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FACTORS AFFECTING SKELETAL MUSCLE PROTEIN SYNTHESIS IN THE
HORSE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

Ashley Leigh Wagner

Lexington, Kentucky

Director: Dr. Kristine L. Urschel, Assistant Professor of Animal and Food Sciences

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

FACTORS AFFECTING SKELETAL MUSCLE PROTEIN SYNTHESIS IN THE HORSE

Skeletal muscle protein synthesis is regulated by the mammalian target of rapamycin (mTOR) signaling pathway. The first objective was to optimize the methodological procedures for assessing mTOR signaling in horses. The response of mTOR signaling (P-Akt Ser⁴⁷³, P-S6K1 Thr³⁸⁹, P-rpS6 Ser^{235/26 & 240/244}, and P-4EBP1 Thr^{37/46} by Western blotting techniques) to meal consumption was determined at three gluteal muscle biopsy depths (6, 8, and 10 cm), and the repeatability of the contralateral side at 8 cm during 5 days of repeated biopsies. There was no effect ($P > 0.05$) of sampling side or biopsy depth on mTOR signaling in mature horses. During repeated biopsies there was an increase ($P < 0.05$) in downstream (P-S6K1 Thr³⁸⁹, P-rpS6 Ser^{235/236 & 240/244} and P-4EBP1 Thr³⁸⁹) mTOR signaling in response to feeding. The second objective was to characterize alterations in mTOR signaling throughout the equid lifespan. Adolescent horses (yearlings and two year olds) studied in the postprandial had a lowered ($P < 0.05$) activation of downstream mTOR signaling with aging. There was a lower ($P < 0.05$) abundance of P-S6K1 Thr³⁸⁹ in aged horses (23.5 years old) than in mature horses (11 years old) during the post-absorptive state. The final objective was to assess mTOR signaling during acute and chronic inflammation. Acute inflammation occurred during 5 days of repeated biopsies, and chronic inflammation is characteristic of the aged. During acute inflammation, characterized by increased muscle mRNA expression of inflammatory cytokines, there was an increase ($P < 0.05$) in downstream mTOR signaling. Chronic inflammation resulted in a decrease ($P < 0.05$) in the abundance of P-S6K1 Thr³⁸⁹. Phenylbutazone was administered to reduce ($P < 0.05$) acute and chronic inflammation in muscle. Phenylbutazone administration during acute inflammation reduced ($P < 0.05$) the activation of downstream mTOR signaling and trended to increase ($P = 0.09$) P-S6K1 Thr³⁸⁹ abundance during chronic inflammation. Whole-body protein synthesis determined using isotope infusion techniques increased ($P < 0.05$) when chronic inflammation was reduced due to phenylbutazone administration. This research provides new standards for muscle biopsy collection when examining

mTOR signaling, and insight into management and feeding practices for adolescent and aging horses.

KEYWORDS: mTOR signaling, protein synthesis, horse, skeletal muscle, inflammation

Ashley L. Wagner, M.S.

December 15, 2011

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CHAPTER I.

The literature review

1.1. INTRODUCTION

The horse is a highly athletic animal that excels in a wide variety of sports that require targeted training of specific muscle groups with the intent of muscle accretion. Skeletal muscle is a unique tissue with a high degree of plasticity in its ability to increase and decrease in size, which is dictated by protein content, and in the horse comprises 50% of body mass (1). However, the horse has received little research attention with regards to protein metabolism and accretion. Rather, the research in the horse has focused on N balance studies, and assessment of ADG during development in order to determine protein and lysine requirements, and characterization of skeletal muscle physiology, specifically fiber types, during exercise and development.

The molecular signaling pathway that regulates protein synthesis has been extensively studied in cell culture, rodents, neonatal piglets, and humans. The mammalian target of rapamycin signaling (mTOR) pathway regulates protein synthesis and has been examined in a single study in the mature horse, where it was demonstrated that the anabolic stimulus of meal consumption increases mTOR signaling (2). These results were in agreement with the numerous studies examining mTOR signaling in response to meal consumption in other mammalian species. The end point of the mTOR signaling pathway is protein synthesis and has been examined in the mature horse following the anabolic stimuli of exercise using isotope techniques, where it was demonstrated that muscle protein synthesis increased to the greatest extent when an

amino acid-glucose mixture was infused in the jugular vein during the recovery period (3). Although the studies examining mTOR signaling and muscle protein fractional synthesis rates have examined the response to anabolic stimuli in various ages of animals, including neonates, adults, and the aged, there has not been a single study in any mammalian species to examine mTOR signaling in response to anabolic stimuli during adolescent development.

The focus of this review is to examine the literature pertaining to the current knowledge of the mTOR signaling pathway, the pathways involved in protein breakdown, whole-body and muscle protein synthesis, acute and chronic inflammation, and to provide a review of the relevant equine literature. Specifically, the upstream and downstream factors involved in the mTOR signaling pathway, and a review of the literature examining the effects of anabolic stimuli throughout the lifespan will be discussed. The methodologies used to examine whole-body and muscle protein synthesis and the literature regarding whole-body and muscle protein synthesis in response to anabolic stimuli throughout the lifespan will also be discussed. Next, there will be a brief discussion of inflammatory signaling, followed by a description of the inflammatory response to both acute and chronic stimuli. The literature review will end with an overview of the equine literature, including an overview of the research pertaining to protein nutrition (digestion and absorption, digestibility, and requirements), characterization of how muscle physiology, specifically fiber typing, changes with development and exercise, and the inflammatory response to acute and chronic inflammation.

1.2. THE MAMMALIAN TARGET OF RAPAMYCIN SIGNALING PATHWAY

The mTOR signaling pathway was initially discovered in yeast in 1991 by Heitman and colleagues (4). In yeast, mTOR is referred to simply as TOR and received its name because it was demonstrated that the protein was inhibited by rapamycin (4). In mammals the mTOR signaling pathway proteins are ubiquitous (5), but the focus of this literature review will focus on mTOR signaling in skeletal muscle (Figure 1.1).

Protein synthesis is limited by both the abundance and efficiency of ribosomes to translate mRNA into protein and the availability of amino acids to form a protein. The efficiency of ribosomes to translate mRNA into a protein is modulated by a series of intracellular signaling cascades that are associated with the mTOR pathway.

1.2.1. *The mammalian target of rapamycin complexes*

mTOR and the complexes it forms are predominately localized to the cytoplasm during inactivated states, but shuttling to the nucleus and lysosomes occurs during various activated states (6), which will be discussed further below. mTOR behaves as the catalytic subunit in 2 complexes: the mTOR complex 1 and 2 (mTORC1 and mTORC2). mTORC1 (Figure 1.2A) consists of 5 proteins: mTOR, mammalian lethal with SEC13 protein 8 (mLST8; also known as G β L), DEP domain-containing mTOR-interacting protein (DEPTOR), regulatory-associated protein of mTOR (RAPTOR), and 40 kDa pro-rich Akt substrate (PRAS40; also known as AKT1S1). G β L acts as a stabilizer for the mTOR-RAPTOR association, and RAPTOR serves to recruit mTOR substrates (7). G β L is also thought to act as a positive regulator for mTORC1 function (8), whereas DEPTOR (9) and PRAS40 (10) may have an inhibitory role; all of which will be further described

below (Section 1.2.2). mTORC2 (Figure 1.2B) is comprised of six proteins, three of which are also found in mTORC1: mTOR, GβL, and DEPTOR. The three remaining proteins that are unique to mTORC2 are rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSIN1; also known as MAPKAP1), and protein observed with RICTOR (PROTOR). RICTOR and PROTOR aid in the assembly of the mTORC2 complex (11). mSIN1 appears to function in the relocalization of the mTORC2 complex towards the cell membrane where it can interact with Akt, a signaling factor within the mTOR signaling pathway (12, 13). The two complexes of mTOR have varied functions and contributions to the mTOR signaling pathway which will be discussed further below (Section 1.2.2). Regardless of complex, the activated form of mTOR is phosphorylated at sites Ser²⁴⁴⁸ and Ser²⁴⁸¹ (14, 15). From this point on mTOR signaling will refer to mTORC1 signaling.

1.2.2. Upstream inputs of the mTOR signaling pathway

Upstream input into the mTOR signaling pathway comes from a variety of sources including: metabolites, hormones, mechanical stimulation, and physiological states. This section of the literature review will be subdivided by these inputs.

1.2.2.1. Insulin and Insulin like growth factor

The insulin and mTOR signaling pathways are joined through protein kinase B (PKB; also known as Akt). Insulin and insulin like growth factor (IGF) bind with their respective receptors, resulting in the autophosphorylation of tyrosine residues. The activation of this insulin receptor tyrosine kinase complex results in the phosphorylation of the insulin receptor substrates (IRS-1, IRS-2, and IRS-3) (16). The IRS act as docking

units for the regulatory subunit of phosphoinositide 3-kinase (PI3K) (16, 17), and together the activated subunit phosphorylates PtdIns(3,4) P_2 / PtdIns(3,4,5) P_3 -dependent kinase 1 (PDK1) on the serine residues. PDK1 then phosphorylates Akt at Thr³⁰⁸ (18). An additional phosphorylation site (Ser⁴⁷³) must also be phosphorylated for Akt to be activated (18). Akt is phosphorylated at Ser⁴⁷³ by the mTORC2 (19, 20). Although the upstream substrates resulting in mTORC2 activation have not been fully elucidated, Akt is phosphorylated at Ser⁴⁷³ in response to insulin and IGF-1(18). Researchers currently theorize that phosphorylation of Ser⁴⁷³ primes Akt for further phosphorylation at Thr³⁰⁸ (21); however, additional research is necessary to confirm this. In summary, insulin and IGF act through the insulin signaling pathway to activate Akt, linking the insulin and mTOR signaling pathways.

There are also several negative effectors that can inhibit activation in the insulin signaling pathway which include protein tyrosine phosphatase-1 β (PTP1 β), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and protein phosphatase 2A(PP2A) (22-24). PTP1 β dephosphorylates both the insulin receptor and IRS, and thus, deactivating the insulin receptor and IRS, which inhibits signaling through the pathway (22, 24). PTEN prevents PI3K from phosphorylating PDK1, inhibiting further signaling in the insulin pathway (22, 24). PP2A dephosphorylates the upstream effector Akt, and the downstream effector, 70 kDa S6 Kinase 1 (S6K1) (22, 24), which will be discussed in detail below (Section 1.2.3.1). Ultimately, for Akt activation to occur through the insulin signaling pathway the activity of these negative effectors must be suppressed.

Akt activation leads to mTOR activation through the inhibition of PRAS40 and tuberous sclerosis 2 (TSC2). PRAS40 interacts with RAPTOR as a component of

mTORC1 and behaves as a negative regulator, inhibiting mTORC1 activity (25). Akt phosphorylation of PRAS40 at Thr²⁴⁶ inhibits PRAS40 activity (10), allowing mTORC1 activation which results in the activation of PRAS40 at Ser¹⁸³ and Ser²²¹ and subsequent disassociation from mTOR (26, 27). Disassociated PRAS40 then binds to the scaffolding protein 14-3-3 (27). Activated Akt phosphorylates TSC2 at four residues (Ser⁹³⁹, Ser^{1086/1088}, Thr¹⁴⁶², and Thr¹⁴²²) (28, 29). TSC2 is a component of the TSC1/2 complex, which acts as a GTPase activating protein (GAP) for the G protein, Ras homologue enriched in brain (RHEB) (30, 31). Because GDP loaded RHEB cannot activate mTOR, the TSC1/2 complex acts as an upstream inhibitor of mTOR. Akt inhibition of TSC2 inhibits the TSC1/2 complex resulting in the inhibition of GAP activity. This keeps RHEB bound to GTP, allowing for the activation of mTOR. PRAS40 and TSC1/2 complex are inactivated by Akt through phosphorylation and mediate the activity of the mTORC1 complex.

Akt influences other cell mediators not involved in the mTOR signaling pathway. Although a detailed description of this is beyond the scope of this literature review, a discussion of Akt would not be complete without it. Activation of Akt results in the translocation of glucose transporter 4 (GLUT4) to the cell membrane (16), and phosphorylates the transcription factors, forkhead box protein O1 (FoxO1) and O3 (FoxO3) (32). The translocation of the 12-transmembrane domain protein, GLUT4, results in the transport of glucose into the cell (33, 34). Alternatively, the FoxO proteins are involved in the molecular signaling pathway regulating protein breakdown (Section 1.3) (35), and in activating the expression of genes regulating apoptosis (36). Thus, Akt influences protein synthesis, protein breakdown, glucose metabolism, and the cell cycle.

1.2.2.2. Glucose

Although glucose does not directly influence mTOR signaling, cellular energy, which is partly derived from glucose, mediates mTOR signaling. Glucose is transported into the cell by GLUT4, which is translocated to the cell membrane by Akt (16). Briefly, ATP is produced from glucose through glycolysis in the cytoplasm, and the Krebs cycle and electron chain transport in the mitochondria. The ratio of AMP to ATP, an indicator of cellular energy status, is allosterically monitored by AMPK. AMPK activity is inhibited when there are elevated levels of ATP because the AMP/ATP ratio is reduced. During times of low energy status, AMP/ATP ratio is increased, activating AMPK. Therefore, any physiological state altering ATP status can affect mTOR signaling, because activated AMPK phosphorylates TSC2 at Thr¹²²⁷ and Ser¹³⁴⁵, causing activation of TSC2 and forms the TSC1/2 complex (37). As previously mentioned, the TSC1/2 complex acts as a GAP for RHEB (30, 31). Activation of the TSC1/2 complex stimulates GAP activity toward RHEB inhibiting mTOR. AMPK can also inhibit mTORC1 through phosphorylating RAPTOR at Ser⁷²² and Ser⁷⁹² inducing the association of RAPTOR and scaffold protein 14-3-3 (38). Any physiological state affecting AMPK status, such as stress and DNA damage, will alter mTOR signaling. DNA damage upregulates AMPK in a p53 dependent manner (39), resulting in inhibition of mTOR signaling. Because AMPK can directly and indirectly inhibit mTORC1, any physiological state affecting ATP status can alter mTOR signaling; therefore, in a glucose rich environment, for example following a meal, AMPK is reduced allowing mTOR signaling.

1.2.2.3 Amino Acids

Intracellular amino acids stimulate mTOR signaling (40), and this stimulation requires a system of amino acid transporters in order to transport extracellular amino acids from blood into the muscle. Although there is not a full understanding of how amino acids stimulate mTORC1 activation, potential mediators have been recognized including mitogen-activated protein 4 kinase kinase kinase kinase (MAP4K3) (41, 42) and PI3K catalytic subunit type 3 (VPS34) (43). MAP4K3 is stimulated by amino acids independent of insulin and is not suppressed by rapamycin, and results in the activation of S6K1, a downstream signaling factor in the mTOR signaling pathway, which will be discussed in detail below (Section 1.2.3.1) (42). Therefore, it is thought that amino acid stimulation of MAP4K3 activates S6K1 independent of mTOR, but this requires further elucidation. Although the mechanistic action of VPS34 on downstream mTOR signaling factors is not fully understood, VPS34 may be the primary modulator of mTOR signaling in response to amino acids (44), and it has been suggested that VPS34 is required for leucine stimulation of S6K1 (45). Another potential mediator is the Rag family of GTPases (46). In the absence of amino acids, the Rag GTPases maintain an inactive conformation, but in the presence of amino acids the Rag GTPases become activated and interact with RAPTOR resulting in mTORC1 relocalization onto the surface of endosomes and lysosomes. This may enable mTORC1 interaction with RHEB and mTORC1 activation (46); however, this requires further elucidation. Even though a mixture of amino acids stimulate mTOR signaling, arginine and leucine have been shown to directly stimulate mTOR signaling. Although the mechanism requires further elucidation, arginine stimulated mTOR signaling in a nitric oxide independent manner

(47). Leucine has been identified as a key amino acid in mTORC1 activation (48-50).

The stimulation of the mTOR signaling pathway by amino acids is a pivotal area of research and may provide insight to more effective diet formulation.

1.2.2.4. Growth factors

Growth factors, such as IGF, epidermal growth factor (EGF), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) can also stimulate mTOR through Akt-independent mechanism (51-53). Stimulation of the extracellular regulated kinase (MEK/ERK) axis through growth factor activation of a small GTPase in the RAS Sarcoma protein subfamily (Ras) and subsequently mitogen activated protein kinase kinase kinase (MAP3K) stimulates MEK and then ERK1/2 to inactivate TSC2 through phosphorylation at Ser⁶⁶⁴ (52). Additionally, Wingless-Type MMTV Integration site family (Wnt) inhibits glycogen synthase kinase 3 β (GSK3 β) through the β -catenin pathway, allowing mTOR activation; however, β -catenin, itself, does not affect mTOR activation (51). The activated form of GSK3 β phosphorylates TSC2 at Thr¹³²⁹, Ser¹³³³, Ser¹³³⁷, and Ser¹³⁴¹ resulting in mTOR inhibition (51). Growth factors, specifically IGF, can also stimulate mTOR signaling in an Akt-dependent manner, which was previously discussed above (Section 1.2.2.1).

1.2.2.5. Other physiological states

Regulated in development and DNA damage response 1 (REDD1; also known as RTP801/DDIT4) is a hypoxia induced gene, and can alter mTOR signaling through several mechanisms including stress (54-56), glucocorticoid treatment (57), and exercise (58). Hypoxia induces REDD1 expression causing TSC2 to dissociate from scaffolding

protein 14-3-3, and allowing TSC1/2 complex activation (55, 56). Energy stress can also act through AMPK dependent and independent mechanisms similar to what is seen during hypoxia, with induced expression REDD1 and subsequent TSC1/2 complex activation (54). Additionally, REDD1 expression is induced following glucocorticoid treatment with dexamethasone leading to mTOR inhibition through TSC1/2 complex activation (59). Immediately following endurance exercise REDD1 expression is elevated (58) and may be responsible for the inhibition of mTOR during exercise; however, these mechanisms have not been fully elucidated.

Another hypoxia induced gene, REDD2 (also known as RTP801L/DDIT4L), also acts as an mTOR signaling inhibitor; however, much less is known about REDD2. Similar to REDD1, REDD2 does not inhibit mTOR signaling through TSC2 activation directly, but through interactions with the scaffolding protein 14-3-3, which results in activation of the TSC1/2 complex. REDD2 expression is stimulated by leucine and mechanical stretch (60), demonstrating that positive and negative signaling components influence the activation of mTOR simultaneously. As a result, in order for mTOR activation to occur, the positive signaling must be greater than the negative signaling.

1.2.2.6. Summary of the upstream activators of mTOR

In conclusion, physiological states such as stress, hypoxia, and exercise, growth factors, and metabolites are all upstream effectors of the mTOR signaling pathway. With the exception of amino acids, all other effectors work through either activating (negative effectors: low energy status, DNA damage, hypoxia) or inhibiting (positive effectors: high energy status, insulin, amino acids, and growth factors) the TSC1/2 complex which

allows for the inhibition or activation of the mTORC1, respectively. Upstream of mTORC1 the insulin and mTOR signaling pathways converge at Akt (Figure 1.3). Additionally, the MEK/ERK axis feeds into the mTOR signaling pathway through the TSC1/2 complex. This series of events leads to the activation of mTORC1.

1.2.3. Downstream effectors of the mTOR signaling pathway

Following mTORC1 activation by RHEB, a series of cell signaling events occurs leading to the formation of the ribosome and the initiation of the translation of mRNA into a protein. Activated mTORC1 causes the phosphorylation of S6K1 at Thr³⁸⁹, Thr²²⁹, Ser⁴⁰⁴, and Thr⁴¹² and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4EBP1) at Thr^{36/47}, Ser⁶⁵, and Thr⁷⁰ through RAPTOR recruitment (61). The function of RAPTOR in mTORC1 is to recruit substrates in order for these substrates to be in close enough proximity for the catalytic activity of mTOR to phosphorylate the substrates (7). This section of the literature review will be subdivided by the downstream effectors.

1.2.3.1. S6K1

The activation of S6K1 through phosphorylation is required for RNA processing and mRNA translation initiation. S6K1 mediates several downstream proteins including: the transcription factor CREM τ (62), the 80 kDa RNA splicing export factor nuclear cap-binding protein (CBP80; also known as NCBP1) (63), Aly/REF-like target (SKAR; also known as POLDIP3) (64), ribosomal protein S6 (rpS6) (65, 66), and eIF4B (67, 68). Of the targets of S6K1, only SKAR, rpS6, and eIF4B have been extensively studied. SKAR is a scaffolding protein that recruits S6K1 to newly synthesized mRNAs and interacts with the exon junction complex that is involved in splicing pre-mRNA (64). S6K1

influence on both CBP80 and SKAR demonstrates the contribution of S6K1 in enhancing translational efficiency gained from splicing of pre-mRNA (63, 64). Translation efficiency is the effectiveness of the ribosome to form a protein from the mRNA and depends on the availability of amino acids. rpS6 is activated through phosphorylation at Ser²³⁵, Ser²³⁶, Ser²⁴⁰, Ser²⁴⁴, and Ser²⁴⁷ by S6K1, and was once suggested to increase translational efficiency of 5' terminal oligopyrimidine (TOP) mRNAs, which encode components of the translation machinery, through ribosomal recruitment (65, 66); however, this theory has been disproven (69). rpS6 activation is necessary for increased protein synthesis, but the mechanism is still unclear (69). rpS6 is also involved in cell proliferation (70) and determining cell growth (69). rpS6 knockout mouse embryo fibroblasts have accelerated cell division due to a shortened G1 phase (70). rpS6 aids in the determination of cell growth through controlling protein synthesis and cell division (69). S6K1 activates the RNA-binding protein, eIF4B, through phosphorylation at Ser⁴²², and is required for ribosomal recruitment to mRNA (68). Activated eIF4B stimulates the ATPase and helicase activities of eIF4A (67), which enhances the translation of mRNAs with some secondary structure because the helicase function can unwind the mRNA secondary structure (71). Overall, S6K1 is thought to increase translational capacity of the cell through translation component enhancement (also known as ribosome biogenesis) (72), and through ribosomal recruitment to mRNA.

1.2.3.2. 4EBP1

mTORC1 activation leads to the phosphorylation of 4EBP1 at Thr³⁷ and Thr⁴⁶, which is thought to prime 4EBP1 for the subsequent phosphorylation at Ser⁶⁵ and Thr⁷⁰ (73). 4EBP1 acts as a regulator for the formation of the eIF4F complex that is required in

the initiation of cap-dependent mRNA translation. The phosphorylation of 4EBP1 causes its dissociation from eIF4E, which allows eIF4E and eIF4G to associate. eIF4E is the rate-limiting translation initiation factor that initiates cap-dependent translation through binding to the Cap structure (m⁷GpppN) at the 5' end of mRNA transcripts (74); therefore, the phosphorylation of 4EBP1 and subsequent dissociation from eIF4E is a key step in translation initiation. The eIF4E/G complex behaves as a scaffolding protein and recruits eIF4A and eIF3; together forming the eIF4F complex (75) that is bound to m⁷GpppN. This complex acts as a mediator of mRNA binding to the 40S ribosomal subunit. Next, eIF4B associates with the eIF4F complex, facilitating mRNA and ribosome association (76). Prior to mRNA recruitment to the 40S subunit, GTP loaded eIF2 bound to Met-tRNA_i is recruited to the 40S subunit (77). This entire complex is referred to as the 43S pre-initiation complex. At this point, the 43S complex scans the mRNA, once the AUG start codon is reached, the GTPase, eIF5, binds to the complex and hydrolyzes eIF2 loaded with GTP to GDP allowing eIF5B to bind to the complex. This series of events causes displacement of the eIF2 and recruitment of the 60S ribosomal subunit, and relocalizes the 60S ribosomal subunit to the 43S complex (77, 78).

1.2.3.3. Elongation of the polypeptide chain

The initiation complex then begins elongating the peptide chain with the association of eukaryotic elongation factor (eEF) 2 (79). eEF2 mediates the translocation step from the “P” to the “A” site of the peptidyl-tRNA, facilitating ribosome movement along the mRNA (53). The “P” and “A” sites are named because they are the sites where the peptidyl-tRNA and the aminoacyl-tRNA bind, respectively. eEF1A bound to GTP

delivers the next aminoacyl-tRNA to the ribosome at the “A” site (80). A second GTP is then needed to remove eEF1A following correct codon, anti-codon recognition (80). Finally, eEF1B assists in the regeneration of eEF1A to its active form, where it can collect another aminoacyl-tRNA (80). This process requires metabolic energy in the form of GTP, where two molecules of GTP are cleaved for every amino acid added to the polypeptide chain.

1.2.3.4. Termination of the elongation of the polypeptide chain

The last step in translation is termination which occurs when the ribosome reaches the stop codon. The eukaryotic releasing factor 1 (eRF1) is activated by eRF3 in a GTP-dependent manner, which results in eRF1 binding to the ribosome and cleaving the bond between the peptide chain and the tRNA (80). This also requires the hydrolysis of GTP (80). The net result is an increase in protein synthesis.

1.2.3.5. Summary of the downstream effectors of mTOR

In conclusion, the activation of mTORC1 results in the downstream activation of S6K1 and 4EBP1 (Figure 1.3). Each of these proteins are key players in forming the initiation complex. S6K1 activates rpS6 and eIF4B. The phosphorylation of 4EBP1 causes it to dissociate from eIF4E, allowing for the association of eIF4E and eIF4G. This series of events initiates the formation of the initiation complex along the mRNA. Initiation is followed by elongation and, once the polypeptide chain is formed, termination. The net result is protein synthesis.

1.2.4. Feedback mechanisms within the mTOR signaling pathway

The interaction of the insulin and mTOR signaling pathways has been previously discussed in regards to activation through these pathways toward translation initiation. These pathways also interact in a negative feedback manner. Hyperactivation of mTORC1 triggers activated S6K1 to inhibit IRS1, resulting in decreased Akt activation and subsequently mTORC1 (81). This negative feedback loop also has consequences for insulin signaling, such as reduced insulin sensitivity and glucose uptake, which may have implications for horses with metabolic syndrome.

1.2.5. Effects of anabolic stimuli on mTOR signaling in mature mammals

Anabolic stimuli such as meal consumption (82-84), amino acid administration (85, 86), insulin (85, 86), or exercise (87-91) increases the activation of translation initiation factors in the mTOR signaling pathway and increases muscle protein fractional synthesis rates. In mature sedentary animals, skeletal muscle protein turnover occurs at a fairly slow rate with slight variations occurring during fasting and feeding, where the net breakdown and synthesis are greater at these times, respectively (92). Fasting has a greater effect of reducing mTOR signaling in muscles primarily composed of fast twitch fibers compared to slow twitch fibers (93). Specifically, during times of short term fasting (18-24 hours) and long term (2-3 days), phosphorylation of Akt and mTOR, respectively, are greater in muscle primarily composed of slow twitch fibers than fast twitch fibers (93). The absence of insulin, glucose, and amino acids during fasting activates AMPK which subsequently activates the TSC1/2 complex which inhibits mTOR activation (Section 1.2.2.2), ultimately suppressing protein synthesis (37, 56, 94).

The difference in mTOR signaling during short and long term fasting where there is an increase in the phosphorylation of Akt and mTOR, respectively, is due to the pro-survival role of autophagy. Upregulation of autophagy in liver, white adipose tissue, and muscle recycles protein, glycogen and lipid stores and releases them as amino acids, glucose, and free fatty acids into the blood stream. These metabolites can then be used by skeletal muscle to cause partial reactivation of mTOR signaling (95), which suppresses autophagy during long term fasting (96). A recent study showed that Gadd34, a protein that is traditionally upregulated during growth arrest and DNA damage, binds to and dephosphorylates TSC2 at Thr¹⁴⁶² during fasting, leading to mTOR suppression (97).

