muscle fiber properties, protein degradation, and tenderness in an Angus-Brahman multibreed herd. Meat Science 135**:** 84-93.

Xu T, Wang C, Shen J, Tong P, & O'Keefe R (2018). Ablation of Dnmt3b in chondrocytes suppresses cell maturation during embryonic development. J Cell Biochem 119**:** 5852-5863.

Yamashita K, & Yoshioka T (1991). Profiles of creatine kinase isoenzyme compositions in single muscle fibres of different types. Journal of Muscle Research & Cell Motility 12**:** 37-44.

Yokota T, Miyagoe-Suzuki Y, Ikemoto T, Matsuda R, & Takeda Si (2014). α1-Syntrophin–deficient mice exhibit impaired muscle force recovery after osmotic shock. Muscle & Nerve 49**:** 728-735.

Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, *et al.* (2001). Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 413**:** 131-138.

Young EA, Abelson J, & Lightman SL (2004). Cortisol pulsatility and its role in stress regulation and health. Frontiers in Neuroendocrinology 25**:** 69-76.

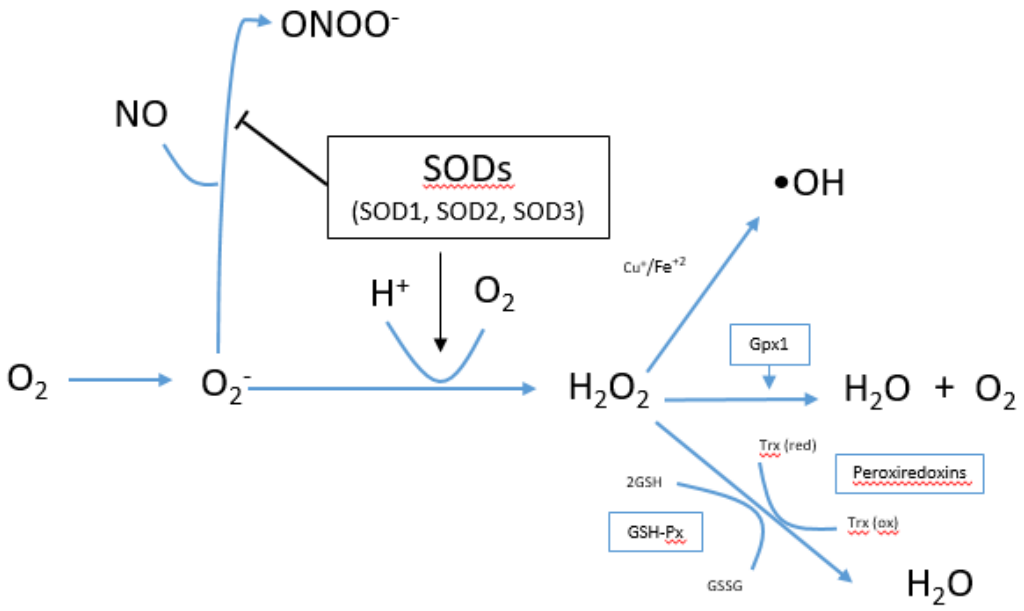
Zavy MT, Juniewicz PE, Phillips WA, & VonTungeln DL (1992). Effect of initial restraint, weaning, and transport stress on baseline and ACTH-stimulated cortisol responses in beef calves of different genotypes. Am J Vet Res 53**:** 551-557.

Zhang S-J, Sandström ME, Aydin J, Westerblad H, Wieringa B, & Katz A (2008). Activation of glucose transport and AMP-activated protein kinase during muscle contraction in adenylate kinase-1 knockout mice. Acta Physiologica 192**:** 413-420.

Zhao X, Yamazaki D, Kakizawa S, Pan Z, Takeshima H, & Ma J (2011). Molecular architecture of Ca2+ signaling control in muscle and heart cells. Channels (Austin) 5**:** 391-396.

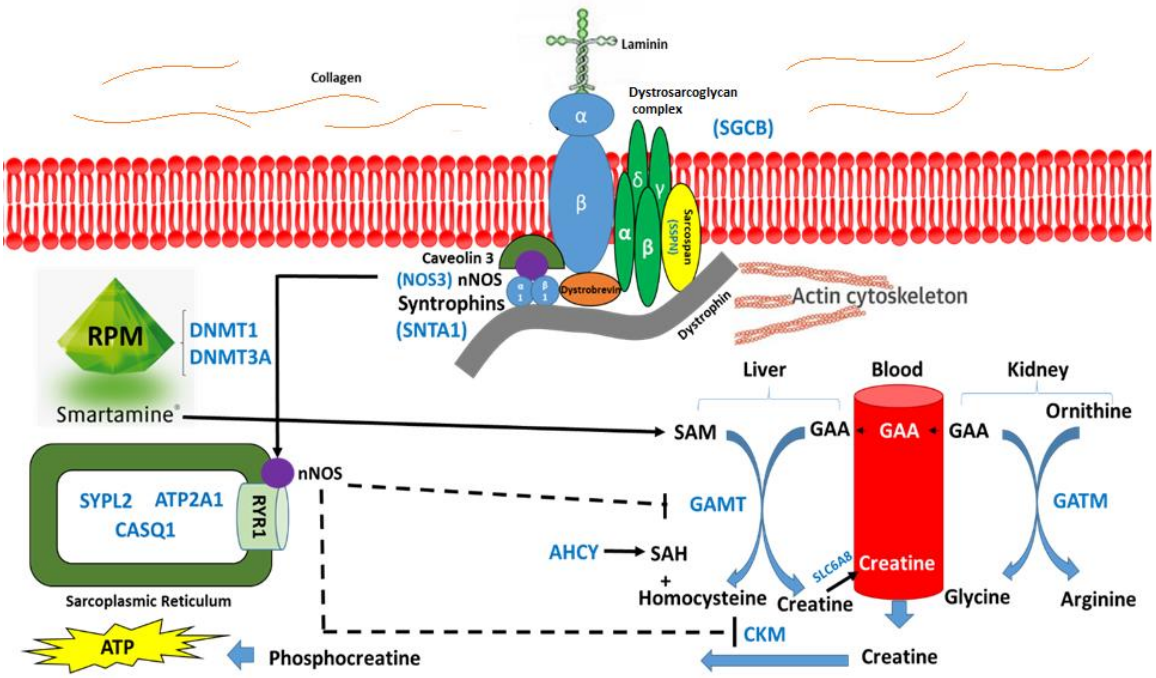
Zuo L, Christofi FL, Wright VP, Bao S, & Clanton TL (2004). Lipoxygenase-dependent superoxide release in skeletal muscle. J Appl Physiol (1985) 97**:** 661-668.

Figure 1. Reactive oxygen species metabolic pathway.



Modified from (Fukai & Ushio-Fukai, 2011)

Figure 2. Proposed muscle fatigue pathway.



