Effects of beta-hydroxy beta-methylbutyrate (HMB) supplementation on *gluteus medius* muscle fiber composition and muscle performance in adult Thoroughbred horses exercising to fatigue on a high-speed treadmill

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ACADEMIC ABSTRACT

Consumption of β-hydroxy β-methylbutyrate (HBM), a leucine metabolite, alters muscle composition and metabolism leading to strength and agility improvements in human athletes. To determine if HMB affects athletic performance and muscle function in horses, Thoroughbred geldings were fed a control (CON; n=5) or HMB (n=6) supplement (30 mg/kg/day) for 6 weeks prior to completing a standardized exercise test (SET). Gluteus medius (GM) muscle samples were obtained before the SET for fiber-typing and venous blood was collected before and immediately upon completion of the SET for lactate measurements. Heart rate (HR), biceps femoris (BF) and semitendinosus (ST) surface electromyograms, and fore- and hindlimb metacarpophalangeal joint angles were captured for the duration of the SET. Results demonstrate that HMB supplementation increased ($P < 0.05$) the percentage of type IIA muscle fibers in the GM with a corresponding decrease ($P < 0.05$) in type IIX fibers. The percentage of type I fibers was unaffected by diet. Supplementation with HMB did not result in any significant effects on performance, muscle function or biomechanical properties by comparison to CON. Increasing treadmill speed resulted in an increase ($P < 0.05$) in stride length and maximal extension angle of the fore fetlock, and a shortening ($P < 0.05$) of the stance phase of the gait cycle. Integrated EMG (iEMG) increased ($P < 0.05$) with increasing treadmill speeds for both the BF and ST, with the BF exhibiting greater iEMG values than the ST. In summary, HMB increased the percentage of type IIA fibers which did not translate into immediate, improved athletic performance.

KEYWORDS: β-hydroxy β-methylbutyrate, equine, exercise physiology, myofiber

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GENERAL AUDIENCE ABSTRACT

Muscles depend on their fibers, innervation, energy supply, and blood flow to contract. Failure to meet one or more of these requirements precludes muscle tissue from performing work, situation termed fatigue. Identification of fatigue indicators is of interest to the horse industry for a number of reasons, including horse and human safety, prevention of unnecessary expenses, and general public opinion of the sport disciplines. Diet supplementation with legal, performance-enhancing compounds is of interest to riders and horse owners alike. Molecules such as beta-hydroxy beta-methylbutyrate (HMB) improve muscle function, protein synthesis, and muscle tissue repair. Assessment of the athletic capacity and performance of horses by evaluating fatigue indicators favors responsible training regimes. Techniques to achieve this goal include muscle sampling, biochemical, electromyographic, and biomechanical analysis.

We hypothesized that dietary supplementation of HMB would have positive effects on the athletic performance of horses. This study evaluated the effects of 45-day HMB supplementation on muscle fiber composition, muscle performance, and rates of fatigue in adult Thoroughbred horses by use of a high-speed treadmill. Muscle biopsies, blood lactate, high-speed video captures, and electromyography were analyzed. These analyses revealed that HMB supplementation increased the number of fatigue-resistant fibers in muscles but caused no substantial, immediate improvements on the athletic performance of horses.

KEYWORDS: beta-hydroxy beta-methylbutyrate, equine, exercise physiology, muscle

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DEDICATION

To my mom, Mariane Busse, for her steadfast and unconditional support, and to my dear deer, Jess, for saving me. Auch an alle meine Freunde und Freundinnen, die noch bei mir stehen. Por la paciencia inagotable de mis amigos. Merci mille fois.

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ATP, adenosine triphosphate

BCAA, branched chain amino acid

BF, biceps femoris

bpm, beats per minute

EMG, electromyography

GM, gluteus medius

HMB, β-hydroxy β-methylbutyrate

iEMG, integrated electromyography

MCPJ, metacarpophalangeal joint

MdF, median frequency

mTOR, mechanistic target of rapamycin

MyHC, myosin heavy chain

RMS, root mean square

sEMG, surface electromyography

SET, standardized exercise test

ST, semitendinosus

TCA, tricarboxylic acid

TTE, time to exhaustion

V200, speed at HR of 200 bpm

VO_{2max}, maximal oxygen consumption

CHAPTER I

INTRODUCTION

Musculoskeletal injuries are the major cause for turnover in training stables, precluding 19-33% of affected horses from returning to training in the United States. Training failure and early wastage of Thoroughbred horses results in a diminished economic return for the owners. A median of $23,000 USD difference in economic return at 2-year-old sales has been previously reported (Hernandez and Hawkins 2001; Stover 2003). The horse industry in the United States generates over $102 billion USD in yearly economic impacts for the U.S. national economy; over $26 billion (>25.4%) of these are related to the racing segment (American Horse Council 2005). Maximizing performance and reducing wastage are key elements to maintaining this industry (both commercial and academic), and its peripheral associated fields.

Nutrition in plays an integral role in the life of the successful athlete and the racehorse is no exception. Depending on boarding facilities, diet, training and performance level, nutrition alone accounts for 31-60% of an athlete's stabling costs (Guelph 2013). Adequate nutrition during a horse's early life stages should be maximized to promote optimal musculoskeletal growth and prevent developmental orthopedic diseases. In adult athletes, however, nutrition aims to enhance performance, reduce recovery times, replenish substrate stores, and improve tissue repair (Firth et al. 2004; Geor 2006; Harris 2009). The global supplement industry had a market consisting of over $75 million USD by 2018, with an annual growth rate predicted to reach over $100 million USD by 2027. Both North America (e.g. United States) and Europe (e.g. United Kingdom, Germany) hold major shares of this market. This is driven by the consumer's heightened concerns about efficacy and safety of alimentary supplements, as well as increasing numbers of athletes engaging in high-performance equestrian disciplines (Transparency Market

Research 2019). The interest of owners, riders, and trainers in gaining a competitive advantage makes the industry susceptible to production and advertisement of goods without proper evidence of their claimed effects. A majority of ergogenic supplements and nutraceuticals claim to boost performance, hasten recovery, or prevent joint disorders (Geor 2006).

Ergogenic supplements seek to increase an individual's capacity to perform work by providing a beneficial compound or set of molecules. The leucine metabolite β-hydroxy β-methylbutyrate (HMB), or hydroxymethylbutyrate, may prevent muscle damage in athletes performing high intensity exercise and aid in its repair and recovery (Nissen et al. 1996; Ostaszewski et al. 2012) and has gained traction as ergogenic aid. The ergogenic effects displayed by HMB are not seen in other branched chain amino acids (BCAA) such as valine and isoleucine, bolstering the notion that leucine and HMB are responsible for these effects (Holecek et al. 2009). As a supplement, HMB has been extensively studied in humans (Nissen et al. 1996; Kaczka et al. 2019), but only recently been reported as a possible equine dietary supplement (Miller et al. 1998; Ostaszewski et al. 2012).

The project tested the hypothesis that supplementation with calcium beta-hydroxy beta-methylbutyrate monohydrate in adult horses will have a positive impact on their athletic performance by delaying the onset of fatigue and prompting a myofiber transdifferentiation of myofibers towards more fatigue-resistant fiber types in the gluteus medius muscle, within the scope of increasing general athletic performance.

LITERATURE REVIEW

Beta-hydroxy beta-methylbutyrate

The molecule β-hydroxy β-methylbutyric acid (HMB) is a bioactive leucine metabolite with ergogenic capabilities, and approximately 5% of ingested leucine is converted to HMB. The metabolite is formed by transamination of leucine to α-ketoisocaproate (KIC) in muscle tissue and subsequent oxidation of KIC to β-hydroxy β-methylbutyrate in the liver (Nissen and Abumrad 1997; Arazi et al. 2018). Beta-hydroxy beta-methylbutyrate is then transformed into different molecules including cholesterol, beta-hydroxybutyrate, beta-methylglutaconyl-CoA, and isovaleryl-CoA, with a small percentage lost in urine (Nissen et al. 1996; Arazi et al. 2018). In its free acid form, ingested HMB is absorbed faster by the proximal gastrointestinal tract and possesses an elimination half-life approximately 20% longer than HMB in its calcium-conjugated counterpart (Fuller et al. 2015; Hu et al. 2020). This compound presents valuable traits to different fields such as exercise physiology, orthopedics, oncology, neurology, gerontology, and nutrition in a variety of species (Zanchi et al. 2011; Rahman et al. 2014; Szcześniak et al. 2015; Holeček 2017; Arazi et al. 2018; Engelen and Deutz 2018; Suryawan et al. 2020). The molecule, found naturally in minor quantities in plants such as alfalfa, asparagus and grapefruit, displays anabolic properties on protein synthesis including activation of mechanistic target of rapamycin (mTOR) protein kinase, stimulation of cholesterol biosynthesis pathways, decreased activity of apoptotic pathways, and increased satellite cell (SC) proliferation *in vitro* (Wilson et al. 2008; Szcześniak et al. 2015; Brook et al. 2016; He et al. 2016; Barrientos et al. 2017; Kaczka et al. 2019). Beta-hydroxy beta-methylbutyric acid and its calcium-conjugated salts have been proposed to be beneficial to muscle metabolism in humans, including increases in muscle strength, improvement in aerobic capacity, and faster returns to normal

performance after over-training (Nissen et al. 1996; Lamboley et al. 2007; Wilson et al. 2014). This compound is commercially available as over-the counter supplements in different presentations for human consumption, and as top-dress powdered supplement for animal use. Recent studies in exercising Thoroughbred horses have shown decreased muscle damage after exercise, improved performance, and faster recovery times (Ostaszewski et al. 2012). To attain ideal anti-catabolic effects, a period of HMB consumption prior to damage-inducing exercise has been described in humans (Wilson et al., 2013) but this timeframe has yet to be established in racehorses. Studies in rats and chicken models have shown a decrease in muscle proteolysis by 80% and increase in protein synthesis by 20%. Immune response, particularly lymphocyte blastogenesis, was also positively affected by HMB in *in vitro* studies in an ovine model and human model (Nissen and Abumrad 1997). With extensive research being done on rats and humans, HMB has been reported to inhibit the ubiquitin-proteasome system, phosphorylate the mechanistic target of rapamycin (mTOR), increase insulin-like growth factor 1 (IGF-1), improve cell membrane integrity through cholesterol synthesis, and stimulating muscle satellite cells to proliferate and differentiate (He et al. 2016; Kaczka et al. 2019; Suryawan et al. 2020).

Muscle biology and protein synthesis

Protein synthesis is achieved by phosphorylation of the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), fulfilling homeostatic and growth control roles (Saxton and Sabatini 2017; Zhang et al. 2019). Studies have reported HMB-mediated activation of mTORC1 through different mechanisms than those used by leucine. Leucine inactivates the inhibitory complex Sestrin2-GATOR2 while increasing RagA-mTOR and RagC-mTOR complex formation, whereas HMB does not interact with those mediators. These reports also confirm that

protein synthesis mediated by leucine and HMB do not participate in growth-factor-mediated mTORC1 activation pathways (Wolfson et al. 2016; Lima-Soares et al. 2017; Davis et al. 2018; Suryawan et al. 2020). Protein breakdown is hindered by inhibition of the ubiquitin proteasome system through a decrease in the expression of the 20S catalytic proteasome α subunits and down-regulation of the ubiquitin-conjugating E_{14k} enzyme (Smith et al. 2005). Expression of the p42 and MSS1 subunits of the 19S proteasome regulatory complex is attenuated by HMB in MAC16 cells (Smith et al. 2005; Tanaka 2009). Beta-hydroxy beta-methylbutyrate shows a favorable effect in the cholesterol metabolism of tissues including muscle and the immune system (Zanchi et al. 2011; Lima-Soares et al. 2017). The branched chain amino acid (BCAA) metabolite acts as a direct precursor of cholesterol in the form of beta-hydroxy beta-methylglutaryl-CoA (HMG-CoA). Cholesterol, a sterol precursor of steroid hormones, vitamin D, and bile acids, is an indispensable structural element in membranes of animal cells and can comprise up to 55% of the lipid bilayer. Here it plays essential roles including decreasing membrane fluidity, stabilization of vesicles, and fusion capabilities of a membrane, as well as intracellular transport and signaling roles. (Barrientos et al. 2017; Lee et al. 2017). Use of HMB as a substrate for intracellular synthesis of cholesterol is particularly important in tissues that rely on *de novo* synthesis of cholesterol (Nissen et al. 2000). Besides the intake and local utilization of cholesterol, muscle tissue is able to reverse its cholesterol transport and has been hypothesized to participate in cholesterol homeostasis at a systemic level (Muscat et al. 2002). Availability of HMB as substrate for cholesterol synthesis contributes to restore damaged or synthesize new cell membranes; key processes during repair of the sarcolemma after exercise or production of new mitochondria, respectively (Nissen and Abumrad 1997; Arazi et al. 2018). Mitochondrial biogenesis is influenced by HMB via peroxisome proliferator-activated receptor gamma co-

activator 1-alpha (PGC-1α), favoring the transition of fibers towards the oxidative-capable, mitochondria-rich type I myofibers (He et al. 2016). Downstream activation of the myocyte enhancer factor-2 (Mef2) and nuclear factor of activated T-cels (NFAT) genes enhances type I myofibril expression through binding to the *slow troponin 1* locus in mice (Naya et al. 2000; He et al. 2016). In supplemented individual conversion of HMG-CoA into Acetoacetyl-CoA within the mitochondria is enhanced and provides additional Acetyl-CoA by action of acetyl-CoA acetyltransferase and allows for the tricarboxylic acid cycle (TCA) to continue over extended periods of time (Nissen and Abumrad 1997). Higher concentrations of precursors for oxidative phosphorylation and energy production translates to a delay in adenosine triphosphate (ATP) depletion and the onset of acute fatigue. Insufficient supply of oxygen forces the muscle to produce energy under anaerobic conditions by utilizing glycolytic pathways in the cytoplasm.

Higher concentrations of available precursors (such as creatine, glycogen and fatty acids) for use in oxidative phosphorylation, paired with increased mitochondrial numbers, favor a delayed onset of acute fatigue and increased workload capacity (Ament and Verkerke 2009; Arazi et al. 2018). Favoring a shift in skeletal muscle fiber types towards slow, oxidative fibers (type I fibers) is beneficial in stamina-based disciplines (e.g. endurance racing), whereas a shift towards fast, fatigable, glycolytic fibers (type IIX fibers) improves performance in speed-based activities (e.g. racing, reining) (Barlow et al. 1984; Rivero et al. 1993; Rivero and Hill 2016). In type IIX fibers the cross-sectional area is greater than their type I counterparts, implying not only a different myosin heavy chain (MyHC) expression, but a higher volume of content (Paul and Rosenthal 2002). Adaptation to different exercise stimuli can manifest as myofiber remodeling with metabolic changes to tolerate longer periods of physical activity, or fiber hypertrophy to increase power output while retaining the biochemical properties of the fiber (Serrano et al.

2000). Hypertrophy of muscle tissue achieve greater strength by expanding the number of myofibrils within the sarcolemma of the myofiber. Unlike other tissues, skeletal muscle hypertrophies by expanding intracellular volume and fusion of satellite cells. Tissues comprised of mononucleated cells increase their cytoplasm-to-nucleus ratio instead (Paul and Rosenthal 2002). Muscle adaptation to increasing workloads is one of several mechanisms to prevent musculoskeletal injury. Muscle injury-regeneration events involve a cascade of intricate signaling processes including release of inflammatory cytokines and intracellular contents by damaged cells (e.g. tumor necrosis factor-α, interferon-γ, insulin-like growth factor-1, interleukin-10), gene expression and activation of transcription factors such as mTOR, MRF4, Myf5, MyoD, MYOG, MYMK, p38-MAPK, DUSP1, among others upregulation of membrane proteins (e.g. myomaker, c-MET), activation, proliferation and differentiation of SC populations, and fusion of myoblasts with damaged myofibers (Goh and Millay 2017; Siles et al. 2019).