Meal consumption results in the stimulation of the mTOR signaling pathway (82-84). Following meal consumption, there is an elevation in amino acids, free fatty acids, glucose, and insulin concentrations in the blood stream. As mentioned above (Section 1.2.2), amino acids and insulin stimulate mTOR signaling. Therefore, many studies have been performed using clamps to stimulate a fed state (98) which have demonstrated increased mTOR signaling. Intravenous clamps maintain circulating levels of a desired metabolite or hormone at a set state. Regardless of nutrient content, ingestion of any type of meal will increase circulating insulin. Therefore, in order to determine if both amino acids and insulin are required to stimulate mTOR leading to protein synthesis, reduced amino acid hyperglycemic hyperinsulinemic hyperlipidemic clamp studies were performed, which demonstrated that insulin alone does not sufficiently stimulate mTOR to lead to increased protein synthesis (85, 86), but Akt is activated (86). This phenomenon may be explained due to a lack of available amino acids to form a protein or due to insufficient translation initiation. Additionally, insulin stimulates vasodilation

which promotes protein synthesis through increasing the flow of available nutrients, such as amino acids, to the muscle (99). This mechanism has not been fully elucidated, but does not appear to require Akt activation (99).

Amino acids alone can stimulate the mTOR signaling pathway and subsequently protein synthesis. The increase in protein synthesis due to amino acids is not affected by the method of delivery of amino acids (oral or infusion) (100). Because leucine has been demonstrated as the key amino acid in regulating mTOR signaling, many studies have focused on supplementing leucine. Even though elevating leucine ingestion past 0.14g/kg in mature rodents resulted in a near maximal stimulation of protein synthesis, the activation of mTOR signaling factors appears to be dose dependent (101). Additionally, the ingestion of carbohydrate and amino acid mixture enriched in leucine seems to stimulate mTOR signaling through AMPK-TSC1/2 complex axis, likely from the carbohydrate portion of the mixture (102), and S6K1 and 4EBP1 due to the amino acid and leucine component in resting humans (103). This study also demonstrated that this leucine enriched mixture decreased the phosphorylation of eEF2 which promotes elongation and stimulated muscle protein fractional synthesis rates (103). Regardless of delivery method, amino acids stimulate protein synthesis at the molecular points of translation and elongation.

During exercise, skeletal muscle contractile activity, which can also be thought of as mechanical stimulation, increases. Mechanical stimulation of skeletal muscle fiber (104) can occur through the stimulation of muscle fibers in the laboratory, or subjects performing aerobic or resistance exercise, the result is still some form of mechanical stimulation. Mechanical stimulation increases the activity of the mTOR signaling

pathway. Elevated protein synthesis through activation of the mTOR signaling pathway can lead to an enlargement in muscle fibers (105). Following acute resistance exercise in rodents, there is almost an immediate (5-10 min post exercise bout) increase in mTOR signaling factors: phosphorylation of Akt, 4EBP1, and rpS6 and the association of eIF4E to eIF4G (87). The activation of mTOR itself has had mixed reports in response to mechanical stimulation, where there are reports of no effect on the phosphorylation of mTOR at Ser²⁴⁸¹ (88, 89), an increase in the phosphorylation of mTOR at Ser²⁴⁴⁸ (91), or no effect on the phosphorylation of mTOR at Ser²⁴⁴⁸ (87). However, the downstream effectors have been shown to increase in phosphorylation (61, 65, 67), and these proteins are associated with an increase in the rate of muscle protein synthesis (81). Although the mechanism still requires elucidation, it is currently accepted that there is an increase in the downstream effectors (90, 106, 107) and subsequent protein synthesis (108) following the mechanical stimulation of skeletal muscle. It has been demonstrated that activation of the downstream effectors due to mechanical stimulation is independent of PI3K/Akt signaling (90). However, it appears that mechanical stimulation activates the downstream effector, rpS6 through an mTORC1 independent mechanism. Following mechanical stimulation there is an increase in MEK/ERK signaling (90), which activates p 90 ribosomal S6 kinases (RSK). RSK activate rpS6 through phosphorylation of Ser²³⁵ and Ser²³⁶ (90, 107). Because mTORC1 inhibitor rapamycin can inhibit S6K1 phosphorylation following mechanical stimulation, and not rpS6 phosphorylation, it appears that protein synthesis due to mechanical stimulation is regulated by both the MEK/ERK signaling pathway and the mTOR signaling pathway (90).

The adaptation of skeletal muscle to hypertrophy following exercise has been shown to be fiber type dependent, where fast twitch fibers (Type II) are more susceptible to adaptation than slow twitch fibers (Type I) (109). This can be partly explained by the fact that muscle groups containing greater proportion of Type II compared to Type I fibers have a greater phosphorylation of S6K1 in response to resistance exercise (110). Additionally, maximal lengthening contraction stimulates S6K1, rpS6, and MAP3K greater in Type II fibers compared to Type I, which not only further demonstrates fiber type differences, but also indicates the role of MEK/ERK in contraction driven protein synthesis (111). Another explanation may be that the majority of muscle groups that are predominately slow twitch fibers are mainly used in posture and this continuous mechanical stimulation is less effective than intermittent mechanical stimulation at activating the mTOR pathway (112, 113).

Both aerobic (endurance) and resistance exercise increase mTOR pathway signaling and subsequent protein synthesis (106, 108). However, following resistance exercise the phosphorylation of S6K1, rpS6, and myofibrillar muscle fraction synthesis rates were higher than following endurance exercise in untrained humans (108). Humans who have been trained for resistance exercise have elevated phosphorylation of Akt and eIF4E, and increased myofibrillar protein fractional synthesis rates following a bout of resistance exercise compared to following a bout of endurance exercise in endurance-trained individuals (108). Additionally, mitochondrial protein fractional synthesis rates in the muscle are elevated to the same extent following a bout of endurance or resistance exercise in untrained individuals; however, in trained individuals muscle mitochondrial protein fractional synthesis rates are greater following endurance exercise (108). The

mechanistic differences in which endurance and resistance exercise stimulate the mTOR signaling pathway may explain the differences demonstrated by Wilkinson and colleagues (108). Endurance exercise results in AMPK inhibition of TSC2, while resistance exercise elevates phosphorylation of Akt and subsequent TSC2 phosphorylation (114). A recent study in untrained subjects, following a bout of aerobic exercise the phosphorylated forms of Akt, TSC2, and 4EBP1 were greater in the skeletal muscle of these subjects than in those subjects performing the resistance exercise (106). However, a few studies (115, 116) in humans have indicated an additional upstream mechanism (MEK/ERK) that results in the stimulation of mTOR during resistance exercise, but this has yet to be demonstrated under endurance exercise conditions.

The combination of consuming a high protein meal either before (117) or after (118) an endurance exercise bout has been demonstrated to have greater activation of mTOR signaling than either stimulus alone. However, the effect of resistance exercise on the activation of mTOR signaling appears to be benefitted from the ingestion of a high protein meal following exercise, but not prior to exercise (82, 119, 120). In addition to activating mTOR signaling, elevated MAPK signaling occurs when a high protein meal is consumed following resistance exercise (82). Thus, combining anabolic stimuli (exercise and feeding) results in a further increase in mTOR signaling.

Both amino acid supplementation and exercise have been demonstrated to increase the mRNA expression of several amino acid transporters in skeletal muscle (121-123). One hour following ingestion of essential amino acids, mRNA expressions of the amino acid transporters LAT1/SLC7A5, CD98/SLC3A2, SNAT2/SLC38A2, and PAT1/SLC36A1 were elevated (121). This led to increased protein abundance of

LAT1/SLC7A5 and SNAT2/SLC38A2 3 hours following the ingestion of essential amino acids (121). Resistance exercise had similar effects on amino acid transporters, but the time course was delayed. The mRNA expression of the amino acid transporters CD98/SLC3A2, PAT1/SLC36A1, and CAT1/SLC7A1 increased 6 hours following resistance exercise (122), and the mRNA expression of LAT1/SLC7A5 increased 3 hours following resistance exercise (122). This led to increased protein abundance of LAT1/SLC7A5 and CD98/SLC3A2 at 6 and 24 hours post-resistance exercise, respectively (122). These studies (121-123) also examined VPS34, mTOR, S6K1, rpS6, and eIF4G markers of mTOR signaling, which were elevated following the respective anabolic stimulus used in each study indicating that the elevated expression of amino acid transporters may be dependent on mTOR signaling; however, this requires further elucidation.

1.2.6. mTOR signaling response to anabolic stimuli throughout the life span

As previously mentioned (Section 1.2.2.4), growth factors affect the activation of translation initiation, and thus, during the life stage where there are increased (neonatal and development) or decreased (aging) circulating growth factors (124), there are alterations in the response of mTOR signaling and subsequently protein synthesis to anabolic stimuli. The neonatal period is characterized by the highest growth rate during the lifespan with skeletal muscle showing the largest increase in mass (125). Elevated growth rate in the neonate is a result of protein synthesis rates being higher than protein degradation rates. However, with post-natal maturation, protein synthesis rates gradually decrease (125-127) until both synthesis and degradation are equal in non-growing adult muscle (128).

The activation of translation initiation factors (129-131) and protein synthesis (132, 133) in skeletal muscle are more responsive to feeding during early postnatal life and declines with development. Insulin infusion can stimulate muscle protein synthesis and whole-body protein synthesis during a period of fasting in weaned rats (134) and fetal sheep (135), respectively. However, insulin alone does not stimulate protein synthesis in adult animals or humans (85, 86, 136), which indicates that muscle protein synthesis response may be developmentally regulated. This was further demonstrated by examining the insulin signaling pathway activation in response to feeding where the activation of insulin receptor, IRS-1, IRS-2, PI3K (137) and Akt (23) are greater in the 7 day old neonatal pig compared to the 26 day old. Additionally, increasing insulin elevates protein synthesis and the activation of insulin and mTOR signaling factors in a dose-dependent manner, even when amino acid concentrations are low (138-140).

Elevated rates of protein synthesis during the neonatal period are a result of an increased efficiency of dietary amino acids used for protein accretion (141). Activated mTOR signaling and protein synthesis due to amino acid supplementation decreases with development, similar to the response of insulin and feeding (138, 142). Of all of the essential amino acids, leucine, has received the most attention, because leucine alone has been shown to stimulate mTOR signaling (143), while the other branched chain amino acids cannot stimulate mTOR signaling alone (49). Leucine, individually, does not elicit the same increase in mTOR signaling activation as when it is given with a balanced amino acid mixture (48), which may be partly attributed to the fact that the ability of leucine or any other anabolic stimuli to stimulate protein synthesis is dependent on the availability of amino acids needed for synthesis of a protein (50).

The negative regulators of insulin and mTOR signaling, PTP1B, PTEN, PP2A, and TSC2, are less active in the skeletal muscle of neonates, but the activity of these negative regulators increases with age (23, 144). However, the inhibition of mTOR signaling due to AMPK did not appear to be developmentally regulated when the stimulation was feeding (23). Feeding also inhibits TSC2 activation in the skeletal muscle of neonates, and this is attenuated with age (23).

Not only are the positive factors of the insulin and mTOR signaling pathways more active during neonatal development, but there is also a greater total abundance of these factors in skeletal muscle. The abundance of the insulin receptor, PDK-1, and Akt in skeletal muscle decreases with development (131, 137). The abundance of mTOR and RAPTOR also decreases with postnatal development, which results in a decreased association of the two proteins (23, 129, 145). However, the abundance of downstream signaling factors such as S6K1, rpS6, and 4EBP1 do not change with development (146). In addition to the highly responsive mTOR signaling pathway, skeletal muscle of neonates has been reported to have a higher concentration of ribosomes which decreases with age (127, 147), and may also contribute to the elevated protein synthetic rates because of a higher efficiency to synthesize proteins. Overall, the skeletal muscle of the rapidly growing neonate is physiologically adept to synthesize protein efficiently and thus accrete muscle mass.

Aging is characterized by the involuntary loss of muscle mass and strength which is referred to as sarcopenia. Sarcopenia has been partially attributed to a multitude of factors which include a decline in physical activity and a less than optimal diet in the aging human population (148-150). Sarcopenia is a result of an imbalance of protein

synthesis and breakdown. The increase in muscle protein fractional synthesis rates in response to anabolic stimuli such as exercise (149, 151, 152), amino acids (149, 153), insulin (154), or meal consumption (152, 153) is decreased in the aged compared to the younger adult, and may be due to dysregulation of signaling factors in the insulin and mTOR signaling pathways (155, 156). Meal consumption in the aged does not increase protein synthesis or activate the mTOR signaling pathway to the same magnitude as in the young adult (155-157); however, when the meal is supplemented with leucine there is a restoration in the anabolic effect of feeding (158). This may indicate that amino acid supplementation, specifically leucine, plays a larger role in stimulating protein synthesis than insulin in the aged. Physiological hyperinsulinemia does not stimulate mTOR signaling in aging skeletal muscle as it does in young adult skeletal muscle (159, 160); however, at supraphysiological levels of hyperinsulinemia mTOR signaling in the aging skeletal muscle is restored (159). This may indicate that insulin resistance with aging may at least partially be responsible for sarcopenia (161). Additionally, aerobic exercise (162, 163), amino acid supplementation (164), and vaso-dilation (165) can improve the anabolic response of mTOR signaling to hyperinsulinemia. Thus, activation of the mTOR signaling pathway in aging skeletal muscle appears to behave similar to young adult skeletal muscle when stimulated by multiple anabolic stimuli rather than just one. This also holds true for resistance exercise, which more effectively stimulates mTOR signaling in the elderly if amino acids are supplemented following the exercise bout (166). However, amino acid supplementation prior to a bout of resistance exercise does not enhance muscle protein synthesis relative to resistance exercise alone (120). Similar to the young adult, aging skeletal muscle has increased mRNA expression of several

amino acid transporters (LAT1/SLC7A5, CD98/SLC3A2, PAT1/SLC36A1, CAT1/SLC7A1) following resistance exercise; however, the expression of these amino acid transporters remain elevated for a longer period of time in the aged skeletal muscle (122). This may mechanistically explain how adding amino acid supplementation to resistance exercise stimulates the activation of mTOR signaling pathway to the same extent as in the young adult skeletal muscle.

It has been stated that mTOR signaling and protein synthesis decrease with aging; however, the signaling factor that appears to be the most influenced by aging is S6K1. The activation of S6K1 in response to various anabolic stimuli such as meal consumption (129, 145, 156), amino acids (142, 155-157), insulin (142, 156, 162), and exercise (157, 162) consistently decreases throughout development and into aging. Additionally, the activation of S6K1 in the post-absorptive state declines during development (142, 145) and from mature to aging adults (155). The influence on aging is also demonstrated in the time course response of S6K1 compared to the other mTOR signaling factors. With aging, the activation of the mTOR signaling factors to exercise and amino acids is delayed except for the activation of S6K1, even though the abundance is still lowered in the aged (157). It has also been demonstrated that the total abundance of S6K1 declines during aging (155). The response of S6K1 to various anabolic stimuli during aging indicates that it may be the most influenced by aging.

Even though it is currently accepted that there is an overall decline with aging in the activation of mTOR signaling and protein synthesis in response to anabolic stimuli, the research that supports this only examines the neonatal phase and post-adulthood aging. This leaves a hole in the literature for the peri-pubertal, and post-pubertal slow

growth phase occasionally referred to as adolescence. In order to be sure that mTOR signaling and protein synthesis decline throughout the lifespan this life phase must not be ignored. Unfortunately, due to the restrictions of common research models, this phase has been overlooked. In the humans there are regulations making it difficult to collect muscle tissue from healthy children under the age of 18 years; whereas, the time of the adolescent phase is extremely brief in both rodents and pigs, making it difficult to implement experimental conditions. As a result, this phase has received little to no research attention.

1.3. THE MOLECULAR MARKERS OF PROTEIN BREAKDOWN AND THE INTERACTION WITH mTOR SIGNALING

A review of the literature pertaining to muscle mass accretion would not be complete without mentioning protein breakdown. Although the focus of this dissertation has been on protein synthesis, muscle mass cannot be accreted without the rates of muscle protein synthesis being greater than the rates of muscle protein breakdown. Protein breakdown, similar to protein synthesis, has associated pathways that can be studied to improve our understanding of the process. The main pathways involved in protein breakdown are the lysosome, ubiquitin-proteasome, and calcium dependent pathways, and apoptosis (167); however, skeletal muscle contains a limited number of lysosomes, and the contribution of lysosomal proteases and cathepsins to proteolysis or myofibrillar protein degradation is limited (168). The lysosomal system is mainly responsible for the degradation of organelles and membrane proteins. The ubiquitin-proteasome pathway is responsible for the degradation of most of the skeletal muscle proteins (169), and requires muscle specific ubiquitin ligases, such as muscle-RING-

finger protein 1 (MuRF1) and atrogin-1/MAFbx (169-171). There are three ubiquitin ligases involved in the addition of ubiquitin to a protein substrate: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-ligating enzyme (172). The E3 ubiquitin ligases confer substrate specificity (172), and muscle has the specific ubiquitin ligases (169-171), mentioned earlier. Many sarcomeric proteins are degraded through the calcium dependent pathway due to ubiquitous calpains 1 and 2, and muscle specific calpain 3 (168, 173). Within skeletal muscle, satellite cells, which are muscle stem cells, are the most susceptible to apoptosis, which activates the caspases (174) and is regulated by the transcription factor, forkhead box proteins (FoxO) (175). Caspase-3 may be involved in the initiation of the degradation of actin (176). FoxO also regulates the expression of atrogin-1 (177), which is a muscle specific E3 ubiquitin ligase (171). The molecular components of these pathways such as MuRF1, atrogin-1, calpain 3, and FoxO can be studied as indicators of protein degradation.

Many of the mTOR signaling proteins interact with the main molecular components of protein degradation. The active form of Akt inhibits FoxO through phosphorylation, which recruits 14-3-3 proteins preventing relocalization to the nucleus (175). Additionally, FoxO is inhibited by the phosphorylation of serum- and glucocorticoid regulated kinase by mTORC2 and PDK1 (21). Caspase 3 may also be inhibited by the activated form of Akt (177), but further elucidation is required. Nuclear factor κ B (NF- κ B) is stimulated by inflammatory cytokines, and may play a role in inactivating Akt and increasing the expression of MuRF-1 (177); however further research is warranted. Additionally, activation of mTOR inhibits atrogin-1,

independently of FoxO (178); however this mechanism requires further elucidation.

Therefore, Akt and mTOR play a pivotal role in both protein synthesis and degradation.

Similar to protein synthesis, protein breakdown is altered during various physiological states such as aging (35, 179), and during postprandial and post-absorptive states (180). During development, muscle protein metabolism is elevated, and rates of both muscle protein synthesis and breakdown are increased, with a net result of protein synthesis (181). Research examining the molecular markers of protein degradation is limited; however, it has been determined that caspase-3 and caspase-9 are downregulated during development in the skeletal muscle of rodents (182). This is an area that requires further elucidation during development; however, aging rodents have elevated proteasome content and activity and free ubiquitin and ubiquitylated protein concentrations in skeletal muscle compared to younger mature rodents (179). The aged also have higher abundance of FoxO than the mature counterparts (35). Additionally, ubiquitination rate is greater in the post-absorptive state than the postprandial state in the aged rodent whereas the mature rodents are not affected by physiological state (180). Overall, it appears that there is an increase in the molecular markers of protein degradation throughout the lifespan.

Although there is much work still to be done examining these interactions between the molecular markers of protein synthesis and breakdown, it appears that protein breakdown and protein synthesis are both regulated by the activation of Akt (175, 177) and mTOR (178). Additionally, with aging there is an elevation in the molecular markers of protein degradation (179-181), which is pronounced during the post-

absorptive state (180). Ultimately, in order to study protein accretion effectively, both protein synthesis and degradation need to be examined.

1.4. WHOLE-BODY AND MUSCLE PROTEIN SYNTHESIS:

METHODOLOGIES AND THE EFFECT OF ANABOLIC STIMULI

1.4.1. Isotope methodologies

Up to this point the literature review has focused on the mTOR signaling pathway and how it results in protein synthesis. Studying the mTOR signaling pathway provides insight to researchers as to whether or not a potential exists for an increase in the rate of muscle protein synthesis. Isotopic measurements are used to quantify rates of protein synthesis. Both whole-body and muscle protein synthesis rates are typically measured using the infusion of a stable amino acid isotope, for example [1-¹³C]phenylalanine. By measuring phenylalanine flux and rates of phenylalanine oxidation to carbon dioxide (CO₂) during [1-¹³C]phenylalanine infusion, researchers can estimate the rate of whole-body protein synthesis using a stochastic approach (Figure 1.4). This estimate of whole-body protein synthesis is based on the principle that phenylalanine flux is equal to the rate that phenylalanine enters (from the diet and protein breakdown) and exits (through oxidation to CO₂, use in protein synthesis and conversion to tyrosine) the free amino acid pool, and is calculated from the dilution of the isotope by unlabeled phenylalanine in the plasma (183). The calculated difference of measured phenylalanine converted to CO₂ and flux results in non-oxidative disposal, which is an indirect measure of whole-body protein synthesis, because non-oxidative disposal includes the rate of phenylalanine used for protein synthesis and phenylalanine conversion to tyrosine. If researchers supply both phenylalanine and tyrosine at equal levels to the different treatment groups, then it is

generally assumed that the phenylalanine conversion to tyrosine should also be equal among treatment groups; therefore, an increase in phenylalanine oxidation indicates a decrease in whole-body protein synthesis. During these infusion procedures, the rate of muscle protein synthesis can also be measured, through the collection of muscle biopsies, during and at the end of the isotope infusion and measuring the rate of [1-¹³C]phenylalanine incorporation into muscle protein (184). Although phenylalanine was used during this explanation of isotope methodologies, researchers can use any stable indispensable amino acid isotope, as long as the carboxyl group is released as ¹³CO₂ in the breath as the amino acids are catabolized.

1.4.2. Alterations in whole-body protein metabolism in response to anabolic stimuli

Whole-body protein synthesis is the sum of all of the individual tissue rates of protein synthesis and reflects the overall change in protein synthesis occurring during the physiological state that is being studied. This makes it a useful tool to understand what is occurring on a large scale basis.

Whole-body protein metabolism is affected by anabolic stimuli such as meal consumption (185, 186) and exercise (187). During the post-absorptive state, isotope tracer oxidation is less than following the consumption of a high protein meal (186), indicating that feeding increases the amino acid flux. The additional amino acids that enter the free amino acid pool following meal consumption (dietary intake) that are not utilized for protein synthesis or conversion to other metabolites are oxidized; therefore, during the postprandial state amino acid isotope tracer oxidation is elevated because there is an excess of free amino acids in the plasma pool. However, during the post-absorptive state the opposite is true, there is a reduction in free amino acids in the plasma pool

because amino acid entry is coming entirely from protein degradation rather than dietary intake, as is the case of the postprandial state. Because there is a reduction in the free amino acid pool, there is not an excess of free amino acids, which results in a decrease in amino acid oxidation. If flux is unchanged then, the increase in amino acid oxidation typically results in a decrease in protein synthesis; however, during the post-absorptive and postprandial periods, flux is not equal. Non oxidative disposal can then be derived from the difference in flux and oxidation (Section 1.4.1). In addition to a reduction in amino acid oxidation during the post-absorptive state, there is a reduction in protein synthesis in comparison to protein breakdown (186, 188, 189). As a result, protein balance is in a negative state, where protein degradation is occurring at a higher rate than protein synthesis. However, the opposite is true during the postprandial state, where non-oxidative disposal is greater than release from protein breakdown (186, 188, 189), which results in a positive protein balance, because there is a greater rate of protein synthesis than protein degradation. Overall, during the postprandial state there is a net increase in whole-body protein synthesis leading to protein accretion, and a net loss of whole-body protein during the post-absorptive period. In a mature, sedentary, individual this cyclical balance of protein gain and loss during the postprandial and post-absorptive states throughout the day is equal, resulting in no accretion of protein.

Whole-body protein metabolism is altered both during and following an exercise bout. Because most exercise bouts last only a short period of time (minutes to hours), many researchers hypothesize that post-exercise changes in whole-body protein metabolism are of greater physiological relevance than changes occurring during the exercise state. As a result, there is an abundance of literature examining the post-exercise

effects on whole-body protein metabolism, whereas studies of whole-body protein metabolism during exercise are limited and typically examine endurance or aerobic type exercise. There are conflicting reports regarding changes in whole-body protein breakdown during aerobic exercise, where it has been demonstrated to be elevated (190-192) or remain unchanged (193, 194). However, all of these reports are in agreement that there is an increase in amino acid tracer oxidation (190, 192, 194), which may be derived from an increase in protein degradation. The majority of these studies used leucine as the amino acid tracer, which is extensively catabolized in skeletal muscle. Additionally, whole-body protein synthesis has been demonstrated to both decrease (190, 192) and remain unchanged (193) during aerobic exercise. This provides supporting evidence for a negative protein balance during exercise, where there is an overall loss in whole-body protein. The authors of these reports attribute two possible tissue systems for the increase in whole-body protein breakdown during aerobic exercise: the gastrointestinal tract (191), and skeletal muscle (190). Reports on post-exercise alterations in whole-body protein metabolism are also conflicting; however, this may be primarily due to differences in the types of exercise. Whole-body protein synthesis is elevated and unchanged following aerobic (190, 195) and resistance (196) type exercise, respectively. Whole-body protein breakdown has been demonstrated to decrease (190) or remain unchanged (195) from pre-exercise resting rates following aerobic exercise. Additionally, whole-body protein breakdown is unchanged from resting rates following resistance type exercise (196, 197). Thus, it appears that following resistance exercise there is no change in whole-body protein metabolism, whereas, following endurance exercise there appears to be an increase in protein accretion. The majority of these studies, regardless of exercise type,

examine individuals exercising during the post-absorptive period, which may attribute to a negative protein balance and may not be physiologically relevant to the common practices of athletic humans or horses. Therefore, additional research is warranted examining the effects of exercise on whole-body protein metabolism during the postprandial state.

Skeletal muscle is responsible for 25% of whole-body protein synthesis (198, 199), and fat free mass plays a key role in whole-body protein synthesis (187, 200, 201). Specifically, differences in the fat free mass of human subjects accounts for up to 87% of the variation seen in whole-body protein kinetics (187). For this reason, some researchers theorize that the reduction in whole-body protein synthesis during the postprandial state seen in the aged may not be due to aging specifically, but rather due to a reduction in the fat free mass in this population (185). Regardless of whether the reduction in whole-body protein synthesis in the aged population in response to the anabolic stimuli of meal consumption is actually due to aging or a product of the increase in fat mass, it is certain that there are differences in whole-body protein metabolism in the frail aged population and younger mature adults, which may suggest a higher protein requirement (202). However, there is no effect of age on whole-body protein synthesis during the post-absorptive state (186). Unfortunately, studies examining whole-body protein metabolism in children and adolescents are limited, due to the ethical constraints in studying this population (203), compared to healthy children of the same age. It has been determined that children with diabetes have elevated whole-body protein degradation and amino acid oxidation (203). Further research is warranted to examine

whole-body protein metabolism in a research model for healthy human children and adolescents.