Rumen-protected methionine provided by Smartamine® will incorporate additional methyl groups required for creatine synthesis in the liver by guanidinoacetate N-methyltransferase (*GAMT*). Glycine amidinotransferase, mitochondrial (*GATM*) is involved in creatine biosynthesis, whereby it catalyzes the transfer of a guanido group from L-arginine to glycine, resulting in guanidinoacetic acid (GAA), the immediate precursor of creatine. In skeletal muscle, the dystroglycan complex works as a transmembrane linkage between the extracellular matrix and the cytoskeleton. α-dystroglycan is extracellular and binds to laminin in the basement membrane, while β-dystroglycan is a transmembrane protein and binds to dystrophin. Dystrophin binds to intracellular actin fibers. In this way, the dystroglycan complex, which links the extracellular matrix to the intracellular actin fibers, is thought to provide structural integrity in muscle tissues. Neuronal form of nitric oxide synthase (nNOS) binds to caveolin binding motif producing traslocation of nNOS to the cytosol. If nNOS binds to Ryanodine receptor channel (*RYR1*) present in the sarcoplasmic reticulum, it produces a destabilization of the sarcoplasmic reticulum membrane and a consequent Ca^{++} leak that will affect the regular function of relaxation and contraction of muscles (Bellinger *et al.*, 2009; Westerblad & Allen, 2011). Furthermore, nNOS inhibits *GAMT* and the enzyme creatine kinase present in muscle (*CKM*) responsible for the conversion of creatine to phosphocreatine, which releases the energy (ATP) required to mitigate muscle fatigue.

Figure 3. Overhead and transversal view of skeletal muscle biopsy area on a beef heifer.

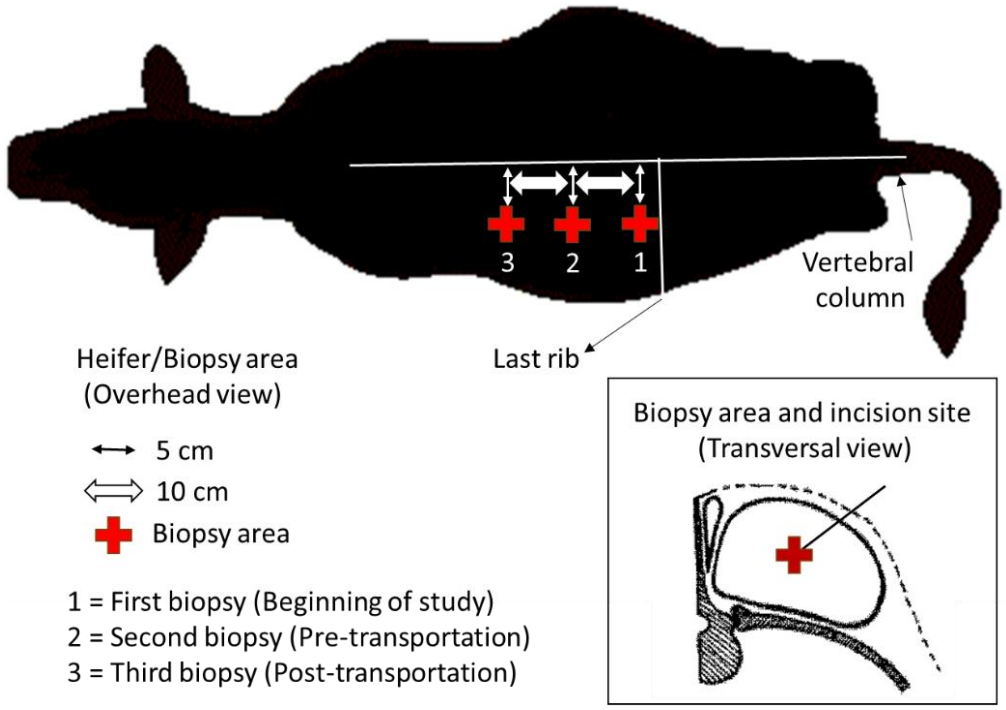
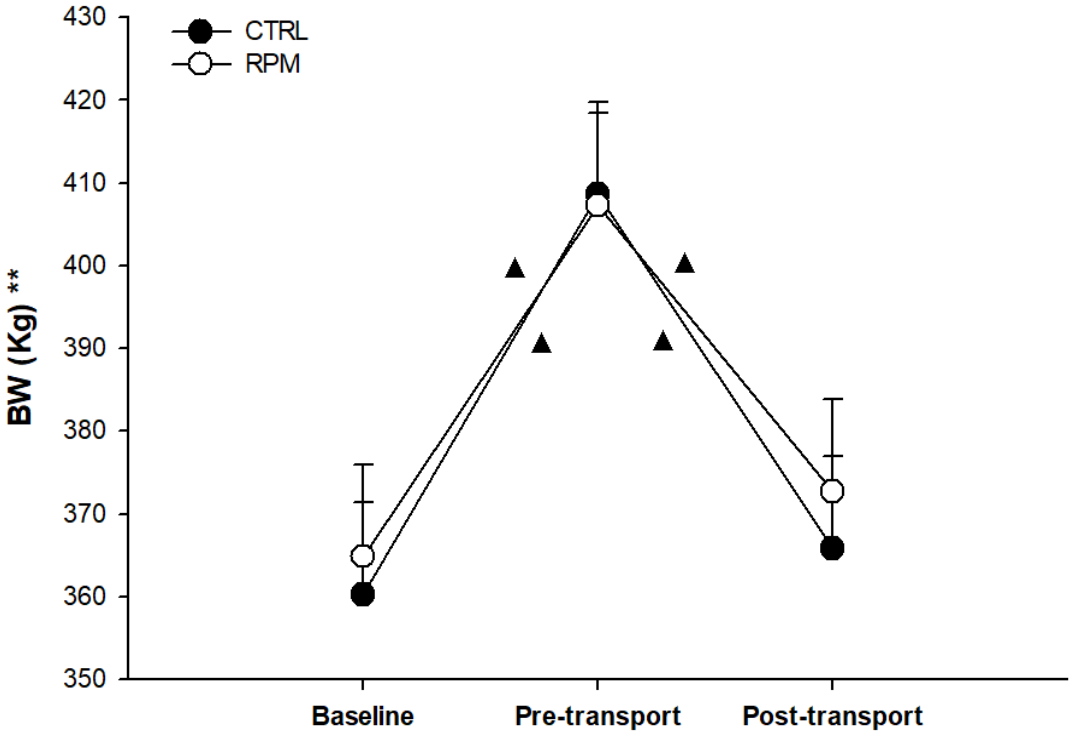
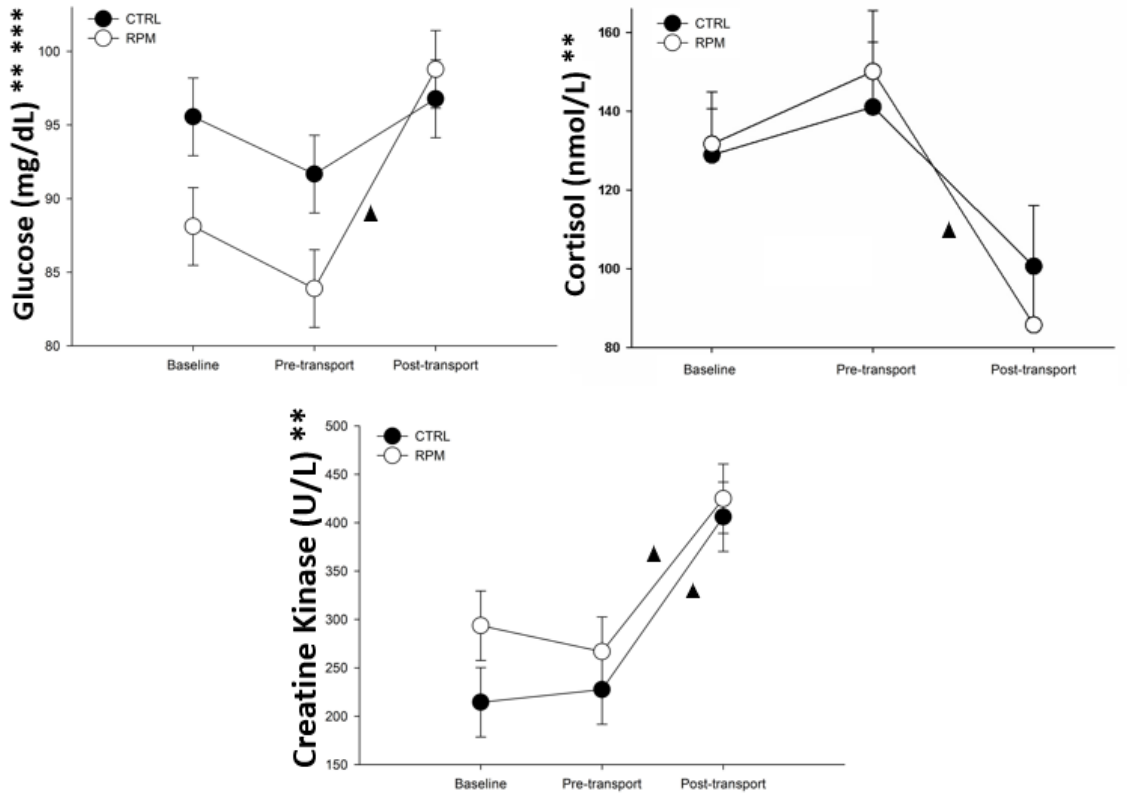