Muscle tissue in the equine comprises up to 55% of total body weight, distributed proximally to the center of mass to aid in efficient locomotion (Valberg 2014). Mammalian skeletal is composed by an heterogenous pool of fibers, frequently classified as muscle fibers as type I, IIA, IIX or IID, or IIB alludes to the predominant physiological properties of the fiber given by their MyHC isoform content. (Serrano et al. 2000; Talbot and Maves 2016; Schiaffino 2018). Muscle fiber types are commonly categorized glycolytic and oxidative metabolic properties, or a combination of the latter two as types I, IIA, IIX/D and IIB (Snow and Guy 1976), by their speed and fatigue index as fatigue resistant or fatigable (Burke 1978), or by contraction rates as slow twitch (10-30 Hz) and fast twitch (30-70 Hz) (Feher 2012). In the equine species IIB fibers are absent from skeletal muscle (Rivero et al. 1996). Equine adult skeletal muscle predominantly expresses MyHC-7 in type I fibers, MyHC-2 in type IIA fibers, and MyHC-1 in type IIX myofibers, with the potential to co-express more than

one isoform simultaneously ("hybrid" fibers). Slow-twitch, fatigue-resistant type I fibers have lower contraction frequencies, lower power output and increased mitochondrial numbers and oxidative capacity. These fibers primarily express MyHC-7. This myosin heavy chain beta isoform makes them better suited for stamina-based activities such as endurance running, and repeated or extended isometric muscle contractions, as well as every-day maintenance of posture. Eventual fatigue of these myofibers can be central (neural or psychological) or peripheral (motor unit involved) in nature (Kawai et al. 2009). More fatigable, fast-twitch, type II fibers have opposite attributes, displaying less mitochondria and using glycolysis as its main source of energy. Expression of MyHC-1 and 2 in these fibers is abundant and not mutually exclusive. Higher percentages of type II myofibers provide muscles with increased ability for short bursts of high-powered motions, single explosive contractions such as weightlifting or sprinting (Kawai et al. 2009). Fatigue of these fibers is most commonly peripheral in nature and short lived due to a rapid energy (ATP and substrates) depletion but can also be afflicted by central fatigue (Ament and Verkerke 2009; Wan et al. 2017). Expression of MyHC-4 within type IIB fibers does not commonly occur in large mammals despite the responsible gene *MYH4* being present in the DNA sequence (Brearley et al. 2021). On rare occasions, isolated, single-fiber expression of MyHC-3 (abundantly expressed by embryonic myofibers) within equine adult muscle tissue occurs (unpublished data). In the horse, only fibers of type I, IIA, IIX, as well as intermediate hybrids, have been identified (Lindner et al. 2002; Yamano et al. 2002). Plasticity of muscle tissue allows for structural or metabolic adaptations to take place in response to external demands such as exercise programs, or reverse in cases of detraining (Serrano et al. 2000). Fiber composition will shift towards the oxidative end of the fiber spectrum (Revold et al. 2010), and transcriptome changes can be observed after a single bout of high-intensity exercise (Bryan et al. 2017). Increased numbers of oxidative-capable fibers (I, IIA, IIAX hybrids also called

IIC translates into more efficient delivery of energy (Billeter et al. 1980; Rivero et al. 1996). Higher numbers of total mitochondria and upregulation of rate-controlling genes such as NADH dehydrogenase and mitochondrial respiratory chain complex I, improve oxidative phosphorylation (Bryan et al. 2017).

Energy required for the interaction between myosin and actin in a ratchet-like contractile mechanism is provided by metabolization of ATP via aerobic oxidative (slower, efficient) or anaerobic glycolytic (faster, inefficient) pathways (McMiken 1983). Energy sources available for muscle include free ATP, creatine, molecules of lipid origin such as esterified fatty acids and triglycerides, and carbohydrates including glucose and intramuscular deposits of glycogen (McMiken 1983; Van Hoven 2006). Higher concentrations of available precursors (such as creatine, glycogen and fatty acids) for use in oxidative phosphorylation, paired with increased mitochondrial numbers, favor a delayed onset of acute fatigue and increased workload capacity (Ament and Verkerke 2009; Arazi et al. 2018). Theoretically, increases in oxidative capacity of muscle tissue would delay fatigue onset or lessen the impact of energy depletion on the onset of fatigue. Favoring a shift in skeletal muscle fiber type composition towards slow, fatigue-resistant, oxidative fibers (type I myofibers) is beneficial in stamina-based disciplines (e.g. endurance racing), whereas a shift towards fast, fatigable, glycolytic fibers (type IIX myofibers) improves performance in speed-based activities (e.g. racing, reining) and strength-based disciplines (e.g. show jumping, eventing) (Barlow et al. 1984; Rivero et al. 1993; Denoix 2013; Rivero and Hill 2016). In type IIX fibers the cross-sectional area is greater than that of their type I and IIA counterparts, implying not only a different myosin heavy chain (MyHC) expression, but also a higher total volume of content (Paul and Rosenthal 2002).

Biomechanical assessment: electromyography and kinematics

Electromyography (EMG) has been extensively used in multiple animal species before (Valentin and Zsoldos 2016). Electromyography has been used in humans to assess athletic performance, research of neuromuscular activity, and diagnosis of neurological and musculoskeletal disorders in a clinical setting. Despite some intrinsic limitations of animal research (Reaz et al. 2006), electromyography has gained traction in the muscular evaluation of different species in the last few decades (Valentin and Zsoldos 2016). Numerous variables and analyses derived from electromyographical data collected in a static or dynamic setting have been described as useful in racehorses (Takahashi et al. 2018; Williams 2018). Successive recruitment of motor units will occur until all available motor units are activated in an attempt met demands of force generation. The recruitment order follows the size principle and begins with the smaller, highly excitable motor units, progressing towards the larger motor units. Consecutive action potentials causing contractions of the muscle fiber before relaxation is allowed will ultimately cause a state of tetany and a maximal, sustained generation of force. This phenomenon is known as summation of force (Feher 2012). Direct visualization of EMG data allows for descriptive assessment of onset, duration, offset, and timing of myoelectrical activity, as well as electrode placement, noise, and artifacts if bilateral evaluation of a muscle is performed. Analysis of EMG signals often require noise removal by use of high- and low-pass filters, and subsequent rectification to obtain a positive signal that fluctuates according to the strength of motor unit action potentials. Integrated EMG (iEMG) provides a way to evaluate summation of muscle workload between different exercise timepoints. The area under the curve of the integrated EMG trace provides a "volume" of the work performed, proportionately increasing with contraction intensity (Williams 2018). Furthermore, iEMG has been employed to

determine fatigue rate in human muscle using individual motion cycles (Malinzak et al. 2001). Median frequency will increase as activity commences plateauing at approximately 60-70 Hz for the equine species indicating activation of fast fibers. As muscle fatigue progresses, fast-twitch fibers stop contracting effectively while slow-twitch fibers remain active, thus shifting the median frequency towards 30 Hz (Colborne et al. 2001a; Reaz et al. 2006; Phinyomark et al. 2012).

Kinematic evaluation describes to the motions of an individual performing an action. Determination of the positioning of an object of interest can be in a bi-dimensional or tri-dimensional space. Multiple methods for achieving this exist and include infrared motion tracking in three dimensions, bidimensional reflective videography, traditional marker-less videography, and both digital and physical goniometry (Back et al. 1997). Gathering of data for this purpose allows for vector decomposition, determining angles, velocities and acceleration of the objects of interest, namely a marker on a known anatomical structure (Buchner et al. 1994). Joint angles will tend to be consistent within the same gait and speed, while suffering alterations towards fatigue as compensatory mechanisms kick in, allowing the organism to maintain a desired speed or force demand (Edouard et al. 2018). Evaluation of these motions in contrast with comparable, known values of normality allow for interpretation of the individual's status in respect of other variables (Colborne et al. 2001b; Clayton and Hobbs 2017). Such variables can include lesions, training, supplementation, or drug effects, among other factors. Small changes in proximal areas of the body can have significant effects on distal portions, as well as distant, unrelated parts of the body (Hardeman et al. 2019; Hardeman et al. 2020).

Exercise and response to fatigue

Exercising and muscular conditioning in the horse aim at increasing force output, improving aerobic metabolic capacity, increase in glycogen muscle deposits, optimization of muscle fiber composition, structural adaptation of tissues (Van Hoven 2006; Gerard et al. 2013). Simply put; improving speed, strength, and stamina (Rivero 2007; Rogers et al. 2007). Degree and nature of muscular response to exercise will depend on two main factors: status of muscle at the beginning of training (breed, age, sex, fitness level), and type of stimulus (intensity, duration, frequency, type) (Rivero 2007). Different exercise regimens will cause distinct structural and biochemical adaptations in the muscle that will subsequently modify the physiological traits of speed, endurance, and strength, until conditioning is achieved (Serrano et al. 2000; Rivero 2007; Leisson et al. 2008). Muscle adaptation to exercise will follow two paths: increasing individual fiber size irrespective of the number of nuclei (hypertrophy) with local microvasculature remodeling (Paul and Rosenthal 2002; Rivero 2007) resulting in increased power output capacity, and metabolic transitioning with changes in mitochondrial numbers and fiber-type shifts where a decrease in muscle fatigability is primarily seen (Rivero 2007).

Muscle fatigue, commonly defined as a decrease in the force-generating capacity of muscle tissue, has been divided in central and peripheral components (Fowler 2002; Pedersen et al. 2004; Mueller-Wohlfahrt et al. 2013). Fatigue has also been previously termed anaerobic fatigue and can be subdivided in two different components, depending on the etiopathogenesis, as neurogenic and muscular (McMiken 1983; Gerard et al. 2013). Neurogenic acute fatigue depends on neurotransmitter depletion or electrolyte imbalances disrupting membrane potentials such as in endurance conditions (McMiken 1983). Muscular acute fatigue relies on depletion of local energy substrate, and local accumulation of metabolites such as lactate (McMiken 1983).

Central fatigue refers to events occurring in the central nervous system and structures located proximally to the neuromuscular junction that decrease neural drive to the muscle through different mechanisms. Conversely, peripheral fatigue involves alteration to components of the neuromuscular junction; motor axons, endplate, and innervated muscle fibers (Ament and Verkerke 2009; Wan et al. 2017). Muscle fatigue encompasses a wide array of signs that ultimately presents as the incapacity of the tissue to continue performing work at a given rate or intensity, a loss of power output, and electrical or contractile disturbances. Muscle fatigue signs in humans has been described as longer reaction times, decreased coordination and motor control precision, reduced force generation capacity, and lower performance (Kellis and Liassou 2009). Similarly, signs of muscle fatigue in horses manifest as decreased stride frequency, increased stance times, increased maximum metacarpophalangeal joint (MCPJ) extension angle, stride lengthening with increased suspension time, increased vertical range of motion, general decrease in speed, fasciculations (post activity), muscle soreness, tenderness, and reluctancy to move (Foreman 1998; Colborne et al. 2001a; Cifrek et al. 2009; Pugliese et al. 2020). If muscular work is continued, fatigue will progress to a state of exhaustion in which a stiff gait, muscle stiffness and cramping are present (Foreman 1998). Continued strenuous work beyond the homeostatic capabilities of an individual can progress into moderate to severe multisystemic and hemodynamic disturbances, as well as onset of exertional heat illness (Brownlow and Mizzi 2021). Thermal stress originated from internal sources (e.g. muscle contractions, metabolism) or external sources (e.g. ambient temperature, excessive insulation) hasten fatigue onset and can lead to early exhaustion by reaching critical core temperatures of ≥40° Celsius (Brownlow and Mizzi 2020). Once this threshold is crossed, a drastic drop in performance can be observed as a safety mechanism to prevent homeostatic failure (Schlader et al. 2011). Blood circulation plays a

major role in heat dissipation originating from non-superficial tissues such as organs, muscle, and brain (Hodgson et al. 1993; Brownlow and Mizzi 2020). Increased skin vascular demand for thermoregulating competes with muscle tissue demands for a finite cardiac output, fostering dyshomeostasis (Brownlow and Mizzi 2021). Heat production above the body's dissipation capabilities rapidly increases core temperature and decreases the cardiovascular system's efficiency creating a negative feedback loop (Ament and Verkerke 2009; Schlader et al. 2011). Decreased blood flow to peripheral tissues, including muscle and central nervous system, further favor glycolytic process and central thermoregulatory dysregulation, respectively (Krogh 1919; Ament and Verkerke 2009). The central nervous system in mammals is particularly susceptible to hyperthermia (Hodgson et al. 1993). Hyperthermia-induced alterations to the nervous system in humans will manifest, in a continuum, as fatigue, weakness, altered behavior, disorientation, encephalopathy, coma, and ultimately, death (Brownlow and Mizzi 2021). Specialized anatomical structures to relieve CNS hyperthermia have been evolved by some prey species (e.g. carotid rete in artiodactyls), but such anatomical adaptations have yet to be confirmed in the equine (Brownlow and Mizzi 2020).

Energy metabolism

L-Lactate is the levorotatory isomer of lactic acid and an intermediate reservoir supplying pyruvate needed in oxidative phosphorylation. Synthesis of lactate occurs constantly in aerobic and anaerobic conditions, but its production is enhanced in the absence of oxygen. Elevated lactate concentrations can have different significances and meanings, based on concentrations and physiological context, increasing proportionately to a workload (Munsters et al. 2014). In anaerobiosis the TCA and oxidative phosphorylation are halted. Glycolysis then takes over as a fast but inefficient process for supplying energy via lactate dehydrogenase activity by causing an increase in NAD+ through the oxidation of NADH, allowing for the respiratory chain to continue, although at a lower efficiency rate (Harris and Snow 1988; Rogatzki et al. 2015; Sun et al. 2017; Ferguson et al. 2018; Brooks 2020). This reaction takes place in multiple tissues including blood, brain, muscle tissue with the purpose of supplying substrate for the respiratory chain (Brooks 2020). Lactate is continuously recycled into ATP, CO_2 and H_2O under aerobic conditions whereas it accumulates locally and systemically under anaerobic conditions (Brooks 2020). Increases in lactate concentrations cause a momentary metabolic acidosis by increased hydrogen concentration that is buffered by bicarbonate ions (Brooks 2020). In higher concentrations, lactate diffuse to the interstitial space and will be transported via systemic circulation to the liver where it will be converted to pyruvate and then to glucose following Cori's cycle. This conversion allows the muscles to utilize glucose and partially replenish ATP allowing for further muscle activity (McMiken 1983). Once the tipping point between respiration and glycolysis (the lactate threshold) is crossed, hydrogen ion concentrations increase, lowering the pH as a result. This acidosis is dampened by buffering hydrogen ions with bicarbonate and generating carbonic acid that exothermically dissociates into carbon dioxide and water. Excess

CO_2 is exhaled during breathing, process relying on adequate lung perfusion and ventilation to achieve maximal efficiency (Ament and Verkerke 2009; Ferguson et al. 2018). Lactate fulfills its metabolic purposes as a precursor of gluconeogenesis and as source of energy for mitochondrial oxidation by means of the intracellular lactate shuttle, as well as a signaling purpose by acting as an autocrine, paracrine, and endocrine molecule. High concentrations of lactate prompt a surge in reactive oxygen species inside the cells, an increased expression of angiogenic factors, and synthesis of sirtuins (Sir2 family) responsible for cellular homeostasis involved in mitochondrial function regulation. Lactate-mediated modulation of mitochondrial biogenesis occurs as an adaptive response to energy deficient conditions. This mechanism is paradoxically responsible for suppressing adipose lipolysis by binding to the hydroxycarboxylic acid receptor 1 (HCAR-1) G-protein, further impairing energy supply. Lactate is presumed to have an epigenetic influence on the genome by lactylation of lysine residues on histones, thus modifying gene expression and protein synthesis (He et al. 2016; Brooks 2020).

STUDY HYPOTHESIS

The hypothesis of this study was that supplementation of a balanced diet with calcium beta-hydroxy beta-methylbutyrate monohydrate would increase a horse's athletic performance by delaying the onset of fatigue and elicit a myofiber type shift increasing the percentages of oxidative fibers and expression of their associated myosin heavy chain (MyHC) isoforms.