1.4.3. Alterations in muscle protein fractional synthesis rates in response to anabolic stimuli

Skeletal muscle comprises approximately 50% of body weight, but only contributes to 25% of whole-body protein synthesis (198, 199). As a result, changes in muscle protein synthesis may only be detected at the whole-body level if they are dramatic. Furthermore, if another tissue decreases its rate of protein synthesis and the muscle increases its rate of protein synthesis, then there would be no net effect measured at the whole-body level. Because of this, it is useful to measure skeletal muscle protein synthesis directly. In Section 1.2, the anabolic stimuli that increase muscle protein fractional synthesis rates were discussed. There have been numerous studies (103, 204-206) examining feeding-induced changes in muscle protein synthesis and breakdown, which highlight the more dramatic elevation in muscle protein synthesis compared to muscle protein breakdown (Section 1.3). Because of the alterations in protein synthesis and breakdown in response to anabolic stimuli, it can be stated that within protein metabolism the overall limitation is within the regulation of protein synthesis. Muscle protein synthesis increases following meal consumption in adult humans (103, 204-206) and neonatal pigs (133, 207), but eventually slows and returns to basal levels approximately 240 min post meal consumption, as demonstrated in the neonatal piglet (207). Muscle protein synthesis is greatly affected by extracellular levels of amino acids following meal consumption; however, the effects of amino acids on muscle protein synthesis is graded until saturation (208). Regardless of age, the muscle protein synthesis

response to graded intakes of amino acids has been demonstrated to be curvilinear, with a plateau at 0.13 g/kg body weight of indispensable amino acids in humans (155). However, it is important to note that the postprandial rates of muscle protein synthesis at any given concentration of amino acids for younger adults (~28 years old) was greater than for older humans (~70 years old) (155). There are no age related differences between adults and aged humans in muscle protein synthesis during the post-absorptive state (155-157). It was previously mentioned that whole-body protein synthesis is affected by fat free mass and this also holds true for muscle protein synthesis, where muscle protein fractional synthesis rates in lean young adult (20 years old) and aged lean men (75 years old) following the co-ingestion of a protein and leucine supplement are not different (209).

Exercise may influence protein metabolism to a greater extent in muscle than in any other tissue in the body. However, similar to whole-body protein metabolism, different types of exercise affect muscle protein fractional synthesis rates differently. Myofibrillar protein fractional synthesis rates are elevated and remain unchanged from pre-exercise resting rates following a bout of resistance and aerobic type exercise, respectively, in previously untrained muscle (108). However, mitochondrial protein fractional synthesis rates were elevated from pre-exercise resting rates following a bout of exercise, regardless of exercise type, in previously untrained muscle (108). Other reports (210, 211) examining mixed muscle protein fractional synthesis rates immediately following aerobic type exercise are conflicting with reports of no change (210) and increases (211) compared to pre-exercise rates. Wilkinson and colleagues (108) suggest that these conflicts are due to differences in exercise intensity, increased AMPK (Section

1.2.2), time of sampling, and only examining mixed muscle rather than mitochondrial and myofibrillar fractional synthesis rates, which may have concealed the changes in mitochondrial fractional synthesis rates. Additionally, the increased mitochondrial fractional synthesis rates following aerobic exercise (108) correspond to increased mitochondrial content with aerobic training (212, 213).

In addition to the bout of exercise, the training status of an individual can alter both resting and post-exercise muscle protein fractional synthesis rates. Resting myofibrillar fractional synthesis rates were elevated following 10 weeks of resistance training (108). However, a single bout of resistance exercise increased myofibrillar fractional synthesis rates to the same extent in trained and untrained individuals (108). Resistance training did alter resting mitochondrial fractional synthesis rates, and the increase in mitochondrial fractional synthesis rates seen with a single bout of resistance exercise in untrained individuals was not present when trained individuals performed a single bout of resistance exercise (108). Ten weeks of aerobic exercise training did not alter myofibrillar or mitochondrial fractional synthesis rates at rest or following a bout of aerobic exercise, compared to pre-training rates (108). The authors hypothesized that the untrained individual may be in a heightened state of responsiveness to the anabolic stimuli of exercise (108).

As with other anabolic stimuli, such as feeding, the response of muscle protein fractional synthesis rates to an exercise bout is altered with aging. Reports have indicated a delayed response of mixed muscle protein fractional synthesis rates in aged subjects (3-6 hours following an exercise bout) compared to adults (1-3 hours following an exercise bout) (157). However, the use of resistance exercise to increase muscle mass and

strength is recommended in older individuals for the prevention or delay of sarcopenia (214-216). Furthermore, combining anabolic stimuli, specifically consuming amino acids following a bout of resistance exercise increases mixed muscle protein fractional synthesis rates beyond the effects of amino acid supplementation or exercise alone (119, 217, 218), and may prove to be useful in reducing sarcopenia in the aging population; however, further research is warranted.

1.4.4. Summary of the alterations in whole-body and muscle protein metabolism in response to anabolic stimuli

The use of isotope infusion protocols is a useful and commonly practiced technique in human research examining both whole-body and muscle protein synthesis. Both whole-body and muscle protein synthesis in response to anabolic stimuli such as meal consumption (103, 157, 202) and exercise (157, 211), declines with age. However, aging does not alter whole-body and muscle protein synthesis during the post-absorptive state (122, 155, 186). Additionally, at least a portion of the variation in both whole-body and muscle protein synthesis are attributed to differences in fat free mass of the subjects (187). Overall, using isotope infusion protocols to study whole-body and muscle protein synthesis allows researchers to examine protein metabolism of the entire subject and at the tissue level. When these techniques are used in addition to molecular tools to determine mTOR signaling, then information about the whole system can be studied from the cellular to the whole-body level.

1.5. AN OVERVIEW OF THE LITERATURE ON INFLAMMATION

RELATING TO THE RESEARCH IN THIS DISSERTATION:

MECHANISMS OF INFLAMMATORY SIGNALING, THE EFFECTS OF ACUTE AND CHRONIC INFLAMMATION IN CIRCULATION AND SKELETAL MUSCLE, AND THE REDUCTION OF ACUTE AND CHRONIC INFLAMMATION THROUGH NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

1.5.1. Inflammatory signaling

Inflammation is a physiological response to a variety of stimuli such as infection, tissue damage, exercise, and aging. Inflammation can occur as an acute or chronic process depending on the stimulation. For example, the inflammatory response to a disease or aging can be chronic, whereas the response to infection, exercise and tissue damage from a muscle biopsy is typically acute. Both acute and chronic inflammation will be discussed below (Section 1.5.3-1.5.4), but first, this review of inflammation will examine inflammatory cytokine production (Figure 1.5).

There are a number of inflammatory cytokines that play a pivotal role in the development of both chronic and acute inflammation. A cytokine is a small (molecular weights range from 8 to 40,000 d) nonstructural signaling protein, and an inflammatory cytokine is a cytokine that mediates inflammation. Examples of cytokines include the interleukins (-1,-6,-8, -10, -12,-18), tumor necrosis factor- α (TNF- α), and interferon γ (IFN- γ). When tissue damage (which we will define as anything that elicits an inflammatory response such as exercise, infection or a muscle biopsy) occurs, mast cells that are located in the damaged tissue degranulate, resulting in the release of pro-

inflammatory cytokines, histamine, and eicosanoids (219). Histamine, eicosanoids, and the initial pro-inflammatory cytokines move from the damaged tissue into circulation, resulting in neutrophil migration from bone marrow to circulation. Full infiltration of neutrophils into the damaged tissue occurs within 6 hours (219). Neutrophil migration to the damaged tissue occurs as a result of mediators of acute inflammation from the vascular endothelial cell release of E- and P-selectin which are endothelial adhesion molecules (219, 220). Once at the site of damage, neutrophils begin to tether to the vascular endothelium and release macrophage inflammatory proteins. These macrophage inflammatory proteins attract and activate macrophages to the site of damage (219, 220). Macrophages arrive at the site of tissue damage 5-6 hours later. Activated macrophages secrete IL-1, IL-6, and TNF- α (221, 222). The effects of these inflammatory cytokines are similar: induction of localized fever, synthesis of acute phase inflammatory proteins in the liver, increased vascular permeability, and T and B cell activation (219, 221, 222). Both IL-1 and TNF- α also increase adhesion molecules on vascular epithelium, fibroblast proliferation, and induce IL-6 and IL-8 secretion, which are potent chemoattractors of neutrophils and can be produced by almost all cell types (219, 221, 222). The stimulation of platelet production and immunoglobulin synthesis are unique effects of the interleukins and IL-6, respectively (219, 221, 222). IFN- γ also activates macrophage and monocyte secretion of inflammatory cytokines, and is produced by activated CD4⁺, CD8⁺, and natural killer cells (223). The overall response of inflammatory cytokines to tissue damage in circulation is to draw attention to the site of tissue damage, and stimulate macrophage migration into the damaged tissue.

Inflammatory cytokines and macrophages migrate into the damaged tissue, where activated macrophages secrete IL-1, IL-6 and TNF- α . These inflammatory cytokines then activate I κ B kinase complex (I κ KB), which phosphorylates NF- κ B (224). Even though IL-1, IL-6, and TNF- α are secreted together in the body, cell culture work with C2C12 myocytes treated with TNF- α indicates that I κ KB activation is very susceptible to TNF- α alone (225). Although NF- κ B is phosphorylated, activation does not occur until I κ B is ubiquitinated and targeted to the proteasome for degradation (224). Activated NF- κ B interferes with mTOR signaling and stimulates further production of inflammatory cytokines, as described in Section 1.3. Overall this entire inflammatory response typically results in the involvement of the components of the adaptive immune response (T and B cells), although it can occur without it (as described above). For the purposes of this literature review, where the focus of inflammation is due to exercise and aging, the involvement of the adaptive immune response is limited and therefore will not be discussed.

Until this point, the focus has been on the pro-inflammatory response; however, some of the pro-inflammatory cytokines also have anti-inflammatory properties. Additionally, there are many cytokines that function in suppressing the inflammatory response, and are appropriately referred to as anti-inflammatory cytokines. The role of anti-inflammatory cytokines is to regulate the pro-inflammatory response so that healing can occur without resulting in further tissue damage. The role of IFN- γ as a pro-inflammatory cytokine was discussed above; however, IFN- γ suppresses the production of IL-1 in macrophages (226), and inhibits IL-1 activity through stimulating the production of IL-1 receptor antagonist (227) and type II IL-1 receptors (222, 228), which

binds the receptors of IL-1, inhibiting interaction of IL-1 and its receptor. IFN- γ also inhibits IL-18 function through the stimulation of IL-18 binding protein gene induction (229, 230). This may serve as a negative feedback loop because IL-18 induces IFN- γ production (231). Additionally, IFN- γ disrupts the stimulation of IL-8 production by TNF- α (232) and IL-1 (233), and reduced IL-8 expression has been associated with impaired TNF- α induced NF- κ B activation (234). Because of the involvement of NF- κ B in potentially reducing protein synthesis through interactions with mTOR signaling (Section 1.3), this may be a regulatory mechanism in the protection of protein loss; however, further research is necessary. Other major anti-inflammatory cytokines include transforming growth factor- β (TGF- β) and IL-10. TGF- β inhibits NF- κ B activation through the decreased production of IL-8, IL-1 and TNF- α . Additionally, TGF- β blocks differentiation of naive CD4⁺ and CD8⁺ cells (235), which when activated secrete IFN- γ , a pro-inflammatory cytokine that stimulates the macrophage secretion of IL-1 and TNF- α . IL-10 is secreted by anti-inflammatory macrophages, T, B, dendritic, and mast cells, and neutrophils and eosinophils (236) in response to IL-12 (237), IL-6 (238), IL-27 (239), and TGF- β (240). IL-10 acts in a negative feedback loop inhibiting the secretion of IFN- γ , TNF- α , IL-1 and IL-2 (241-243). Therefore, following a pro-inflammatory response, IL-10 secretion is induced and serves to mitigate inflammation, possibly inhibiting potentially harmful effects of inflammation (protein loss) on tissue.

1.5.2. Summary of inflammatory signaling

There are two types of inflammatory cytokines, pro-and anti-inflammatory cytokines, which stimulate and suppress inflammation, respectively. Some of the main

pro-inflammatory cytokines include: IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, TNF- α , and IFN- γ . The major anti-inflammatory cytokines include: IL-10, TGF- β , and IFN- γ . IFN- γ is a unique cytokine with both pro- and anti-inflammatory properties. When tissue is damaged, there is a rise in circulating pro-inflammatory cytokines which results in the migration of macrophages into the damaged tissue, resulting in the production of more pro-inflammatory cytokines. In relation to protein metabolism, the increase in pro-inflammatory cytokines in the damaged tissue activates NF- κ B, which interacts with mTOR signaling and may reduce protein synthesis in the damaged tissue. Anti-inflammatory cytokines regulate this process and are secreted in response to elevated circulating pro-inflammatory cytokines. The purpose of anti-inflammatory cytokines is to protect the tissue from potentially harmful effects that may be induced by prolonged inflammation.

1.5.3. Factors affecting acute inflammation

In addition to acute illness, acute inflammation can occur following muscle biopsy collection or a bout of exercise. Acute inflammation is characterized by a local increase in inflammatory cytokines that persists for no longer than 24 hours (244, 245). There may also be an increase in systemic inflammatory cytokines which does persist longer than 24 hours (244, 245).

The effect of percutaneous muscle biopsy collection on muscle and circulating inflammatory cytokines has been examined. From a methodological standpoint, the use of a single incision for the collection of repeated biopsies separated by 30 min is not advised because of alterations in the stress related ERK1/2 signaling pathway (246),

which is affected by inflammatory cytokines (247). Unfortunately, research examining the effects of using of a single incision site for the collection of repeated biopsies on muscle inflammatory cytokines is limited, but previous work (246) demonstrating an increase in the activation of the ERK1/2 pathway, suggests that there may be an increase in muscle inflammatory cytokines, even though muscle inflammatory cytokines were not examined. Thus, there is a need to study the effects of the collection of repeated biopsies from a single incision site on muscle inflammatory cytokines. Further research is also necessary to determine if the use of single incision site for multiple biopsies induces a greater inflammatory response than the use of multiple incision sites for additional biopsies. In humans, the collection of two percutaneous muscle biopsies from separate incision sites on the right leg immediately and 30 minutes following the collection of the first biopsy did not result in differences in IL-6 mRNA expression (248). An additional biopsy was collected from the left leg 31 minutes following the incision on the right leg of the subjects in this study and again no differences in IL-6 mRNA expression were reported (248). However, in a biopsy collected from the right leg 123 minutes after the initial biopsy, from a new incision site, there was a 1.8 fold increase in IL-6 mRNA expression (248). The results from this study indicate that the collection of multiple biopsies from the separate incision sites can be performed within a 30 minute period without altering IL-6 expression. Additionally, the collection of repeated biopsies using a new incision site for each consecutive biopsy in humans has demonstrated an increase in the percentage of neutrophils in circulation at 30 minutes and up to 24 hours following the initial biopsy from the percentage of circulating neutrophils at the time of the initial biopsy (245). It was also determined that the expression of IL-1 β was elevated in the

muscle 30 minutes after the collection of the initial muscle biopsy, and IL-1 α expression in skeletal muscle was elevated at 30 minutes and up 6 hours following the initial biopsy (245). However, in horses there was no change in muscle or circulating inflammatory cytokine mRNA expression (IFN- γ , IL-1, IL-6, and TNF- α) for 24 hours following the initial biopsy when a separate incision site was used for each biopsy (244). This apparent disconnect between human and equine skeletal muscle requires further investigation to determine whether it is a result of different research protocols or due to different physiological mechanisms in the two species.

The vast research examining the effects of aerobic exercise on inflammation varies in both tissue collection methods and in the inflammatory markers studied; however, it is clear that a bout of aerobic exercise elicits an acute inflammatory response. The percentage of neutrophils and monocytes in circulation were elevated at 6 hours and on the fourth day following a bout of aerobic exercise (245). Circulating levels of IL-6 are elevated for 24 hours following a plyometric exercise (249), which is aerobic exercise that involves rapid deceleration followed by rapid acceleration in the opposite direction, and for at least 4 hours (last sampling point) following an incremental aerobic exercise test (250). Other pro-inflammatory cytokines, TNF- α and IL-1 β , were also elevated in circulation for 3 hours and 30 minutes, respectively, following aerobic exercise (250). In addition, the anti-inflammatory cytokine, IL-10, and the functional blocker of IL-1, IL-1 receptor antagonist, are elevated in circulation following aerobic exercise for at least 4 hours, which was the last sampling point in that study (250). Unfortunately, the literature examining exercise induced skeletal muscle inflammation is limited; however, IL-1 β has been demonstrated to increase in skeletal muscle 30 minutes following an aerobic

exercise bout (245). Although the majority of this work has been performed in humans, performance of an incremental exercise test in horses increased IFN- γ , IL-6, and TNF- α in skeletal muscle 1 hour, 30 minutes, and immediately following exercise, respectively, whereas skeletal muscle IL-1 mRNA expression remained unchanged from pre-exercise values (244). The authors of this study also examined circulating inflammatory cytokines and demonstrated an increase in IL-1, TNF- α , and IFN- γ from 2 to 6 hours, at 6 hours, and immediately following exercise, respectively; however, there was no change in the mRNA expression of circulating IL-6 following exercise (244). Although species may play a role in the time course elevations of the respective inflammatory cytokines, which needs further investigation, it is certain that a bout of aerobic exercise induces an acute inflammatory response.

The role of exercise induced inflammation is currently thought to be a result of muscle damage in the form of disrupted contractile structures (251, 252) and permeabilization of the sarcolemma (253, 254). Thus, the body of literature has been focused on bouts of strenuous aerobic exercise with limited investigation of resistance exercise, which has been the focus of the majority of exercise studies on mTOR signaling and protein synthesis. As a result of the interaction of downstream inflammatory signaling components (Section 1.5.1) and mTOR signaling factors (Section 1.2) needs to be studied in response to resistance exercise. However, it has been determined that 3 hours after a bout of resistance exercise, circulating mRNA expression of TNF- α , IL-1 β , IL-6, and IL-8 were elevated from pre-exercise levels, despite no change in the mRNA expression in skeletal muscle of these inflammatory cytokines (255).

1.5.4. Factors affecting low grade chronic inflammation

Aging is characterized by chronic low grade inflammation which has been coined “inflamm-aging” (256). Low grade chronic inflammation results from increased concentrations of circulating inflammatory cytokines (257-259) and a prolonged acute inflammatory response to infections and tissue damage in the aged, compared to their younger counterparts (260-262). Compared to the healthy, mature population, there is an elevated expression of pro-inflammatory cytokines, such as TNF- α (263–265) and IL-6 (263, 266, 267), increased concentration of acute phase proteins, for example, C-reactive protein, which is associated with the acute inflammatory response (268), and high neutrophil count (269) in the circulation of the human aging population. Additionally, there is an increase in anti-inflammatory cytokine, IL-10, and anti-inflammatory mediator, IL-1 receptor antagonist, in human aging circulation (270). There is an overall increase in circulating inflammatory cytokines with aging.

Low grade chronic inflammation has been associated with the loss of skeletal muscle mass, referred to as sarcopenia, in the aging population (271-273). The mRNA expression of circulating TNF- α and the concentration of circulating soluble TNF- α receptor have been inversely correlated with hand grip (274) and muscular strength (275), respectively, in aged humans (older than 85 years of age). Elevated serum IL-6 concentrations have been correlated with decreased muscle strength in the aged (271). Additionally, reduced muscle strength that has been attributed to increased circulating IL-6 and TNF- α has also been correlated with decreased muscle mass in the aged (273). As a result, low grade chronic inflammation is thought to be a partial cause of reduced skeletal muscle mass in the elderly.

The alterations in skeletal muscle inflammatory cytokines and their relationship to sarcopenia have not been as well characterized as the elevations in the circulating inflammatory cytokines in the aged population. Current research on the role of IL-1 β and TNF- α mRNA expression in skeletal muscle in the aged is inconclusive (276-278), and there is no difference in skeletal muscle mRNA expression of IL-6 and TGF- β between young and aging humans (276, 278, 279). However, the skeletal muscle mRNA expression of IL-6 receptor has been demonstrated to increase with aging (280), which may indicate that cells are more sensitive to IL-6. TNF- α has been demonstrated to be a greater stimulus of satellite cell apoptosis in aged compared to young rodents (174, 281). Although the expression of muscle inflammatory cytokines in the aged varies, it appears that there may be an increased sensitivity to muscle inflammatory cytokines in aged skeletal muscle fibers and satellite cells.

The association of skeletal muscle inflammatory cytokines with sarcopenia has primarily been studied using cell culture techniques. C2C12 myotube cell culture studies have shown that IL-1 β stimulates IL-6 mRNA expression, resulting in the activation of p38 MAPK, NF- κ B, atrogen-1 and MuRF-1, leading to proteolysis (282, 283). TNF- α reduces mTOR signaling in human myoblast (284) and C2C12 myoblasts (285-287). Although human studies in this area are limited, it has been demonstrated that increased TNF- α mRNA expression in the skeletal muscle is inversely correlated with muscle protein synthesis following resistance exercise in the aged (288). These studies indicate that sarcopenia may be a result of increased inflammatory cytokines interfering with the signaling pathways for muscle protein synthesis, while stimulating the pathways of muscle protein degradation.

In addition to the increase in pro-inflammatory cytokines with aging, there is also an increase in anti-inflammatory cytokines. The skeletal muscle of aged men shows increased mRNA expression of both IL-10 and IL-1 receptor antagonist, compared to younger men. These anti-inflammatory cytokines may be produced in the circulation and then migrate to skeletal muscle, because the number of anti-inflammatory macrophages in skeletal muscle is less in the aged compared to younger adult humans; however, further research is needed to determine this. Alternatively, it is possible that the increased skeletal muscle anti-inflammatory cytokines in the aged are due to elevations in the activity of anti-inflammatory macrophages in skeletal muscle. The elevated expression of anti-inflammatory cytokines in skeletal muscle is thought to be a physiological attempt to reduce pro-inflammatory cytokines in the aged.

Low grade chronic inflammation in the aged is thought to contribute to delayed skeletal muscle regeneration following injury (289-291). Skeletal muscle damage can occur after a bout of exercise (Section 1.5.3), and because the aged are in a state of chronic inflammation, it is possible that the capacity of the immune cells to respond to additional inflammatory stimuli is limited. The reported changes in inflammation that occur following a bout of exercise in the aged are conflicting due to differences in the age and physical condition of the elderly subjects studied. This results in differences in the extent of chronic inflammation, and differences in the mode and intensity of the aerobic exercise bout, which may lead to differences in the acute inflammatory response. It has been demonstrated that 4-6 hours following a bout of aerobic exercise, there is a decrease in circulating neutrophils (292, 293) and IL-6 (294) concentration in aged humans compared to their mature counterparts. In contrast, no difference in the post-aerobic

exercise (6 hour) circulating neutrophil count and an increase in IL-6 concentration were found when comparing aged and mature humans (295). The conflicting results of these studies may be a result of the intensity of aerobic exercise. Additionally, circulating IL-1 receptor antagonist and TGF- β protein concentrations increased similarly in both age groups following a bout of aerobic exercise (294, 295), indicating that the anti-inflammatory response to acute inflammatory stimuli is not altered with low grade chronic inflammation in the aged.

Another characteristic of low grade chronic inflammation in the aged is an increase in the time it takes for skeletal muscle regeneration to occur following tissue damage. This prolongs the muscle damage associated with exercise in the aged compared to their younger counterparts. Reduced macrophage infiltration of skeletal muscle in aged compared to younger mature men following exercise (276, 278) may play a pivotal role in inhibiting muscle regeneration. Reports of the effects of aging on the muscle inflammatory cytokine response to acute inflammatory stimuli of exercise vary. Skeletal muscle TNF- α mRNA expression increases similarly in both mature and aged humans (276) or remains unchanged (296). mRNA expression of IL-6 in aged skeletal muscle is increased (297), decreased (276), or is similar to mature counterparts (278), following exercise. There is either a decrease (278, 298) or similar increase as seen in mature adult skeletal muscle (276) in the mRNA expression of IL-1 β in aged skeletal muscle following exercise. There has been less examination of aged skeletal muscle anti-inflammatory cytokine response to exercise, but it has been determined that IL-1 receptor antagonist mRNA is unchanged following exercise regardless of age, and IL-10 is lower in skeletal muscle of aged compared to mature men following exercise (278). In order to

improve our understanding of the response of aged skeletal muscle to acute inflammation cause by exercise, additional research is necessary which should attempt to standardize for the degree of pre-study low grade chronic inflammation in the aged subjects and standardize the experimental exercise tests.

1.5.5. Summary of acute and chronic Inflammation

Acute inflammation is a response to inflammatory stimuli such as an infection, tissue damage, or exercise and typically persists for a 24 hour period. Chronic inflammation can be a result of a disease state or aging. Following exercise, there is an increase in circulating and skeletal muscle inflammatory cytokines which is thought as a necessary step for satellite cell proliferation and regeneration of damaged skeletal muscle, and will be discussed in detail below (Section 1.5.6). Inflamm-aging occurs in the elderly, where there are persisting increased levels of both circulating and skeletal muscle inflammatory cytokines. This is thought to be at least partially responsible for sarcopenia, through the increased activation of NF- κ B and the signaling pathways of protein degradation (Section 1.3) and reduction in the activation of the protein synthesis pathways. Additionally, chronic inflammation alters the acute inflammatory response and affects the muscle's ability to recover from acute tissue damage, such as exercise. This is thought to be a result of a decreased capacity of immune cells to increase inflammatory cytokines in both circulation and skeletal muscle in response to exercise; however, the results from these studies are extremely variable and require further research. Ultimately, the interaction of inflammation and protein synthesis is complicated, where the effects of acute and chronic inflammation on protein synthesis appear beneficial and deleterious, respectively, and require further elucidation.

1.5.6. Effect of acute and chronic inflammation on satellite cells

Satellite cells have been mentioned throughout this dissertation, but to this point have not been fully explained. Satellite cells are undifferentiated myogenic stem cells that have the ability to re-enter the cell cycle, generating new muscle fibers or providing new myonuclei during postnatal growth (299, 300). Additionally, satellite cells have the ability to generate daughter cells that can become new satellite cells (301). Satellite cells are located outside of the sarcolemma, under the basal lamina of the muscle, laying parallel or obliquely to the long axis of the fiber (302). A number of molecular markers have been proposed to identify satellite cells have been the topic of recent review (303), and are an alternate method to the more classical method of using toluidine blue staining and a light microscope to examine the high heterochromatin myonuclei (304). Some of these molecular markers include: the membrane-bound neural cell adhesion molecule (N-CAM/CD56/Leu-19), which is a cell-surface glycoprotein localized to satellite cells, the Ca^{++} -dependent muscle specific cadherin (M-cadherin), the myocyte nuclear factor (MNF), the receptor for the hepatocyte growth factor (HGF), and the paired box protein 7 (PAX7), a transcription factor that plays a role in embryonic patterning and organogenesis (303). However, many of these molecular markers are expressed during both the quiescent and activated state and there is currently no consensus on the best marker to label activated versus quiescent satellite cells.

Satellite cell content in humans varies between muscle groups (305-308), the amount of physical activity (305-307) and the age (307, 308) of the individual. Satellite cells are activated (proliferated) following a single bout of exercise, regardless of exercise type, for up to 8 days (309). Typically activated satellite cells will proliferate

following exercise, but terminal differentiation does not always occur (309). Satellite cells are also altered following exercise training where, regardless of exercise type, training enhances the satellite cell content in the skeletal muscle of human athletes (306, 307, 310, 311). However, the satellite cell response to low intensity aerobic exercise training is greater than for high intensity resistance training (307). Overall, a single bout of exercise increases satellite cell proliferation and exercise training increases satellite cell content in skeletal muscle.

The alterations in satellite cell activity following exercise are thought to be a result of tissue damage, and the release of inflammatory cytokines and growth factors following exercise. Section 1.5.3 discussed the changes in inflammatory cytokines following exercise as a result of exercise induced tissue damage. It has been demonstrated that IL-6 and TNF- α are involved in the regulation of satellite cell proliferation in rodent skeletal muscle (312, 313). Additionally, the administration of non-steroidal anti-inflammatory drugs (NSAID) to humans and animal models negatively affects satellite cell proliferation, differentiation and fusion (314-318). Thus, the increase in inflammatory cytokines during acute inflammation is a beneficial process enhancing skeletal muscle repair, and in the case of exercise this process coincides with elevated mTOR signaling and subsequent protein synthesis.