Figure 4. Effect of RPM supplementation on body weight (kg) at Baseline, Pre- and Post-transportation.



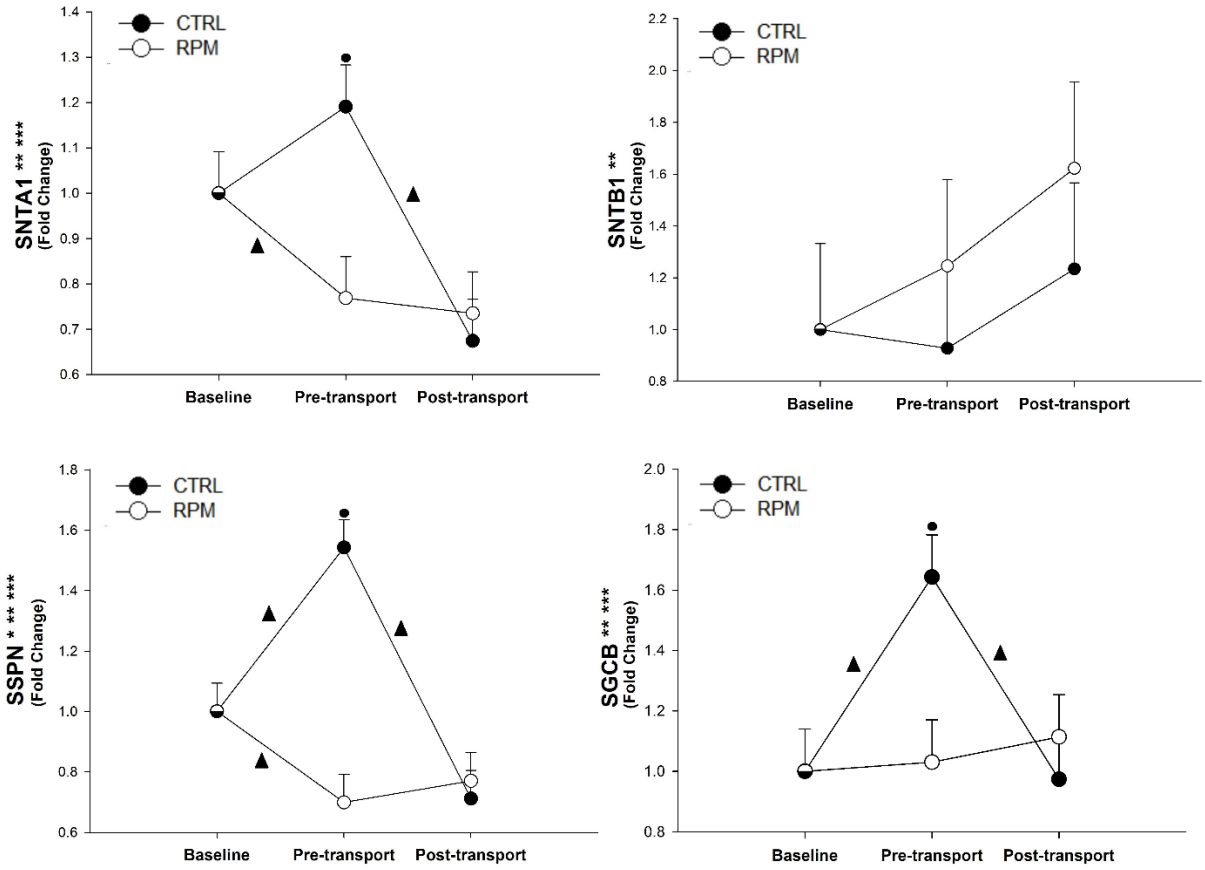
Statistically significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × time interaction (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 5. Mean serum glucose, cortisol, and creatine kinase concentration for CTRL and RPM heifers at Baseline, Pre-transportation and Post-transportation.