CHAPTER II

Beta-hydroxy beta-methylbutyrate (HMB) supplementation to adult Thoroughbred horses increases type IIA fiber content in the gluteus medius without direct improvement of muscle performance during intense high-speed treadmill exercise

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ABSTRACT

Consumption of β-hydroxy β-methylbutyrate (HBM), a leucine metabolite, alters muscle composition and metabolism leading to strength and agility improvements in human athletes. To determine if HMB affects athletic performance and muscle function in horses, Thoroughbred geldings were fed a control (CON; n=5) or HMB (n=6) supplement (30 mg/kg/day) for 6 weeks prior to completing a standardized exercise test (SET). Gluteus medius (GM) muscle samples were obtained before the SET for fiber-typing and blood was collected before and immediately upon completion of the SET for lactate measurements. Heart rate (HR), biceps femoris (BF) and semitendinosus (ST) surface electromyograms, and fore- and hindlimb metacarpophalangeal joint angles were captured for the duration of the SET. Results demonstrate that HMB supplementation increased ($P < 0.05$) the percentage of type IIA muscle fibers in the GM with a corresponding decrease ($P < 0.05$) in type IIX fibers. The percentage of type I fibers was unaffected by diet. Supplementation with HMB did not result in any measurable effects on performance, muscle function or biomechanical properties by comparison to CON. Increasing treadmill speed resulted in an increase ($P < 0.05$) in stride length and maximal extension angle of the fore fetlock, and a shortening ($P < 0.05$) of the stance phase of the gait cycle. Integrated EMG (iEMG) increased ($P < 0.05$) with increasing treadmill speeds for both the BF and ST, with the BF exhibiting greater iEMG than the ST. In summary, HMB increased the percentage of type IIA fibers which did not translate into improved athletic performance.

INTRODUCTION

Beta-hydroxy β-methylbutyrate (HMB), alternatively referred to as hydroxymethylbutyrate, is a metabolite of leucine used by athletes as an ergogenic aid. Oral supplementation strategies in conjunction with resistance training or a combination of skill and strength training results in an increase in lean body mass (LBM), power (watts) and speed in adult men (Wilson et al. 2014; Durkalec-Michalski et al. 2017). The gains in LBM correlate with a reduction in skeletal muscle protein degradation, and a modest increase in muscle protein synthesis (Nissen et al. 1996; Wilkinson et al. 2018). The protein accretion effects are duplicated in pigs and rats consuming HMB (He et al. 2016). Improved body composition in response to HMB also is linked to altered systemic hormone and cytokine profiles. Minor increases in growth hormone occurs in rats fed HMB and improved insulin kinetics are observed in both rodents and humans in response to the diet supplement (Kaczka et al. 2019). Pigs administered HMB show improved LBM coincident with greater concentrations of serum interleukin-15 and fibroblast growth factor-21, both myokines involved in fat metabolism and decreased fat deposition (He et al. 2016; Duan, Li, et al. 2018). Thus, the means by which HMB positively affects muscle form and function is multifaceted and includes protein turnover and enhanced metabolism.

High intensity exercise, such as that experienced by racehorses, results in fatigue that can lead to injuries and lost training time (Boston and Nunamaker 2000). An improved capability to identify the early onset of muscle fatigue may limit tissue damage, thereby allowing for the design of athlete-specific training and conditioning programs. Biomechanical gait analysis of horses working on a high-speed treadmill reveals an increase in stride length, decrease in stance time and an increase in fetlock joint angle of (Corley and Goodship 1994; Wickler et al. 2006;

Cruz et al. 2018). The general changes in kinematic parameters are duplicated by horses working on dirt racetracks with a decrease in stride length occurring with fatigue (Morrice-West et al. 2020; Takahashi et al. 2021). Changes in biomechanical properties of the limbs are reflective of increasing surface electromyography (sEMG) activity with speed and the resulting decline in muscle activation as fatigue develops (Colborne et al. 2001a; Robert et al. 2001; St. George et al. 2019). However, sEMG activities vary with a muscle's location in the body and its role in propulsion or skeletal stabilization (Takahashi et al. 2018; Takahashi et al. 2020).

The hypothesis that HMB improves skeletal muscle oxidative metabolism leading to fatigue resistance and improved kinematic parameters was tested in adult, unfit Thoroughbred geldings. Our results reveal that HMB supplementation can increase the percentage of type IIA muscle fibers in the gluteus medius, but this does not translate into detectable changes in hindlimb muscle activation or performance.

MATERIALS AND METHODS

Diet and Husbandry

All horse protocols were reviewed and approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use committee. Twelve Thoroughbred geldings (7.3 ± 0.8 yrs and 527.2 ± 6.9 kg BW) were deemed sound and clinically healthy before initiating activity. The horses were housed in dry lot pens in groups of three, with ad libitum access to water and mineral supplements. Horses were individually fed for 42 d a commercial grain product at 0.25% of BW in two meals daily with a topdress control (CON; 1.8 g L-glutamine, 350 mg vitamin C, 104 I.U. vitamin E, Platinum Performance, Buellton, CA; n=5) or treatment (HMB; CON mix containing 12.5 g Ca-HMB, Platinum Performance; n=6) supplement. Hay was provided at 1.75% of BW to each pen (Table 2-1). Diets met the energy requirements for adult, sedentary horses. Horses were rotated through each pen (n=4) at 10-day intervals. One horse in the CON group failed to complete the feeding trial due to complications unrelated to the experiment.

Exercise Protocol

Horses were fitted with a heart rate (HR) monitor (V800, Polar, Bethpage, NY) prior to exercise on a highspeed treadmill (EquiGym, Paris, KY). Each horse performed an incremental standardized exercise test (SET) until fatigue was reached (Allen et al. 2016). The SET consisted of 2 m/s for 3 min, 4 m/s for 4 min at a three-degree incline, 6 m/s for 1 min, 8 m/s for 1 min, and 10 m/s for 1 min. Incremental 1 m/s/min steps were then applied until fatigue ensued. Fatigue was defined as the moment when the horse could no longer maintain its position on the treadmill in response to moderate encouragement.

Sample Retrieval

Blood was obtained by venipuncture into sodium fluoride / potassium oxalate-containing grey-top tubes (BD Vacutainer, Franklin Lakes, NJ) from the jugular vein alternating between sides to decrease risk of phlebitis. Collection times included pre-exercise, and at 0 min, 20 min, 60 min and 24 h post-exercise. Plasma was isolated by centrifugation at 1,500 x g for 10 min at 4°C and stored at -80°C until further analysis. Plasma lactate concentration was determined colorimetrically (Lactate Assay Kit, Sigma Aldrich, St. Louis, MO).

Muscle biopsies were obtained from the gluteus medius (GM) muscle in the standing, sedated horse (Xylazine 0.8 mg/kg, VetOne, Boise, ID). Location for percutaneous access to the ventral compartment of the GM was identified by tracing an imaginary line between the tuber coxae and sacrococcygeal joint, and establishing the site between the first third and the center point of this line (Wagner et al. 2013). Retrieval of biopsies was performed using a vacuum assisted biopsy system equipped with 10 G x 100 mm biopsy needles (BD EleVation Breast Biopsy System, BD, Franklin Lakes, NJ) through a 10-mm-wide stab incision after aseptic preparation of the area. Muscle destined for molecular analysis was wrapped in aluminum foil and snap-frozen in liquid nitrogen. Biopsies destined for histological analysis were embedded in optimal cutting temperature media (Tissue-Plus O.C.T. Compound, Fisher Scientific, Waltham, MA) and frozen in a controlled manner using liquid nitrogen over 45 seconds to prevent sample disruption and fracturing of the OCT block. Muscle biopsies were cut into 10 µm sections using a cryostat and mounted on glass slides (SuperFrost Plus, Fisherbrand, Pittsburgh, PA), allowed to dry for 30 min, and stored at -80°C.

Electromyography

A 1-cm^2 area near the middle portion of each biceps femoris (BF) muscle and semitendinosus (ST) muscle was clipped and shaved to the skin, then fitted with two 50mm flexible cloth ECG surface electrodes (Nissha Medical Technologies, Buffalo, NY) applied with cyanoacrylate glue (Gorilla Glue Company, Cincinnati, OH). A small blob of conductive gel was placed under the sensor to improve electrode contact and data capture. Electrodes were aligned with the muscle fibers leaving an inter-electrode distance of approximately 50 mm. Electromyographic activity was recorded for the entirety of the trial for each individual. Electromyographic data was processed using SEMG Analyzer software suite (BTS Bioengineering Corporation, Quincy, MA) using 3rd order Butterworth low- and high-pass filters set at 450 Hz and 40 Hz, respectively (St. George et al. 2018).

Immunohistochemistry

Frozen slides were allowed to equalize at room temperature and washed in PBS three times for 5 min each. Fixation of samples was performed using pre-chilled 1:1 methanol and acetone solution at -20°C for 15 min. Hydrophobic rings were drawn around each muscle sample and the slides were further rinsed in PBS again using the same procedure. Incubation of cryosections with anti-MyHC I (1:200 dilution, BA-D5, Developmental Studies Hybridoma Bank, Iowa City, IA), and anti-MyHC IIA (1:100 dilution, A4.74, Developmental Studies Hybridoma Bank) antibodies for 60 min in a humidified chamber at room temperature. Antibodies were diluted in 1% horse serum blocking solution. Secondary antibody mix contained goat anti-mouse Alexa Fluor 488 IgG_1 (1:1,000, Thermo Fisher, Waltham, WA) and goat anti-mouse Alexa Fluor 555 IgG_2b (1:1,000, Thermo Fisher) antibodies, in addition to 4′,6-diamidino-2-phenylindole (10 µg/mL, Thermo Fisher), and wheat germ agglutinin Alexa Fluor 647 conjugate (2 µg/mL, Thermo Fisher). After 30 min of secondary incubation, slides were rinsed in PBS, coverslipped in 30% glycerol in PBS (v/v) and sealed with clear nail polish. Fiber identification and image acquisition of immune complexes was performed using an inverted epifluorescent microscope (Ti Eclipse, Nikon, Melville, NY) with shutter speed controlled by NIS Elements software (Nikon, Melville, NY).

Kinematic Evaluation

A set of eight 2-cm polystyrene hemispherical retroreflective markers were placed on the center of rotation of joints in the sagittal plane of the distal, and proximal fore- and hindlimbs as described elsewhere (Leach and Dyson 1988). Footage of the entirety of the trial was recorded using a Quintic High-Speed LIVE camera (Quintic Consultancy Ltd, Birmingham, UK) with a f/1.2, 8-48 mm zoom lens (Computar H6Z0812, CBC America, Cary, NC) at a capture rate of 150 frames per second. Quantitative biomechanical data was collected by sampling consecutive strides using Quintic Biomechanics Video Analysis Software (v.31, Quintic Consultancy Ltd., Birmingham, UK). For each speed step, 10 s of footage was isolated for individual analysis. Five consecutive strides were assessed for angular and linear measurements. Datasheets for each parameter were exported from the EMG analysis software and compiled using Microsoft Excel (Office 365, Microsoft Corporation, Redmond, WA).

Statistical Analysis

Biomechanical and iEMG data were analyzed as a mixed model, 2-way ANOVA with speed and diet as fixed effects. Sidak's multiple comparisons with a pooled variance was performed with significance established at $P < 0.05$. Median frequency (MdF) was analyzed in a similar manner with physiological endpoint and muscle as the fixed effects. Simple linear regression was performed using root mean square (RMS) of the EMG voltage. Slopes were considered different at $P < 0.05$. Body weight, V200 (speed at which HR reaches 200 beats per minute (bpm)), time to exhaustion (TTE), blood lactate and fiber types were analyzed by two-tailed, unpaired student T-test assuming Gaussian distribution and equal standard deviations. Significance was established at $P < 0.05$. In all instances, a trend was defined as $0.05 \leq P < 0.10$.

RESULTS

Physiological parameters were examined before and after a standardized exercise test (SET) session to exhaustion. Supplementation with HMB did not affect either bodyweight, blood lactate concentration or V200 (Table 2-2). As expected, maximal HR remained unchanged (Table 2-2).

The effects of HMB on muscle composition were evaluated by conventional fiber typing using antibodies specific for myosin heavy chain (MyHC) isoforms I and IIA (Fig. 2-1A). Enumeration of the immunostained type I fibers demonstrated no differences (Fig. 2-1B). An increase ($P < 0.05$) in percentage of type IIA fibers with a concomitant decrease ($P < 0.05$) in the percentage of type IIX fibers was noted in response to HMB (Fig. 2-1 C–D). Cross-sectional area of the various fiber types was unaffected by diet (Fig. 2-1E).

The impact of altered IIA:IIX myofiber content was assessed by surface EMG of the biceps femoris (BF) and semitendinosus (ST) muscles during performance of the SET. The root mean square (RMS) of the average EMG amplitude was linear regressed from 2 m/s through 11 m/s, the average V200 and predicted maximal oxygen consumption (VO_{2max}), to compare muscle activation in response to increasing speed (Fig. 2-2). Results demonstrate no effect of supplement on rate of muscle activation for either the left BF or ST (Figs. 2-2A,C). The right BF of horses supplemented with HMB tended ($P = 0.06$) to reach maximal muscle activation at a lesser workload (speed) than those receiving the CON (Fig. 2-2B). By contrast, the right ST of HMB supplemented horses tended ($P = 0.07$) to reach maximal muscle activation sooner than CON (Fig. 2-2D).

Median frequency, the frequency wherein the EMG wavelet is bisected equally, was recorded for the right BF and ST during the SET. Although the BF MdF was greater ($P < 0.05$) than the corresponding value in the ST, no effect of diet was noted at either 11 m/s or upon completion of the SET (Fig. 2-2). Integrated EMG is the area under the curve of the rectified EMG signal expressed in voltage per unit of time. As workload and muscle activation increased during the SET, amplitude of the EMG wavelet increased resulting in greater ($P < 0.05$) iEMG values (Table 2-3). No effect of HMB supplementation was evident on these values. There was no difference between right side and left side BF or ST activation. Integrated EMG values for the BF and ST differed ($P < 0.05$) from one another at speeds greater than 2 m/s (Fig. 2-3).

To determine if HMB affects biomechanical properties as a horse nears fatigue, gait and fetlock goniometric parameters were measured during the SET. Results reveal no effect of HMB on any aspect of the gait cycle or fetlock extension (Table 2-4). With increasing speed, stride length increased ($P < 0.05$) with a reduction ($P < 0.05$) in time spent in the stance phase (Table 2-4). The extension angle of the loaded front fetlock increased ($P < 0.05$) with speed, while no differences were detected during maximal extension of the rear fetlock during the stance phase of the gait cycle (Table 2-4).

DISCUSSION

Beta-hydroxy β-methylbutyrate may act as a nutrient repartitioning agent due to its ability to redirect fatty acids away from storage depots to the mammary gland (Nissen et al. 1994; Flummer and Theil 2012) and skeletal muscle (Duan, Zhang, et al. 2018; Zhong et al. 2019). A greater supply of fatty acids to muscle can stimulate mitochondria biogenesis and boost oxidative phosphorylation, key factors in determining fiber type (Garcia-Roves et al. 2007). Piglets fed HMB demonstrate an increase in *MyHC IIb* mRNA, total fast MyHC and increased type II fiber cross-sectional area (Wan et al. 2016). In a similar manner, mice supplemented with the leucine metabolite exhibited a reduction in *MyHC I* and *IIA* mRNA and an increase in *MyHC IIB* mRNA that translated into a greater amount of total fast MyHC proteins (Zhang et al. 2020). By contrast, men ingesting HMB exhibited a reduction in the percentage of type IIX fibers in the vastus lateralis with a tendency for an increase in the percentage of the more oxidative type IIA fibers (Jakubowski et al. 2019). Our results more closely reflect those in young men with a shift away from the type IIX fiber toward a type IIA fiber. It is interesting to note that in rodents and pigs, species where *MyHC IIb* is expressed in locomotive muscles, HMB supports a transition toward a more glycolytic fiber type while in species that do not express the *IIB* gene (humans, horses) appears to act in an opposite manner. This divergent responses may be related to evolution of modes of ambulation, role as predator or prey in foraging interactions, or purely coincidental as a limitation of the current study is the absence of supporting mitochondria number and enzymatic activities (citrate synthetase, succinate dehydrogenase) pointing conclusively towards improved oxidative phosphorylation driven ATP generation.