The alterations in circulating and muscle inflammatory cytokines during low grade chronic inflammation that is associated with aging have been discussed in detail in Section 1.5.4. In that section, it was briefly mentioned that in aging rodents, the increase in TNF- α stimulates satellite cell apoptosis (174, 281). As a result, the number of satellite cells is lowered in the elderly compared to the younger mature rodents (307,

319). Additionally, in humans the proliferative capacity of the satellite cells of the aged is decreased compared to the mature counterparts (320). Together these studies indicate that low grade chronic inflammation reduces the number and activity of satellite cells in the aging population. Non-damaging exercise (aerobic and resistance) has been shown to increase satellite cell proliferation in the aged (305, 309, 311), and if a training regimen is implemented, the satellite cell pool can be replenished in this population (310).

1.5.7. Summary of the effects of acute and chronic inflammation on satellite cells

Satellite cells are myogenic stem cells that are activated by inflammatory cytokines. During an acute inflammatory response, there is an increase in satellite cell proliferation indicating a beneficial effect of inflammation. However, during low grade chronic inflammation there is a reduction in both satellite cell content and activity, which can be combated by non-damaging exercise training.

1.5.8. Reducing inflammation with non-steroidal anti-inflammatory drugs

1.5.8.1. Mechanism of action of non-steroidal anti-inflammatory drugs

NSAID include a range of drugs that inhibit prostaglandin formation, for example aspirin, ibuprofen, mefenamic acid, and phenylbutazone (321). Although the entire mechanism in which NSAID reduce inflammatory cytokines has not been elucidated, it has been the topic of several reviews (321-323). Briefly, NSAID reduce inflammation by blocking cyclo-oxygenase (COX) enzymatic activity. As a result, the production of prostaglandin E₂ (PGE₂) from arachadonic acid is nearly ceased (321, 323). Both COX-2 and PGE₂ regulate the activation of NF- κ B (322, 323); however this mechanism is still unclear. Because NF- κ B is also a regulator of inflammatory cytokine release (Section

1.5.1) (322, 323), the reduction in COX activity is believed to be the mechanism whereby NSAID reduce inflammatory cytokines. There are two different isoforms of the COX: COX-1 and COX-2. Both forms of COX are expressed in the majority of tissues in the body, including skeletal and cardiac muscle, gastrointestinal tissue, kidney, brain and epithelial cells. COX-2 expression in these tissues is minimal unless activated by inflammatory cytokines (324), whereas, COX-1 is highly expressed in gastrointestinal tissue. As previously mentioned, both COX-1 and -2 are involved in the formation of PGE₂ from arachadonic acid. PGE₂ has a physiological protective role in gastrointestinal tissue, behaves as a vaso-dilator, and contributes to erythema, and hyperalgesia (321, 323). Because of the role of PGE₂ in gastrointestinal tissue, when PGE₂ production is ceased in the presence of NSAID, gastric ulcer formation may occur.

Prolonged NSAID administration has been implicated in the development of gastric ulcers in several species (323, 325), including horses (326). In the stomach, the COX-1 enzyme is generally expressed at a higher level than the COX-2 enzyme, and the resulting PGE₂ plays an important protective role. PGE₂ in the stomach suppresses TNF- α expression, neutrophil adherence, and epithelial cell apoptosis (323). As a result the overall function of PGE₂ in the stomach is to prevent the formation of gastric ulcers. However, when COX-1 is inhibited or in the presence of gastric ulcers, COX-2 becomes upregulated (327), due to an increase in inflammatory cytokines to the tissue. The increase in COX-2 activity results in an increase in PGE₂ formation, which is an attempt to suppress further tissue damage. Unfortunately, most NSAID do not selectively inhibit the activity of COX-2 and as a result, both COX-1 and COX-2 in the stomach are inhibited (323), reducing PGE₂ production. The COX enzymes also regulate the

formation of other prostaglandins from arachadonic acid. These prostaglandin are involved in the regulation of bicarbonate, mucous, and acid secretion, and accelerate ulcer healing (323). As a result, extended NSAID administration can result in gastric ulcers in many mammalian species.

1.5.8.2. Reducing acute inflammation with NSAID

NSAID are commonly used to reduce the discomfort associated with muscle damage caused by strenuous exercise. NSAID administration post-exercise has been extensively studied to characterize post-exercise physiology and as a mechanism to eliminate inflammatory effects on physiological responses to exercise. Following any type of exercise, skeletal muscle COX-2 mRNA expression and protein content are elevated (255, 328, 329) compared to at rest, and post-exercise NSAID administration reduced the elevated levels of COX-2 mRNA expression and protein content (329, 330). Oral administration of a NSAID during 12 weeks of resistance exercise training resulted in skeletal muscle COX-1 protein abundance that was lower than in an exercised group that received a placebo in place of NSAID (331). Furthermore, oral administration of a herbal supplement (curcumin), with properties functionally similar to a NSAID, reduced muscle IL-1 β , IL-6, and TNF- α mRNA expression in rodents following exercise (332). The local infusion of a NSAID post-exercise in humans reduced the skeletal muscle mRNA expression of TNF- α , but did not alter the skeletal muscle mRNA expression of TGF- β and IL-1 β (333), nor was there an increase in macrophage or neutrophil infiltration (316). Because the increase in inflammatory cytokines following exercise may be necessary for muscle repair post-exercise (334), and inflammatory cytokines play a regulatory role in satellite cell proliferation (312, 313), the administration of NSAID

with the goal of reducing inflammation may be preventing muscle recovery by reducing satellite cell proliferation (316). Further research is required in this area to determine if the benefits of NSAID during an acute inflammatory response outweigh the possible consequences.

1.5.8.3. Reducing chronic inflammation with NSAID

The study of the use of NSAID to reduce circulating and skeletal muscle inflammatory cytokines in the aged has been limited because of the long term harmful effects of NSAID use, such as gastric ulcers (323). However, the use of NSAID administration as a tool to study the association behind aging related inflammatory cytokines and the development of sarcopenia has been investigated. In aged rodents, 5 months of oral NSAID administration (ibuprofen) reduced circulating levels of IL-6 and IL-1 β , while increasing skeletal muscle mass and muscle protein fractional synthesis rates (335). Therefore, future research is needed to fully elucidate these mechanisms and then develop strategies to reduce both circulating and skeletal muscle inflammatory cytokines in order to promote the maintenance of muscle mass with old age.

1.5.8.4. Summary of reducing inflammation with NSAID

NSAID reduce inflammatory cytokines through the inhibition of the enzymatic activity of COX, which suppresses the activation of NF- κ B and inhibits the release of inflammatory cytokines. Although the prostaglandins produced by COX mediate inflammatory cytokines through NF- κ B, there are also many beneficial physiological effects of these prostaglandins throughout the body. In the stomach, the prostaglandins produced by COX-1 play an important role in protecting the stomach from the

development of gastric ulcers. However, because most NSAID do not inhibit a specific COX isoform, the beneficial effects of prostaglandins are also affected by NSAID administration and prolonged NSAID administration has been associated with the development of gastric ulcers (323). The administration of NSAID during acute inflammation reduces circulating and muscle inflammatory cytokines, which coincides with a reduction in satellite cell proliferation. The latter may impair the recovery of skeletal muscle during acute inflammation (Section 1.5.6). The literature examining NSAID reduction of inflammatory cytokines during chronic inflammation is limited due to the side effects of extended NSAID use; however, NSAID administration has been demonstrated to reduce circulating inflammatory cytokines in the aged. Ultimately, further research is warranted to determine whether there are other methods that can be used over a longer period to reduce chronic inflammation without causing side effects such as gastric ulcers.

1.6. AN OVERVIEW OF THE EQUINE LITERATURE RELATING TO THE RESEARCH IN THIS DISSERTATION: PROTEIN NUTRITION AND METABOLISM, AND MUSCLE PHYSIOLOGY THAT MAY ALTER PROTEIN METABOLISM

In comparison to cell culture models, rodents, neonatal piglets, and humans, there is only limited data regarding muscle protein metabolism in the horse. However, there is considerable research in horses concerning protein digestibility and utilization, crude protein requirements, muscle biology and development, and inflammation and the review of the literature in horses will focus on these areas.

1.6.1. Protein nutrition in the horse

1.6.1.1. Dietary indispensable and dispensable amino acids in the horse

Although the protein requirement as reported by the NRC (336) is written for crude protein (CP), the horse actually has requirements for individual amino acids, the building blocks of protein. CP is a calculation of total nitrogen (N) in the feed, where CP is equal to N times 6.25 (336). This calculation is derived from the fact that, by weight, protein is 16% N. Horses, like other mammals, are believed to require 9 dietary indispensable amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and, under certain physiological states, an additional 6 conditionally indispensable amino acids (arginine, cysteine, glutamine, glycine, proline, and tyrosine) may be required. Arginine is a conditionally indispensable amino acid because the rate of arginine synthesis in many growing mammals is insufficient to meet its needs, and is indispensable during illness or injury due to the role of arginine in immune function as a precursor to nitric oxide. Cysteine and tyrosine can be synthesized from dietary methionine and phenylalanine, respectively, if supplied in sufficient quantities. During times of illness or physiological stress, glutamine becomes indispensable, and glycine and proline are also indispensable during the growth period. The remaining amino acids are considered dietary dispensable amino acids because of their ability to be synthesized in sufficient enough quantities to meet the body's needs (alanine, asparagine, aspartate, glutamate, and serine).

1.6.1.2. Protein digestion in the horse

For the most part, amino acids are consumed in the equine diet as part of whole proteins, which must be broken down to individual amino acids or small peptides for absorption. In the horse, dietary protein is digested in the foregut, similar to other nonruminant species, and this process has been the topic of several chapters and entire books (337-339). In short, prior to consumption of a meal, the vagal release of acetylcholine stimulates the upper gastro-intestinal tract, initiating saliva secretion in the mouth, and hydrochloric acid, pepsinogen, and sodium bicarbonate secretion from the stomach. The equine stomach is unique in that it has a section referred to as the *margo plicatus* where the squamous epithelial mucosa lacks the mucous-sodium bicarbonate barrier to buffer the high acidity. This is the area where gastric ulcers and erosion tends to occur (340), and gastric ulcers do occur at a higher rate in horses compared to other nonruminants. The stomach has four main secretions, hydrochloric acid, pepsinogen, sodium bicarbonate, and mucus, which begin enzymatic digestion of proteins. Hydrochloric acid is secreted from the parietal cells resulting in an increase in the acidity of the stomach. The reduced pH denatures dietary protein, unfolding it to a linear polypeptide chain, and at pH 4 causes pepsinogen, the zymogen (inactive enzyme) of pepsin, to become pepsin. This mechanism protects the cells of the stomach from pepsin trying to break the peptide bonds involving aromatic amino acids (phenylalanine, tryptophan, and tyrosine) of proteins bound to cell membranes: pepsin is only in its active form when there is protein from feedstuffs available for enzymatic digestion. Pepsinogen is secreted from chief cells, and once it is in the active form it begins cleaving the peptide bonds involving aromatic amino acids, resulting in some oligopeptides and short

polypeptide chains which will be further enzymatically digested in the small intestine.

As previously mentioned, sodium bicarbonate is also secreted in the stomach from epithelial mucous cells and acts to buffer the stomach from the acidic pH.

During the initial digestion of dietary proteins in the stomach, the vagal release of cholecystokinin in the exocrine pancreas stimulates release of other zymogens. Although this causes the release of a number of enzymes from the pancreas, the ones involved in protein digestion (trypsinogen, chymotrypsinogen, and procarboxypeptidase A and B) are secreted from the acinar cells. These zymogens and sodium bicarbonate, produced by the duct cells, enter the duodenum, the first section of the small intestine, where enteropeptidase, secreted from the duodenal crypts of Lieberkühn, converts trypsinogen into the active form, trypsin. Together enteropeptidase and trypsin convert chymotrypsinogen, and procarboxypeptidase A and B to chymotrypsin and carboxypeptidase A and B, respectively. Trypsin is a serine protease that cleaves at the carboxyl side of lysine and arginine, except when followed by proline. Chymotrypsin is an endopeptidase that cleaves peptide amide bonds where the carboxyl side of the bond is an aromatic amino acid. Carboxypeptidase A and B are exopeptidases, cleaving peptide bonds at either the C- or N-terminus of the oligopeptides and short polypeptide chains that have entered the duodenum from the stomach. As a result, oligopeptides, di- and tri-peptides, and free amino acids enter the jejunum, the second section of the small intestine.

The di-, tri-peptides are absorbed via peptide transporter 1 (PepT1) located on the apical side of the enterocytes in the jejunum (341). It is important to note that PepT1 transporters are also located in the enterocytes of the ileum, resulting in some di-, tri-

peptide absorption; however, the majority of PepT1 transporter expression is in the jejunum, and therefore, most di-, tri-peptide absorption occurs in the jejunum (341). Some of these di-, tri-peptides are broken down to free amino acids in the cytosol of the enterocyte cells, while others remain intact. For the oligopeptides and di-, tri-peptides that do not get transported into the enterocytes by PepT1, further digestion occurs at the enterocytes brush border membrane, by aminopeptidases and endopeptidases. Although these peptidases are located throughout the entire length of the small intestine, the jejunum and ileum possess the highest activities (342). PepT1 has not yet been characterized in the equine gastrointestinal tract, but the mRNA expression of PepT1 was high in the small intestine, decreased in the colon, and not detectable in the cecum of another hind-gut fermenter, the rabbit (343). Additionally, PepT1 activity has been suggested in equine jejunum through increased current flow through equine jejunal membrane when incubated with a dipeptide (344).

For the di-, tri-peptides not transported into the enterocytes by PepT1 and oligopeptides, further digestion occurs at the brush border membrane, resulting in free amino acids. Free amino acids are transported into the enterocytes by a variety of apical transporters, specific for certain types of AA. The free amino acids transported into the cytosol of the enterocyte cells can be further metabolized by the enterocytes, used for enterocyte protein synthesis, or transported across the basolateral membrane and into portal vein. Both apical and basolateral membrane amino acid transporters are specific to the amino acids transported, based on the amino acid properties such as size, polarity, and charge (345). The majority of amino acid transporters do not require energy; however, sodium dependent transporters require ATP because the co-transport of sodium and

amino acid requires the removal of sodium out of the enterocyte via a sodium/potassium-ATPase (345). Some amino acid transporters are unique to the apical (system names: ASC, B⁰, B^{0,+}, b^{0,+}, IMINO, X_{AG}⁻) or basolateral membrane (system names: A, L, y⁺, y^{+L}) while others can be found on both membranes of the enterocyte (system name: T) (345, 346). The study of amino acid transporters in the equine gastrointestinal tract has been limited. However, glutamine transport has been demonstrated across the basolateral membrane from the bloodstream to the enterocytes where it is proposed to be used as a key energy substrate in the equine enterocyte (347). This study was useful in that it examined the amino acid flow, so although Duckworth and colleagues (347) did not determine the amino acid transport system, their study indicated that there is a glutamine transporter on the basolateral membrane of the enterocyte transporting from the bloodstream into the enterocyte. More recently, Woodward and colleagues (348) have examined the distribution of the mRNA of specific amino acid transporters along the intestinal tract of mature horses, where the mRNA expression of the apical b^{0,+} system was expressed similarly throughout the jejunum, ileum, cecum and colon. The mRNA expressions of the basolateral y⁺ and basolateral medium affinity L transport systems were higher in the small intestine than in the large intestine, while the low affinity L transport system mRNA expression was greater in the cecum than the jejunum (348). These results indicate that, at least for the b^{0,+} system, there may be similar absorption throughout the jejunum, ileum, cecum and colon; however, because the basolateral y⁺ and basolateral medium affinity L transport were greater in the small intestine, this indicates that the greatest absorption of amino acids that contributes to whole-body protein metabolism occurs in the small intestine due to the greater mRNA expression of

basolateral amino acid transporters and therefore, a greater ability to transport the absorbed amino acids from the enterocytes into circulation. Because there was expression of some transport systems in the cecum and colon, this study (348) also indicates that there may be some amino acid absorption in the large intestine; providing a mechanism for the absorption of microbial amino acids. However, additional research is needed to characterize the presence and function of amino acid transport systems in the cecum and colon.

Not all of the dietary protein that is consumed is completely digested and then absorbed in the small intestine. The dietary protein that bypasses digestion and/or absorption in the small intestine and reaches the cecum and large intestine can be metabolized by the residing microbial population. Although little is known of the microbial protein metabolism in the equine hindgut, it has been demonstrated that the microbial population of the equine hindgut can degrade and utilize dietary protein, partially degraded protein and ammonia and urea that have bypassed small intestine absorption (349). Once absorbed by the microbe, peptides are cleaved into free amino acids for microbial protein synthesis or degraded to the carbon skeleton and ammonia. The carbon skeleton can be utilized for energy while the ammonia can be used to synthesize amino acids or secreted by the microbe (350). Because microbial digestion occurs proximal to digestion and absorption in the ruminant, microbial protein can be digested and absorbed similar to dietary protein in nonruminants. However, microbial digestion occurs distal to digestion and absorption in the horse, and therefore microbial proteins cannot be absorbed similarly to dietary proteins due to anatomical constraints. As a result, researchers have attempted to increase the understanding of the physiological

mechanisms contributing to possible amino acid absorption and quantify the microbial amino acid contribution to whole-body amino acid metabolism in the horse.

The use of isotope tracers has led researchers to believe that some microbially synthesized amino acids may end up in circulation. Infusing ^{15}N labeled cecal bacterial protein into the cecum of an anaesthetized pony, resulted in ^{15}N labeled amino acids in circulation (351). There was less than 10% labeled amino acid recovery in the tissues of ponies fed ^{15}N urea (352). However, the infusion of ^{14}C labeled bacteria or protein into the cecum of ponies showed no ^{14}C labeled amino acids in the circulation (353). These studies show that a small amount of N is being absorbed in the large intestine; however, the fact that the carbon backbone of these amino acids was not absorbed may indicate that microbial amino acids are not being absorbed, but rather a N compound such as ammonia is (352, 354, 355). Once ammonia is absorbed, it can be used to form dispensable amino acids. However, urea supplementation to horses has not been found to be beneficial if the indispensable amino acid requirements are not met (355).

Even though amino acid transport systems have been found on the apical membrane of the large intestine of horses (348), the isotope tracer work (mentioned above) and *ex vivo* studies do not currently support the idea of large amounts of indispensable dietary amino acids being absorbed in the large intestine and contributing to whole-body protein metabolism. Minimal amounts of alanine (356), arginine (357), histidine (357), leucine (358), and lysine (357) have been demonstrated to cross the equine colonic mucosa in *ex vivo* experiments. However, a great deal of ammonia is transported across the mucosal membrane of the equine colon (357). As a result, it is generally accepted that ammonia that is transported across the apical membrane of the

equine large intestine contributes to protein metabolism; however, this is an area where additional research is needed.

1.6.1.3. Protein digestibility in the horse

It was mentioned above (Section 1.6.1.1) that horses actually require amino acids, but the current requirements according the NRC (336) are for crude protein and lysine. Unfortunately, not all protein is created equal with regards to digestibility and quality. Quality refers to the amino acid composition of the protein in relation to the animal's amino acid requirements, whereas digestibility refers to the amount of dietary protein that disappears, and is presumably absorbed, along the gastrointestinal tract.

Studies in horses examining protein digestibility vary extensively in the method of measuring digestibility, age of horse, and feedstuffs examined. The method of measuring digestibility is crucial when comparing these studies, because it accounts for how much protein has been digested at which point along the gastrointestinal tract (337). The simplest measurement is apparent total tract digestibility and is determined by the difference of the N intake (feed) and N output (feces) divided by the N intake (337). This does not correct for endogenous losses such as sloughed cells, microbes and microbial protein, and typically underestimates protein absorption. Correction factors that have been determined for endogenous losses range from 0.72 - 0.91 mg endogenous nitrogen/kg dry matter consumed, due to differences in total intake and diet composition (359-362). When endogenous losses are accounted for in the calculation of protein digestibility, it is no longer coined apparent digestibility, but rather true digestibility (337). Because it is believed that the majority of protein is digested and amino acids are

absorbed proximal to the cecum, pre-cecal digestibility has been a tool used by researchers in an attempt to more accurately determine protein absorbed as amino acids (337). Again, the endogenous losses must be accounted for, and the correction factors for pre-cecal endogenous N losses that have been reported to range from 1.8 - 5.8 mg endogenous nitrogen/kg dry matter consumed (359-362).

Diet composition and intake affect endogenous losses and protein digestibility. The NRC (336) has reported estimates of total tract apparent nitrogen digestibility and pre-cecal apparent nitrogen digestibilities of 79 and 51%, respectively. Concentrates tend to have higher pre-cecal digestibility than forages (360-365). The differences in protein digestibility and endogenous losses between diets high in forage or concentrate are thought to be a factor of the bulkiness and slow rate of movement through the gastrointestinal tract of diets primarily composed of forage, where there are higher endogenous losses than diets that have a greater proportion of concentrate. Specifically, pre-cecal apparent digestibilities of concentrates range from 48 - 72% depending on the type of concentrate (360, 362) and pre-cecal apparent digestibilities of forages range from 1.3 - 21% (361). The pre-cecal true digestibilities of concentrates and forages range 59 - 62% (364) and 27 - 42% (361), respectively. Additionally, protein intake also affects digestibility and endogenous losses. As CP intake increases, there is a reduction in endogenous N losses relative to intake, resulting in greater apparent nitrogen digestibility (365). However, increasing CP intake from low to moderate does not alter true CP digestibility, but increasing CP intake to high levels reduces true and apparent pre-cecal digestibility (360). Others claim that regardless of dietary N concentration, true total tract digestibility ranges between 0.7 - 0.9 (337). Overall, compared to forage-based

diets, endogenous losses and protein digestibility, regardless of type (true or apparent), appear to be reduced and elevated, respectively, in diets that are primarily composed of concentrates in mature horses.

The discussion of protein digestibility has focused on CP digestibility; however, it was previously mentioned that horses actually have requirements for amino acids. It has been determined that the majority of amino acids have pre-cecal apparent digestibilities that range from 0.3 - 0.6 (366). Similar to what was seen with increasing CP intake, apparent amino acid digestibility is increased with increasing amino acid intake (367). True and apparent amino acid digestibilities range for the individual amino acids from 52.8 - 86.3% for glycine and leucine, respectively (367). Additionally, physiological state may alter amino acid digestibilities, gestating mares were reported to have higher amino acid digestibilities (368) than those reported in foals (367). This is an area where additional research is warranted.

The growing horse has received less attention in the area of protein digestibility than the mature horse, and it appears that the growing (~6 months old) horse may have a decreased capacity to digest and absorb protein than their mature counterparts. Weaned foals (3 - 7 months old) and yearlings consuming a primarily concentrate diet (<60%) had apparent total tract N digestibilities of 35 - 45% (369) and 80% (370), respectively. The apparent total tract N digestibility reported in the weaned foals is lower than the estimate for mature horses by the NRC (336); however, the apparent total tract N digestibility reported in the yearlings was comparable to that of mature horse. This may indicate that there are developmental limitations in protein digestibility; however, it is

necessary to elucidate the time course changes in protein digestibility that occur during development.

1.6.1.4. Protein and amino acid requirements in the horse

There are several methods for expressing protein requirements of horses, which are used in various locations across the world. In the United States, protein requirements are expressed as amounts of CP and lysine required, and have been detailed in the recent NRC (336). CP and digestible CP are used to express the protein requirements of horses in Japan and Germany, respectively. In France, protein requirements of horses are expressed in “horse digestible crude protein” or “Matières Azotées Digestibles Cheval” (MADC). A detailed examination of all of these systems is beyond the scope of this dissertation. However, this section will provide a brief overview of the US system, including a discussion of the research that was used to determine the requirements, and a short comparison of the US to the French system.

1.6.1.4.1. *Protein and amino acid requirements based on physiological status in the horse*

Nutrient requirements are specific to the physiological state of the animal. The NRC (336) separates these physiological states into: Maintenance, Growth, Pregnancy, Lactation, and Exercise. Examples of the protein and lysine requirements of a 600 kg mare at various physiological states according to the NRC (336) are provided in Table 1.1. The focus of this section will be to discuss the factors affecting protein requirements in the various physiological states and some of the research that led to the development of those requirements.

Maintenance requirements for protein are generally the amount of protein consumption needed by the animal in order to support the daily processes of life. Although animals at maintenance are making and degrading protein at the same rate and for this reason should be able to “recycle” amino acids released from protein degradation for use in protein synthesis, this system is not perfectly matched and even at maintenance dietary amino acids are needed. “Losses” of amino acids in animals in the maintenance state include endogenous losses in the gastrointestinal tract, cutaneous losses, and amino acids from proteins that have been post translationally modified (for example: amino acids that are methylated) and can no longer interact with their respective tRNA to be used for protein synthesis. The maintenance requirements have been calculated from linear regression and broken line analysis of means from the numerous N balance studies that have been conducted in mature horses, receiving a variety of feedstuffs. The CP requirement has been identified based on the N intake where N retention is zero. However, the minimum CP requirement was actually set slightly higher than zero because the majority of N balance studies do not account for endogenous losses of hair, skin and sweat; thus overestimating N retention (336). As a result, the CP of average maintenance has been derived as: $BW \times 1.26g \text{ CP/kg BW/d}$ (336). The requirements of amino acids during maintenance have not been determined due to lack of data; however, based on the amino acid content of common feedstuffs fed to horses and the limiting amino acids of other nonruminants, mainly swine (371), it is thought that the first three limiting amino acids may be lysine, threonine, and methionine. The lysine requirement during maintenance has been calculated as 4.3% of CP requirement (336). Linear regression of N balance studies that reported the diet composition were used to determine

the lysine intake required to obtain zero N retention. Then, broken-line analysis was used to determine the lysine intake at which N retention plateaued (336). Additionally, in horses receiving diets supplemented with lysine and threonine, the amino acid supplementation resulted in lower plasma urea N concentrations in mature and aged horses, compared to horses receiving the unsupplemented diet (372), providing additional support that lysine and/or threonine are likely candidates for limiting amino acids in the mature horse.

During growth, the maintenance requirements must initially be met in order to sustain the physiological processes and maintain the current weight. Then, the growing horse requires additional amino acids (protein) to support the tissue (for example, muscle) accretion that occurs when an animal is growing. The protein requirements of growing horses have been derived from N balance studies (369, 373) and the use of ADG measurements (369, 374, 375) with varying protein or amino acids intakes provided by the diet. The latter has been a classical tool to study protein requirements extensively used in growing horse. When the protein or amino acid intake provided by the diet is below requirements, increases in intake will result in an increased ADG. Once the required level has been met, ADG will not increase with increasing intake of the amino acid or protein. The means of data collected in these studies were fitted to broken-line analysis and then extrapolated to a mature body weight to incorporate the changes in body weight with age (336). This provided a total CP requirement for a certain aged horse at a specific weight. In order to determine the separate requirements of maintenance and growth, the maintenance requirements must be subtracted from the total requirements (336). The CP requirement of growing horses has been determined using a

factorial approach and based on the assumption that gain is 20% protein: $(BW \times 1.44 \text{ g CP/kg BW}) + ((ADG \times 0.20)/\text{Efficiency of use of dietary protein})/0.79$ (336). The efficiency of use of DP is estimated from 50% in 4-6 month old horses to 30% in yearlings and older growing horses (336).

There have also been some studies examining the effects of amino acid supplementation, mainly lysine, on ADG in growing horses (369, 375-378), where it has been concluded that lysine is vital for growth. When lysine is supplemented to poor quality protein diets of linseed meal (369) and brewer's dried grains (376), the ADG was equivalent to high protein quality diets of milk and soy bean meal, respectively. Additionally, ADG was not altered in horses fed low CP diets that were supplemented with lysine compared to higher CP diets (375). Using linear regression and broken-line analysis from the means of the previously mentioned studies, the NRC determined that the lysine requirement of growing horses was 4.3% of CP requirements (336). Additionally, supplementing low protein diets with lysine and threonine resulted in lower plasma urea N concentrations, indicating threonine may be the second limiting amino acid in growing horse diets (374).