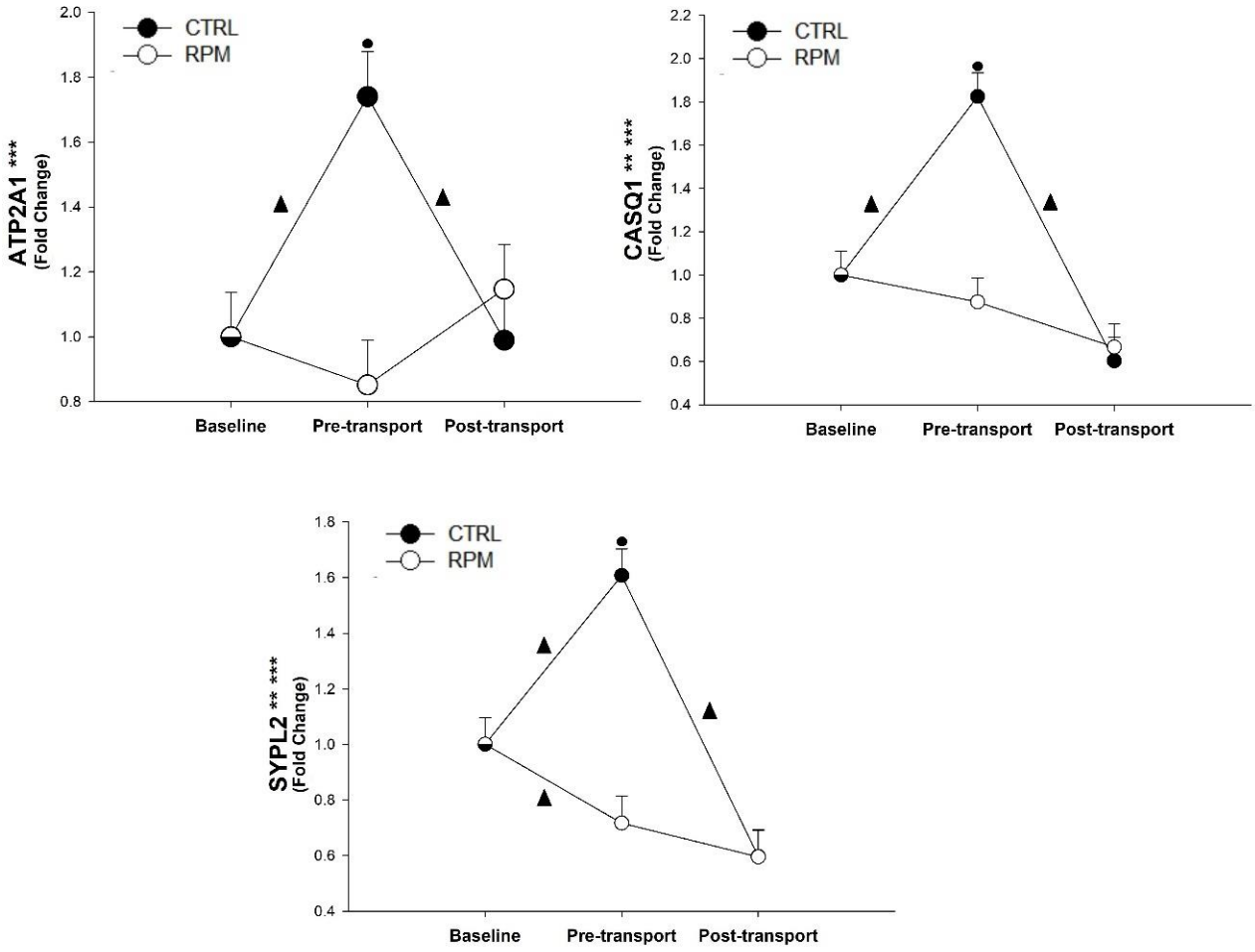
Statistically significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × time interaction (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 6. Expression of genes related to the Dystrophin-Glycoprotein complex in *Longissimus dorsi* muscle of beef heifers that received rumen-protected methionine (RPM) for 45 days before transportation and control heifers (CTRL) that did not received RPM.



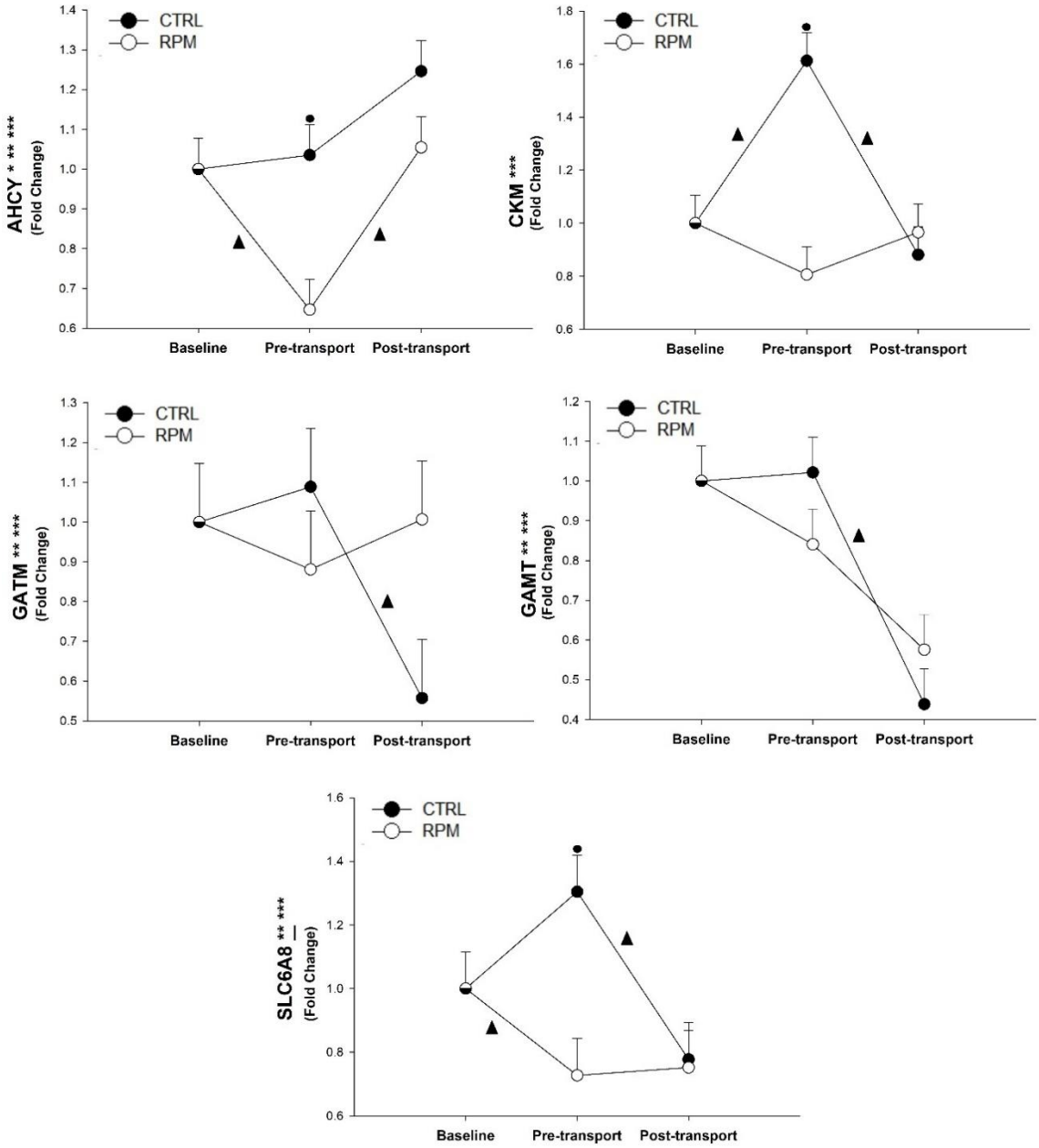
Statistically significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × time interaction (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($P < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($P < 0.05$) between treatments at the same time point.