A greater percentage of oxidative muscle fibers is associated with improved athletic performance in horses (Rivero and Hill 2016). Although horses fed HMB contained more type IIA fibers in the GM, this did not translate to improved V200 during the SET. The absence of a measurable effect may be related to the use of unfit geldings. Fit men participating in an integrated strength and endurance training program demonstrated an increase in anaerobic power that was associated with a tendency for improved VO_{2max} (Durkalec-Michalski et al. 2017). The modest performance gains in men may be secondary effects related to reduced muscle damage and improved recovery times (Gepner et al. 2018). A recent meta-analysis supports a role for HMB in lowering blood creatine kinase and lactate following a bout of exercise (Rahimi et al. 2018). Similar to humans, Thoroughbred racehorses in training exhibited lower post-exercise blood lactate and creatine kinase in response to HMB co-supplementation (Ostaszewski et al. 2012). No changes in post-exercise blood lactate in the horses receiving HMB herein reflects a difference in exercise parameters. Blood lactate immediately post-exercise were greater than 10 mM which are strikingly different than the 4 mM values reported by others (Ostaszewski et al. 2012) and point to a more intense bout of work. Future efforts using fit horses may help unravel the value of HMB to a training program.

Recognizing the early stages of fatigue would allow to limit tissue damage due to overtraining and exertional exercise. Surface EMG provides insight into the neuromuscular activities controlling contraction and muscle function. It may represent a less invasive and more precise method of detecting early onset fatigue. The tool does come with some limitations that include loss of skin contact leading to signal artifacts, complete dislodgement of the device from the moving animal, and subcutaneous fat interference (Williams 2018). From a physiological perspective, the value of sEMG to detect fatigue has been questioned owing to the multifaceted

nature of the condition and confounding factors (electrolyte imbalance, neural drive, etc.) affecting myoelectric activity (Vigotsky et al. 2018). The horses performed exercise to exhaustion leaving no doubt that fatigue had been reached thus, diminishing the value of sEMG activity upon completion of the SET. Our objective was to detect changes in sEMG prior to exhaustion that may be a sign of impending fatigue. Median EMG frequencies for both the ST and BF align with those reported by others (Takahashi et al. 2018; Takahashi et al. 2020) and remained stable over the course of the SET, arguing that muscle activation and proportion of recruited fibers was largely unaffected. Thus, it was not unexpected that the RMS slopes for CON and HMB BF and ST muscles were similar leading up to V200. Comparison of the EMG amplitudes at each of the SET's predefined speed steps further supports no deficits in muscle activation. It is noteworthy, however, that the BF iEMG at the various speeds is nearly double than that of the ST. The BF, ST and semimembranosus muscle form the hamstring and work cooperatively to provide propulsion to the horse. Given the ST and BF both contain 85-90% type II muscle fibers (Kawai et al. 2009), the difference in iEMG may be attributed to larger motor units found in the BF which is responsible for greater power output. Collectively, our results indicate that noticeable altered muscle activity, as measured with sEMG, did not occur immediately prior to fatigue.

Biomechanical gait assessment during exercise offers a non-invasive means of gauging performance-related declines. Consistent with previous reports, an increase in stride length, decrease in stance phase time and increased fetlock joint angles were found with increasing treadmill speed (Corley and Goodship 1994; Muñoz et al. 1997; Johnston et al. 1999). Variations in these parameters can correlate with fatigue. Increased extension at the fetlock during stance phase of the gait cycle suggests a decreased force-output capacity in the flexor muscles of the

digit. Fatigued muscles are incapable of performing the eccentric contractions necessary to counteract the load placed on the hoof during this phase of the step, thus causing overextension of the distal limb (Thorpe, Clegg, et al. 2010) Components such as the accessory ligaments of the digital flexor tendons (proximal and distal check ligaments) protect tendons and muscles from overextension of the digit by absorbing excessive loading. Risk of injury increases when these structures are subjected to a single load exceeding their physiologic capacity (ultimate tensile strength), or when repetitive, less forceful overloading events occur causing cumulative fibril microdamage (Thorpe, Stark, et al. 2010)

Supplementation with HMB did not alter any kinematic parameter during the SET corroborating the EMG and blood biochemistry results that the metabolite offers no performance gains under these experimental conditions. There was a tendency for fetlock angles to have a diet and speed interaction effect. This result, however, should be viewed with caution as the horses had prior racing careers that may have caused structural conditioning of ligaments and tendons, thus showing subtle differences in fetlock angles.

In conclusion, HMB supplementation to adult, unfit horses results in an increase in type IIA fibers at the expense of type IIX fibers. The gain in fast twitch, fatigue-resistant fibers does not directly translate into biomechanical or athletic performance improvements in the absence of exercise or ancillary stimuli.

ACKNOWLEDGEMENTS

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TABLES AND FIGURES

Table 2-1. Chemical composition of feedstuffs, dry matter basis.

Component	Concentrate	Hay
Dry Matter, %	91.4	92.6
Crude protein, %	18.3	15.7
Acid detergent fiber, %	25.3	38.4
Neutral detergent fiber, %	40.6	62.6
Starch, %	8.6	0.7
Fat, %	12.4	3.4
Ash, %	7.7	6.5
Calcium, ppm	12,190	4,200
Copper, ppm	226	9
Iron, ppm	346	116
Magnesium, ppm	4,890	2,330
Manganese, ppm	217	90
Phosphorus, ppm	8,710	4,600
Potassium, ppm	14,240	33,100
Sodium, ppm	2.235	60
Zinc, ppm	226	27
Calculated DE, Mcal/kg DM	3.02	2.08

Table 2-2. Effects of CON and HMB supplementation[1] to adult horses for 42 d on performance parameters[2].

	CON	HMB
Bodyweight, kg	531.6 ± 3.7	530.3 ± 3.2
Blood lactate post-exercise, mM	14.1 ± 1.4	14.0 ± 1.4
HR_{max}, bpm[3]	208.0 ± 6.0	209.0 ± 7.0
V200, m/s	10.7 ± 0.3	11.3 ± 0.2
Time to exhaustion, min	12.1 ± 0.3	12.4 ± 0.3

[1] Horses were fed a CON supplement (3.5 g L-glutamine, 700 mg vitamin C, 209 I.U. vitamin E) or CON supplement containing 15 g of β-hydroxy β-methylbutyrate (HMB).

[2] Means and SEMs presented.

[3] bpm, beats per minute.

Table 2-3. Integrated EMG values (mean ± SEM) for the right (R) and left (L) biceps femoris (BF) and (ST) of adult Thoroughbred geldings performing an incremental exercise test[1].

Speed, m/s	RBF, µV/s	LBF, µV/s	RST, µV/s	LST, µV/s
2	0.906 ± 0.035	0.732 ± 0.032	0.254 ± 0.011	0.248 ± 0.004
4	5.188 ± 0.171	4.561 ± 0.092	1.945 ± 0.101	1.386 ± 0.038
6	9.336 ± 0.332	8.368 ± 0.135	3.636 ± 0.083	2.686 ± 0.005
8	12.311 ± 0.780	10.773 ± 0.170	**4.890 ± 0.194**	3.985 ± 0.026
10	15.081 ± 0.859	13.470 ± 0.039	**5.905 ± 0.343**	5.716 ± 0.042
11	18.394 ± 1.327	17.176 ± 0.440	8.109 ± 0.166	7.661 ± 0.128
12	21.821 ± 1.806	20.364 ± 0.690	10.641 ± 0.486	9.626 ± 0.140
13	24.497 ± 1.973	24.332 ± 0.534	12.676 ± 0.781	11.939 ± 0.941

[1] All values within a column differ from one another at $P < 0.05$ except those in bold.

Table 2-4. Biomechanical properties of adult horses supplemented with HMB for 42 d performing an incremental exercise test[1].

	Control, m/s				HMB, m/s				SEM[3]	*P*-value[2]		
	10	11	12	13	10	11	12	13		D	S	D x S
Stride length, m	5.50	5.66	5.95	6.29	5.45	5.53	6.01	6.41	0.22	0.926	0.006	0.903
Stance, msec	127.00	119.01	111.90	106.57	126.93	118.05	111.46	106.02	2.91	0.799	<0.001	0.946
Swing, msec	400.54	390.77	388.11	392.44	387.44	389.29	387.66	384.67	13.61	0.806	0.179	0.079
FF stance[4], deg	244.98	245.06	247.51	248.73	242.95	245.44	246.34	245.93	3.48	0.921	0.003	0.078
RF stance[4], deg	247.19	247.71	248.17	248.83	246.60	248.39	248.80	249.36	3.33	0.875	0.125	0.179

[1] Adult geldings were supplemented with Control (CON, 3.5 g L-glutamine, 700 mg vitamin C, 209 I.U. vitamin E) or CON containing 15 grams of β-hydroxy β-methylbutyrate (HMB) daily for 42 d, followed by an incremental exercise test to exhaustion.

[2] D, diet; S, speed; D x S, diet by speed interaction.

[3] Largest SEM presented.

[4] Maximal posterior angle of right front fetlock (FF) or right rear fetlock (RF).

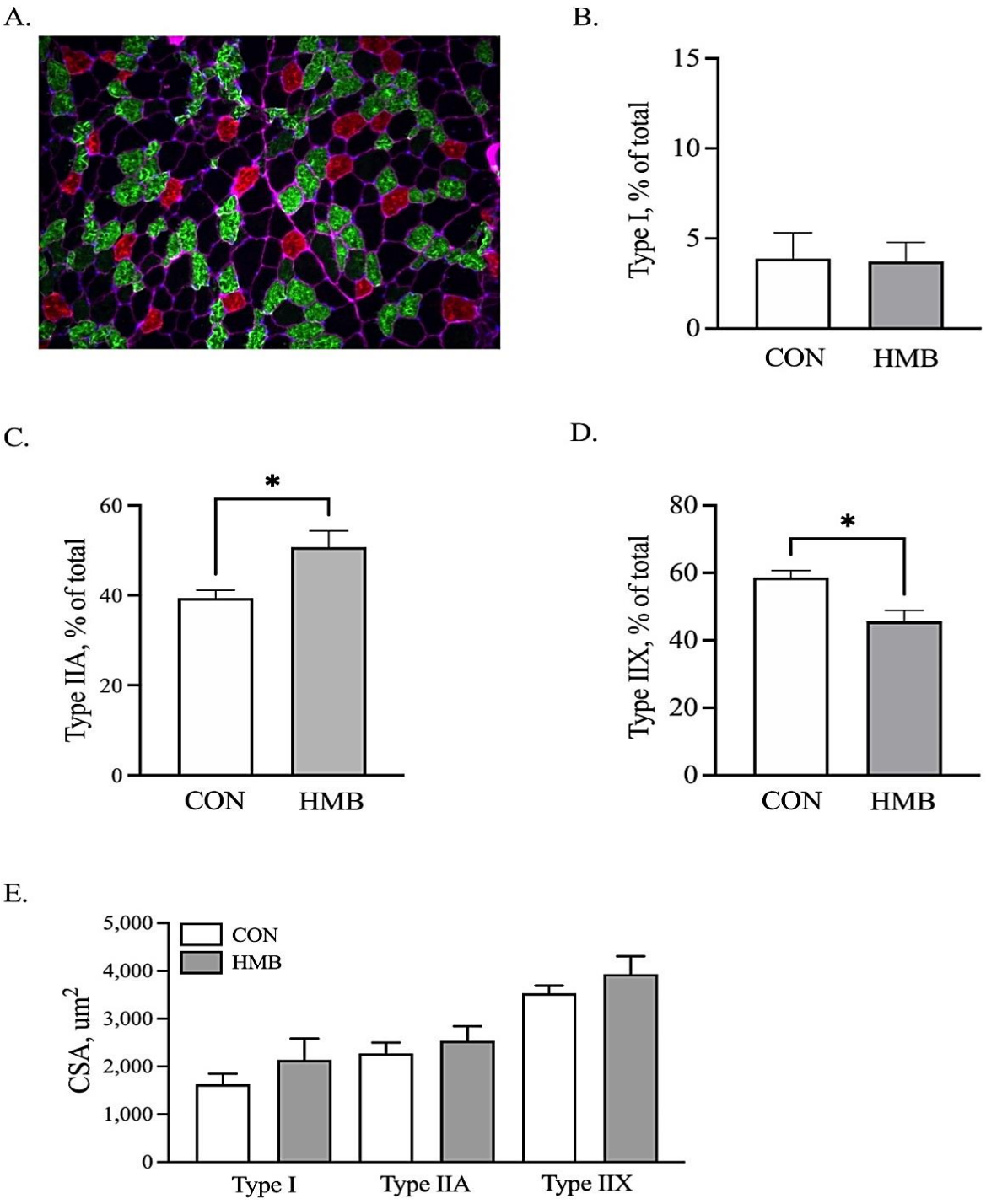

Figure 2-1. Immunofluorescence results of gluteus medius muscle biopsies. (**A**) Composite, false-colored, multilayer image of a gluteus medius immunostained 10 µm section. Red: stained type I myofibers (BA-D5 antibody). Green: stained type IIA fibers (A4.74 antibody). Black: unstained type IIX myofibers. Fuchsia: myofiber membrane (WGA). Blue: not visible, cell nuclei (DAPI). (**B**) Type I fibers, percental averages. (**C**) Type IIA fibers, percentual averages. (**D**) Type IIX fibers, percentual averages. (**E**) Cross sectional area per fiber type per group, mm^2.

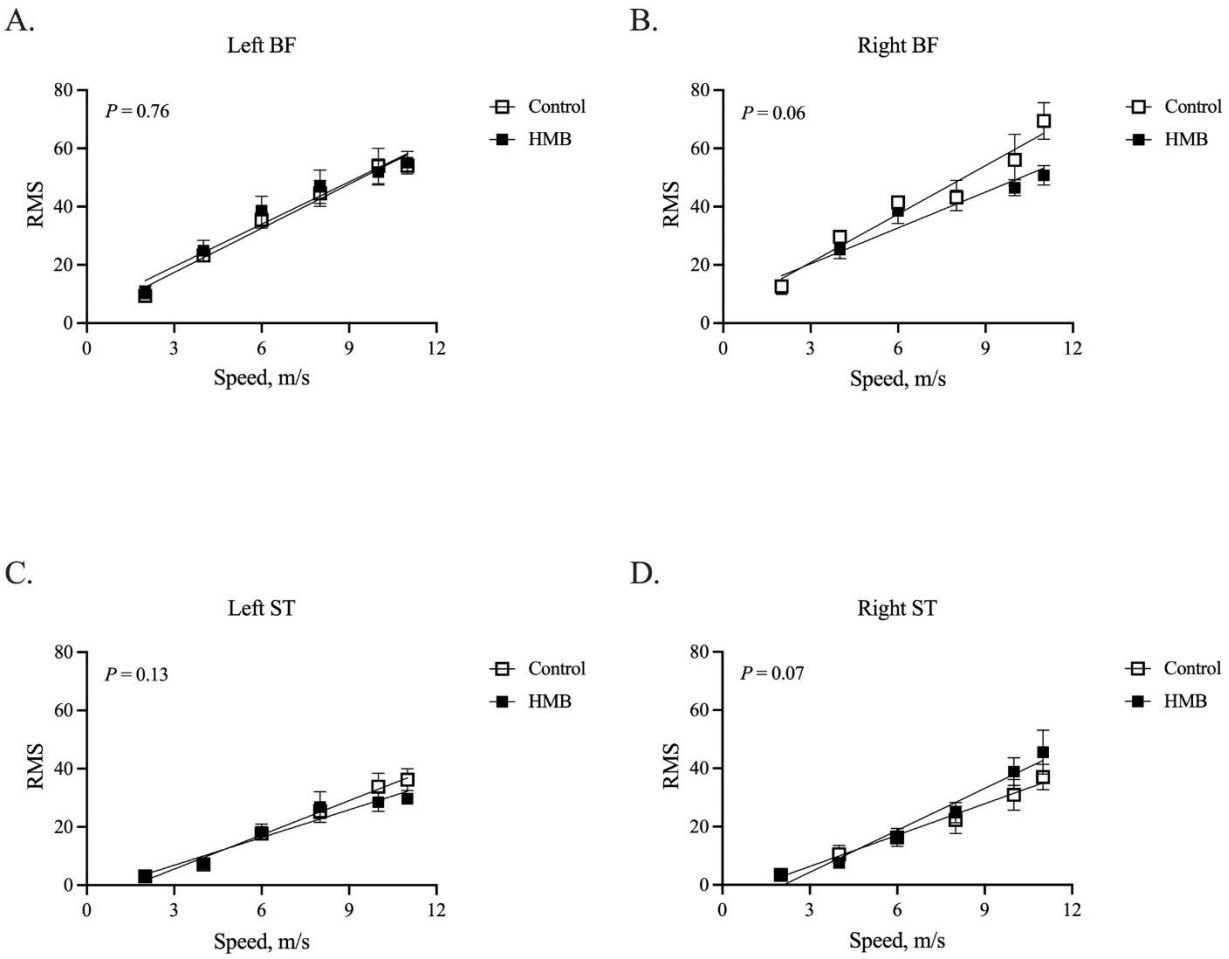

Figure 2-2. Average RMS values at different speeds for two propulsive muscle groups of the hindlimb. Averages per group shown, significance set at $P < 0.05$. (**A**) and (**B**) Biceps femoris muscle (BF). (**C**) and (**D**) semitendinosus muscle (ST).