There is minimal research regarding protein requirements during gestation in horses due to the possible unwanted effects, such as fetal loss or stunted growth of the foal, of consuming a protein (or amino acid) deficient diet during pregnancy. From the minimal research in this field, there have been more studies examining protein requirements during mid to late compared to early gestation. Similar to the growing animal, the maintenance requirements must be met and there are additional protein requirements for increased protein synthesis to support fetal and placental growth, as well

as for the production and secretion of placental fluids. However, because there is not a large ADG during early pregnancy, the NRC (336) has concluded that protein requirements are only slightly higher than maintenance: $BW \times 1.26 \text{ g CP/kg BW/d}$. Linear regression of fetal weight gain was performed in horses (379) and ponies (380), which in addition to cow data, were used in non-linear regression analysis to determine fetal growth rates. The NRC (336) then assumed that fetal composition is equivalent to neonatal composition. From this, the NRC (336), could calculate the amount of CP deposited per day in horses and ponies. Using a factorial approach of protein efficiency the CP requirements were determined for gestating mares from 5 months through parturition: $(BW \times 1.26 \text{ g CP/kg BW/d}) + (\text{fetal gain in kg}/0.5)/0.79$ (336). The 0.5 comes from the estimation of 50% efficiency of use of dietary protein in pregnancy, and 0.79 is representative of the 79% digestibility of the protein (336). It is also important to note that the calculated CP of fetal gain does not account for protein needs of the uterus (336). Additionally, due to the lack of data on amino acid requirements, the NRC suggests that the same estimation of lysine requirements during pregnancy as maintenance (336).

Rather than the ADG studies that were used to estimate protein requirements in the growing horse, milk production was used for the determination of protein requirements beyond maintenance during lactation. In addition to the maintenance protein requirements, lactating mares require additional dietary protein to support the synthesis of milk protein. Broken-line analysis of the CP content of milk produced throughout gestation found that milk protein concentration plateaus at day 22 of gestation (336). This led the NRC (336) to determine the CP requirement during the first month of

lactation to be greater than the rest of lactation. Again, the NRC (336) used a factorial approach incorporating 50% efficiency of utilization and 79% protein digestibility to determine the lactating mare CP requirement: $(BW \times 1.44 \text{ g CP/kg BW/d}) + (\text{milk production kg/d} \times 50 \text{ g CP/kg milk})$. The amino acid requirements during lactation have been suggested by Wickens and colleagues (381) and are based on the amino acid to lysine ratio in milk. The NRC (336) accepts this suggestion (kg milk/d multiplied by 3.3.g lysine per kg milk), but adds the maintenance requirement for lysine.

Additionally, if any of these physiological classes (adult maintenance, gestating, lactating, or growing) of horses undergo exercise, there will be an additional requirement above what has already been discussed. The NRC (336) has classified exercise requirements by intensity (light, moderate, heavy, and very heavy), which have been classified based on heart rate and oxygen utilization above rest. Correction factors have been developed from the literature to account for protein synthesis for muscle growth and to repair damaged muscle following exercise (336). Additional correction factors have also been determined based on the percentage of body weight lost in sweat at each intensity of exercise (336). Again, the NRC (336) used a factorial approach to determine the CP requirement of exercising horses based on the 50% efficiency of utilization and 79% protein digestibility: $(BW \times \text{muscle gain}) + (((BW \times \text{sweat loss} \times 7.8 \text{ g/kg})/0.50)/0.79) + \text{the maintenance requirement for protein (336)}$. It is also important to note that at very high intensities of exercise, a substantial increase in lean gain may also increase the maintenance protein requirements. The lysine requirement of exercising horses has been determined using broken-line analysis of the means of data from N

retention studies that reported diet composition and intake and lysine intake and was determined as 4.3% CP requirement (336).

1.6.1.4.2. Protein requirement systems

The French system expresses protein requirements in MADC, which has some similarities to digestible CP, the method used by the Germans. MADC is based on the assumptions that protein quality in terms of amino acid composition and site of digestion (pre- versus post- cecal) affect the availability of the amino acids to the animal. The advantage of this system compared to the US and German systems is that differences in small intestinal availability of amino acids in various feeds are accounted for.

Additionally, this system more accurately estimates forage amino acid availability than digestible CP because the physical bulkiness of forages result in a decrease in the amino acids available for small intestinal absorption from the forage (382, 383). From a scientific perspective, this system has many advantages to the US system because it more accurately assesses the availability of amino acids for absorption; however, the lack of knowledge of digestible CP and amino acid availability in many of the common feedstuffs fed to horses make it difficult to use this system.

1.6.1.5. Summary of protein nutrition in the horse

Although there is still a vast amount of research to be performed in the horse, regarding protein nutrition (digestion, digestibility, and requirements), the scientific knowledge base has improved substantially from the previous (384) to the current (336) NRC which were published 18 years apart. The majority of protein digestion and absorption occurs in the small intestine of horses. The primary contribution of the

hindgut to protein metabolism is through the absorption of ammonia produced by the hindgut microbes. The NRC (336) provides both CP and lysine requirements for horses at the various physiological states, which have been derived using linear regression and broken-line analysis from the means of the available studies, followed by a factorial approach considering efficiency of utilization and protein digestibility. Ultimately, there is still a lot left to elucidate and further research is necessary in all of these areas of protein nutrition in order to better feed and manage these animals.

1.6.2. Muscle biology and development in the horse

The horse is an athletic animal with nearly 50% of body mass composed of skeletal muscle (1). Thus, there is a considerable amount of research over the past century that has focused on equine skeletal muscle. The majority of equine skeletal muscle research has focused around the characterization and possible treatments of disease related states, such as metabolic syndrome (385-387), pituitary pars intermedia dysfunction also referred to as Cushing's disease (388, 388, 389, 389), exertional rhabdomyolysis also referred to as tying-up (390-394), hyperkalaemic periodic paralysis also referred to as HYPP (395-398), neuromuscular disorders (399-402), polysaccharide storage myopathy also referred to as PSM (403-405) and the combination of these disorders (406-408). However, a detailed examination of the alterations of skeletal muscle physiology and metabolism during these disease states is beyond the scope of this literature review. Rather, this section will focus on the sizeable body of literature examining the characterization of skeletal muscle fiber type, the alterations of skeletal muscle physiology and metabolism during development, exercise (409-414), and exercise during development (411, 415-419).

1.6.2.1. Characterization of skeletal muscle fiber types

1.6.2.1.1. A historical introduction

Researchers have been examining skeletal muscle fiber types for well over a century. The characterization of fiber types began in 1865 with the correlation of a greater blood supply to red muscle compared to white muscle, and was followed by the demonstration that contraction velocity was faster in the white muscle compared to the red muscle (420). With advances in technology in the mid 1880s to the late 1910s, researchers began histological studies of skeletal muscle demonstrating a greater number of mitochondria in red compared to white muscle fibers (420).

The development of a correlation between velocity of muscle shortening to ATPase activity when the muscle was with or without load in both vertebrates and invertebrates (421) led to the discovery of a link between morphological and histochemical characteristics to physiological properties of the fiber. This link was displayed in the motor units of the cat gastrocnemius (422). Burke and colleagues (422) categorized fibers as fast twitch fatigue sensitive (FF), fast twitch fatigue resistant (FR), and slow twitch fatigue resistant (S). Higher glycogen concentrations, lactate dehydrogenase activity, and myofibrillar ATPase activity were seen in FF and FR compared to S fibers. This connection opened the door to the current thought of fiber types characterized as slow twitch being primarily oxidative, and fast twitch fibers being primarily glycolytic.

1.6.2.1.2. Description of the fiber types

Most mammalian twitch muscles can be divided into two categories: slow (Type I) and fast (Type II) twitch. Fast twitch fibers can be further subdivided into three subcategories: fast oxidative-glycolytic (FOG; Type IIA), fast-glycolytic (FG; Type IIB), and an intermediate fiber (Type IIX, Type IIC, or Type IID).

Slow twitch fibers are characterized with a red color, slow contraction speed and duration of contraction, and a high oxidative capacity. Type I fibers are well vascularized providing increased blood flow for the delivery of O₂ which increases the oxidative capacity. To further aid in their oxidative capacity, slow twitch fibers contain high concentrations of mitochondria and myoglobin. An increased number of mitochondria are present to allow for higher capacity to efficiently operate the Krebs cycle and electron chain transport to produce ATP for work. The high concentration of myoglobin provides O₂ storage for increased production of ATP. Slow twitch fibers also have a decreased glycolytic capacity and glycogen content.

Fast twitch Type IIA fibers are also described as red in color, but are faster in contraction speed and duration of contraction than slow twitch fibers. Type IIA fibers have both oxidative and glycolytic capacities. As a result, these fibers have many of the same properties as both slow twitch Type I and fast twitch Type IIB fibers.

Fast twitch Type IIB fibers are characterized as a white colored muscle fiber with the fastest contraction speed. These fibers utilize glycolytic metabolism for the production of energy for work. As a result, they are poorly vascularized with a decreased need for O₂ because of their reliance on anaerobic metabolism. In addition, Type IIB

fibers have low concentrations of myoglobin and mitochondria. Glycolytic metabolism, unlike oxidative phosphorylation, takes place in the sarcoplasm not the mitochondria; hence a decreased need for mitochondria in Type IIB fibers.

Fast twitch Type IIX, or occasionally referred to as IIC or IID, fibers are considered an intermediate fiber between the fast twitch types IIA and IIB. Type IIX fibers have both glycolytic and oxidative capabilities, but are faster in speed of contraction than Type IIA fibers. As a result, this fiber type has been poorly distinguished until the use of myosin heavy chain isoforms as a tool to differentiate between the various fiber types.

1.6.2.1.3. *Fiber Type Differentiation as a Result of Myosin Heavy Chain Isoforms*

Myosin is one of the most prominent proteins in the muscle and comprises the majority of the thick filament. The myosin molecule is composed of six different proteins: two heavy chains (myosin heavy chains; MHC) and four light chains (myosin light chains; MLC; (423). Both MHC and MLC components of myosin occur as several uniquely distinct isoforms; all of which are coded by different genes (424). In fact, there are eight different MHC isoforms found in skeletal and cardiac mammalian muscle (425). It has been shown that slow and fast twitch fibers contain different isoforms of MHC (426). The MHC-I isoform is expressed in slow fibers, and hydrolyzes ATP slowly. As a result, the cross bridge cycle is slow which leads to a slow velocity of contraction.

Within the last 25 to 30 years, it has been shown that there are three myosin isoforms found in Type II fibers: MHC-IIA, MHC-IIB, and MHC-IIX or -IID.

Traditionally, it was thought that there were only two fast twitch fiber types (IIA and

IIB), and the IIX fibers were being mistakenly categorized as IIB. The mistaken identity of the IIX fiber was a result of the experimental methods of the time. The use of methods such as ATPase histochemical reactions resulted in the distinction between slow and fast twitch fibers in the middle gluteus muscle of horses (411). On the basis of oxidative capacity, a reflection of NADH-diaphorase staining (427) equine skeletal muscle fibers have been classified as slow twitch (comparable to Type I), fast twitch with high oxidative capacity (comparable to Type IIA), and fast twitch with low oxidative capacity (comparable to Type IIB) (411). This disconnect in nomenclature between and among species continued until modern day techniques, such as gel electrophoresis and immunoblotting became regularly available, which provided a greater distinction in these fiber types (428-430). Definitive evidence that MHC-IIX was a separate protein and not a product of post-translational modification of MHC-IIB was realized when the cDNA of MHC-IIX mRNA was isolated. All three Type II MHC isoform cDNAs display different restriction endonuclease maps, indicating their derivation from three separate genes. In addition, each isoform contains a unique 3' untranslated sequence which has been used to develop specific probes for the use in *in situ* hybridization studies (424).

1.6.2.1.4. Classical methods of equine fiber typing

Traditional methods of fiber typing that have been used in rodents and humans have been utilized in horses for nearly 50 years (411). Generally, the study of equine skeletal muscle requires muscle biopsies in order to obtain muscle samples, and these are most commonly collected from the gluteus medius muscle using the percutaneous needle biopsy technique (411). The gluteus medius muscle is commonly used due to the role of this muscle in both locomotion and posture (411). The use of myofibrillar ATPase,

NADH-diaphorase, and periodic acid-Schiff (PAS) as a marker of contraction speed correlation, oxidative capacity, and glycogen distribution, respectively, provided a basis for the determination of slow twitch, fast twitch high oxidative, and fast twitch slow oxidative. Other traditional determinates are the use of biochemical analyses of metabolites in the muscle: glycogen, pyruvate, lactate, ATP, creatine phosphate, glucose and glucose-6-phosphate. Lastly, the use of enzyme activities as a marker of glycolytic and oxidative capacities can aid in the determination of fiber type. These techniques have provided the evidence for delineation of equine skeletal muscle into three major fiber types (I, IIA, and IIB (431)).

1.6.2.1.5. Electrophoretic and immunohistochemical methods for equine fiber typing

After the identification of the myosin heavy chain isoforms (I, IIA, IIB, and IIX) in rodents and humans, gel electrophoresis was then used to examine equine skeletal muscle (413). The rat costal diaphragm was used as a control because it contains all four of the myosin heavy chain isoforms (432). Gel electrophoresis revealed three MHC isoforms; two of which co-migrated with the Type I and IIA isoforms of the rat. The third equine isoform migrated between the IIX and IIB rat isoforms; but showed a mobility closer to that of the IIX. These results provided a new question to be solved: were the fast oxidative fibers previously classified as Type IIB in horses actually Type IIB or IIX fibers containing MHC-IIB or -IIX with a different molecular weight due to post-translational modifications or were they some newly discovered fiber type containing a unique MHC isoform? Further examination of the MHC isoform at the protein level revealed that the rat and equine MHC-IIX isoform has identical antigenic and mATPase determinants (412). This evidence suggests that the MHC-IIB protein is

not functional at the protein level in horses. Additional evidence of the absence of the MHC-IIB isoform in equine skeletal muscle was provided with the use of PCR and *in situ* hybridization techniques, which allowed researchers to isolate both mRNA and cDNA for MHC-I, -IIA, and -IIX isoforms (433). The mRNA isolation was performed based on the conserved sequences of exon 40 in both fast and slow isoforms (424).

Additional proof for the absence of the Type IIB fiber Type In horses came with nucleotide sequencing of the equine diaphragm, semitendinosus and longissimus thoracis muscles (409). It could be speculated that if there was an expression of a IIB fiber in the horse it would be seen in the diaphragm because it has been shown that in rat and mice all four MHC isoforms are expressed in the diaphragm (432). cDNA synthesis and sequencing and PCR analysis was done on all of the MHC genes. The equine MHC isoforms were found to contain 5980, 5982, and 5984 base pairs for IIA, IIX, and I, respectively. These identified sequences coded for MHC isoforms that contain 1937, 1938, and 1935 amino acids for the IIA, IIX, and I proteins, respectively. The MHC-IIA and -IIX isoforms are similar both in the number of amino acids and in the overall structure; however, there are differences in the functional regions of the two proteins. Within the functional region of the MHC-IIA protein there are two amino acid deletions compared to the functional region of the MHC-IIX protein. Overall, this study demonstrated that there are three functional MHC proteins, -I, -IIA, and -IIX.

During the examination of the equine diaphragm, semitendinosus and longissimus thoracis muscles, Chikuni and his colleagues (409) amplified a 596 base pair fragment of the MHC 3' region using MHC-IIB amplification primers. This fragment contained a similar sequence to the other MHC isoforms, but differed in an additional adenine

nucleotide. This additional nucleotide resulted in a frame shift, and thus, a shortened translation. Chikuni and his colleagues (409) referred to this MHC-IIB fragment as the equine MHC-IIB pseudogene because the gene exists, but cannot be translated into a functional protein.

1.6.2.1.6. *The large animal theory and the implications of an absent functional MHC-IIB isoform*

The absence of the MHC-IIB isoform in horses is thought to be due to the large animal theory, which explains the absence of a functional MHC-IIB isoform because of differences in velocity of shortening, metabolic energy consumption, and the ability of bones to resist force in large animals compared to small on the basis of body size. For example, smaller animals must take a larger number of steps in order to cover the same distance as larger animals due to their shorter stride length. An animal's force-velocity curve must be appropriate for the speed at which they move: larger animals having a slower velocity of shortening than their smaller counterparts (434). In addition, with the decreased stride length of smaller animals, more energy must be consumed in order to meet their increased velocity of shortening in comparison to the large animal (435).

The final component of the large animal theory is that with increases in body size, there are added stresses on bone due to increased muscle size. The ability of bones to resist force depends on the ratio of bone to muscle cross sectional area, which decreases in proportion to animal weight. In an effort to adjust for this, the limbs of large animals vary in size, and are more closely aligned with the ground reaction force in a size-dependent manner (436).

The lack of the MHC-II B isoform in horses may indicate an evolutionary elimination due to components of the large animal theory. The entire sequence of the MHC-I, -II A, and -II X isoforms are highly conserved in mammals; therefore, the equine MHC isoform sequences showed high identities to the porcine, bovine and human MHC isoforms (409). The fact that horses contain a “gene” for MHC-II B isoform that lacks expression due to an early termination as a result of a frame shift from the additional adenine may further indicate the evolution of the species.

During the course of history, the modern horse has evolved from a small (0.4m) 4-toed Hyracotherium to the large (1.6m) hooved animal seen today. This small equine ancestor and its progeny (3-toed, 0.6m Meshippus and 3-toed, 1m Merychippus) may have contained the functional MHC-II B isoform. These ancestral precursors to the modern horse are related to the pig and other ungulates who express the MHC-II B isoform (409). This would indicate that ancestral animals of the orders Perissodactyla and Artiodactyla may have kept all four MHC isoforms, and the horse and cattle (others are still to be determined) lost the functionality of the MHC-II B isoform. The absence of the MHC-II B isoform in horses may be a result of an evolutionary need to decrease bone stress due to velocity of contraction as the animal evolved to have an increased body size.

1.6.2.2. Implications of breed and sampling depth on equine skeletal muscle fiber type

Fiber type distribution in an individual muscle within a horse is extremely variable, and is greatly influenced by breed of horse. The percentage of Type I fibers in the middle gluteus muscle is the highest in Warmblood type horses, and decreases in ponies, Arabians, Thoroughbreds, and Quarter Horses (414). Alternatively,

Thoroughbreds express the greatest percentage of Type II fibers, followed by Quarter Horses, Arabians, ponies, and Warmblood type horses (414). Of the Type II fibers, Thoroughbreds have the greatest percentage of IIA type fibers, followed by Arabians, Quarter Horses, ponies and Warmblood type horses (414). The Quarter Horse possesses the greatest percentage of IIX type fibers and the proportion is lower in Arabians, Thoroughbreds, ponies and Warmblood type horses. Although variation exists, these differences are narrow with the range of Type I fibers in the above breeds from 7 - 31%, Type IIA from 37 - 51%, and Type IIX from 32 - 45% (414). Additionally, breed related differences are detectable as early as the first day of life (437, 438). It is also important to note, that these studies did not use modern methodologies of immunohistochemistry or MHC isoform separation through electrophoresis.

Regardless of fiber typing method, age and breed of the horse, variation exists within a single skeletal muscle. Because the gluteal muscle is the most commonly sampled, the majority of data is from this muscle and has shown to vary with depth. The proportion of Type IIX fibers decreases and Type I fibers increases with depth in mature horses (413, 439-442) and foals (439) in the gluteal muscle. This is merely a function of the muscle; the gluteus muscle is used in both locomotion and posture. The deeper portion of the muscle is used more for posture than the shallower portion of the muscle.

1.6.2.3. Changes in skeletal muscle physiology during development and aging

The proportion of muscle fiber type shifts during development. During neonatal growth in humans and rodents, there are shifts in the relative proportions of MHC-neonate, MHC-I, MHC-IIA and MHC-IIX in skeletal muscle, with increases in all forms,

except MHC-neonate which vanishes with time. However, the appearance of MHC-II isoforms is delayed in comparison to MHC-I (432, 443-445). Similar shifts occur in MHC protein concentration and mRNA expression during the first year of life in Dutch Warmbloods (410, 446-448) and Thoroughbreds (449). The proportions of MHC-I and MHC-IIA are increased and MHC-IIX is decreased at 24 months of age, compared to 12 months of age in Dutch Warmblood horses (448, 450). Correlation coefficient analysis was performed on MHC isoforms throughout the equine lifespan (from birth to 30 years of age), where it was determined that MHC-I dramatically decreases, MHC-IIA slightly increases, and MHC-IIX remains unchanged (451). However, further research is warranted to determine the specific time course for these shifts in MHC fiber type expression.

Because fiber type is associated with glycolytic and oxidative capacity, alterations in fiber type during development and aging also lead to alterations in glycolytic and oxidative capacity. Glycolytic and oxidative capacity are measured through the expression and activity of enzymes involved in the anaerobic (glycolysis) and aerobic (Kreb's cycle and oxidative phosphorylation) production of ATP, respectively (448). Markers of oxidative capacity are the enzymes succinate dehydrogenase (SDH) and citrate synthase (CS) and the mitochondrial complexes involved in oxidative phosphorylation. The enzymes phosphofructokinase (PFK) and lactate dehydrogenase (LDH) are involved in the glycolytic production of ATP, which may also result in the production of lactic acid. There is an increase in the activities of several of the oxidative enzymes during growth (452-454), with subsequent declines in oxidative capacity with old age in humans and rodents (455, 456). In horses, SDH activity does increase during

the first 2 years of life (446, 448, 449); however, the effects of maturation on CS activity appears to be dependent on breed (438, 448). CS activity in Standardbreds and Quarter horses ceases to decrease earlier in development (~14 weeks) (438) than Dutch Warmbloods (~22 weeks) (448). Correlation coefficient analysis has been performed on the enzymes involved in oxidative capacity throughout the equine lifespan (birth to 30 years of age), where it was determined that CS decreases (451), while LDH increases (438, 451). This data indicates that shifts in MHC throughout the lifespan coincide with shifts in oxidative capacity.

The gluteal muscle amino acid profile has been examined during the first year of life in horses, where it was demonstrated that glutamine was the most abundant free α -amino acid in the muscle up to 3 months of age (457). As foals grow from 3 to 12 months of age, the concentrations of alanine, glutamate and glycine were greater than that of glutamine in the skeletal muscle of mature Standardbreds (457). Additionally, the β -amino acid, taurine is extremely abundant in equine skeletal muscle (2, 458) and has been shown to decrease with from 6 to 12 months of foal development (457). However, mature equine skeletal muscle has taurine concentrations that compare to 6 month old foal (2, 458). In rodents, taurine protects skeletal muscle from reactive oxygen species post-exercise (459), and has been demonstrated to increase skeletal muscle force when taurine is supplemented to the diet (460). Glutamate is the most abundant free α amino acid in mature skeletal muscle, followed by alanine, glutamine and glycine (2, 458). Carnosine, the di-peptide of histidine and β -alanine, is present in extremely high quantities in adult equine skeletal muscle (458, 461), where it buffers lactic acid buildup; however, it has not been examined in developing equine skeletal muscle. The amino acid

composition of aged equine skeletal muscle is also limited; therefore, further examination of the amino acid profile throughout the lifespan is necessary. The elevated abundance of free α -amino acids in the rapidly growing skeletal muscle of the foal compared to the mature horse indicates the increased amino acid availability for protein synthesis in order to accrete muscle during this rapidly growing life stage.

1.6.2.4. Changes in skeletal muscle physiology following exercise

Because of the athletic ability of the horse, there is a considerable amount of literature regarding the physiological changes to skeletal muscle following a bout of exercise (415, 462-464) and exercise training (416-419, 465, 466). However, these studies vary in the age of horses used, and the intensity of exercise and duration of training. This subsection of the literature review will focus on the alterations in MHC content, oxidative and glycolytic capacity, and free amino acid content.

The recruitment pattern of muscle fiber types in mammals has been well characterized, with prolonged exercise there is a shift in the fibers used from Type I to Type IIX fibers; however this recruitment does not hold true during short high intensity exercise bouts common in racehorses. Following an 800 and 1200 m race, the recruitment of Type IIA and IIX fibers were reduced in the equine gluteal muscle based on myosin ATPase activity, and glycogen depletion did not occur in Type I fibers (464). An exercise bout, regardless of intensity (462, 463) or track slope (462) will recruit all MHC fibers to a similar extent if the exercise bout is a minimum of 8 minutes long. However, during shorter bouts of exercise (4 minutes), there is a higher recruitment of all MHC fibers in moderate (60% VO_2 max) to high (80% VO_2 max) intensity workouts than

extremely high intensity (100% VO_2max) workouts (463). Glycogen can be stored in all fiber types (Section 1.6.2.1.2), and is typically not depleted in Type I fibers during short duration high intensity exercise (464). Glycogen depletion following a bout of exercise is affected by intensity (462, 463), but not track slope (462), where the intensity of the exercise coincides with the degree of glycogen depletion. Overall, these results indicate that during short duration high intensity workouts similar to that seen in horse racing there is a similar recruitment of fiber type which is not what is characteristic of prolonged exercise.

Duration and intensity alters fiber type recruitment during an exercise bout, but also alters the MHC protein content following prolonged training. Duration of exercise, regardless of intensity, there is altered MHC protein composition following exercise, where MHC-I, -IIA, and -IIX were greatest with 15, 25, and 5 minute exercise bouts, respectively, after 22 days of training (418). Additionally, following 22 days of training at moderate (velocity at which blood lactate concentration is 2.5 mmol/L ($V_{2.5}$)), and high (V_4) intensities, regardless of the duration of each training bout, MHC-I, -IIA, and -IIX content was higher following the V_4 , V_4 , and $V_{2.5}$ intensity bout, respectively (418). Overall, 22 days of training, regardless of bout duration or intensity caused MHC-I, -IIA, and -IIX content to remain unchanged, increase, and decrease, respectively (418). Short duration high intensity training for 8 months has been reported to have no effect on MHC content, which the authors' attributed to the superficial sampling site of 2 cm within the gluteal muscle (417), which was confirmed when the authors' performed another short duration high intensity training for 22 days and alterations in MHC content were detected at a sampling depth of 6 cm within the gluteal muscle (418). Incline training does not

appear to alter MHC content or oxidative capacity: 16 weeks of sloped track training increased SDH activity and MHC-IIA content to a similar extent as 16 weeks of flat track training (415). Ultimately, with moderate to high intensity short term training protocols, there is a shift in the proportion of fast twitch fibers mainly towards MHC-IIA and an increase in glycolytic and oxidative capacities compared to pre-training.

Endurance type racing is another sport that horses commonly participate in. As a result, this type of exercise training has been examined, where following 90 days of endurance type training there was a shift in MHC content towards a greater percentage of MHC-I fibers at the expense of –IIX fibers (466). Similar results were seen following endurance training by Rivero and colleagues (416) and Serrano and colleagues (419). Additionally, capillarity associated with Type I and IIA fibers has been demonstrated to increase following endurance type training (416, 419). Following endurance type training, oxidative capacity is increased (CS activity and glycogen storage as markers), and glycolytic capacity is decreased (LDH and PFK activities as markers) (419). Endurance type training results in increased MHC content of Type I fibers and oxidative capacity.

Breed has been determined to greatly affect MHC content and also plays a role in the susceptibility of MHC recruitment to training. For example, the Arabian, who were bred for endurance type exercise, are more predisposed to MHC conversion toward MHC-I following endurance type training (416). However, additional research is needed to examine the susceptibility of MHC recruitment of a breed like the Thoroughbred, which was bred for high intensity short duration exercise, following endurance type exercise training.

Intramuscular amino acid concentrations are altered by exercise. When muscle biopsies were collected 30 minutes following a maximal exercise test, alanine, leucine and lysine concentrations increased from pre-exercise values; however, glutamate and taurine concentrations decreased (458). Following a submaximal exercise test, alanine concentrations increased immediately and returned to pre-exercise levels 18 hours later (467). The authors did not detect differences in any other amino acid due to the high degree of variability, which they attributed to the wide age range of 2.5 to 6 years (467). The younger horses may have been growing at a slow rate, whereas the older horses were probably not still developing. Additionally, the increases in alanine post-exercise is most likely due to the involvement in the Cori cycle (339), and the decrease in taurine indicates that the exercise protocol used in this study created reactive oxygen species, which through the indirect measurement of taurine concentration implies that taurine was used to suppress these reactive oxygen species (459). Additional research is necessary to determine if alterations in intramuscular amino acids following exercise are influenced by duration and intensity.