Figure 7. Expression of genes located at the sarcoplasmic reticulum in *Longissimus dorsi* muscle of beef heifers that received rumen-protected methionine (RPM) for 45 days before transportation and control heifers (CTRL) that did not received RPM.



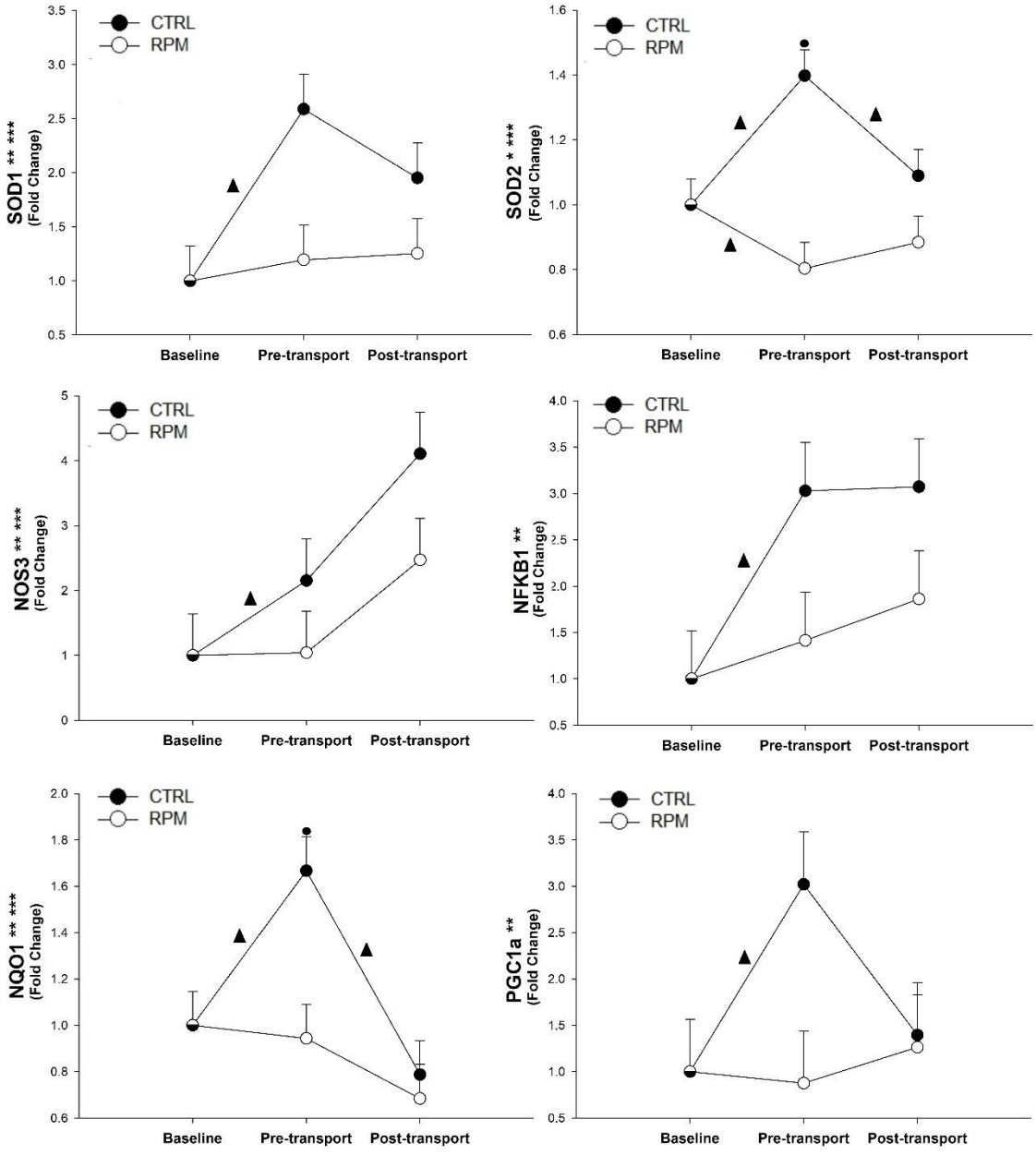
Statistically significant differences where declared at P < 0.05 and tendencies at P > 0.05 and < 0.1. Treatment × time interaction (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($P < 0.05$) between two time point for the same treatment, symbols (●) denote significant differences ($P < 0.05$) between treatment at the same time point.

Figure 8. Expression of genes related to the creatine synthesis pathway in *Longissimus dorsi* muscle of beef heifers that received rumen-protected methionine (RPM) for 45 days before transportation and control heifers (CTRL) that did not received RPM.