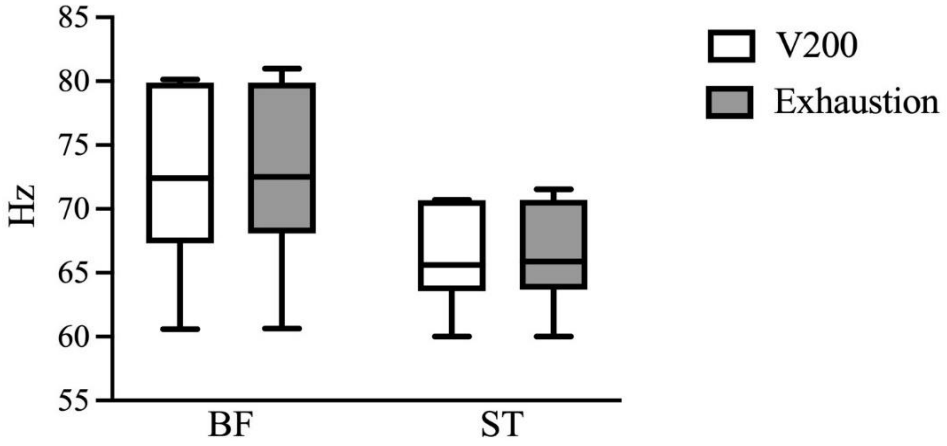

Figure 2-3. Median frequency (MdF) values for biceps femoris and semitendinosus muscles. Speed of 200 bpm of heart rate (V_{200}) and speed at exhaustion are shown in white and grey, respectively.

CHAPTER III

Study implications and future research opportunities

Results of the present report evaluating HMB supplementation in adult, unfit Thoroughbred horses suggest potential ergogenic effects under these incremental exercise conditions. Contrary to our hypothesis, HMB supplementation did not have a substantial effect on the athletic performance of the study population. To the author's best knowledge, this is the first report of myofiber-type shift from type IIX myofibers to type IIA fibers in response to HMB supplementation. Beta-hydroxy beta-methylbutyrate, metabolite of the BCAA leucine, was found to have a statistically significative effect on myofiber composition of the GM, but no effect on post-exercise blood lactate concentrations, V200, or time to exhaustion (TTE). The goal of an ergogenic compound is to safely improve the functional capacity of a work-limiting tissue. Previously described dosages of HMB and frequency of administration to horses (30 mg/kg BM/day, split in two equal servings) used by Ostaszewski and collaborators (2012) slightly differ from the optimal recommended posology for humans of 38 mg/kg BM/day, split in three equal servings (Wilson et al. 2013; Lima-Soares et al. 2017). Both of these differ as well as from those reported in other species including rodents, swine, bovine, ovine, and poultry (Szcześniak et al. 2015). Despite numerous reports of varying efficacy rates, the use of HMB for its anticatabolic properties on protein metabolism has not been limited to athletic environments, but also to human patient care during recovery from musculoskeletal injury, chronic wasting conditions, sarcopenia, and cancer. With this in mind, parallel applications in animal care and husbandry offer potentially interesting research opportunities.

Proteins have heterogenous synthesis rates (Balagopal et al. 1997), possibly explaining why fiber transdifferentiation happens in a gradual, progressive manner. Exercise-stimulated myofibers will increase in size to meet force demands, provided intervals between training sessions allow for recovery and overcompensation to take place (Rivero 2007; Rogers et al. 2007). If no concurrent stimulation (e.g. exercise) happens parallel to HMB supplementation or other ergogenic stimuli, expression of different MyHC isoforms might replace previous myosin chains in the contractile apparatus without increasing the total number of sarcomeres per fiber (Rivero et al. 2007; Schiaffino et al. 2015). This gradual replacement of sarcomeric components, similar to that reported by Schiaffino and collaborators (2015), can explain the heterogeneous populations of hybrid fibers such as IIAX fibers expressing different proportions of MyHC isoforms (Rivero et al. 1996; Yamano et al. 2005; Bloemberg and Quadrilatero 2012). In our particular case, cross-sectional area remained constant after myofiber transdifferentiation from type IIX to IIA, further bolstering this notion. Previous studies in rodents have shown good correlation between myofiber types and their glycolytic or oxidative activity (Zhang et al. 2020). Similar behavior is likely to occur in equine muscle tissue, and results of this suggest maximal force output was compromised and traded for endurance-capabilities. Ergogenic supplementation in the absence of exercise might have latent, macroscopically unnoticeable effects requiring for mechanical stimuli (e.g., exercise) to manifest its maximal potential (e.g., growth, increased number of sarcomeres, increased force output, decreased glycolytic rates, etc.). Athletic performance is not only affected by muscle composition, but also by cardiovascular capacity, musculoskeletal suitability, familiarity with the activity to be performed, mental disposition, skill, and environmental factors, among others.

Muscle architecture and ultrastructure of the GM has been well-characterized over the years, underscoring the importance of adequately matching frames of reference used with the age, breed, and fitness level of the individuals being studied (Bruce and Turek 1985; Wood et al. 1988; López-Rivero et al. 1992; Bruce et al. 1993; Valette et al. 1999; Lindner et al. 2002; Wagner et al. 2013). During this study, the retrieval of muscle biopsies by using a commercially available, handheld, vacuum-assisted method proved to be effective in the equine, with a similar efficacy rate as those reported in other species including humans (Barthelemy et al. 2020) and swine (Zhao et al. 2018), although consistent placement of the biopsy needle in the GM is crucial to retrieve comparable samples originating from the same muscle compartment.

Fatigue has been proposed to be a centrally regulated anticipatory response with homeostatic preservation as its ultimate goal (Noakes and St Clair Gibson 2004). This central governor limits work preventing hypoxic conditions from developing in one or more vital organs such as the heart and brain (Noakes et al. 2001). Control of oxygen demand is indirectly attained by regulating the number of motor units recruited, thus mitigating V_{O2} needs of peripheral tissue and prioritizing central organ perfusion (Noakes et al. 2001). In humans, the central governor model, as proposed by Noakes, weaves together feedback factors based on real-time sensory stimuli, and feedforward mechanisms (e.g. anticipation and previous experiences), ultimately preventing homeostatic catastrophic failure (Noakes 2012). Implication of such elements cannot be completely ruled out in an equine fatigue model and should be taken into consideration during instrument design or assessment of individual behavior. Increases in muscle mitochondrial content (induced or naturally occurring) could be partially responsible for increased rates of heat generation despite improved respiration function; consequently, hastening attainment of critical core temperatures, onset of fatigue, and systemic disturbances.

Mitochondria have been also implicated as regulators of the transdifferentiation of myofibers (Venhoff et al. 2012). With that in mind, the HMB-mediated upregulated expression of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1 α) can be considered a responsible for an indirect myofiber shift from fast-twitch, glycolytic IIX fibers towards more oxidative and fatigue-resistant IIA fibers (He et al. 2016). Measurement of V_{200} (running speed at a HR of 200 bpm) is a valuable tool to evaluate aerobic exercise capacities in equines (Kobayashi et al. 1999). Its use has been reported in multiple disciplines involving different breeds including Thoroughbreds and Standardbreds, and under field or laboratory (high-speed treadmill) conditions (De Mare et al. 2017). High correlation between V_{200} and HR allows for monitoring efficacy of a training protocol, and prevention of catastrophic sport injuries by keeping activity intensities within the exercise capacity of the horse (Kobayashi et al. 1999; Rivero 2007; De Mare et al. 2017).

Execution of equine-based studies face numerous challenges compared to human studies on homologous models. Isolated, "on-demand" muscle contractions and motions can be easily obtained from a human being, whereas a similar, targeted evaluation in the horse is often unfeasible. Thus, analysis of a specific muscle involves assessing the entire horse in motion, where complex interactions between multiple muscles are unavoidable. This introduces confounding elements such as offsetting the onset of fatigue of target muscle, crosstalk during electromyographic evaluation, and kinematic changes to relieve the amount of work performed by a fatigued muscle or muscle group. During spatial recruitment of myofibers, increasingly numerous motor unit action potentials (MUAPs) align with each other and experience constructive interference (Feher 2012). This summation of individual wave amplitudes results in a single wave of greater local amplitude when they are in-phase, as seen during RMS

evaluation. Changes in the wavelet envelope can be observed during sEMG captures and post-processing, providing an additional qualitative visual reference during data capture and serving as quantitative indicators of motor unit recruitment while comparing different stages of an exercise bout using both raw and filtered data.

Median frequency (MdF) is used to monitor onset of fatigue in healthy muscle, or as a diagnostic tool in pathological conditions. In events of muscle fatigue MdF tends to shift towards lower frequencies (Liu et al. 2014). In humans, MdF frequencies lower than 70 Hz are associated with dystonic musculature. Concomitantly, an increase in the area under curve of total power in the low frequency range can be observed in those cases (Go et al. 2014). In this study, MdF values remained stable around the expected frequencies of approximately 70-75 Hz for both BF and ST. These experimental conditions did not elicit MdF variations the on BF and ST after maximal frequencies were reached. The stable frequencies on these muscles suggest that the assessed muscles did not suffer from neural fatigue as was initially conjectured, and the absence of any MdF shift correlates with the anticipated steady increase of iEMG values seen throughout the study (Phinyomark et al. 2012). This indicates that electromyographic evaluation of different muscle groups is needed to determine if fatigue originates peripherally from a component of the locomotor system. Additionally, incomplete datasets due to excessive noise or electrode detachment should be excluded from the analysis; aggressive measures to mitigate sensor detachment or noise-related artifacts should be taken whenever possible.

Due to their critical role in action potential transmission, the sodium-potassium pump (Na^+/K^+-ATPase) and electrolyte disturbances in the muscle have been recently proposed to play a dual role in muscle fatigue in humans (McKenna et al. 2008). Local Na^+, K^+, and Cl^- perturbations may have a protective, force-enhancing effect during early exercise, whereas

extensive disturbances contribute depolarization of the sarcolemma and T-tubules, precluding further myofiber contraction. In early phases of exercise, increased Na^+/K^+-ATPase activity contributes to maintain Na^+ and K^+ concentrations and membrane excitability, delaying fatigue. Conversely, during maximal contraction, exercise-induced inhibition of Na^+/K^+-ATPase via accumulation of K^+ in the T-tubules impairs membrane potential transmission, leading to muscle fatigue (Pedersen et al. 2004; McKenna et al. 2008). Equine sweat contains important concentrations of Cl^-, Na^+, and K^+ (140-301 mmol/l, 110-249 mmol/l, 28-53.1 mmol/l, respectively), thus is considered nearly isotonic relative to plasma. As such, a massive loss of fluids occurs aggravating pre-existing electrolyte imbalances during prolonged submaximal exercise, complicating proper muscle action potential transmission (Foreman 1998; Spooner et al. 2010). Combination of these two mechanisms is conceivably responsible for abrupt development of muscle fatigue in the horse during intense, maximal activity. After intense work, acidotic sarcoplasmic conditions hinder the response of the contractile apparatus to Ca^{2+}, and concurrent membrane depolarization (through K^+ accumulation in the T-system) limit a muscle's capacity to contract. However, intracellular acidosis also exerts a paradoxical protective effect on myocontractility. This is achieved by decreasing the Cl^- permeability of the sarcolemma, decreasing in turn the inward Na^+ current required to propagate an action potential thus facilitating contraction in advanced stages of muscle activity (Pedersen et al. 2004). These interactions can be further modified depending on the nutritional status of the individual, as lower concentrations of cholesterol in the sarcolemma can negatively impact excitation-contraction coupling by modulating Cav1.1 and subsequent Ryanodine receptor type 1 (RyR1) activation (Barrientos et al. 2017). Decreased membrane cholesterol concentrations also impairs

insulin-mediated glucose uptake by GLUT4 translocation at the level of T-tubules, affecting local replenishment of energy substrates (Yang et al. 2016; Barrientos et al. 2017).

Extensor muscles of the hip play a crucial role on quadrupedal forward propulsion (Denoix 2013). The vertebral and pelvic heads of the ST acts by extending the hip during the stance phase and promote extension of the hock joint and flexion of the stifle joint during swing phase (Payne et al. 2005). The BF possesses a high power output potential, acting as extensor of the hip in the stance phase, and as stifle flexor and hock extensor during the swing phase (Payne et al. 2005; Rivero and Hill 2016). The BF and ST, unlike other deeper muscles (e.g. GM), provide advantages such as muscle size and superficiality, which improves EMG data quality by reducing cross-talk (Valentin and Zsoldos 2016). In this study the GM was deliberately excluded from electromyographic analysis due to the confounding presence of the gluteus superficialis muscle (GS) partially covering the middle portion and insertion of the GM. The origin of the GM is covered by the gluteal fascia, while the GS covers the middle portion (area of interest for EMG evaluation) and insertion of the GM. With this in mind, and the detrimental interference-generating effect of cross-talk during different gaits, which can render these measurements inaccurate, our team opted for selection of different propulsive muscle groups (BF, ST) (St. George et al. 2019).

Contrary to the method used by Pugliese and colleagues, who attached electrogoniometers to the distal limb, this study relied on high-speed videography for joint angle determination (Pugliese et al. 2020). This method proved useful to evaluate each individual without risking instrument displacement, although occasional reflective marker detachment can occur. Behavior of the front fetlock angular displacement in this study echoes the expected behavior of the anterior MCPJ joint during proximal muscle fatigue as described in a previous study (Thorpe,

Clegg, et al. 2010). Concomitantly to greater MCPJ angles, increased stride length, shortened stance phase times occurred as treadmill speed increased, as reported elsewhere (Corley and Goodship 1994; Muñoz et al. 1997; Johnston et al. 1999; Thorpe, Clegg, et al. 2010). Signs of lameness, otherwise concealed by proper stabilization by unfatigued muscles, might become evident when the athlete begins to use different, supporting muscle groups as a compensatory mechanism for relieving fatigue while remaining in movement (Foreman 1998). Similarly, fatigue-induced kinematic changes can predispose to injuries by altering dynamic joint stability as previously reported in a human knee model (Ortiz et al. 2010) and on equine front distal limbs (Thorpe, Clegg, et al. 2010). Horses in the CON group did not extend their forelimbs as much as HMB group during the suspension phase late in the SET, suggesting the CON group could not effectively exert the force needed to anteriorly extend their front limbs compared to the HMB group. Increased durations of swing phases found in the HMB group asserts that they are either using compensatory mechanisms to counteract fatigue effects or experienced fatigue later than their CON counterparts.

In contrast to over-ground evaluations, this project can count the use of a high-speed treadmill and a modest-sized, non-shod study population as potential limitations. Utilizing an incremental, volitional, fatigue-based exercise protocol in laboratory settings makes results more vulnerable to individual-related variations (e.g. temperament, previous experiences) than under-saddle evaluations in racetrack conditions (Geor 2006). Barefoot horses can display minor but significant differences in the forces exerted by the distal limb of the horse during locomotion compared to properly shod animals (Roepstorff et al. 1999). These factors warrant further studies of the effect of HMB on fit equine athletes in training. Development of more instruments that correlate physiologic parameters to equine performance potential is still warranted. Mathematical

models and score systems incorporating V200, $V_{fatigue}$, HR_{max}, TTE, and other aerobic capacity indicators could concisely quantify breakpoints in the fatigue continuum, fitness status, and approximate muscle compositions. These tools would allow for improved research approaches as well as advancing racetrack safety standards and stud farm genetic selection.