1.6.2.5. Changes in skeletal muscle physiology following exercise during development and aging

The desire to prepare young prospects for show or race has led horse owners to place yearlings in training in order to prepare them for the 2 year old races and in hand futurity events for show horses. As previously discussed (Section 1.6.2.3), there are many physiological alterations occurring in skeletal muscle of horses during this stage of rapid growth and development. These normal physiological changes can also be influenced by exercise. Developmental alterations during the first year of life (increase in

Type I and IIA and decrease in Type IIX) are less substantial in foals that were housed in the pasture (receiving ad libitum exercise) compared to those housed in a stall (receiving no exercise) (448). However, there was no effect of the differences in housing induced exercise on capillarity CS or SDH activity (448). Dingboom and colleagues (446) found that the developmental change in fiber type was not altered throughout the first year of life in foals that were housed in a stall (receiving no exercise), or those housed in a stall but forced to exercise. The differences in these studies may be a factor of the intensity and duration of exercise involved. However, growing horses in a moderate exercise training program that included 6 trips over a 3 fence combination 2 days/week from weaning to 3 years of age, had a higher percentage of MHC-I and –IIA at 2 and 3 years of age compared to non-exercising 2 and 3 year old horses (450). This coincided with a greater developmental increase in CS activity in the training group compared to the non-exercising control group (450). However, short duration high intensity exercise training for 16 weeks in 2 year old Thoroughbreds did not alter MHC content, SDH or PFK activities (468). These studies indicate that there is an effect of exercise duration, intensity and training period on the developmental changes in muscle biology; however, further research is needed to examine the effects of exercise training on the alterations in MHC content that occur during development.

The beneficial effects of exercise in the aged have been demonstrated in humans; however, there is limited data on the effects of exercise in the aging horse. Exercise training over a period of 10 weeks in aged horses improved VO₂max and plasma lactate concentrations (469). This training period also increased MHC-IIA and –IIX content in the semimembranosus and triceps muscles, respectively (469). Additionally, CS activity

was increased in the triceps muscle (469). Implementation of an exercise protocol during aging appears to improve oxidative capacity, and potentially strengthen muscles which may prevent injury; however additional research is needed.

1.6.2.6. mTOR related signaling in equine skeletal muscle

Currently there are only two studies in horses to examine mTOR signaling (2) or other signaling pathways that may directly influence mTOR signaling (470). The first study examined the effect of exercise on the mitogen activated protein kinase (MAPK) family, which has three subdivisions: the ERK1/2, MAPK, and c-Jun NH₂ terminal kinase (JNK). The effects of ERK1/2 and MAPK on mTOR signaling have been discussed in detail above (Section 1.2.2.4). JNK is also a stress-activated MAPK cascade that is activated during oxidative stress, and by inflammatory cytokines and growth factors. Ten minutes following a submaximal exercise test, the phosphorylation of p38 MAPK was elevated in both the vastus lateralis and pectoralis descendens muscles (470). The authors of that study (470) chose the vastus lateralis because of the involvement of this hind limb muscle in forward movement and the pectoralis descendens because of the role in posture (470). The phosphorylation of MAP3K, MAPK activated protein kinase 2 (an intermediate signaling factor in the MAPK signaling cascade), and JNK were elevated 10 minutes following a submaximal exercise test in the vastus lateralis (470). However, the phosphorylation of ERK was not affected by exercise in either muscle examined (470). Activation of these signaling factors following exercise could potentially lead to increased protein synthesis through inhibiting TSC1/2 complex (Section 1.2.2.4). Following the consumption 4g/kg of a high protein pelleted feed (90 minutes), there was a greater abundance of the phosphorylated forms of both 4EBP1 and rpS6 in the gluteal

muscle of mature horses than during the post-absorptive state (2). However, feeding status did not alter the phosphorylation of Akt (2). This data indicates that following the anabolic stimulus of meal consumption muscle protein synthesis may increase.

1.6.2.7. Muscle protein turnover

To date, only a single study has examined skeletal muscle protein synthesis in the horse using isotopes. Skeletal muscle protein synthesis was examined after a single bout of high intensity exercise in long 2 year old Thoroughbreds that had been in short duration high intensity training once a week for 3 months (3). Matsui and colleagues (3) found that muscle protein synthesis across the hind leg increased to the greatest extent following exercise when an amino acid-glucose mixture was infused in the jugular vein during the recovery period compared to the recovery period of the individual infusion of saline, amino acids, and glucose. Infusion of amino acids alone also increased muscle protein synthesis (3). However, infusion of glucose alone or saline did not increase protein synthesis during the 120 minutes recovery period. Skeletal muscle protein degradation across the hind leg was also measured in this study, were there was an initial decrease in muscle degradation rates during the first hour following exercise in the amino acid and amino acid-glucose mixture infused horses (3). During the second hour of recovery there was no difference in rates of muscle protein degradation in any of the infused groups; however, during hours 3 and 4 of recovery the rate of muscle protein degradation in the amino acid-glucose mixture group was elevated (3). This study was a great step forward in understanding muscle protein metabolism in the exercising horse. However, the author's did not examine the effects of pre- to post-exercise on skeletal muscle protein synthesis, and the exercise bout that was used during the experimental

period was less intense than the weekly training exercise protocol, which was not typical of a competition situation. Further research is necessary to determine the effects of a more intense exercise protocol, similar to that seen in the industry.

1.6.2.8. Summary of muscle biology and development

Because the horse is a highly athletic animal, there is an extensive amount of research pertaining to skeletal muscle development and exercise. The developmental changes in MHC content throughout development are similar to other species; however the recruitment of fibers during and following exercise differs from other species mainly due to the duration and intensity of the exercise bout. Additional factors that influence fiber type recruitment and content include duration and intensity of exercise training and breed and age of the horse. To date a single study has examined the anabolic stimulus of exercise on skeletal muscle protein synthesis, and another study that characterized mTOR signaling in response to feeding. This area of research requires further examination, which will be addressed in subsequent chapters of this dissertation.

1.6.3. *Acute and chronic inflammation in the horse*

Inflammatory signaling in response to diseases and laminitis has been the focus of much of the equine work in this area. Although the studies are limited, this subsection of the literature review will focus in the areas of exercise induced inflammation and inflamm-aging in the horse.

1.6.3.1. Characterization of the acute inflammatory response to exercise in the horse

Exercise induced inflammation was briefly discussed in (Section 1.5.3), but the focus of this section will be on the literature in the horse. There have been reports detailing the increased susceptibility of viral infection following exercise (471, 472); however, the reports detailing the inflammatory response following a bout of exercise or exercise training will be discussed here.

Reports of exercise induced inflammation in horses vary due to sampling methods and times, exercise protocols and assay methods. Following prolonged acute exercise, leukocyte expression of TNF- α and IL-1 β are elevated at 23 and 30 days of-high intensity exercise training, but IL-2, -4, -6, or -10 mRNA expression was not affected (473). However, immediately following a maximal exercise test, serum concentrations of TNF- α and PGF2 α were elevated and returned to pre-exercise levels at 2 hours (474). In circulating leukocytes, IL-6 and TNF- α expression was greatest 2 hours following maximal exercise test with no alterations in leukocyte IL-1 β expression (474). Neutrophil counts have also been demonstrated to be elevated in the blood immediately following maximal exercise and remain elevated for 6 hours following exercise (475). However, 24 hours post-maximal exercise test, the mononuclear cells obtained from circulation did not have differences in the mRNA expression of IL-12, -4, and IFN- γ (476). Although sampling times and exercise protocols differ, it is certain that there is an acute increase in circulating inflammatory cytokines following exercise.

This led to a valuable study to determine the 24 hour time course response of inflammatory cytokines in circulation to a maximal exercise test in horses. This study

reported increased mRNA expression of IL-1 from 2 to 6 hours, TNF- α at 6 hours, and IFN- γ immediately following the maximal exercise test (244). However, there were no alterations in the circulating mRNA expression of IL-6 over the 24 hour period (244). Because samples were only collected prior to, immediately following, 30 minutes, 1, 2, 6, and 24 hours following exercise, any alterations in circulating mRNA expression of IL-6 may have been missed.

There has been minimal investigation in the inflammatory response to exercise in equine skeletal muscle. The 24 hour time course inflammatory response has been examined following a maximal exercise test, demonstrated an increase in the mRNA expression of IFN- γ at 1 hour, IL-6 at 30 minutes, and TNF- α immediately following the maximal exercise test (244). However, the authors did not report any alterations in the skeletal muscle mRNA expression of IL-1 during the 24 hour period following the maximal exercise test (244). One implication of this study is that the overall increases in skeletal muscle mRNA expression of inflammatory cytokines to maximal exercise test appear to occur more rapidly than increases in circulating inflammatory cytokines.

1.6.3.2. Characterization of chronic inflammation in the aging horse

Throughout the lifespan, horses exhibit changes in circulating inflammatory cytokines as a result of growth, obesity and aging. Serum C-reactive protein, an acute inflammatory phase protein, increases from birth to 12 months of age, but then decreases when the horses reach maturity, around 4 to 5 years of age (477). However, research examining inflammatory cytokines in circulation of growing horses is limited. Similar to humans (478-480), obesity in horses is characterized by a mild state of chronic

inflammation, and elevations in mRNA expression of TNF- α , IL-1, and IL-6 may play a role in the development of insulin resistance (481). Additionally, aging in horses is characterized as inflamm-aging, where there is an increase in circulating inflammatory cytokines. The relative expression of IL-6 to IL-10 and TNF- α to IL-10 is elevated in peripheral blood mononuclear cells of aged horses compared to adult horses (482). Not only is there an increase in circulating inflammatory cytokines in aging horses (IL-1 β , IL-15, IL-18, and TNF- α), the number of actively secreting IFN- γ labeled lymphocytes and TNF- α labeled lymphocytes are also elevated (483). There is also an increase in the number of circulating CD5 and CD4 T cell subsets in the aged horse (483). Obesity in the aged horse further alters circulating cytokines; however, diet restriction which led to a decrease in bodyweight did lower circulating inflammatory cytokines (484). Overall, it appears that there may be an increase in inflammation or inflammatory mediators in circulation during development which decreases during the adult phase of life and then increases with post-maturity aging. However, there has not been a specific study aimed at examining inflammatory changes throughout the life span in equine skeletal muscle.

1.6.3.3. Exercise induced inflammation in aged horses

The response of circulating inflammatory cytokines to an acute inflammatory stimuli, exercise, during a state of chronic inflammation, aging, has been examined in circulation in the horse. Horohov and colleagues (485) demonstrated that IL-2 stimulated peripheral blood mononuclear cells cultured from pre- and post- exercise samples collected in young (~8 years old) and aged (~25 years old) horses (485). However, the post-exercise response to IL-2 in the cultured peripheral blood mononuclear cells was

greater in the young horses compared to the aged horses (485). This study indicates that following exercise, older horses are less responsive to an infection than young horses, which may be due to the increased production of inflammatory cytokines; therefore the response is already saturated. However, the acute inflammatory response during low grade chronic inflammation in the aged horse requires additional research.

1.6.3.4. Summary of inflammation in the horse

Horses respond similarly to other mammals to acute and chronic inflammation. Although there is still much work to be done, following exercise there is an increase in circulating and muscle inflammatory cytokines, which differs if the horses have been in a training program or if untrained horses perform a single bout of high intensity exercise. Additionally, horses exhibit inflamm-aging which can be exacerbated by obesity. The effects that these inflammatory states may have on skeletal muscle have not been examined and will be partially addressed in subsequent chapters of this dissertation.

1.7. TABLES

Table 1.1. Requirements of various physiological classes of an estimated mature weight 600 kg mare according to the NRC¹

| Physiological State | ADG or Milk produced in kg/d | Crude Protein Requirement | | Lysine Requirement | |
|--|------------------------------|---------------------------|-----------|--------------------|-----------|
| | | g | g/kg BW/d | g | g/kg BW/d |
| Maintenance | | 756 | 1.26 | 32.5 | 0.05 |
| Weanling Growth (4-6 months old) | 0.94 | 807 | 1.35 | 34.7 | 0.06 |
| Yearling Growth (12-18 months old) | 0.44 | 987 | 1.65 | 42.4 | 0.07 |
| Early Pregnancy (≤ 5 months) | 0.09 | 789 | 1.32 | 33.9 | 0.06 |
| Mid-Pregnancy (6-8 months) | 0.30 | 877 | 1.46 | 37.7 | 0.06 |
| Late Pregnancy (9-10 months) | 0.63 | 1012 | 1.69 | 43.5 | 0.07 |
| Early Lactation (1 month) | 19.6 | 1842 | 3.07 | 101.7 | 0.17 |
| Mid Lactation (2-3 months) | 18.7 | 1799 | 3.00 | 98.9 | 0.16 |
| Late Lactation (4-6 months) | 14.7 | 1597 | 3.66 | 85.5 | 0.14 |
| Light Exercise | | 839 | 1.40 | 36.1 | 0.06 |
| Moderate Exercise | | 921 | 1.54 | 39.6 | 0.07 |
| Heavy Exercise | | 1034 | 1.72 | 44.5 | 0.07 |
| Growth and Exercise (18 month old in light exercise) | 0.34 | 1023 | 1.71 | 44 | 0.07 |

¹Adapted from the NRC 2007 (342)

1.8. FIGURES

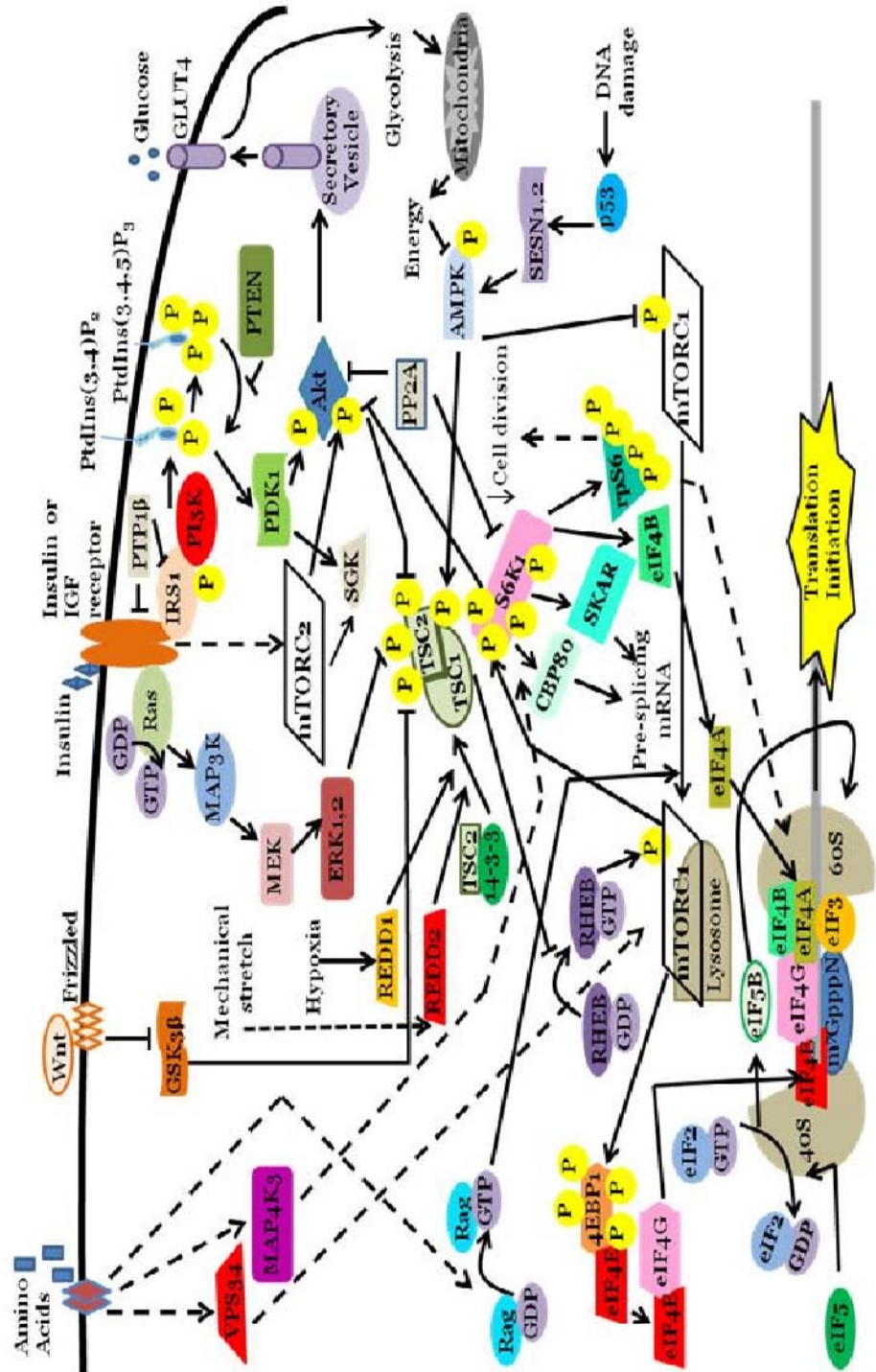
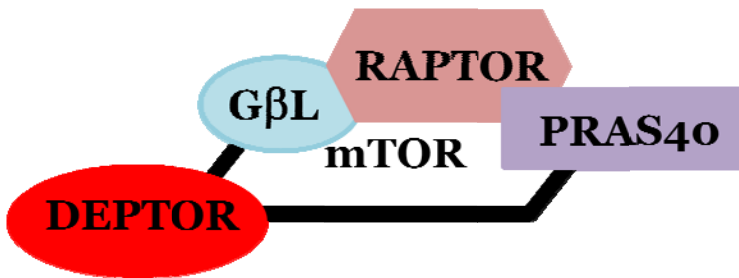


Figure 1.1. Schematic drawing of the mTOR signaling pathway.

Abbreviations: P; phosphorylated; IRS1, insulin receptor substrate 1; PTP1 β , protein tyrosine phosphatase 1 β ; PI3K, phosphoinositide 3-kinase; PtdIns(3,4)P₂/PtdIns(3,4,5)P₃; PDK1, PtdIns(3,4)P₂ / PtdIns(3,4,5)P₃-dependent kinase 1; PTEN, phosphatase and tensin homolog deleted on chromosome 10; SGK, serine/threonine protein kinase; Akt, protein kinase B; PP2A, protein phosphatase 2A; GLUT4, glucose transporter 4; AMPK, adenosine monophosphate kinase; p53, protein 53; GDP, guanosine diphosphate; GTP, guanosine triphosphate; MAP3K, mitogen activated protein kinase kinase kinase; MEK, mitogen extracellular kinase (sometimes referred to as MAPK); ERK1,2, extracellular regulated kinase 1,2; Wnt, Wingless-Type MMTV Integration; GSK3 β , glycogen synthase kinase 3 β ; REDD1,2, Regulated in development and DNA damage response 1,2; TSC1,2, tuberous sclerosis 1,2; 14-3-3, scaffolding protein 14-3-3; VPS34, PI3K catalytic subunit type 3; MAP4K3, mitogen activated protein 4 kinase kinase kinase; Rheb, Ras homologue enriched in brain; Ras, RA_t Sarcoma protein subfamily; mTORC1,2, mammalian target of rapamycin complexes 1 and 2; S6K1, 70kDa S6 kinase 1; rpS6, ribosomal protein S6; eIF, eukaryotic initiation factors (2, 3, 4A, 4B, 4E, 4G, 5); SKAR, Aly/REF-like target; CBP80, 80 kDa RNA splicing export factor nuclear cap-binding protein; 4EBP1, eukaryotic initiation factor 4 eukaryotic binding protein 1; 40S, 40S ribosomal subunit; 60S, 60S ribosomal subunit; m⁷GpppN, Cap structure; an arrow indicates an activation; and a perpendicular line indicates inhibition.

A. mTORC1



B. mTORC2

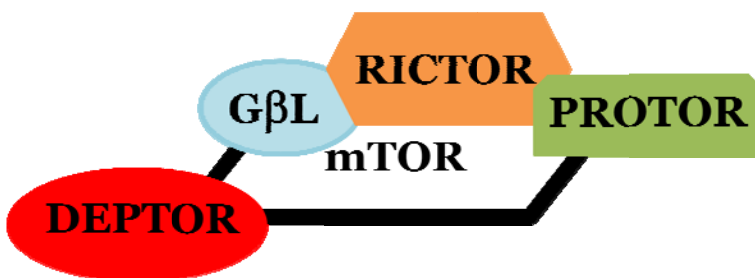


Figure 1.2. Schematic drawing of the mTOR Complexes (A) mTOR Complex 1 (B) mTOR Complex 2.

Abbreviations: mTOR, mammalian target of rapamycin; DEPTOR, DEP domain-containing mTOR-interacting protein; GβL, mammalian lethal with SEC13 protein 8; RAPTOR, regulatory-associated protein of mTOR; PRAS40, 40 kDa pro-rich Akt substrate; RICTOR, rapamycin-insensitive companion of mTOR; PROTOR, protein observed with RICTOR

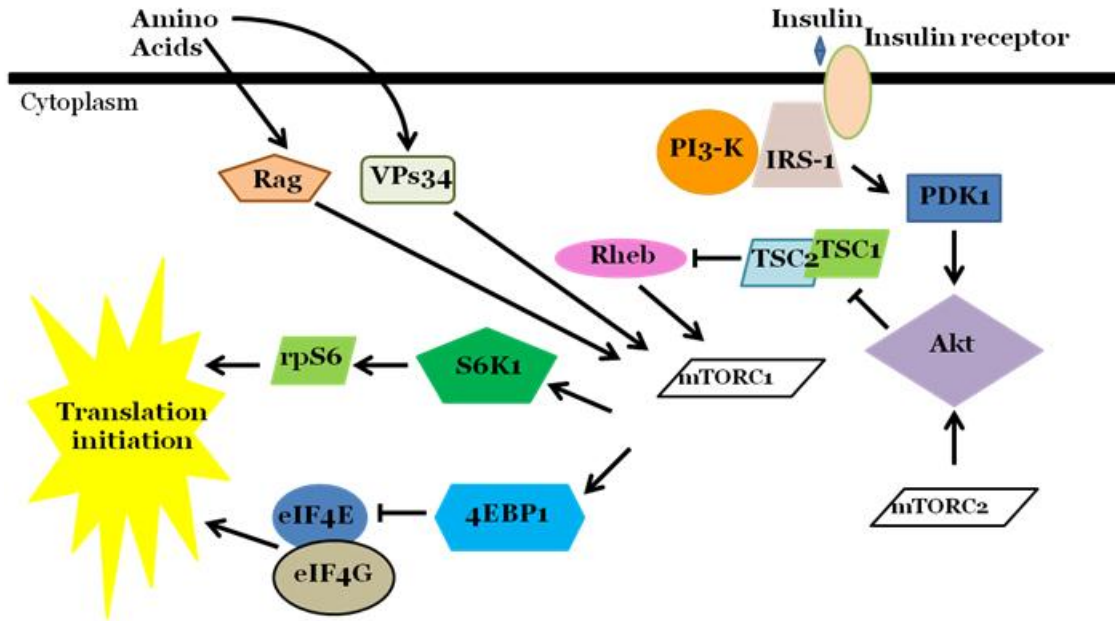
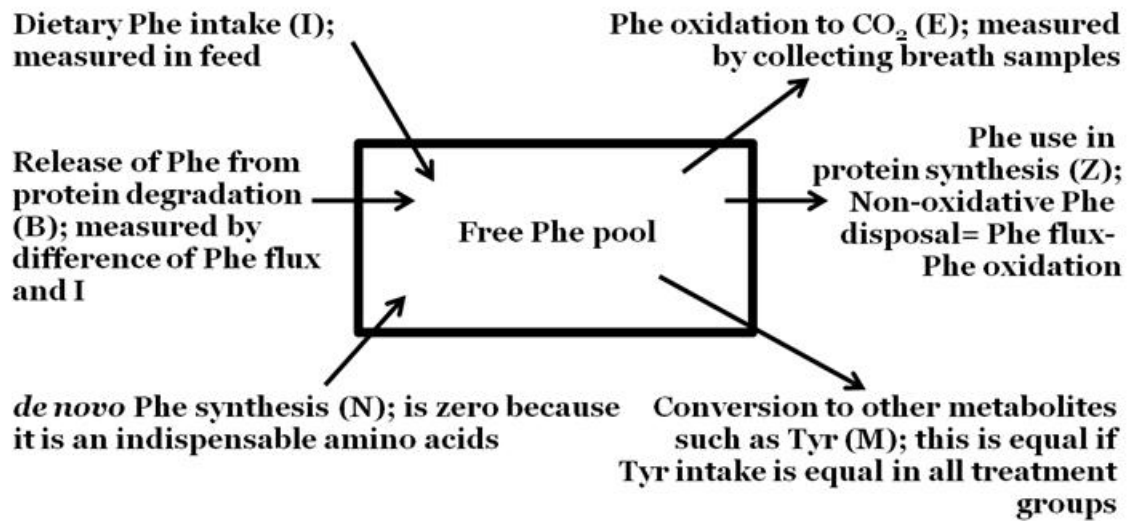


Figure 1.3. Schematic drawing of an abridged version of the mTOR pathway. Abbreviations: IRS1, insulin receptor substrate 1; PI3K, phosphoinositide 3-kinase; PDK1, PtdIns(3,4) P_2 / PtdIns(3,4,5) P_3 -dependent kinase 1; Akt, protein kinase B; TSC1/2, tuberous sclerosis 1,2; Rheb, Ras homologue enriched in brain; mTORC1,2, mammalian target of rapamycin complexes (1,2); S6K1, 70kDa S6 Kinase 1; rpS6, ribosomal protein S6; 4EBP1, eukaryotic initiation factor 4 eukaryotic binding protein 1; eIF4, eukaryotic initiation factor 4 (E, G).

Entry

Exit



Phe flux =
Rate that Phe enters (I + B) and exits (E + Z + M) the free amino acid pool; flux is calculated from the dilution of the isotope by unlabeled phenylalanine in the plasma

Figure 1.4. Schematic drawing of whole-body phenylalanine kinetics.

Chapter II

Rationale and Objectives

2.1. SCOPE OF DISSERTATION

The aims of the research presented in this dissertation were to examine the regulation of protein synthesis through the mTOR signaling pathway in the horse during various physiological states. The horses used in this dissertation varied in age from growing adolescents to aging horses and were studied in the post-absorptive and postprandial states and in states of acute and chronic inflammation. Muscle biopsies were collected in all the studies to examine mTOR signaling responses to growth, aging, acute and chronic inflammation, as well as in the postprandial and post-absorptive states. Additionally, stable isotopes were infused intravenously to study whole-body protein metabolism.

2.2. RATIONALE

As athletic animals, horses' body mass is nearly 50% skeletal muscle (1). Horses rely on skeletal muscle for mechanical and metabolic functions during exercise and as a source of stored protein and amino acids during periods of long term fasting. Although measurements have not been made in the horse, in other mammals skeletal muscle accounts for ~25% of whole body protein synthesis (198, 199), indicating that it is a major site of protein synthesis. In horses, there is an increase in skeletal muscle mass during growth and development, followed by a decrease during post-adulthood aging (486). In other species, these age-related shifts in muscle mass are accompanied by alterations in the response of muscle protein fractional synthesis rates (125-127, 149, 151,

154) and whole body protein metabolism (185, 202, 487) to anabolic stimuli.

Furthermore, age-related changes are present in the activity of the cell signaling pathway referred to as the mammalian target of rapamycin (mTOR) pathway, which is responsible for regulating protein synthesis in response to anabolic stimuli (23, 50, 129, 139, 155-157). Despite the age-related examination of whole body and muscle protein synthesis and the mTOR signaling pathway in other species, there has not been a single study in the horse to quantify these aging-related changes.