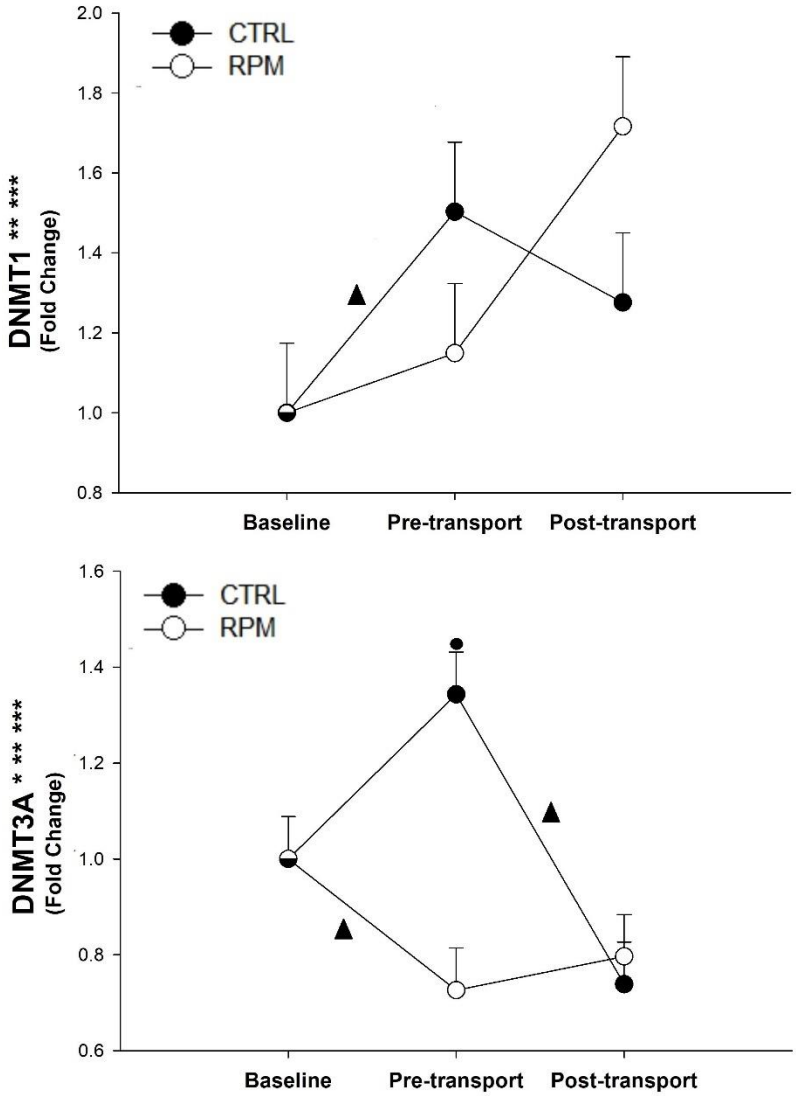
Statistically significant differences where declared at P < 0.05 and tendencies at P > 0.05 and < 0.1. Treatment × time interaction (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($P < 0.05$) between two time point for the same treatment, symbols (●) denote significant differences ($P < 0.05$) between treatment at the same time point.

Figure 9. Expression of genes related to Oxidative Stress in *Longissimus dorsi* muscle of beef heifers that received rumen-protected methionine (RPM) for 45 days before transportation and control heifers (CTRL) that did not received RPM.



Statistically significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × time interaction (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($P < 0.05$) between two time point for the same treatment, symbols (●) denote significant differences ($P < 0.05$) between treatment at the same time point.

Figure 10. Expression of genes related to DNA methylation in *Longissimus dorsi* muscle of beef heifers that received rumen-protected methionine (RPM) for 45 days before transportation and control heifers (CTRL) that did not received RPM.



Statistically significant differences where declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.1. Treatment × time interaction (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($P < 0.05$) between two time point for the same treatment, symbols (●) denote significant differences ($P < 0.05$) between treatment at the same time point.

Table 1. Overall least mean squares values for expression of genes analyzed in *Longissimus dorsi* muscle of Angus-Simmental heifers from CTRL and RPM.

Gene	PRET		POST		SEM	*P* - value		
	CTRL	RPM	CTRL	RPM		Trt	Time	Trt ×Time
ATP2A1	1.7401	0.8517	0.9894	1.1468	0.1382	0.2566	0.7291	0.0001
CASQ1	1.8242	0.8758	0.6034	0.6666	0.1098	0.3006	0.0001	0.0001
GAMT	1.0213	0.8405	0.4383	0.5753	0.0886	0.8909	0.0001	0.0256
GATM	1.0886	0.8811	0.5570	1.0066	0.1473	0.4042	0.0006	0.0031
SGCB	1.6431	1.0302	0.9737	1.1138	0.1399	0.6937	0.0173	0.0001
SNTA1	1.1910	0.7688	0.6745	0.7352	0.0916	0.2734	0.0001	0.0002
SYPL2	1.6068	0.7168	0.5970	0.5952	0.0960	0.1404	0.0001	0.0001
AHCY	1.0355	0.6466	1.2462	1.0545	0.0769	0.0375	0.0001	0.0010
CKM	1.6125	0.8060	0.8812	0.9657	0.1057	0.1108	0.2946	0.0001
DNMT1	1.5030	1.1494	1.2760	1.7166	0.1740	0.9466	0.0003	0.0465
SLC6A8	1.3041	0.7272	0.7776	0.7519	0.1160	0.2223	0.0707	0.0007
SSPN	1.5425	0.6992	0.7114	0.7701	0.0933	0.0498	0.0001	0.0001
DNMT3A	1.3429	0.7260	0.7383	0.7962	0.0881	0.0483	0.0002	0.0001
SOD2	1.3980	0.8041	1.0898	0.8847	0.0802	0.0133	0.8019	0.0001
NQO1	1.6672	0.9437	0.7876	0.6841	0.1467	0.1216	0.0001	0.0008
SOD1	2.5882	1.1948	1.9520	1.2541	0.3226	0.8346	0.0001	0.0386
NOS3	2.1550	1.0438	4.1098	2.4732	0.6390	0.7057	0.0001	0.0382
PGC1a	3.0205	0.8753	1.3952	1.2639	0.5641	0.9955	0.0162	0.1952
SNTB1	0.9281	1.2453	1.2343	1.6226	0.3324	0.3608	0.0250	0.3369
NFKB1	3.0293	1.4143	3.0720	1.8627	0.5216	0.7601	0.0002	0.3900

Table 2. Quantitative real time PCR performance among the 20 genes measured in skeletal muscle samples

Pahtway	Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency (%) [5]	Efficiency[6]	Relative mRNA abundance[7]	1/EΔCt[8]	%
Dystrophin-Glycoprotein complex	**SGCB**	22.948	-0.572	-2.429	0.999	158.043	2.580	1.720	0.003	0.283
	SNTA1	22.972	-0.457	-2.582	0.999	143.948	2.439	1.504	0.002	0.247
	SNTB1	24.521	0.776	-2.614	0.999	141.299	2.413	0.505	0.001	0.083
	SSPN	23.251	-0.203	-2.6705	0.999	136.844	2.368	1.191	0.002	0.196
Sarcoplasmic Reticulum	**ATP2A1**	18.005	-5.467	-2.9325	0.999	119.284	2.193	73.138	0.120	12.022
	CASQ1	18.990	-4.476	-2.549	0.999	146.780	2.468	57.026	0.094	9.373
	SYPL2	21.360	-2.151	-2.526	0.999	148.819	2.488	7.105	0.012	1.168
Creatine Synthesis Pathway	**GAMT**	23.523	0.053	-2.6285	0.999	140.129	2.401	0.955	0.002	0.157
	GATM	25.433	1.982	-2.469	0.999	154.110	2.541	0.158	0.000	0.026
	AHCY	20.991	-2.501	-2.4625	0.999	154.737	2.547	10.367	0.017	1.704
	SLC6A8	23.349	-0.231	-2.597	0.999	142.694	2.427	1.227	0.002	0.202
	CKM	16.517	-7.038	-2.653	0.999	138.195	2.382	449.736	0.739	73.923
DNA methylation	**DNMT1**	26.705	3.035	-2.5305	0.997	148.416	2.484	0.063	0.000	0.010
	DNMT3A	26.194	2.690	-2.4745	0.999	153.584	2.536	0.082	0.000	0.013
Oxidative Stress	**SOD2**	22.588	-1.005	-2.5925	0.999	143.068	2.431	2.441	0.004	0.401
	NQO1	25.987	2.562	-2.3005	0.999	172.075	2.721	0.077	0.000	0.013
	SOD1	23.633	0.077	-2.539	0.998	147.660	2.477	0.933	0.002	0.153
	NOS3	26.751	3.345	-2.3215	0.998	169.622	2.696	0.036	0.000	0.006
	PGC1a	26.318	2.983	-2.596	0.999	142.777	2.428	0.071	0.000	0.012
	NFKB1	26.590	3.103	-2.461	0.999	154.882	2.549	0.055	0.000	0.009
								608.388	1.000	100.000