In conclusion, effects of beta-hydroxy beta-methylbutyrate (HMB) supplementation in untrained horses can influence skeletal muscle composition by increasing type IIA myofiber numbers at the expense of type IIX fibers, although favorable effects on athletic performance are not significantly appreciable at this sample size. Further studies may be warranted to unveil the full extent of its effects and interactions in individuals of varying fitness levels. Exercise physiology and sports medicine would benefit from an updated consensus on the denomination, etiopathogenesis, and assessment methodology of fatigue that is applicable across species, therefore facilitating forthcoming efforts in multidisciplinary comparative research.

APPENDIX A

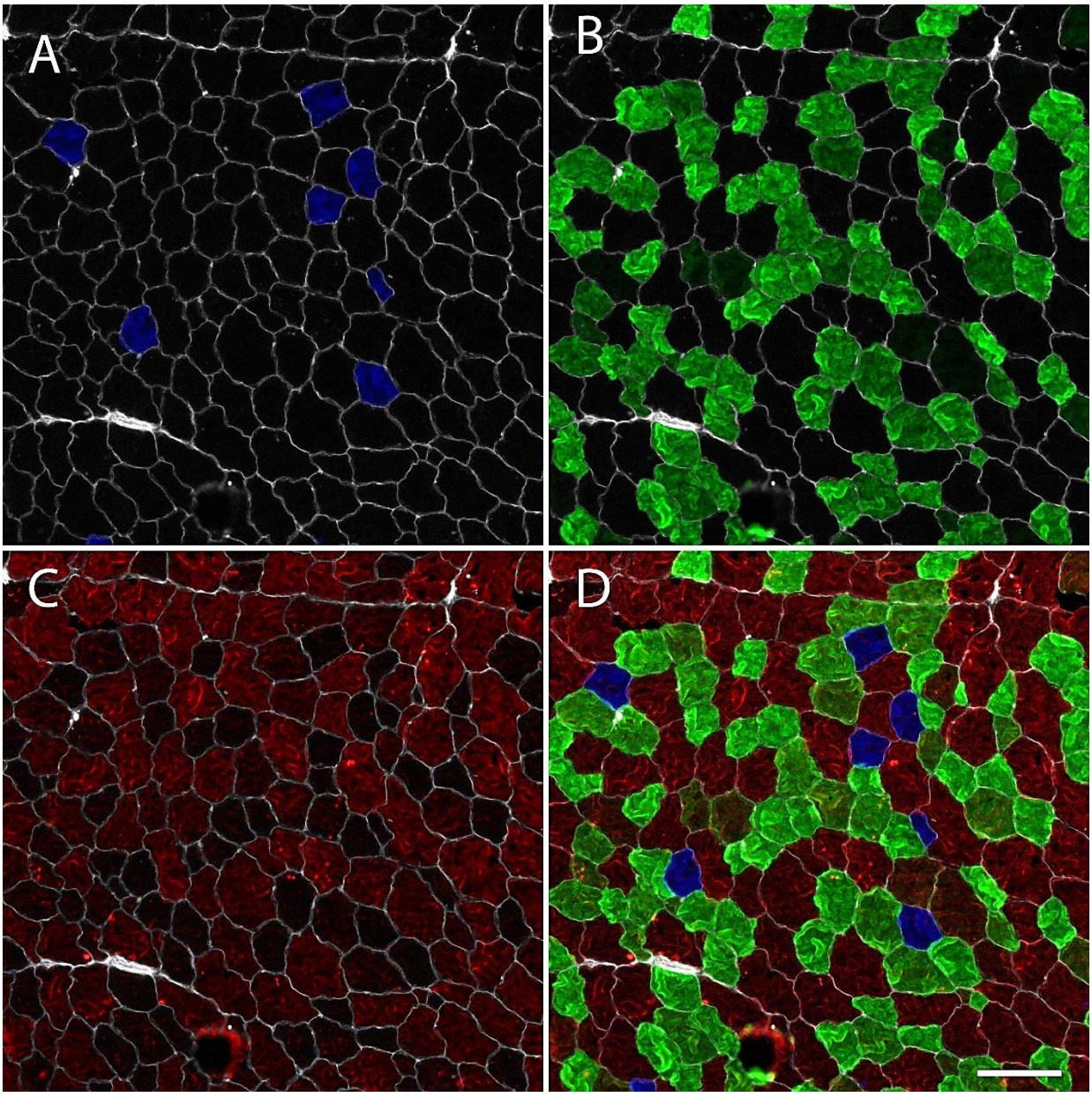

Figure 3-1. Cross-section of equine gluteus medius muscle (GM) stained with antibodies targeting myosin heavy chain isoforms MyHC I, MyHC IIA, and MyHC IIX. Technique performed for fiber-typing method validation, as an additional step to the method described in Chapter II. Primary incubation overnight. Secondary incubation over 60 minutes. Primary and secondary antibodies were sourced from DHSB and Thermo Fisher Scientific, respectively. **A.** Type I fibers; BA-D5, 1:50 dilution. Alexa Fluor 350, 1:100. **B.** Type IIA fibers, A4.74, 1:100 dilution. Alexa Fluor 488, 1:1000. **C.** Type IIX fibers, 6H1, 1:2 dilution. Alexa Fluor 555, 1:1000. **D.** Merged image displaying panels A, B, and C in overlay. Scale bar = 100 µm.

LITERATURE CITED

Allen KJ, van Erck-Westergren E, Franklin SH. 2016. Exercise testing in the equine athlete. Equine Vet Educ. 28(2):89–98. doi:10.1111/eve.12410.

Ament W, Verkerke G. 2009. Exercise and fatigue. Sport Med. 39(5):389–422. doi:10.2165/00007256-200939050-00005.

American Horse Council. 2005. Most comprehensive horse study ever reveals a nearly $40 billion impact on the U.S. economy.

Arazi H, Taati B, Suzuki K. 2018. A review of the effects of leucine metabolite (β-hydroxy-β-methylbutyrate) supplementation and resistance training on inflammatory markers: A new approach to oxidative stress and cardiovascular risk factors. Antioxidants. 7(10):9–13. doi:10.3390/antiox7100148.

Back W, Schamhardtt HC, Barneveld A. 1997. Kinematic comparison of the leading and trailing fore- and. 23:80–83.

Balagopal P, Ljungqvist O, Nair KS. 1997. Skeletal muscle myosin heavy-chain synthesis rate in healthy humans. Am J Physiol - Endocrinol Metab. 272(1 35-1). doi:10.1152/ajpendo.1997.272.1.e45.

Barlow DA, Lloyd JM, Hellhake P, Seder JA. 1984. Equine muscle fiber types: A histological and histochemical analysis of select thoroughbred yearlings. J Equine Vet Sci. 4(2):60–66. doi:10.1016/S0737-0806(84)80083-0.

Barrientos G, Sánchez-Aguilera P, Jaimovich E, Hidalgo C, Llanos P. 2017. Membrane Cholesterol in Skeletal Muscle: A Novel Player in Excitation-Contraction Coupling and Insulin Resistance. J Diabetes Res. 2017. doi:10.1155/2017/3941898.

Barthelemy F, Woods JD, Nieves-Rodriguez S, Douine ED, Wang R, Wanagat J, Miceli MC, Nelson SF. 2020. A well-tolerated core needle muscle biopsy process suitable for children and adults. Muscle and Nerve.(July):1–11. doi:10.1002/mus.27041.

Billeter R, Weber H, Lutz H, Howald H, Eppenberger HM, Jenny E. 1980. Myosin types in human skeletal muscle fibers. Histochemistry. 65(3):249–259. doi:10.1007/BF00493174.

Bloemberg D, Quadrilatero J. 2012. Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. PLoS One. 7(4):e35273. doi:10.1371/journal.pone.0035273.

Boston RC, Nunamaker DM. 2000. Gait and speed as exercise components of risk factors associated with onset of fatigue injury of the third metacarpal bone in 2-year-old Thoroughbred racehorses. Am J Vet Res. 61(6):602–608. doi:10.2460/ajvr.2000.61.602.

Brearley MC, Loczenski-Brown DM, Loughna PT, Parr T, Brameld JM. 2021. Response of the porcine MYH4-promoter and MYH4-expressing myotubes to known anabolic and catabolic agents in vitro. Biochem Biophys Reports. 25:100924. doi:10.1016/j.bbrep.2021.100924.

Brook MS, Wilkinson DJ, Phillips BE, Perez-Schindler J, Philp A, Smith K, Atherton PJ. 2016. Skeletal muscle homeostasis and plasticity in youth and ageing: Impact of nutrition and exercise. Acta Physiol. 216(1):15–41. doi:10.1111/apha.12532.

Brooks GA. 2020. Lactate as a fulcrum of metabolism. Redox Biol. 35(January):101454. doi:10.1016/j.redox.2020.101454.

Brownlow MA, Mizzi JX. 2020. Thermoregulatory capacity of the Thoroughbred racehorse and its relationship to the pathogenesis of exertional heat illness. Equine Vet Educ.:eve.13433. doi:10.1111/eve.13433.

Brownlow MA, Mizzi JX. 2021. Exertional heat illness in Thoroughbred racehorses – Pathophysiology, case definition and treatment rationale. Equine Vet Educ.:eve.13459. doi:10.1111/eve.13459.

Bruce V, Turek RJ. 1985. Muscle fibre variation in the gluteus medius of the horse. Equine Vet J. 17(4):317–21. doi:10.1111/j.2042-3306.1985.tb02508.x.

Bruce VL, Turek RJ, Schurg WA. 1993. Muscle fibre compartmentalisation in the gluteus medius of the horse. Equine Vet J. 25(1):69–72. doi:10.1111/j.2042-3306.1993.tb02905.x.

Bryan K, McGivney BA, Farries G, McGettigan PA, McGivney CL, Gough KF, MacHugh DE, Katz LM, Hill EW. 2017. Equine skeletal muscle adaptations to exercise and training: Evidence of differential regulation of autophagosomal and mitochondrial components. BMC Genomics. 18(1):1–26. doi:10.1186/s12864-017-4007-9.

Buchner HH, Savelberg HH, Schamhardt HC, Merkens HW, Barneveld A. 1994. Kinematics of treadmill versus overground locomotion in horses. Vet Q. 16 Suppl 2:87–90. doi:10.1080/01652176.1994.9694509.

Burke RE. 1978. Motor units: Physiological/histochemical profiles, neural connectivity and functional specializations. Integr Comp Biol. 18(1):127–134. doi:10.1093/icb/18.1.127.

Cifrek M, Medved V, Tonković S, Ostojić S. 2009. Surface EMG based muscle fatigue evaluation in biomechanics. Clin Biomech. 24(4):327–340. doi:10.1016/j.clinbiomech.2009.01.010.

Clayton HM, Hobbs SJ. 2017. The role of biomechanical analysis of horse and rider in equitation science. Appl Anim Behav Sci. 190:123–132. doi:10.1016/j.applanim.2017.02.011.

Colborne GR, Birtles DM, Cacchione IC. 2001a. Electromyographic and kinematic indicators of fatigue in horses: a pilot study. Equine Vet J. 33(S33):89–93. doi:10.1111/j.2042-3306.2001.tb05367.x.

Colborne GR, Birtles DM, Cacchione IC. 2001b. Electromyographic and kinematic indicators of fatigue in horses: a pilot study. Equine Vet J Suppl. 33(33):89–93. doi:10.1111/j.2042-3306.2001.tb05367.x.

Corley JM, Goodship AE. 1994. Treadmill training induced changes to some kinematic variables measured at the canter in Thoroughbred fillies. Equine Vet J. 26(S17):20–24. doi:10.1111/j.2042-3306.1994.tb04867.x.

Cruz AM, Vidondo B, Ramseyer AA, Maninchedda UE. 2018. Effect of trotting speed on kinematic variables measured by use of extremity-mounted inertial measurement units in nonlame horses performing controlled treadmill exercise. Am J Vet Res. 79(2):211–218. doi:10.2460/ajvr.79.2.211.

Davis T, Suryawan A, Fiorotto M. 2018. 184 Supplementation with Leucine and the Leucine Metabolite, Beta-hydroxy-beta-methylbutyrate, Stimulates Muscle Protein Synthesis in Neonatal Pigs via Different Signaling Mechanisms. J Anim Sci. 96(Suppl 3):301–301. doi:10.1093/jas/sky404.662.

Denoix JM. 2013. Biomechanics and Physical Training of the Horse. 1st Editio. Denoix J-M, editor. Boca Raton, FL: CRC Press.

Duan Y, Li F, Guo Q, Wang W, Zhang L, Wen C, Chen X, Yin Y. 2018. β-Hydroxy-β-methyl Butyrate Is More Potent Than Leucine in Inhibiting Starvation-Induced Protein Degradation in C2C12 Myotubes. J Agric Food Chem. 66(1):170–176. doi:10.1021/acs.jafc.7b04841.

Duan Y, Zhang L, Li F, Guo Q, Long C, Yin Y, Kong X, Peng M, Wang W. 2018. β-Hydroxy-β-methylbutyrate modulates lipid metabolism in adipose tissues of growing pigs. Food Funct. 9(9):4836–4846. doi:10.1039/c8fo00898a.

Durkalec-Michalski K, Jeszka J, Podgórski T. 2017. The Effect of a 12-Week Beta-hydroxy-beta-methylbutyrate (HMB) Supplementation on Highly-Trained Combat Sports Athletes: A Randomised, Double-Blind, Placebo-Controlled Crossover Study. Nutrients. 9(7):753. doi:10.3390/nu9070753.

Edouard P, Mendiguchia J, Lahti J, Arnal PJ, Gimenez P, Jiménez-Reyes P, Brughelli M, Samozino P, Morin J-B. 2018. Sprint Acceleration Mechanics in Fatigue Conditions: Compensatory Role of Gluteal Muscles in Horizontal Force Production and Potential Protection of Hamstring Muscles. Front Physiol. 9(November):1–12. doi:10.3389/fphys.2018.01706.

Engelen MPKJ, Deutz NEP. 2018. Is β-hydroxy β-methylbutyrate an effective anabolic agent to improve outcome in older diseased populations? Curr Opin Clin Nutr Metab Care. 21(3):207–213. doi:10.1097/MCO.0000000000000459.

Feher J. 2012. Quantitative Human Physiology. In: Feher J, editor. Quantitative Human Physiology. First Edit. San Diego: Elsevier Inc. p. 270–280.

Ferguson BS, Rogatzki MJ, Goodwin ML, Kane DA, Rightmire Z, Gladden LB. 2018. Lactate metabolism: historical context, prior misinterpretations, and current understanding. Eur J Appl Physiol. 118(4):691–728. doi:10.1007/s00421-017-3795-6.

Firth EC, Rogers CW, Perkins NR, Anderson BH, Grace ND. 2004. Musculoskeletal responses of 2-year-old Thoroughbred horses to early training. 1. Study design, and clinical, nutritional, radiological and histological observations. N Z Vet J. 52(5):261–271. doi:10.1080/00480169.2004.36438.

Flummer C, Theil PK. 2012. Effect of β-hydroxy β-methyl butyrate supplementation of sows in late gestation and lactation on sow production of colostrum and milk and piglet performance. J Anim Sci. 90 Suppl 4(SUPPL4):372–4. doi:10.2527/jas.53971.

Foreman JH. 1998. The exhausted horse syndrome. Vet Clin North Am Equine Pract. 14(1):205–219. doi:10.1016/S0749-0739(17)30220-1.

Fowler WM. 2002. Role of physical activity and exercise training in neuromuscular diseases. Am J Phys Med Rehabil. 81(11 Suppl):S187-95. doi:10.1097/01.PHM.0000029726.80774.83.