There have been three studies (2, 3, 470) in horses to examine muscle protein synthesis, mTOR signaling and another cell signaling pathway that can affect downstream mTOR signaling. However, all of these studies have been performed in the mature horse under various physiological states including postprandial, post-absorptive and post-exercise. Because of the limited number of studies in this area, the optimum methods for muscle biopsy collection, for example the muscle and depth from which the biopsy is collected, and the lag time between biopsies, have not been determined. Currently, there is no standardized method, nor do we know how measurements may be influenced by differences in any of these parameters. Despite the fact that mTOR signaling in various muscles respond differently to anabolic stimuli, there are currently no studies to determine the best sample collection methodology to study mTOR signaling in the horse.

By determining the optimum methods to study mTOR signaling in the horse, and the age-related changes that occur, insight into protein requirements during these life stages may be provided, and allow for the development of better dietary and management

strategies for these animals in the future. This will be extremely important in trying to optimize growth and delay the loss of muscle mass accompanied with aging.

2.3. SPECIFIC HYPOTHESES AND OBJECTIVES

2.3.1. Hypothesis 1.

Hypothesis 1a: mTOR signaling will not be altered by gluteal muscle biopsy collection depth in mature horses in response to feeding. **Hypothesis 1b:** The percentage of myosin heavy chain isoforms will be altered by gluteal muscle biopsy collection depth in mature horses. **Hypothesis 1c:** There will be no variation in the amino acid concentrations due to gluteal muscle biopsy collection depth in mature horses in response to feeding.

The objective of the first study in this dissertation was to determine whether biopsy depth related changes in MHC isoforms were associated with differences in the feeding-induced activation of mTOR signaling in the gluteal muscle of mature horses. Gluteal muscle biopsies were collected 60 minutes following the consumption of 3g/kg of a high protein pellet at 6, 8, and 10 cm below the surface of the skin in mature horses. The gluteal muscle biopsies were then prepared and analyzed for the following: Western blot analysis for mTOR signaling factors (Hypothesis 1a), myosin heavy chain separation analysis (Hypothesis 1b), and reverse phase HPLC for muscle free amino acid concentrations (Hypothesis 1c).

2.3.2. Hypothesis 2.

Hypothesis 2a: The collection of gluteal muscle biopsy samples every 24 hours for 5 consecutive days will reduce mTOR signaling in response to feeding. **Hypothesis 2b:** Administration of a non steroidal anti-inflammatory drug (NSAID) will blunt the effects of gluteal muscle biopsies repeated every 24 hours over the course of 5 days on mTOR signaling. **Hypothesis 2c:** The collection of gluteal muscle biopsy samples every 24 hours for 5 consecutive days will increase mRNA expression of inflammatory cytokines (IL-1 β , IL-6, IL-10, TNF- α , IFN- γ) in skeletal muscle. **Hypothesis 2d:** Administration of a NSAID will reduce mRNA expression of inflammatory cytokines in skeletal muscle during consecutive gluteal muscle biopsies repeated every 24 hours over the course of 5 days.

The objective of the second study of this dissertation was to determine the effects of collecting gluteal muscle biopsies every 24 hours for 5 consecutive days on muscle inflammatory cytokine expression and the activation of mTOR signaling in response to a high protein meal. Gluteal muscle biopsies were collected 60 minutes following consumption of 3g/kg of high protein pellet from 12 mature horses. This process was repeated every 24 hours for the following 4 days. After the initial biopsy was collected 6 of the mature horses were randomly assigned to begin receiving the NSAID drug treatment (1 g of phenylbutazone every 12 hours), and the other 6 horses did not receive any NSAID treatment until the end of the day 5 biopsy. Muscle tissue samples were prepared and analyzed using Western blotting analysis for mTOR signaling (Hypothesis 2a, 2b) and real time PCR analysis to determine the mRNA expression of the inflammatory cytokines (Hypothesis 2c, 2d).

2.3.3. Hypothesis 3.

Hypothesis 3a: There will be an increase in mTOR signaling in the postprandial state of compared to the post-absorptive state in yearlings, two year olds and mature horses. **Hypothesis 3b:** The responsiveness of mTOR signaling to feeding will be greatest in the muscle of yearlings, followed by 2 year olds and will be lowest in mature horses. **Hypothesis 3c:** There will be no differences in mTOR signaling in the post-absorptive state between yearlings, two year olds and mature horses.

The objective of the third study in this dissertation was to determine the effects of feeding following an 18 hour period of feed withholding on the activation of mTOR signaling factors in yearling, two year old and mature horses. Following an 18 hour period feed withholding in yearlings, two year olds, and mature horses, gluteal muscle biopsies were collected 90 minutes following either the consumption of 4g/kg (2g/kg at t=0 min and 2g/kg at t=30 min) of a high protein pellet or after 90 minutes of continued feed withholding (Hypotheses 3a, 3b and 3c). All muscle biopsies were prepared and analyzed for the activation of mTOR signaling using Western blotting procedures.

2.3.4. Hypothesis 4.

Hypothesis 4a: Aged horses (23.5 ± 2.6 y old) will have lower rates of whole-body protein synthesis compared to mature horses (11 ± 2.6 y old). **Hypothesis 4b:** Aged horses will have decreased gluteal muscle mTOR signaling compared to mature horses. **Hypothesis 4c:** Aged horses will have increased gluteal muscle mRNA expression of inflammatory cytokines compared to mature horses. **Hypothesis 4d:** Aged

horses will have higher mRNA expression of inflammatory cytokines in circulation than mature horses.

The objective of the fourth study in this dissertation was to determine the effects of old age on mTOR signaling and whole-body protein synthesis in mature and aged horses, and to further characterize the inflammatory state of the aged horses. Mature and aged horses were studied using a 2 hour primed (14.4 $\mu\text{mol/kg}$), constant (12 $\mu\text{mol/kg/h}$) intravenous infusion of [^{13}C] sodium bicarbonate solution to measure total CO_2 production. Horses then received a 4 hour primed (8.4 $\mu\text{mol/kg}$), constant (6 $\mu\text{mol/kg/h}$) intravenous infusion of [$1\text{-}^{13}\text{C}$]phenylalanine, with blood and breath sampled every 30 minutes, to measure whole-body phenylalanine kinetics (Hypothesis 4a). A gluteal muscle biopsy was collected at the end of the infusion period to determine mTOR signaling (Hypothesis 4b) and the mRNA expression of inflammatory cytokines (Hypothesis 4c) in skeletal muscle. The day before the infusion procedures, a blood sample was collected into a PAXgene tube via venopuncture for the determination of circulating inflammatory cytokine mRNA expression (Hypothesis 4d). mTOR signaling and mRNA expression of inflammatory cytokines were determined using Western blotting and real time PCR techniques, respectively.

2.3.5. *Hypothesis 5.*

Hypothesis 5a: Whole-body protein synthesis will increase in aged (23.5 ± 2.6 y old) horses following 4 weeks of NSAID administration. **Hypothesis 5b:** Gluteal muscle mTOR signaling will increase following 4 weeks of NSAID administration. **Hypothesis 5c:** Gluteal muscle mRNA expression of inflammatory cytokines will be

reduced in aged horses following 4 weeks of NSAID administration. **Hypothesis 5d:** Circulating inflammatory cytokine mRNA expression will be reduced in aged horses following 4 weeks of NSAID administration.

The objective of the final study in this dissertation was to determine the effects of NSAID (phenylbutazone at 2 g/d) administration over 4 weeks on the activation of mTOR signaling and whole-body protein synthesis and skeletal muscle and circulating inflammatory cytokines in aged horses. Aged horses were studied prior to and following 2 and 4 weeks of NSAID administration. Horses were studied using a 2 hour primed (14.4 $\mu\text{mol/kg}$), constant (12 $\mu\text{mol/kg/h}$) intravenous infusion of [^{13}C]sodium bicarbonate solution in order to determine total CO_2 production, followed by a 4 hour primed (8.4 $\mu\text{mol/kg}$), constant (6 $\mu\text{mol/kg/h}$) intravenous infusion of [1- ^{13}C]phenylalanine, with blood and breath sampled every 30 minutes, to measure whole-body phenylalanine kinetics (Hypothesis 5a). A gluteal muscle biopsy was collected at the end of the infusion period to determine the mTOR signaling (Hypothesis 5b) and the mRNA expression of inflammatory cytokines (Hypothesis 5c) in skeletal muscle. On the day before infusion procedures, prior to and following 4 weeks of NSAID administration, a blood sample was collected via venopuncture into a PAXgene tube for the determination of circulating inflammatory cytokine mRNA expression (Hypothesis 5d). mTOR signaling and mRNA expression of inflammatory cytokines were determined using Western blotting and real time PCR techniques, respectively.

Chapter III¹

Gluteal muscle sampling depth does not affect mTOR signaling in response to feeding in mature Thoroughbred mares.

¹At the time of the defense (November 21, 2011), a version of this chapter is currently under review for publication in the Equine Veterinary Journal.

3.1. INTRODUCTION

The collection of percutaneous muscle biopsy samples from the gluteal muscle is commonly used to investigate equine skeletal muscle physiology and metabolism (411); however, there is no standardized depth from which samples are collected. Studies have demonstrated variation in MHC isoform expression across depth in the gluteal muscle of horses (413) and MHC content reflects the metabolic properties of the muscle fiber (425).

Protein synthesis is regulated through the mammalian target of rapamycin (mTOR/mTORC1) signaling pathway. The regulation of mTOR signaling has been the topic of recent review (488). Our laboratory has examined the activation of factors along the mTOR pathway, Akt, rpS6 and 4EBP1, in response to meal consumption in mature horses (2), where it was demonstrated that the anabolic stimulus of meal consumption activates mTOR signaling in the mature horse. In rodents, muscle groups with a greater proportion of type II versus I fibers have a greater abundance of the phosphorylated form of S6K1 in response to resistance exercise (110). However, the effects of MHC proportion on the activation of mTOR signaling factors in response to feeding have not yet been determined.

The objective of this study was to determine whether biopsy depth related changes in MHC isoforms were associated with differences in the feeding-induced activation of mTOR signaling in the gluteal muscle of mature horses.

3.2. MATERIALS AND METHODS

3.2.1. Animals and housing

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (2009-0442). Six Thoroughbred mares (13.4 ± 3.4 yrs; 536 ± 45 kg BW) of moderate body condition (5-7 on a 1-9 scale (489)) and clinically healthy were used in this study. Mares were housed in 3×15 m partially covered dry lot pens with crushed limestone footing, with ad libitum access to both water and salt. Horses were adapted to diet and housing procedures for 1 week prior to sampling.

3.2.2. Experimental procedures

Individual meals were provided twice daily (at 800 and 1500). Diets consisted of alfalfa hay cubes [mean \pm SD; 0.48 ± 0.01 Mcal/kg DE, $17.0 \pm 0.2\%$ CP, $35.2 \pm 0.8\%$ ADF, $44.9 \pm 1.3\%$ NDF, $2.3 \pm 0.1\%$ crude fat, and $9.5 \pm 0.2\%$ ash] and a ration balancer (mean \pm SD; 0.49 ± 0.01 Mcal/kg DE, $14.9 \pm 0.2\%$ CP, $22.5 \pm 0.8\%$ ADF, $43.2 \pm 0.4\%$ NDF, $3.6 \pm 0.1\%$ crude fat, and $12.9 \pm 0.1\%$ ash), provided at a rate of 1.75% and 0.2% of BW/d, respectively.

On the morning of the muscle biopsy, horses were weighed on an electronic scale (TI-500, Transcell Technology Inc., Buffalo Grove, IL) and then fed 3 g/kg BW of a high protein pellet (mean \pm SD; 0.57 ± 0.01 Mcal/kg DE, $36.3 \pm 0.7\%$ CP, $6.8 \pm 0.3\%$ ADF, 13.8

$\pm 0.8\%$ NDF, $3.9 \pm 0.1\%$ crude fat, and $17.2 \pm 0.3\%$ ash) at $t = 0$ min. This high protein pellet is a commercially available feed that was used in a previous study examining mTOR signaling in the horse where the amino acid concentrations of the feed were provided (2). At $t = 60$ min, horses were lightly sedated with xylazine hydrochloride (0.3mg/kg , intravenous [100 mg/mL]), the biopsy site was aseptically scrubbed and anesthetized with a local anesthetic (12mL of 2% lidocaine). Muscle biopsies ($\sim 500 \text{ mg}$) were collected from a single incision in the middle gluteal muscle at depths of 6, 8 and 10 cm below the surface of the skin, using the percutaneous needle biopsy technique (411). Samples were immediately processed for Western blot analysis ($\sim 100 \text{ mg}$) and the remaining muscle was frozen in liquid nitrogen, and stored at -80°C until analysis for amino acid concentration determination and myofibrillar protein preparation.

3.2.3. *Sample analysis.*

3.2.3.1. Amino acids: Muscle free amino acid concentrations were measured using reverse-phase HPLC ($3.9 \times 300 \text{ mm}$ PICO-TAG reverse phase column; Waters, Milford, MA) of phenylisothiocyanate derivatives as previously described (2).

3.2.3.2. Muscle homogenate preparation for Western blot analysis: Muscle homogenates were prepared as previously described (2). Briefly, freshly collected muscle ($\sim 100 \text{ mg}$) was weighed and homogenized over ice in $7 \mu\text{L/mg}$ tissue wet weight of a buffer solution (20 mM HEPES, 2 mM EGTA, 50 mM sodium fluoride, 100 mM potassium chloride, 0.2 mM EDTA and 50 mM β -glycerophosphate; pH 7.4) that contained $20 \mu\text{L/mL}$ of a protease inhibitor (Sigma P8340; Sigma Aldrich, Saint Louis, MO). Homogenized samples were centrifuged at $10,000 \times g$ for 10 min at 4°C and the supernatant was

aliquoted then stored at -80 °C until further analysis. After protein concentrations were determined using a Bradford assay kit (Thermo Scientific, Rockford, IL) modified for a 96 well plate, 50 µL of supernatant was added to 25 µL of a 3X Laemmli buffer (125 mM Trizma® hydrochloride pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 100 mM dithiothreitol, 0.01% (w/v) bromophenol blue). Then, varying amounts of 1X Laemmli buffer were added to each sample/Laemmli buffer mixture to reach a final protein concentration of 2 µg/µL. The sample/Laemmli buffer mixture was boiled for 5 min, and immediately placed on ice prior to gel electrophoresis.

3.2.3.3. Western blot analysis of muscle: The abundance and phosphorylation of 4E-BP1, Akt, S6K1, and rpS6 in the gluteal muscle were determined using Western blot analysis similar to those previously described (2). Proteins in the muscle samples processed and stored in Laemmli buffer were separated in polyacrylamide gels using electrophoresis and then transferred to 0.45 µm PVDF membranes (BioRad, Hercules, CA). Samples were standardized by the amount of protein loaded per well for electrophoresis (20 µg protein was loaded for Akt, rpS6, and 4E-BP1; 30 µg protein was loaded for S6K1). The membranes were blocked in a 5% fat-free milk solution and were then incubated with the appropriate primary antibodies for either 16 h at 4 °C (phosphorylated and total forms of Akt, S6K1, and rpS6, and phosphorylated form of 4E-BP1) or 1 h at room temperature (total form of 4E-BP1).

Individual rabbit polyclonal antibodies (Cell Signaling Technology®, Inc., Boston, MA) were used that recognized total, Ser⁴⁷³, and Thr³⁰⁸ Akt (1:2000 dilution for each); total and Thr³⁸⁹ S6K1 (1:1000 and 1:500 dilutions, respectively); and Ser^{235/236}, and Ser^{240/244} rpS6 (1:2000 dilutions for each). Rabbit monoclonal antibodies (Cell Signaling

Technology®, Inc., Boston, MA) specific to total and Thr^{37/46} 4E-BP1 (1:1000 dilution); and total rpS6 (1:10,000 dilution) were also used. Following washing, membranes were incubated with a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution) (BioRad, Hercules, CA) for 1 h at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). Band densities were quantified as the mean*pixels in a photo editing computer software program (Adobe Photoshop Elements™ Version 8.0; Alpha Innotech, San Leandro, CA).

Gels were run in duplicate, and the average band densities were used to calculate the ratio of phosphorylated to total abundance (Section 3.4.4). In order, to probe for the total versus phosphorylated forms using different primary antibodies, the PVDF membranes were first blotted for the phosphorylated protein abundance and were then stripped for 10 minutes (62.5 mM Trizma® hydrochloride, 2% (w/v) SDS, 0.1 M β-mercaptoethanol, boiling), re-blocked with 5% fat-free milk solution and re-probed for the total protein abundance. Membrane stripping and re-probing served to minimize the inter-assay error that would have resulted if the total and phosphorylated protein abundances had been quantified from different membranes.

Although total Akt and Akt-Ser⁴⁷³, and total and Ser^{235/236} and Ser^{240/244} rpS6 have been previously evaluated in equine skeletal muscle using the rabbit antibodies (2), to the best of the authors' knowledge, the other antibodies used in this study had not been previously validated for horse samples. Therefore, positive control samples (Cell

Signaling Technology®, Inc., Boston, MA) with known reactivity to the antibodies, were loaded and run with all gels (Appendix 2). In addition, prior to performing the western blot analysis for the samples in this study, we confirmed that all antibodies were cross-species specific to these proteins in horses through the use of blocking peptides (Cell Signaling Technology®, Inc., Boston, MA). Primary antibody was mixed with blocking peptide in decreasing ratios of 1:4, 1:1, 1:1/4, and 1:1/16 (antibody volume to blocking peptide volume) to determine the competition of blocking peptide to equine protein for the given antibody. In all cases, we were able to verify that the primary antibodies were reacting with the equine forms of the proteins of interest, because there was a reduced chemiluminescent signal for the protein of interest as increased amounts of the blocking peptide were added to the reaction mixture.

3.2.3.4. Myofibrillar protein preparation: Flash frozen muscle was removed from the -80°C freezer, and allowed to thaw on dry ice. Tissue was then divided into (~50 mg) portions and homogenized with a manual dounce homogenizer over dry ice in 9 µL/mg tissue wet weight of a buffer solution (250mM Sucrose, 25mM NaCl, 20mM Tris; pH 7.4). Homogenized samples were centrifuged at 20,000 x g for 30 min at 4 °C and the supernatant was removed. The pellet was then resuspended in homogenizing buffer (250 µL). After protein concentrations were determined using a Bradford assay kit (Thermo Scientific, Rockford, IL) modified for a 96 well plate, 40 µL of sample was added to 20 µL of a 3X Laemmli buffer (0.15 M Tris, pH 6.8, 6% (w/v) SDS, 75 mM dithiothreitol, 0.06% (w/v) bromophenol blue, 40% (w/v) glycerol). Then, varying amounts of 1X Laemmli buffer were added to each sample/Laemmli buffer mixture to reach a final

protein concentration of 0.2 $\mu\text{g}/\mu\text{L}$. The sample/Laemmli buffer mixture was boiled for 2 min, and immediately placed on ice prior to gel electrophoresis.

3.2.3.5. Separation of MHC Isoforms via electrophoresis: The percentage of MHC isoform in the gluteal muscle was determined using electrophoresis followed by silver staining similar to the methods previously described (490). In short, myofibrillar proteins in the muscle samples processed and stored in Laemmli buffer were separated in polyacrylamide gels using electrophoresis. Samples were standardized by the amount of protein loaded per well for electrophoresis (2 μg of myofibrillar proteins was loaded), and mouse gastrocnemius muscle served as a control on each gel. The electrophoresis ran at 4°C for 40 hours with constant voltage of 70 V using a BioRad Power PAC3000 power supply (Hercules, CA). Membranes were stained using a silver staining kit (Silver Stain Plus Kit; BioRad, Hercules, CA) following manufacturer's directions (Appendix 3). Band densities were quantified as the mean*pixels using a photo editing computer software program (Adobe Photoshop Elements™ Version 8.0; Alpha Innotech, San Leandro, CA).

3.2.4. *Calculations and statistics.*

Individual band densities were corrected for the band density of the positive control (Akt Control Cell Extracts treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA) run on each gel. The averages of the corrected band densities from duplicate gels were averaged, and then expressed as a ratio of the phosphorylated to total forms for each protein. Protein abundance of the mTOR signaling factors was expressed

in arbitrary units, with the ratio of the phosphorylated to total forms of each protein at the 6 cm depth set to 1. MHC isoforms are expressed as a percentage of total MHC.

All data were analyzed using the MIXED procedure of SAS Version 9.2 (SAS Institute Inc., Cary, NC), with statistical significance at $P < 0.05$. When the fixed effects were significant, pre-planned comparisons of least squares means were made using the pdiff test. The dependent variables were analyzed using repeated measures analysis, with depth and block as the fixed effects and horse nested in block as the subject. The variance-covariance matrix was chosen for each analysis based on the lowest value for Schwarz's Bayesian Criterion. Data are presented as means \pm pooled standard error.

3.3. RESULTS

There was a significant effect ($P < 0.05$; Table 3.1) of sampling depth on the muscle concentrations of free alanine, aspartate, glutamate, isoleucine, leucine, methionine, proline and valine.

The phosphorylation of Akt at Ser⁴⁷³ ($P = 0.06$; Figure 3.1), S6K1 at Thr³⁸⁹ ($P = 0.66$; Figure 3.1), rpS6 at Ser^{235/236 & 240/244} ($P = 0.21$; Figure 3.1), and 4EBP1 at Thr^{37/46} ($P = 0.62$; Figure 3.1) was not affected by sampling depth within the gluteal muscle of mature mares 60 min following the consumption of a high protein pellet.

There was a significant effect of sampling depth on the percentage of MHC IIA ($P = 0.03$; Figure 3.2) and IIX ($P = 0.02$; Figure 3.2) in the gluteal muscle of mature mares; however there was no effect ($P = 0.42$; Figure 3.2) of sampling depth on the percentage of MHC I. The percentage of MHC IIA and IIX increased by 27% and decreased by 15%, respectively, from 6 to 10 cm.

3.4. DISCUSSION

This is the first study to examine the effects of gluteal muscle biopsy sampling depth on the activation of mTOR signaling in response to feeding. The results showed no effect of sampling depth on the activation of mTOR signaling, despite changes in the proportion of MHC isoforms. Thus, it appears that if percutaneous muscle biopsy sampling occurs within a 6 to 10 cm range then mTOR signaling in response to feeding can be compared between mature horses.

The collection of gluteal muscle biopsies at 6, 8, and 10 cm below the surface of the skin showed that the proportion of MHC IIA, IIX and I isoforms increased, decreased and remained unchanged, respectively. The changes seen in the proportion of MHC IIA and IIX are consistent with previous reports (413), where the percentage of MHC IIA and IIX isoforms increase and decrease, respectively, from 2 cm to 8 cm in the gluteus muscle (413). However, in the present study we did not see a change in the percentage of MHC I isoforms which has been previously demonstrated to increase with depth (413), and may be due to the sampling protocol in the present study: we collected biopsies at 6, 8 and 10 cm below the surface of the skin rather than a more shallow collection beginning at 2 cm (413). We also did not account for the variation in subcutaneous fat which we have demonstrated to be up to 2 cm in some mature horses (Section 8.1). Additionally, it has been previously demonstrated that extensive variation in fiber type exists between breeds (414), and the present results in Thoroughbreds are consistent with what has been previously measurements of 10-15% of Type I fibers in this breed (414).

The activation of the mTOR signaling in response to feeding was not affected by sampling depth. Although this is the first study to examine biopsy sampling depth in

horses, it is not the first study to look at the association between of MHC isoform content of muscle with mTOR signaling response to anabolic stimuli. In rodents, phosphorylation of mTOR (491) and S6K1 (110) was shown to vary across different muscles in response to contractile activity and the authors suggested that different fiber types (Type II vs. Type I) maybe exhibiting a greater mTOR signaling response. However, the variation in the activation of mTOR signaling factors due to MHC isoforms remains unclear (104), and fiber type is not associated with different rates of protein synthesis in humans (492). Horses, like humans, possess a single MHC I and 2 MHC II isoforms (409), and do not possess a MHC IIB isoform which is present in rodent skeletal muscle (409). It may be that the increased responsiveness to anabolic stimuli is a property that is specific to Type IIB fibers seen in rodents; however, this requires additional research. Additionally, the minimal change in MHC isoforms with depth may explain the lack of an effect of depth on mTOR signaling in response to feeding.

Although sampling depth did not affect the activation of mTOR signaling, the gluteal muscle concentrations of branched chain amino acids (BCAA), alanine, aspartate, glutamate, methionine, and proline were greatest at the 8 cm sampling depth. With elevated concentrations of BCAA, we expected to see greater mTOR signaling (48) at the 8 cm depth, but this was not the case, and these depth-related differences remain unclear. However, from a methodological perspective, these results indicate that when comparing gluteal muscle amino acids of mature horses, researchers need to standardize their biopsy collection procedures by depth.

Conducting muscle biopsies at a depth range of 6 to 10 cm below the surface of the skin did not affect the activation of mTOR signaling in response to consumption of a

high protein pelleted meal, despite differences in the distribution of the MHC isoforms present. Therefore, the activation of mTOR signaling can be compared across this range of depths in mature horses.

3.5. TABLES

Table 3.1: Muscle amino acid concentrations of the gluteus muscle at 6, 8 and 10 cm below the skin in mature mares.

| Amino Acid | 6 cm | 8 cm | 10 cm | Pooled SEM | p-Value |
|----------------------|------------------|------------------|------------------|-------------------|----------------|
| Alanine ¹ | 697 ^a | 944 ^b | 744 ^a | 102 | 0.05 |
| Asparagine | 60 | 60 | 59 | 6 | 0.97 |
| Aspartate | 70 ^a | 94 ^b | 68 ^a | 9 | 0.02 |
| Citrulline | 41 | 50 | 46 | 6 | 0.48 |
| Glutamate | 305 ^a | 442 ^b | 390 ^b | 49 | 0.03 |
| Glutamine | 246 | 303 | 257 | 35 | 0.46 |
| Glycine | 664 | 471 | 664 | 133 | 0.50 |
| Histidine | 58 | 68 | 53 | 8 | 0.24 |
| Isoleucine | 55 ^a | 69 ^b | 56 ^a | 4 | 0.004 |
| Leucine | 105 ^a | 135 ^b | 111 ^a | 10 | 0.007 |
| Lysine | 296 | 189 | 267 | 128 | 0.67 |
| Methionine | 36 ^a | 50 ^b | 39 ^a | 4 | 0.004 |
| Ornithine | 15 | 26 | 17 | 9 | 0.59 |
| Phenylalanine | 48 | 64 | 104 | 29 | 0.39 |
| Proline | 94 ^a | 122 ^b | 100 ^a | 9 | 0.004 |
| Serine | 144 | 150 | 137 | 12 | 0.45 |
| Threonine | 170 | 207 | 173 | 34 | 0.69 |
| Tryptophan | 18 | 20 | 18 | 2 | 0.56 |
| Tyrosine | 61 | 104 | 62 | 24 | 0.36 |

Table 3.1 continued: Muscle amino acid concentrations of the gluteus muscle at 6, 8 and 10 cm below the skin in mature mares.

| | | | | | |
|--------|------------------|------------------|------------------|---|-------|
| Valine | 111 ^a | 148 ^b | 119 ^a | 9 | 0.005 |
|--------|------------------|------------------|------------------|---|-------|

¹ Plasma amino acids concentrations are reported as least square means in $\mu\text{mol/g}$ of wet muscle.

^{abc} Differing letters indicate that values are significantly ($P < 0.05$) different from each other.