1-The median is calculated considering all time points and all heifers. 2-The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each steer. 3-Slope of the standard curve. 4-R2 stands for the coefficient of determination of the standard curve. 5-Efficiency (%) is calculated as [10(-1 / Slope)]-1 x 100. 6-Efficiency is calculated as [10(-1 / Slope)]. 7-Relative mRNA abundance = 1/ Efficiency Median ΔCt. 8-1/EΔCt = relative mRNA abundance/ Σ relative mRNA abundance

Table 3. Gene ID, GenBank accession number, hybridization position, sequence and amplicon size of primers for *Bos taurus* used to analyze gene expression by RT-qPCR.

Gene ID	Accession #	Gene	Primers[1]	Primers (5'-3')[2]	bp[3]
Internal Control					
509768	NM_001025327.2	*MTG1*	F.1163	CAACAAAATGGACCTGGCAGAT	115
		MTG1	R.1265	CTTGACATTTTCATCCTTCACACAGT	
337888	NM_001037443.2	*RPS15A*	F.230	GCAGGCTAAATAAGTGTGGAGTGA	90
		RPS15A	R.319	GGGATGGGAGCAGGTTATTCT	
525680	NM_001037471.2	*UXT*	F. 337	CTGGCAGAAGCTCTCAAGTTCA	100
		UXT	R. 436	GGATATGGGCCTTGATATTCATG	
Dystrophin-Glycoprotein comp					
535372	NM_001102188.1	*SGCB*	F.2978	GAGACTTCAAAGTAGTGTGCCGTAAA	100
		SGCB	R.3077	TTCAAAGTGTCAAGGACAGAGTATTGT	
527488	NM_001075898.2	*SNTA1*	F.1881	CCCATGGCAAGAGACAGAGAA	100
		SNTA1	R.1980	AGAGTAAGGAGGAGAGAGAGGATGAA	
617927	XM_015474564.2	*SNTB1*	F. 991	AAGCCGATTGTTTTCATCATTCAT	105
		SNTB1	R. 1095	AAGCCTCCTTTGCTCAGAGGTA	
613989	NM_001193005.1	*SSPN*	F.237	CCTGCTAGTCAGAGACACTCCATTT	105
		SSPN	R.336	TTCGTCAACTTGATAGGAGACACAA	
Sarcoplasmic reticulum					
518117	NM_001075767.1	*ATP2A1*	F. 2821	GTGTGGACTGTGAGGTCTTCGA	107
		ATP2A1	R. 2927	CTGATTCTCAGACAGGCTGTTGA	
508394	NM_001077877.2	*CASQ1*	F. 561	GGATGGTGGACTCTGAGAAGGA	90
		CASQ1	R. 650	CTTCATCCCCCTTGAAAACATAA	
514665	NM_001075676.1	*SYPL2*	F.389	CCATCATTGTTTCATTCGGCTAT	90
		SYPL2	R.478	TGGTCTTGGAGGTGGATTCATC	
DNA methylation					
281119	NM_182651.2	*DNMT1*	F. 1323	TCTGGTTCAGCAAAGCCGATATAT	105
		DNMT1	R. 1427	ATCAAAACCAGCAATCCACCAT	
359716	NM_001206502.1	*DNMT3A*	F. 5739	AGCGAACCTCATCATTGGAATAA	104
		DNMT3A	R. 5842	GCTGTGAGCTTACTCCTGAGCAA	

[1] Primer direction (F – forward; R – reverse) and hybridization position on the sequence.

[2] Exon-exon junctions are underlined.

[3] Amplicon size in base pair (bp).

Table 3 Continuation. 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[1]	Primers (5'-3')[2]	bp[3]
Creatine synthesis pathway					
508158	NM_001034315.1	*AHCY*	F. 1768	TTCTTGGTTTACAGGTTCATTGGTT	95
		AHCY	R. 1862	GAGCATTCAAACACCCACAGTTC	
286822	NM_174773.4	*CKM*	F. 301	GCTGGTGATGAGGAGTCCTATACG	115
		CKM	R. 415	TCTCATGGTTGAGGTCAGTCTTGT	
515270	NM_001038544.2	*GAMT*	F. 482	GTTCAACTTCATTCGGGACCAT	116
		GAMT	R. 597	TGGTGATGTCTGAATACTTGGTCTTC	
414732	NM_001045878.1	*GATM*	F. 258	ATTGGCTGCTCAGGGAAAGTT	110
		GATM	R.372	TTGTAACCTGGCTTCTTTGTACGA	
282367	NM_174611.2	*SLC6A8*	F.1429	CCTCCTACTACTTCCGATTCCAAA	105
		SLC6A8	R.1533	AGACATACATCCCGCCATCAGT	
Oxidative Stress					
281495	NM_174615.2	*SOD1*	F. 355	TGTTGCCATCGTGGATATTGTAG	105
		SOD1	R. 459	CTGCCCAAGTCATCTGGTTTTT	
281496	NM_201527.2	*SOD2*	F 290	AGAAGGGTGATGTTACAGCTCAGATAG	93
		SOD2	R 382	GATTTGTCCAGAAGATGCTGTGAT	
287024	NM_181037.3	*NOS3*	F. 3944	CTCCGGAAGTATCTTATCTTGAAACC	135
		NOS3	R. 4078	AACTGTAATTGACAGCACTGGCTTAG	
616115	NM_001076409.1	*NFKB1*	F. 2483	GGCACAAGGAGACATGAAACAA	125
		NFKB1	R. 2607	TCAGTATCCCCAGACCTAATTTCTG	
359716	NM_001206502.1	*NQO1*	F. 242	CAATCCCGTCATCTCCAGAAA	
		NQO1	R. 345	TCAGACGGCCTTCTTTATAAGCTAA	
338446	XM_010806009.2	*PGC1a*	F. 1623	TGACAGCGAAGATGAAAGTGATAAA	138
		PGC1a	R. 1760	AATAAAGATTTGGGTGGTGACACA	

[1] Primer direction (F – forward; R – reverse) and hybridization position on the sequence.

[2] Exon-exon junctions are underlined.