Fuller JC, Sharp RL, Angus HF, Khoo PY, Rathmacher JA. 2015. Comparison of availability and plasma clearance rates of β -hydroxy- β -methylbutyrate delivery in the free acid and calcium salt forms. Br J Nutr. 114(9):1403–1409. doi:10.1017/S0007114515003050.

Garcia-Roves P, Huss JM, Han DH, Hancock CR, Iglesias-Gutierrez E, Chen M, Holloszy JO. 2007. Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. Proc Natl Acad Sci U S A. 104(25):10709–10713. doi:10.1073/pnas.0704024104.

Geor RJ. 2006. The role of nutritional supplements and feeding strategies in equine athletic performance. Equine Comp Exerc Physiol. 3(3):109–119. doi:10.1017/ecp200690.

St. George L, Hobbs SJ, Richards J, Sinclair J, Holt D, Roy SH. 2018. The effect of cut-off frequency when high-pass filtering equine sEMG signals during locomotion. J Electromyogr Kinesiol. 43(September):28–40. doi:10.1016/j.jelekin.2018.09.001.

St. George L, Roy SH, Richards J, Sinclair J, Hobbs SJ. 2019. Surface EMG signal normalisation and filtering improves sensitivity of equine gait analysis. Comp Exerc Physiol. 15(3):173–185. doi:10.3920/CEP190028.

Gepner Y, Varanoske AN, Boffey D, Hoffman JR. 2018. Benefits of β-hydroxy-β-methylbutyrate supplementation in trained and untrained individuals. Res Sport Med. 27(2):204–218. doi:10.1080/15438627.2018.1533470.

Gerard M, de Graaf-Roelfsema E, Hodgson DR, van der Kolk JH. 2013. Metabolic energetics. In: Back W, Clayton HM, editors. Equine Locomotion. Second Edi. Philadelphia: Saunders Elsevier. p. 419–441.

Go SA, Coleman-Wood K, Kaufman KR. 2014. Frequency analysis of lower extremity electromyography signals for the quantitative diagnosis of dystonia. J Electromyogr Kinesiol. 24(1):31–36. doi:10.1016/j.jelekin.2013.11.002.

Goh Q, Millay DP. 2017. Requirement of myomaker-mediated stem cell fusion for skeletal muscle hypertrophy. Elife. 6:1–19. doi:10.7554/ELIFE.20007.

Guelph U of. 2013. Annual Horse Expense Sheet. :4. [accessed 2021 Feb 18]. https://www.equineguelph.ca/pdf/infosheets/Cost_Horse_Ownership.pdf.

Hardeman AM, Byström A, Roepstorff L, Swagemakers JH, van Weeren PR, Serra Bragança FM. 2020. Range of motion and between-measurement variation of spinal kinematics in sound horses at trot on the straight line and on the lunge. PLoS One. 15(2):1–19. doi:10.1371/journal.pone.0222822.

Hardeman AM, Serra Bragança FM, Swagemakers JH, van Weeren PR, Roepstorff L. 2019. Variation in gait parameters used for objective lameness assessment in sound horses at the trot on the straight line and the lunge. Equine Vet J. 51(6):831–839. doi:10.1111/evj.13075.

Harris P. 2009. Feeding Management of Elite Endurance Horses. Vet Clin North Am - Equine Pract. 25(1):137–153. doi:10.1016/j.cveq.2009.01.005. \

Harris P, Snow DH. 1988. The effects of high intensity exercise. Equine Vet J. 20(2):109–113.

He X, Duan Y, Yao K, Li F, Hou Y, Wu G, Yin Y. 2016. β-Hydroxy-β-methylbutyrate, mitochondrial biogenesis, and skeletal muscle health. Amino Acids. 48(3):653–664. doi:10.1007/s00726-015-2126-7.

Hernandez J, Hawkins DL. 2001. Training failure among yearling horses. Am J Vet Res. 62(9):1418–1422. doi:10.2460/ajvr.2001.62.1418.

Hodgson DR, McCutcheon LJ, Byrd SK, Brown WS, Bayly WM, Brengelmann GL, Gollnick PD. 1993. Dissipation of metabolic heat in the horse during exercise. J Appl Physiol. 74(3):1161–70. doi:10.1152/jappl.1993.74.3.1161. \

Holeček M. 2017. Beta-hydroxy-beta-methylbutyrate supplementation and skeletal muscle in healthy and muscle-wasting conditions. J Cachexia Sarcopenia Muscle. 8(4):529–541. doi:10.1002/jcsm.12208.

Holecek M, Muthny T, Kovarik M, Sispera L. 2009. Effect of beta-hydroxy-beta-methylbutyrate (HMB) on protein metabolism in whole body and in selected tissues. Food Chem Toxicol. 47(1):255–9. doi:10.1016/j.fct.2008.11.021.

Van Hoven R Den. 2006. Equine exercise physiology - Transforming laboratory studies into practical concepts. Pferdeheilkunde. 22(5):525–530. doi:10.21836/pem20060502.

Hu L, Kristensen NB, Krogh U, Theil PK. 2020. Net Absorption and Metabolism of β-Hydroxy-β-Methyl Butyrate during Late Gestation in a Pig Model. Nutrients. 12(2). doi:10.3390/nu12020561.

Jakubowski JS, Wong EPT, Nunes EA, Noguchi KS, Vandeweerd JK, Murphy KT, Morton RW, McGlory C, Phillips SM. 2019. Equivalent Hypertrophy and Strength Gains in β-Hydroxy-β-Methylbutyrate- or Leucine-supplemented Men. Med Sci Sports Exerc. 51(1):65–74. doi:10.1249/MSS.0000000000001752.

Johnston C, Gottlieb-Vedi M, Drevemo S, Roepstorff L. 1999. The kinematics of loading and fatigue in the standardbred trotter. Equine Vet J Suppl. 30:249–253. doi:10.1111/j.2042-3306.1999.tb05228.x.

Kaczka P, Michalczyk MM, Jastrzab R, Gawelczyk M, Kubicka K. 2019. Mechanism of action and the effect of beta-hydroxy-beta-methylbutyrate (HMB) supplementation on different types of physical performance-A systematic review. J Hum Kinet. 68(1):211–222. doi:10.2478/hukin-2019-0070.

Kawai M, Minami Y, Sayama Y, Kuwano A, Hiraga A, Miyata H. 2009. Muscle fiber population and biochemical properties of whole body muscles in thoroughbred horses. Anat Rec. 292(10):1663–1669. doi:10.1002/ar.20961.

Kellis E, Liassou C. 2009. The effect of selective muscle fatigue on sagittal lower limb kinematics and muscle activity during level running. J Orthop Sports Phys Ther. 39(3):210–220. doi:10.2519/jospt.2009.2859.

Kobayashi M, Kuribara K, Amada A. 1999. Application of V200 values for evaluation of training effects in the young thoroughbred under field conditions. Equine Vet J Suppl. 30(S30):159–162. doi:10.1111/j.2042-3306.1999.tb05208.x.

Krogh A. 1919. The supply of oxygen to the tissues and the regulation of the capillary circulation. J Physiol. 52(6):457–474. doi:10.1113/jphysiol.1919.sp001844.

Lamboley CRH, Royer D, Dionne IJ. 2007. Effects of β-hydroxy-β-methylbutyrate on aerobic-performance components and body composition in college students. Int J Sport Nutr Exerc Metab. 17(1):56–69. doi:10.1123/ijsnem.17.1.56.

Leach DH, Dyson SJ. 1988. Instant centres of rotation of equine limb joints and their relationship to standard skin marker locations. Equine Vet J. 20(Table 1):113–119. doi:10.1111/j.2042-3306.1988.tb04657.x.

Lee J, Yang S, Kiessling V, Tamm LK. 2017. The Role of Cholesterol in Viral Spike Glycoprotein-Mediated Membrane Fusion. Biophys J. 112(3):9a. doi:10.1016/j.bpj.2016.11.078.

Leisson K, Jaakma Ü, Seene T. 2008. Adaptation of Equine Locomotor Muscle Fiber Types to Endurance and Intensive High Speed Training. J Equine Vet Sci. 28(7):395–401. doi:10.1016/j.jevs.2008.05.007.

Lima-Soares F, Cabido CET, Pessôa KA, Cholewa JM, Neves Amorim CE, Zanchi NE. 2017. HMB Supplementation: Clinical and Performance-Related Effects and Mechanisms of Action. Clinical and Performance-Related Effects and Mechanisms of Action. Elsevier Inc. http://dx.doi.org/10.1016/B978-0-12-805413-0.00023-5.

Lindner A, Dag S, Marti-Korff S, Quiroz-Rothe E, López Rivero JL, Drommer W. 2002. Effects of repeated biopsying on muscle tissue in horses. Equine Vet J. 34(6):619–624. doi:10.2746/042516402776180188.

Liu SH, Chang KM, Cheng DC. 2014. The Progression of Muscle Fatigue during Exercise Estimation with the Aid of High-Frequency Component Parameters Derived from Ensemble Empirical Mode Decomposition. IEEE J Biomed Heal Informatics. 18(5):1647–1658. doi:10.1109/JBHI.2013.2286408.

López-Rivero JL, Serrano AL, Diz AM, Galisteo AM. 1992. Variability of muscle fibre composition and fibre size in the horse gluteus medius: an enzyme-histochemical and morphometric study. J Anat. 181 (Pt 1:1–10. http://www.ncbi.nlm.nih.gov/pubmed/1284127%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1259747.

Malinzak RA, Colby SM, Kirkendall DT, Yu B, Garrett WE. 2001. A comparison of knee joint motion patterns between men and women in selected athletic tasks. Clin Biomech. 16(5):438–445. doi:10.1016/S0268-0033(01)00019-5.

De Mare L, Boshuizen B, Plancke L, De Meeus C, De Bruijn M, Delesalle C. 2017. Standardized exercise tests in horses: Current situation and future perspectives. Vlaams Diergeneeskd Tijdschr. 86(2):63–72. doi:10.21825/vdt.v86i2.16290.

McKenna MJ, Bangsbo J, Renaud JM. 2008. Muscle K+, Na+, and Cl- disturbances and Na+-K+ pump inactivation: Implications for fatigue. J Appl Physiol. 104(1):288–295. doi:10.1152/japplphysiol.01037.2007.

McMiken DF. 1983. An energetic basis of equine performance. Equine Vet J. 15(2):123–133. doi:10.1111/j.2042-3306.1983.tb01734.x.

Miller P, Sandberg L, Fuller J. 1998. The effect of supplemental beta-hydroxy-beta-methylbutyrate on training and racing thoroughbreds. Proc 17th Assoc Equine Sport Med. 17:23–4.

Morrice-West A V., Hitchens PL, Walmsley EA, Stevenson MA, Whitton RC. 2020. Training practices, speed and distances undertaken by Thoroughbred racehorses in Victoria, Australia. Equine Vet J. 52(2):273–280. doi:10.1111/evj.13156.

Mueller-Wohlfahrt HW, Haensel L, Mithoefer K, Ekstrand J, English B, McNally S, Orchard J, Van Dijk N, Kerkhoffs GM, Schamasch P, et al. 2013. Terminology and classification of muscle injuries in sport: The Munich consensus statement. Br J Sports Med. 47(6):342–350. doi:10.1136/bjsports-2012-091448.

Muñoz A, Santisteban R, Rubio MD, Vivo R, Aguera EI, Escribano BM, Castrjón FM. 1997. Training as an Influential Factor on the Locomotor Pattern in Andalusian Horses. J Vet Med Ser A Physiol Pathol Clin Med. 44(8):473–480. doi:10.1111/j.1439-0442.1997.tb01133.x.

Munsters CCBM, van Iwaarden A, van Weeren R, Sloet van Oldruitenborgh-Oosterbaan MM. 2014. Exercise testing in Warmblood sport horses under field conditions. Vet J. 202(1):11–19. doi:10.1016/j.tvjl.2014.07.019.

Muscat GEO, Wagner BL, Hou J, Tangirala RK, Bischoff ED, Rohde P, Petrowski M, Li J, Shao G, Macondray G, et al. 2002. Regulation of cholesterol homeostasis and lipid metabolism in skeletal muscle by liver X receptors. J Biol Chem. 277(43):40722–40728. doi:10.1074/jbc.M206681200.

Naya FJ, Mercer B, Shelton J, Richardson JA, Williams RS, Olson EN. 2000. Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. J Biol Chem. 275(7):4545–4548. doi:10.1074/jbc.275.7.4545.

Nissen S, Faidley TD, Zimmerman DR, Izard R, Fisher CT. 1994. Colostral milk fat percentage and pig performance are enhanced by feeding the leucine metabolite beta-hydroxy-beta-methyl butyrate to sows. J Anim Sci. 72(9):2331–7. doi:10.2527/1994.7292331x.

Nissen S, Sharp R, Ray M, Rathmacher JA, Rice D, Fuller JC, Connelly AS, Abumrad N. 1996. Effect of leucine metabolite β-hydroxy-β-methylbutyrate on muscle metabolism during resistance-exercise training. J Appl Physiol. 81(5):2095–2104. doi:10.1152/jappl.1996.81.5.2095.

Nissen S, Sharp RL, Panton L, Vukovich M, Trappe S, Fuller JC. 2000. β-hydroxy-β-methylbutyrate (HMB) supplementation in humans is safe and may decrease cardiovascular risk factors. J Nutr. 130(8):1937–1945. doi:10.1093/jn/130.8.1937.

Nissen SL, Abumrad NN. 1997. Nutritional role of the leucine metabolite β-hydroxy β-methylbutyrate (HMB). J Nutr Biochem. 8(6):300–311. doi:10.1016/S0955-2863(97)00048-X.

Noakes TD. 2012. Fatigue is a brain-derived emotion that regulates the exercise behavior to ensure the protection of whole body homeostasis. Front Physiol. 3 APR(April):1–13. doi:10.3389/fphys.2012.00082.

Noakes TD, Peltonen JE, Rusko HK. 2001. Evidence that a central governor regulates exercise performance during acute hypoxia and hyperoxia. J Exp Biol. 204(18):3225–34.

Noakes TD, St Clair Gibson A. 2004. Logical limitations to the “catastrophe” models of fatigue during exercise in humans. Br J Sports Med. 38(5):648–9. doi:10.1136/bjsm.2003.009761.

Ortiz A, Olson SL, Etnyre B, Trudelle-Jackson EE, Bartlett W, Venegas-Rios HL. 2010. Fatigue effects on knee joint stability during two jump tasks in women. J strength Cond Res. 24(4):1019–27. doi:10.1519/JSC.0b013e3181c7c5d4.

Ostaszewski P, Kowalska A, Szarska E, Szpotański P, Cywinska A, Bałasińska B, Sadkowski T. 2012. Effects of β-Hydroxy-β-Methylbutyrate and γ-Oryzanol on Blood Biochemical Markers in Exercising Thoroughbred Race Horses. J Equine Vet Sci. 32(9):542–551. doi:10.1016/j.jevs.2012.01.002.

Paul AC, Rosenthal N. 2002. Different modes of hypertrophy in skeletal muscle fibers. J Cell Biol. 156(4):751–760. doi:10.1083/jcb.200105147.

Payne RC, Hutchinson JR, Robilliard JJ, Smith NC, Wilson AM. 2005. Functional specialisation of pelvic limb anatomy in horses (Equus caballus). J Anat. 206(6):557–574. doi:10.1111/j.1469-7580.2005.00420.x.

Pedersen TH, Nielsen OB, Lamb GD, Stephenson DG. 2004. Intracellular acidosis enhances the excitability of working muscle. Science (80-). 305(5687):1144–1147. doi:10.1126/science.1101141.

Phinyomark A, Thongpanja S, Hu H, Phukpattaranont P, Limsakul C. 2012. The Usefulness of Mean and Median Frequencies in Electromyography Analysis. In: Naik GR, editor. Computational Intelligence in Electromyography Analysis - A Perspective on Current Applications and Future Challenges. InTechOpen.

Pugliese BR, Carballo CT, Connolly KM, Mazan MR, Kirker-Head CA. 2020. Effect of Fatigue on Equine Metacarpophalangeal Joint Kinematics—A Single Horse Pilot Study. J Equine Vet Sci. 86:102849. doi:10.1016/j.jevs.2019.102849.