[3] Amplicon size in base pair (bp).

Table 4. 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.

Internal control	
MTG1	CGACCGAAATACCACCCTTGGAAGAGAGGCATAAAACATGTTTGTTTTTCCAACCTGTGTGAAGGATGAAAATGTCAAGA
RPS15A	GACTGATATGAGTGTCACTCATAGTATCCTAGGATAGTGGCAGAATAACCTGCTCCCAGTCCCAACTCCACACTATTATTACGGTACGTTACGCATG
UXT	CGACCGAAATACCACCCTTGGAAGAGAGGCATAAAACATGTTTGTTTTTCCAACCTGTGTGAAGGATGAAAATGTCAAG
Dystrophin-Glycoprotein complex	
SGCB	GAGTCGGAATACATATCCCATTTAGTCATTTAAATATAACAATACTCGATGTCCTAGTACACTTTGAAAA
SNTA1	CGATCGCTCTCTGACTCACCTACCTGTGGCATCAGGGTTCTCTTCATCCATCGTCTCGTCCTCCTTACTCATAA
SNTB1	CTGGCTAGCTAGATACAGACCEGGGGTTGGGIGGGCTGGAGCAGGGGGGTGAATEACTCTGAGCAAAGGCAGGTCTAG
SSPN	GACGTCATGTTCTGCTAGTGCTATCTCGGTTTGTTTATGCTTTGTGTCTCCTATCAAGTTGACGAC
Sarcoplasmic reticulum	
ATP2A1	GCATCAGTCAGTGTGGACAGCGTATCGGTCACGTACTCGTCGTCACTGGATCGCACGAGTCGATCGACGACTGACGGTCTCAATGCGAG TCACTGTGCCTGTGAGCGAGAGTCGTACGCATCGAAGACCTCACAGTCCACACATA
CASQ1	GTCCATACCTGGACGTACTCAGAGGACAGCGTTTTATGTTTTCTAGGGGGATGAAGATCCCTTCTCTCCTTTCCTCAGAGCCACACAATCCAA
SYPL2	CGTGTACGGTCAGTAGTAGAGTCCCCTCGTGTACGATGATCCACCTCCAAGACCA
Creatine synthesis pathway	
AHCY	GATCAGTCAGAGTAGAGGATCTCTGCTCAAGGGTGTGAGGGAACTCGCTGGGTGTTTGAATGCATCA
CKM	GCATGACCTCTGACTATCATCAGGACGGCACTGGGGGCTCAACCCACAGTACAAGCACAAGGACTGTACCTCAACCATGAGACACCGTATAGGAC
GAMT	GGCTGCTGTGACAGGGGTTCTCACCTACAGTTACCTCACCTCCATGGGGAGAGCTAATGAAGACCAAGTATTCTAGTACAGTCACCAATAGAGGG
GATM	TACTGCAGCATGAGCTGCGGATTCATTCGAGCTGGAGAGATATCATTCGCTCAAAGAAGCCAGGTTTACAAACTTTTCCCTGAGCAGCCAATAAAA
SLC6A8	GACACGGACTCGTTGCACATCTGCTTTGTCATCGACCTCTCCATGGTGACTGATGGCGGGATGTATGTTCTTAA
DNA methylation	
DNMT1	CGATACATCTCGCAGTGTATTATGGCAAAATTTTGGCATAAACGAATGGTGGATTGCTGGTTTCTGATAA
DNMT3A	GACTATTGTGTAAAAAAAATTACCAGCTTTGAATCCTTTGCCCTTGCCCGGGGTAAGCTCCCCCGCAAA
Oxidative stress	
SOD1	CTAGTACATTCTCAGGGAATATCATCACTGGCGCACGATGGTGGTCTATGAAAAACAGATGACTGCGGTCAGANACAATATCCACGATGGCAACAA
SOD2	CCGTTGCCCGGTCGTGGTAGTCTACGGGGTGCCATATCAATCACAGCATCTTCGTGACAAATCATAGAA
NOS3	GTATCTATCATTCATATTTATTATTGAGATACCATAAGAGACTGGACCAGAAGTTAGGAGACCTATCTAAGA
NFKB1	CGTGGATGCAGCTGCAGCTCTACAGTGCTAGAATCTGATCAGACAAAAACTGGGCTA
NQO1	GCTAGTACGTAGGACGGGGACTTTCAGTATCCTGCCGAGACTGTTTTAGCTTATAAAGAAGGCCGTCCTGA
PGC1a	CGTATCTGATGCACGCAGTCTATTCATTGTTCGATGTGTCGCCTTCTTGTTCTTCTTTTAACTCTCCGTGTGAGAGATTCTGTGTC

Table 5. Sequencing results of genes using BLASTN from NCBI (http://www.ncbi.nlm.nih.gov) against nucleotide collection (nr/nt) with total score.

Gene	Best hit in NCBI
AHCY	Bos taurus adenosylhomocysteinase (AHCY), mRNA
ATP2A1	Bos taurus ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1 (ATP2A1), mRNA
CASQ1	Bos taurus calsequestrin 1 (CASQ1), mRNA
CKM	Bos taurus creatine kinase, M-type (CKM), mRNA
DNMT1	Bos taurus DNA methyltransferase 1 (DNMT1), mRNA
DNMT3A	Bos taurus DNA methyltransferase 3 alpha (DNMT3A), mRNA
GAMT	Bos taurus guanidinoacetate N-methyltransferase (GAMT), mRNA
GATM	Bos taurus glycine amidinotransferase (GATM), mRNA
MTG1	Bos taurus mitochondrial ribosome associated GTPase 1 (MTG1), mRNA
NFKB1	Bos taurus nuclear factor kappa B subunit 1 (NFKB1), mRNA
NOS3	Bos taurus nitric oxide synthase 3 (endothelial cell) (NOS3), mRNA
NQO1	Bos taurus NAD(P)H quinone dehydrogenase 1 (NQO1), mRNA
PGC1α	PREDICTED: Bos taurus PPARG coactivator 1 alpha (PPARGC1A), transcript variant X1, mRNA
RPS15A	Bos taurus ribosomal protein S15a (RPS15A), mRNA
SGCB	Bos taurus sarcoglycan beta (SGCB), mRNA
SLC6A8	Bos taurus solute carrier family 6 member 8 (SLC6A8), mRNA
SNTA1	Bos taurus syntrophin alpha 1 (SNTA1), Mrna
SNTB1	PREDICTED: Bos taurus syntrophin beta 1 (SNTB1), mRNA
SOD1	Bos taurus superoxide dismutase 1 (SOD1), mRNA
SOD2	Bos taurus superoxide dismutase 2 (SOD2), mRNA
SSPN	Bos taurus sarcospan (SSPN), mRNA
SYPL2	Bos taurus synaptophysin like 2 (SYPL2), mRNA
UXT	Bos taurus ubiquitously expressed prefoldin like chaperone (UXT), mRNA