Rahimi MH, Mohammadi H, Eshaghi H, Askari G, Miraghajani M. 2018. The Effects of Beta-Hydroxy-Beta-Methylbutyrate Supplementation on Recovery Following Exercise-Induced Muscle Damage: A Systematic Review and Meta-Analysis. J Am Coll Nutr. 37(7):640–649. doi:10.1080/07315724.2018.1451789.

Rahman A, Wilund K, Fitschen PJ, Jeejeebhoy K, Agarwala R, Drover JW, Mourtzakis M. 2014. Elderly persons with ICU-acquired weakness: the potential role for β-hydroxy-β-methylbutyrate (HMB) supplementation? J Parenter Enter Nutr. 38(5):567–75. doi:10.1177/0148607113502545.

Reaz MBII, Hussain MS, Mohd-Yasin F. 2006. Techniques of EMG signal analysis: Detection, processing, classification and applications. Biol Proced Online. 8(1):11–35. doi:10.1251/bpo115.

Revold T, Mykkänen AK, Karlström K, Ihler CF, Pösö AR, Essén-Gustavsson B. 2010. Effects of training on equine muscle fibres and monocarboxylate transporters in young Coldblooded Trotters. Equine Vet J. 42(Suppl. 38):289–295. doi:10.1111/j.2042-3306.2010.00274.x.

Rivero J-LL. 2007. A scientific background for skeletal muscle conditioning in equine practice. J Vet Med A Physiol Pathol Clin Med. 54(6):321–32. doi:10.1111/j.1439-0442.2007.00947.x.

Rivero J-LL, Talmadge RJ, Edgerton VR. 1996. Myosin heavy chain isoforms in adult equine skeletal muscle: An immunohistochemical and electrophoretic study. Anat Rec. 246(2):185–194. doi:10.1002/(SICI)1097-0185(199610)246:2<185::AID-AR5>3.0.CO;2-0.

Rivero JLL, Hill EW. 2016. Skeletal muscle adaptations and muscle genomics of performance horses. Vet J. 209:5–13. doi:10.1016/j.tvjl.2015.11.019.

Rivero JLL, Ruz A, Martí-Korff S, Estepa JC, Aguilera-Tejero E, Werkman J, Sobotta M, Lindner A. 2007. Effects of intensity and duration of exercise on muscular responses to training of thoroughbred racehorses. J Appl Physiol. 102(5):1871–1882. doi:10.1152/japplphysiol.01093.2006.

Rivero JLL, Serrano AL, Henckel P, Aguera E. 1993. Muscle fiber type composition and fiber size in successfully and unsuccessfully endurance-raced horses. J Appl Physiol. 75(4):1758–1766. doi:10.1152/jappl.1993.75.4.1758.

Robert C, Valette JP, Denoix JM. 2001. The effects of treadmill inclination and speed on the activity of three trunk muscles in the trotting horse. Equine Vet J. 33(5):466–472. doi:10.2746/042516401776254745.

Roepstorff L, Johnston C, Drevemo S. 1999. The effect of shoeing on kinetics and kinematics during the stance phase. Equine Vet J Suppl. 30:279–285. doi:10.1111/j.2042-3306.1999.tb05235.x.

Rogatzki MJ, Ferguson BS, Goodwin ML, Gladden LB. 2015. Lactate is always the end product of glycolysis. Front Neurosci. 9(FEB):1–7. doi:10.3389/fnins.2015.00022.

Rogers CW, Rivero JLL, van Breda E, Lindner A, Sloet van Oldruitenborgh-Oosterbaan MM. 2007. Describing workload and scientific information on conditioning horses. Equine Comp Exerc Physiol. 4(1):1–6. doi:10.1017/S1478061507727408.

Saxton RA, Sabatini DM. 2017. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 168(6):960–976. doi:10.1016/j.cell.2017.02.004.

Schiaffino S. 2018. Muscle fiber type diversity revealed by anti-myosin heavy chain antibodies. FEBS J. 285(20):3688–3694. doi:10.1111/febs.14502.

Schiaffino S, Rossi AC, Smerdu V, Leinwand LA, Reggiani C. 2015. Developmental myosins: Expression patterns and functional significance. Skelet Muscle. 5(1):1–14. doi:10.1186/s13395-015-0046-6.

Schlader ZJ, Stannard SR, Mündel T. 2011. Exercise and heat stress: Performance, fatigue and exhaustion - A hot topic. Br J Sports Med. 45(1):3–5. doi:10.1136/bjsm.2009.063024.

Serrano AL, Quiroz-Rothe E, Rivero JLL. 2000. Early and long-term changes of equine skeletal muscle in response to endurance training and detraining. Pflugers Arch Eur J Physiol. 441(2–3):263–274. doi:10.1007/s004240000408.

Siles L, Ninfali C, Cortés M, Darling DS, Postigo A. 2019. ZEB1 protects skeletal muscle from damage and is required for its regeneration. Nat Commun. 10(1):1–18. doi:10.1038/s41467-019-08983-8.

Smith HJ, Mukerji P, Tisdale MJ. 2005. Attenuation of proteasome-induced proteolysis in skeletal muscle by β-hydroxy-β-methylbutyrate in cancer-induced muscle loss. Cancer Res. 65(1):277–283.

Snow DH, Guy PS. 1976. Percutaneous Needle Muscle Biopsy in the Horse. Equine Vet J. 8(4):150–155. doi:10.1111/j.2042-3306.1976.tb03327.x.

Spooner HS, Nielsen BD, Schott HC, Harris PA. 2010. Sweat composition in Arabian horses performing endurance exercise on forage-based, low Na rations. Equine Vet J. 42(SUPPL. 38):382–386. doi:10.1111/j.2042-3306.2010.00208.x.

Stover SM. 2003. The epidemiology of thoroughbred racehorse injuries. Clin Tech Equine Pract. 2(4):312–322. doi:10.1053/j.ctep.2004.04.003.

Sun S, Li H, Chen J, Qian Q. 2017. Lactic acid: No longer an inert and end-product of glycolysis. Physiology. 32(6):453–463. doi:10.1152/physiol.00016.2017.

Suryawan A, Rudar M, Fiorotto ML, Davis TA. 2020. Differential regulation of mTORC1 activation by leucine and β-hydroxy-β-methylbutyrate in skeletal muscle of neonatal pigs. J Appl Physiol. 128(2):286–295. doi:10.1152/japplphysiol.00332.2019.

Szcześniak KA, Ostaszewski P, Fuller JC, Ciecierska A, Sadkowski T. 2015. Dietary supplementation of β-hydroxy-β-methylbutyrate in animals - a review. J Anim Physiol Anim Nutr (Berl). 99(3):405–417. doi:10.1111/jpn.12234.

Takahashi Y, Mukai K, Matsui A, Ohmura H, Takahashi T. 2018. Electromyographic changes in hind limbs of thoroughbreds with fatigue induced by treadmill exercise. Am J Vet Res. 79(8):828–835. doi:10.2460/ajvr.79.8.828.

Takahashi Y, Mukai K, Ohmura H, Takahashi T. 2020. Do Muscle Activities of M. Splenius and M. Brachiocephalicus Decrease Because of Exercise-Induced Fatigue in Thoroughbred Horses? J Equine Vet Sci. 86:102901. doi:10.1016/j.jevs.2019.102901.

Takahashi Y, Takahashi T, Mukai K, Ohmura H. 2021. Effects of Fatigue on Stride Parameters in Thoroughbred Racehorses During Races. J equine Vet Sci. 101:103447. doi:10.1016/j.jevs.2021.103447.

Talbot J, Maves L. 2016. Skeletal muscle fiber type: using insights from muscle developmental biology to dissect targets for susceptibility and resistance to muscle disease. Wiley Interdiscip Rev Dev Biol. 5(4):518–534. doi:10.1002/wdev.230.

Tanaka K. 2009. The proteasome: overview of structure and functions. Proc Jpn Acad Ser B Phys Biol Sci. 85(1):12–36. doi:10.2183/pjab.85.12.

Thorpe CT, Clegg PD, Birch HL. 2010. A review of tendon injury: why is the equine superficial digital flexor tendon most at risk? Equine Vet J. 42(2):174–80. doi:10.2746/042516409X480395.

Thorpe CT, Stark RJF, Goodship AE, Birch HL. 2010. Mechanical properties of the equine superficial digital flexor tendon relate to specific collagen cross-link levels. Equine Vet J. 42(Suppl. 38):538–543. doi:10.1111/j.2042-3306.2010.00175.x.

Transparency Market Research. 2019. Global equine supplement products market to reach US $102.3 Mn by 2027; rise in demand for protein/amino acid supplements to boost market. https://www.transparencymarketresearch.com/pressrelease/equine-supplement-products-market.htm.

Valberg SJ. 2014. Muscle anatomy, physiology, and adaptations to exercise and training. In: Hodgson DR, Mckeever KH, Mcgowan CM, editors. The Athletic Horse: Principles and practice of equine sports medicine. Second Edi. Missouri: Saunders Elsevier. p. 174–201. https://linkinghub.elsevier.com/retrieve/pii/B9780721600758000216.

Valentin S, Zsoldos RR. 2016. Surface electromyography in animal biomechanics: A systematic review. J Electromyogr Kinesiol. 28:167–183. doi:10.1016/j.jelekin.2015.12.005.

Valette JP, Barrey E, Jouglin M, Courouce A, Auvinet B, Flaux B. 1999. Standardisation of muscular biopsy of gluteus medius in French trotters. Equine Vet J Suppl. 30:342–344. doi:10.1111/j.2042-3306.1999.tb05246.x.

Venhoff N, Lebrecht D, Pfeifer D, Venhoff AC, Bissé E, Kirschner J, Walker UA. 2012. Muscle-fiber transdifferentiation in an experimental model of respiratory chain myopathy. Arthritis Res Ther. 14(5):1–11. doi:10.1186/ar4076.

Vigotsky AD, Halperin I, Lehman GJ, Trajano GS, Vieira TM. 2018. Interpreting signal amplitudes in surface electromyography studies in sport and rehabilitation sciences. Front Physiol. 8(JAN). doi:10.3389/fphys.2017.00985.

Wagner AL, Urschel KL, Lefta M, Esser KA. 2013. Effect of gluteus medius muscle sample collection depth on postprandial mammalian target of rapamycin signaling in mature Thoroughbred mares. Am J Vet Res. 74(6):910–917. doi:10.2460/ajvr.74.6.910.

Wan H, Zhu J, Su G, Liu Y, Hua L, Hu L, Wu C, Zhang R, Zhou P, Shen Y, et al. 2016. Dietary supplementation with β-hydroxy-β-methylbutyrate calcium during the early postnatal period accelerates skeletal muscle fibre growth and maturity in intra-uterine growth-retarded and normal-birth-weight piglets. Br J Nutr. 115(8):1360–9. doi:10.1017/S0007114516000465.

Wan JJ, Qin Z, Wang PY, Sun Y, Liu X. 2017. Muscle fatigue: General understanding and treatment. Exp Mol Med. 49(10):e384-11. doi:10.1038/emm.2017.194.

Wickler SJ, Greene HM, Egan K, Astudillo A, Dutto DJ, Hoyt DF. 2006. Stride parameters and hindlimb length in horses fatigued on a treadmill and at an endurance ride. Equine Vet J Suppl. 38(36):60–4. doi:10.1111/j.2042-3306.2006.tb05514.x.

Wilkinson DJ, Hossain T, Limb MC, Phillips BE, Lund J, Williams JP, Brook MS, Cegielski J, Philp A, Ashcroft S, et al. 2018. Impact of the calcium form of β-hydroxy-β-methylbutyrate upon human skeletal muscle protein metabolism. Clin Nutr. 37(6):2068–2075. doi:10.1016/j.clnu.2017.09.024.

Williams JM. 2018. Electromyography in the Horse: A Useful Technology? J Equine Vet Sci. 60:43-58.e2. doi:10.1016/j.jevs.2017.02.005.

Wilson GJ, Wilson JM, Manninen AH. 2008. Effects of beta-hydroxy-beta-methylbutyrate (HMB) on exercise performance and body composition across varying levels of age, sex, and training experience: A review. Nutr Metab. 5(1):1–17. doi:10.1186/1743-7075-5-1.

Wilson JM, Fitschen PJ, Campbell B, Wilson GJ, Zanchi N, Taylor L, Wilborn C, Kalman DS, Stout JR, Hoffman JR, et al. 2013. International Society of Sports Nutrition Position Stand: beta-hydroxy-beta-methylbutyrate (HMB). J Int Soc Sports Nutr. 10(1):6. doi:10.1186/1550-2783-10-6.

Wilson JM, Lowery RP, Joy JM, Andersen JC, Wilson SMC, Stout JR, Duncan N, Fuller JC, Baier SM, Naimo MA, et al. 2014. The effects of 12 weeks of beta-hydroxy-beta-methylbutyrate free acid supplementation on muscle mass, strength, and power in resistance-trained individuals: A randomized, double-blind, placebo-controlled study. Eur J Appl Physiol. 114(6):1217–1227. doi:10.1007/s00421-014-2854-5.

Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR, Sabatini DM. 2016. Sestrin2 is a leucine sensor for the mTORC1 pathway. Science (80-). 351(6268):43–8. doi:10.1126/science.aab2674.

Wood CH, Ross TT, Armstrong JB, Hall DC. 1988. Homogeneity of muscle fiber composition in the M gluteus medius of the horse. J Equine Vet Sci. 8(4):294–296. doi:10.1016/S0737-0806(88)80053-4.

Yamano S, Eto D, Kasashima Y, Hiraga A, Sugiura T, Miyata H. 2005. Evaluation of developmental changes in the coexpression of myosin heavy chains and metabolic properties of equine skeletal muscle fibers. Am J Vet Res. 66(3):401–405. doi:10.2460/ajvr.2005.66.401.

Yamano S, Eto D, Sugiura T, Kai M, Hiraga A, Tokuriki M, Miyata H. 2002. Effect of growth and training on muscle adaptation in Thoroughbred horses. Am J Vet Res. 63(10):1408–1412. doi:10.2460/ajvr.2002.63.1408.

Yang S, Kreutzberger AJB, Lee J, Kiessling V, Tamm LK. 2016. The role of cholesterol in membrane fusion. Chem Phys Lipids. 199(3):136–143. doi:10.1016/j.chemphyslip.2016.05.003.

Zanchi NE, Gerlinger-Romero F, Guimarães-Ferreira L, de Siqueira Filho MA, Felitti V, Lira FS, Seelaender M, Lancha AH. 2011. HMB supplementation: clinical and athletic performance-related effects and mechanisms of action. Amino Acids. 40(4):1015–1025. doi:10.1007/s00726-010-0678-0.

Zhang Q, Duplany A, Moncollin V, Mouradian S, Goillot E, Mazelin L, Gauthier K, Streichenberger N, Angleraux C, Chen J, et al. 2019. Lack of muscle mTOR kinase activity causes early onset myopathy and compromises whole-body homeostasis. J Cachexia Sarcopenia Muscle. 10(1):35–53. doi:10.1002/jcsm.12336.

Zhang Y, Yang M, Zhou P, Yan H, Zhang Z, Zhang H, Qi R, Liu J. 2020. β-Hydroxy-β-methylbutyrate-Induced Upregulation of miR-199a-3p Contributes to Slow-To-Fast Muscle Fiber Type Conversion in Mice and C2C12 Cells. J Agric Food Chem. 68(2):530–540. doi:10.1021/acs.jafc.9b05104.

Zhao L, McMillan RP, Xie G, Giridhar SGLW, Baumgard LH, El-Kadi S, Selsby J, Ross J, Gabler N, Hulver MW, et al. 2018. Heat stress decreases metabolic flexibility in skeletal muscle of growing pigs. Am J Physiol - Regul Integr Comp Physiol. 315(6):R1096–R1106. doi:10.1152/ajpregu.00404.2017.

Zhong Y, Song B, Zheng C, Li F, Kong X, Duan Y, Deng J. 2019. α-Ketoisocaproate and β-hydroxy-β-methyl butyrate regulate fatty acid composition and lipid metabolism in skeletal muscle of growing pigs. J Anim Physiol Anim Nutr (Berl). 103(3):846–857. doi:10.1111/jpn.13077.

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