3.6. FIGURES

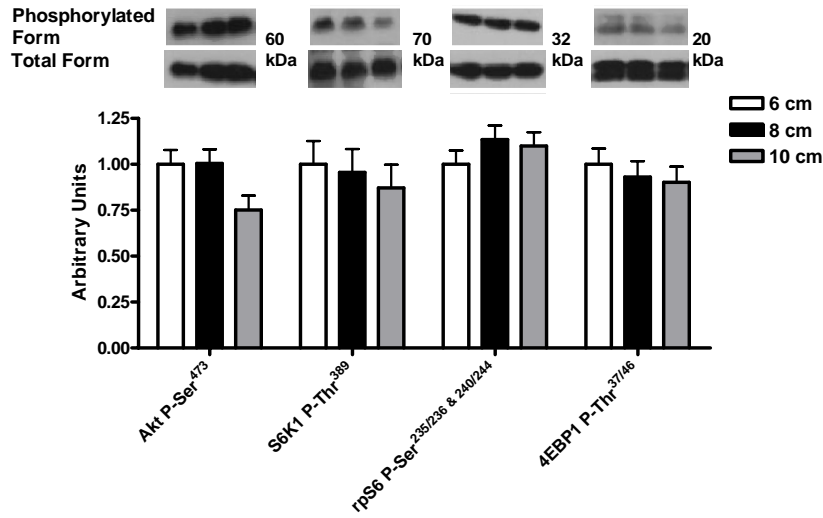


Figure 3.1. Gluteal muscle phosphorylation of Akt at Ser⁴⁷³, S6K1 at Thr³⁸⁹, rpS6 at Ser^{235/236 & 240/244}, and 4EBP1 at Thr^{37/46} at 6, 8, and 10 cm below the surface of the skin within the gluteus medius muscle of mature mares 60 min after consuming 3g/kg of a high protein pelleted feed. The phosphorylated forms of the translation initiation factors was corrected by the respective total form abundance, with the value for mature horses set at 1.0 AU. Values are least square means ± pooled SE, n=6 per depth. Representative images of the immunoblots are shown above.

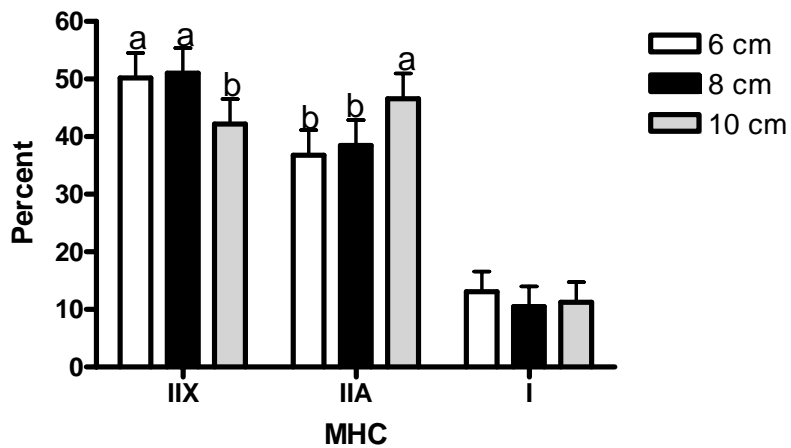


Figure 3.2. Percentage of MHC isoforms at 6, 8, and 10 cm below the surface of the skin within the gluteus medius muscle of mature mares 60 min after consuming 3g/kg of a high protein pelleted feed. Values are least square means \pm pooled SE, n=6 per depth.

^{ab}Different letters indicate a significant depth effect ($P < 0.05$).

Chapter IV

Repeated muscle biopsies over a 5 day period increases mTOR signaling in equine skeletal muscle

4.1. INTRODUCTION

Repeated percutaneous needle muscle biopsies, over the span of hours or days, are a common practice in human and equine studies examining various factors affecting muscle metabolism and physiology. However, the literature examining the effects of repeated biopsies on muscle metabolism and physiology is limited. Inflammatory changes in the muscle are induced by aging (493-495), exercise (244, 245, 255), and muscle biopsy collection (248). In many studies, the effects of repeated biopsies on muscle inflammation has been confounded by the inclusion of a simultaneous exercise treatment (244, 245, 255, 332). Multiple biopsies have been reported to increase both systemic and muscle inflammatory cytokines 30 (245) and 126 minutes (248) following an initial biopsy. However, repeated biopsies over a 24 hour period in equine skeletal muscle did not affect systemic or muscle inflammatory cytokines. This disconnect between human and equine skeletal muscle, in addition to the absence of non-exercise related studies, necessitated further investigation.

Protein synthesis is regulated through the mammalian target of rapamycin (mTOR) signaling pathway. There are numerous reviews (21, 488, 496) examining the regulation of this pathway by anabolic stimuli. Briefly, insulin stimulates the mTOR signaling pathway through the phosphorylation of Akt (18, 19, 21), which inhibits the activation of several mTOR inhibitors (18, 19, 21). Amino acids also stimulate the

mTOR signaling pathway through the activation of a series of signaling proteins which subsequently activate mTOR (45, 46, 497). mTOR activation results in the phosphorylation of two downstream signaling proteins, ribosomal S6 Kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein-1 (4EBP1) (146, 496).

Phosphorylation of S6K1 activates ribosomal protein S6 (rpS6) (498), a component of the 40S ribosomal subunit (498). Phosphorylation of rpS6 and 4EBP1 result in the activation of the translational equipment, allowing for protein synthesis to occur (146, 496).

Numerous studies have shown that the activation of these key proteins in the mTOR signaling pathway in rodents (499-501), humans (99, 502, 503), and pigs (504-506) correlate with an increase in the fractional muscle protein synthesis rates.

The use of multiple biopsies to examine protein synthesis rates and the mTOR signaling pathway typically coincide with an exercise treatment, making it impossible to separate the effects of exercise from repeated biopsy. These studies typically occurred during approximately a 24 hour period where the initial biopsy was collected and then subsequent biopsies are obtained following an exercise regimen or during isotope infusion techniques over a several hour period. Protein synthesis rates do not change with multiple muscle biopsies collected between 60, 210 and 240 minutes of a 4 hour period (507) and during hourly biopsies over a 6 hour period (508). However, no study has examined the effects of repeated muscle biopsies on muscle protein synthesis rates after 6 hour, and there has been no study to examine the effects of repeated biopsies on the activation of mTOR signaling factors. It is crucial to study protein synthesis and mTOR signaling during a longer period following inflammatory stimuli because in humans inflammation has shown to remain elevated up to 72 hours following exercise

(509). Inflammation may impact the responsiveness of the mTOR signaling pathway and subsequently protein synthesis rates in response to anabolic stimuli. In aged rodents with systemic inflammation, the administration of a NSAID was able to increase muscle protein synthesis (335). Therefore, it may be possible to mitigate changes in inflammation and mTOR signaling due to repeated biopsies using NSAID administration. The objective of this study was to determine the effects of multiple biopsies over the course of 5 consecutive days on muscle inflammatory cytokine expression and the activation of mTOR signaling in response to a high protein meal.

4.2. MATERIALS AND METHODS

4.2.1. Animals and housing.

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (2009-0442). Twelve Thoroughbred mares were obtained from the University of Kentucky Animal and Food Sciences' Maine Chance Farm. All mares were classified as mature (average age 13.4 ± 3.4 ; range 10-19 y old), of a moderate body condition (body condition score 5-7, scale 1-9 (489)), and clinically healthy. Mares were on a regular vaccination, anthelmintic, and farrier schedule prior to inclusion on this study. For the duration of the study mares were housed in 3×15 m partially covered dry lot pens with crushed limestone footing. Ad libitum access to both water and salt was provided throughout the study. Horses were adapted to diet and housing procedures for 1 week prior to the initiation of study procedures.

4.4.2. Feeding procedures.

Individual meals were provided twice daily (at 800 and 1500). Diets consisted of alfalfa hay cubes and a ration balancer; and were designed to meet the NRC (336) requirements for mature horses with an approximate body weight (BW) of 600 kg and average maintenance needs. The alfalfa hay cubes [mean \pm SD; 0.48 \pm 0.01Mcal/kg DE, 17.0 \pm 0.2% crude protein (CP), 35.2 \pm 0.8% acid detergent fiber (ADF), 44.9 \pm 1.3% neutral detergent fiber (NDF), 2.3 \pm 0.1% crude fat, and 9.5 \pm 0.2% ash] and ration balancer (mean \pm SD; 0.49 \pm 0.01Mcal/kg DE, 14.9 \pm 0.2% CP, 22.5 \pm 0.8% ADF, 43.2 \pm 0.4% NDF, 3.6 \pm 0.1% crude fat, and 12.9 \pm 0.1% ash) were provided at a rate of 1.75% and 0.2% of BW/d, respectively, for the duration of the study. Samples of the alfalfa hay cubes, ration balancer, and high protein pellet, which was offered on sampling days, were collected and sent to Dairy One Forage Laboratory (Ithaca, NY) for nutrient analysis.

4.2.3. Experimental design and procedures.

The current study was executed as a 2-way factorial design with day (1-5) and NSAID administration (+NSAID or -NSAID) as the fixed effects. The twelve mares were divided into two blocks of 6 horses, with 3 horses on each treatment in each block to ease sampling procedures.

On the morning of day 1, following the adaptation period, horses were weighed on an electronic scale (TI-500, Transcell Technology Inc., Buffalo Grove, IL) and fed a high protein pellet (mean \pm SD; 0.57 \pm 0.01Mcal/kg DE, 36.3 \pm 0.7% CP, 6.8 \pm 0.3% ADF, 13.8 \pm 0.8% NDF, 3.9 \pm 0.1% crude fat, and 17.2 \pm 0.3% ash) at 3g/kg. This high protein pellet is a commercially available feed that was used in a previous study examining

mTOR signaling in the horse where the amino acid concentrations of the feed were provided (2). Pre-feeding (t = -15 min) and post-feeding (t = 60 minute after feed was offered) jugular vein blood samples were collected via venapuncture into evacuated glass tubes (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) containing sodium heparin. Blood samples were immediately centrifuged at 1,500 x g for 10 minutes at 4°C. Aliquots of plasma samples were frozen at -20°C until the time of analysis. At t = 60 min, horses were placed in equine stocks (Priefert® Rodeo & Ranch Equipment, Mount Pleasant TX), and were lightly sedated with xylazine hydrochloride (0.3mg/kg, intravenous [100mg/mL]). Muscle biopsies (~500 mg) were collected from both the right and left gluteal muscles, at a standardized site from a depth of 8cm (Chapter III) in the middle gluteal muscle by use of the percutaneous needle biopsy technique (411). Biopsies on day 2 – 5 were collected using separate incisions at least 1 cm apart from all previous biopsy sites. Samples were immediately processed for Western blot analysis (~100 mg) and quantitative real time (qRT)-PCR (~80 mg), as described below, and the remaining muscle was frozen in liquid nitrogen, and stored at -80°C until analysis. Phenylbutazone (NSAID) was administered orally after the initial biopsy at 1 g every 12 hours for the next consecutive 5 day in the +NSAID group, and was not administered in the -NSAID group. This procedure was repeated every 24 hr for the next 4 d. At the end of the sampling period on day 5, all horses received phenylbutazone (2g/day) for the next 3 days to alleviate any discomfort or inflammation that may have been associated with the procedures. All mares were then returned to the Department of Animal and Food Sciences Maine Chance Farm Research herd at the end of the study procedures.

4.2.4. *Sample analysis.*

4.2.4.1. Plasma glucose and insulin: Plasma glucose concentrations were determined enzymatically using a YSI 2300 STAT Plus™ Glucose and Lactate Analyzer (YSI Inc., Life Sciences, Yellow Springs OH) (Appendix 4.1). A Coat-A-Coat RIA® kit (Siemens Healthcare Diagnostics Inc., Deerfield IL) was used to determine plasma insulin concentrations (Appendix 4.2).

4.2.4.2. Amino acids: Both plasma free amino acid concentrations (Appendix 1.1 – 1.2) and total feed amino acid (Appendix 1.4 – 1.5) content were measured using reverse-phase HPLC (3.9 × 300 mm PICO-TAG reverse phase column; Waters, Milford MA) of phenylisothiocyanate derivatives as previously described (2).

4.2.4.3. Western blot analysis of muscle samples: The abundance of the total and phosphorylated forms of Akt, S6K1, rpS6, and 4E-BP1 in the gluteal muscle homogenates were determined using electrophoresis followed by Western blotting techniques (Chapter III)

4.2.4.4. RNA isolation and qRT-PCR. Freshly collected muscle (~ 80 mg) was weighed, submerged in *RNAlater* (Qiagen Inc., Valencia, CA), and stored at -20°C until further analysis. At time of analysis, muscle was homogenized into the RNA-Stat60 solution (Tel- Test, Friendswood, TX) using the bead beating technique (510). Total RNA was then isolated and quantified using phenol-chloroform extraction and quantified using spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). Finally, reverse transcription reactions were performed as previously described (244, 484), using 0.5 µg of each RNA sample and reverse transcription master mix (Promega, Madison, WI).

These reactions were incubated at 42°C for 15 minutes and 95°C for 5 minutes. cDNA samples were stored at -20°C until analyzed by qRT-PCR.

The mRNA expression of pro-inflammatory cytokines, IFN- γ , IL-1 β , IL-6 and TNF- α , and the anti-inflammatory cytokine IL-10 were measured in cDNA samples using equine specific intron-spanning primer/probe sets (244). A gene expression master mix solution containing: 6.25 μ L nuclease-free water (Qiagen), 1.25 μ L 20 \times assay mix for primer/probe set of interest (Applied Biosystems, Foster City, CA), and 12.5 μ L Taqman™ (Applied Biosystems, Foster City, CA) reacted in 384 well plates with 5 μ L of cDNA. All reactions were run in duplicate under the following PCR conditions: 95°C for 10 minute followed by 45 cycles of 95°C for 15s and 60°C for 60s in an Applied Biosystems 7500 sequence detection system (Appendix 5). Differences in RNA isolation and cDNA construction between samples were corrected with the use of an internal control, β -glucorindase, for each sample (244, 484).

4.2.5. *Calculations and statistical analysis.*

Protein abundance of the mTOR signaling factors is expressed in arbitrary units, which is the ratio of the phosphorylated to total forms for each protein (Chapter III). The $\Delta\Delta$ CT method (484) was used to calculate relative changes in mRNA expression from the initial biopsy. The calibrator for individual cytokines was set as the mean Δ CT averaged for all horses at day 1. These results are expressed as the relative quantity (RQ), which is calculated as $2^{-\Delta\Delta$ CT}.

All data were analyzed using the mixed procedure of SAS Version 9.2 (SAS Institute Inc., Cary, NC), with statistical significance and trends considered if $P < 0.05$ and $0.05 < P < 0.10$, respectively. When the fixed effects were significant, pre-planned comparisons of least squares means were made using the pdiff test.

The dependent variables plasma glucose, insulin, and amino acid concentrations were analyzed using repeated measures analysis, with day, NSAID treatment, day*NSAID treatment interaction, and block as the fixed effects and horse nested within treatment*block as the random subject. Baseline concentration was also included in the model as a covariate because it significantly ($P < 0.05$) influenced the post-meal sample. Biopsy side (left or right) was initially included in the model for each of the mTOR signaling proteins, but was not significant ($P > 0.05$) and was removed from the model. Each of the mTOR signaling proteins and the expression of individual inflammatory cytokines were analyzed using repeated measures analysis, with day, NSAID treatment, day*NSAID treatment interaction, and block as the fixed effects with horse nested within treatment*block set as the random subject. For all repeated measures analysis, the variance-covariance matrix was chosen for each analysis based on the lowest value for Schwarz's Bayesian Criterion. Data are presented as means \pm pooled standard error unless otherwise noted.

4.3. RESULTS

4.3.1. Plasma glucose, insulin, and amino acid concentrations

Plasma glucose concentrations were significantly affected by the interaction of NSAID treatment*day ($P = 0.05$; Table 4.1), where at 60 minutes following the

consumption of a high protein pelleted meal the plasma glucose concentrations decreased over the course of the 5 day sampling period in the +NSAID treatment group ($P < 0.05$), and did not change in the -NSAID group ($P > 0.10$). Plasma insulin concentrations were not affected ($P > 0.10$; Table 4.1) by day, NSAID treatment, or day*NSAID treatment interaction.

There was a significant effect ($P < 0.05$; Table 4.1) of treatment on plasma concentrations of isoleucine, and proline. There was a statistical trend for an effect of treatment on the plasma concentrations of leucine ($P = 0.10$), and valine ($P = 0.10$; Table 4.1). Day of sampling significantly affected ($P < 0.05$; Table 4.1) plasma concentrations of alanine, and threonine. There was a significant ($P = 0.04$; Table 4.1) day*NSAID treatment interaction on the plasma concentrations of asparagine.

4.3.2. Muscle mTOR signaling factors

The phosphorylation of Akt at Ser⁴⁷³ decreased significantly with day of biopsy ($P < 0.0001$; Figure 4.1), regardless of treatment; however, the +NSAID group decreased from day 1 to 2, and the -NSAID group decreased from day 1 to 3. Akt P-Ser⁴⁷³ was not affected by treatment or treatment*day interaction ($P > 0.10$). There was a significant treatment*day interaction on the phosphorylation of S6K1 at Thr³⁸⁹ ($P = 0.003$; Figure 4.2), where the +NSAID group had a transient increase from day1 during days 2 and 3 and returned to baseline by day 4, and the -NSAID group had a greater Thr³⁸⁹ S6K1 on day 5 than on day 1. The phosphorylation of S6K1 at Thr³⁸⁹ was significantly affected by day of sampling ($P = 0.02$), but not treatment ($P > 0.10$). There was a significant interaction of treatment*day on the phosphorylation of rpS6 at Ser^{235/236 & 240/244} ($P <$

0.001; Figure 4.3) with a transient decrease from day 1 in the +NSAID group on days 4 and 5, and an increase from day 1 with collection of each subsequent biopsy in the – NSAID group. The phosphorylation of rpS6 at Ser^{235/236 & 240/244} was significantly affected by both day of sampling (P = 0.005) and treatment (P = 0.05). There was a time*treatment interaction on 4EBP1 at Thr^{37/46} (P = 0.02; Figure 4.4) with no change due to day in the +NSAID group and a gradual increase at day 5 from day 1 in the – NSAID group. There was a trend (P = 0.06) for an effect of sampling day on the phosphorylation of 4EBP1 at Thr^{37/46} by sampling day, and no effect of treatment (P > 0.10).

4.3.3. Muscle inflammatory cytokines

IL-1 β mRNA expression was significantly affected by sampling day (P = 0.01) and there was a trend (P = 0.05) for an effect of the treatment*day of sampling interaction (Table 4.2). IL-1 β mRNA expression did not change (P > 0.10) in the +NSAID group with day, but dramatically increased (P = 0.001) in the –NSAID group on day 5. IFN- γ mRNA expression was significantly affected (P = 0.003; Table 4.2) by day, with no change (P > 0.10) in the +NSAID group, and a decrease (P = 0.006) with day in the – NSAID group. However, there was no effect of the treatment*day of sampling interaction (P > 0.10) on the expression of IFN- γ mRNA, indicating the main effect of sampling day was primarily due to changes in the –NSAID group. There was a trend for an effect of day on IL-10 mRNA with a transient increase in the +NSAID group; however, there was no effect of treatment, or the interaction of treatment*day of

sampling ($P > 0.10$). There was no effect ($P > 0.05$) of day, treatment, or treatment*day of sampling interaction for IL-6 (Table 4.2) and TNF- α (Table 4.2).

4.4. DISCUSSION

To the best of our knowledge, this is the first study to examine the effects of multiple gluteal muscle biopsies over 5 consecutive days on local inflammation and on the activation of mTOR signaling in response to a high protein meal. The primary finding of this research was that repeated biopsies over 5 consecutive days may be obtained without eliciting changes in downstream mTOR signaling response to feeding due to increases in inflammation when NSAID are administered. However, when NSAID are not administered, 5 days of consecutive biopsies altered muscle IL-1 β and IFN- γ and elevated the activation of the downstream mTOR signaling proteins in response to feeding. Thus, the administration of NSAID appeared to reduce the local inflammation induced by the collection of repeated biopsies and prevented changes in the downstream mTOR signaling response to feeding.

Reports are varied on the effects of repeated biopsies on local inflammation due to the frequency and number of biopsies collected, the method of biopsy collection, and the site of biopsy. In the current study, IFN- γ and IL-1 β skeletal muscle mRNA expression decreased and increased, respectively, from the initial to the fifth biopsy in the -NSAID group. However, a previous study in horses found no change in the mRNA expression of IFN- γ or IL-1 β with repeated biopsies over a 24 hour period (244), consistent with our results. In human, there was an initial elevation in the protein expression of IL-1 β 30 minute following the first biopsy, but this increase did not persist

over the next 7 days (245). The biopsy sites in the present study were more closely spaced (1 cm) than the previous study (2 cm) (245); therefore, our finding of elevated IL-1 β by day 5 may have been due to the closer spatial arrangement to the previous biopsy sites. Although IFN γ mRNA expression was elevated on days 1 and 2, it then decreased on day 3, remaining constant until day 5 in the -NSAID group. IFN- γ functions as a key regulator of other pro-inflammatory parameters, causing an up regulation of TNF- α (511) and NF- κ B (512), and inhibiting IL-1 β (513). Therefore, the increase in IL-1 β seen on day 5 in the -NSAID group may be regulated by the decrease in IFN- γ expression observed in the current study. The mechanisms surrounding the decrease in IFN- γ expression in the -NSAID group requires further elucidation.

In the current study, TNF- α mRNA expression was not altered with the collection of biopsies over 5 consecutive days in the -NSAID group, which is in agreement with previous work in horses over a 24 hour period (244) and humans over a 7 day period (245). IL-6 mRNA expression has been shown to increase during 7 days of repeated biopsies (245) and with acute repeated biopsies through separate incision sites (248) in humans, but this was not seen in the present study or in previous studies in horses (244). IL-6 production may be more responsive in human skeletal muscle than equine skeletal muscle; however, this requires further investigation. In the present study, the absence of an effect of repeated biopsies on the mRNA expression of IL-6 and TNF- α may have been due to the decline in IFN- γ mRNA expression by day 5 because IFN- γ plays a crucial role in the stimulation of the production of IL-6 and TNF- α from monocytes and macrophages (511, 513).

Although other studies have looked at the effectiveness of NSAID at reducing the inflammatory response caused by exercise, this is the first study to determine the effects of NSAID administration on inflammatory cytokines using repeated biopsies to elicit inflammation. Oral administration of a herbal supplement with NSAID properties in rodents reduced muscle IL-1 β , IL-6, and TNF- α mRNA expression following exercise (332), although in humans only TNF- α mRNA expression is reduced following post-exercise NSAID infusion (333). Intravenous NSAID infusion in horses appeared to reduce the systemic inflammatory response of lipopolysaccharide (LPS) infusion; however, TNF- α was the only inflammatory cytokine examined (514). Although the methods of NSAID administration, cause of inflammatory response, site of inflammation, and inflammatory cytokine examined were different between the three studies, the current study also showed that NSAID administration ameliorates the effects of repeated biopsies over 5 consecutive days on inflammatory cytokines in the gluteal muscle of mature horses through the absence of a change in mRNA expression of the interleukins (-6, -1 β , and -10), TNF- α , and IFN- γ in the skeletal muscle of the +NSAID group during 5 days of biopsies.

All animals studied were mature, sedentary and consuming the same meal for 5 consecutive days; therefore, we did not expect a change in the activation of downstream effectors or protein synthesis, as long as the repeated biopsies and/or NSAID administration were not influencing muscle metabolism. There were no differences in the downstream mTOR signaling factors in the +NSAID group showing that any changes in muscle inflammation or metabolism caused by the repeated biopsies were mitigated by the NSAID. However, in the absence of NSAID administration, the daily collection of

muscle biopsies for 5 days increased the activation of the downstream mTOR signaling proteins, S6K1, rpS6, and 4EBP1, by 22, 73, and 117%, respectively. The increase in mTOR signaling is suggestive of an increase in rates of muscle protein synthesis in the –NSAID group (500, 502, 505), although isotopic confirmation is necessary.

Following a bout of exercise there is an increase in muscle inflammatory cytokine gene expression (244, 255, 509) and the activation of mTOR signaling proteins (515) for up to 72 hours. It is thought that the increase in inflammatory cytokines following exercise is necessary for muscle repair post-exercise (334) because inflammatory cytokines are regulators of satellite cell proliferation (312, 313). A similar phenomenon may have occurred during the current study, where there was an increase in the activation of 4EBP1, S6K1, and rpS6 and the pro-inflammatory cytokine, IL-1 β , in the –NSAID group after 5 days of biopsy collection. Additionally, when a NSAID was administered locally following eccentric exercise there was a reduction in satellite cell proliferation (316), but protein synthesis was unaltered (333). However, when NSAID are administered orally there is a reduction in post-exercise protein synthesis (516). Therefore, the increase in IL-1 β mRNA expression in the –NSAID group in the current study which coincided with an increase in all of the downstream mTOR signaling factors may be necessary for muscle to repair; however, further research is needed to confirm this. Overall the findings from the –NSAID group may indicate that future experiments using repeated biopsies over the course of several days to examine the activation of mTOR signaling proteins in response to anabolic stimuli may require a control group or NSAID treatment to account for changes in mTOR signaling not related to the experimental treatments.

Regardless of treatment, the activation of Akt over the course of 5 days of repeated biopsies was reduced. Akt is activated through insulin signaling (18, 19), and circulating insulin levels were variable in the present study regardless of treatment group, which may indicate that the phenylbutazone dose (1g every 12 hours) was not sufficient to elicit treatment differences. Hepatic activation of NF- κ B, an inhibitor of Akt phosphorylation (517), has been associated with systemic insulin resistance in mice (518). Although we did not examine NF- κ B in the present study, Akt activation may have been inhibited through NF- κ B due to insufficient inhibition by phenylbutazone (519), or through NF- κ B altered insulin sensitivity. Additionally, the reduction in Akt activation could indicate a reduction in insulin sensitivity associated with the repeated biopsies that could not be mediated by the administration of an NSAID.

Although there was a reduction in the activation of Akt with repeated biopsies, there was an increase in the activation of all of the downstream mTOR signaling factors in the -NSAID group. This may indicate that although Akt was inhibited and perhaps insulin signaling, other factors such as amino acids were sufficiently stimulating the activation of mTOR and subsequently S6K1, rpS6 and 4EBP1, which is consistent with the results of a previous study (2) performed by our lab.

The reduced activation of NF- κ B by phenylbutazone (519) may allow for an increase in the activation of downstream effectors. Administration of ibuprofen during low grade chronic inflammation in aging rodents ameliorated the reduction in muscle protein synthesis normally associated with the aging population (335), however, there was no effect on mTOR signaling factors. During an acute inflammatory response

following exercise, the administration of local (333) and oral (516) NSAID following exercise did not alter and reduced protein synthesis, respectively. In the present study, there was no difference in the activation of the downstream signaling factors between day 1 and day 5 in the +NSAID group and an elevation in the –NSAID group indicating that NSAID ameliorated the effect of repeated biopsies. This is consistent with the response following exercise. Overall, our results indicate that the administration of a NSAID during an acute inflammatory response due to the collection of multiple biopsies maintained protein synthesis at levels equal to rates prior to the collection of the initial biopsy.

Conducting muscle biopsy procedures every 24 hours for up to 5 days increases muscle inflammatory cytokine gene expression, specifically IL-1 β , and increases the activation of S6K1, rpS6, and 4EBP1 in response to a meal when NSAID are not administered. However, if repeated biopsies are collected over 5 days with oral administration of a NSAID, then the increase in inflammatory cytokines is ameliorated and the activation of the downstream effectors of the mTOR signaling factors are not different over the 5 day period. Additionally, repeated biopsy collection every 24 hours over the course of 5 days resulted in a reduction in the activation of Akt regardless of NSAID. Therefore, a control group may be a useful tool when studying animals using repeated percutaneous needle muscle biopsies.

4.5. TABLES

Table 4.1: Plasma metabolite concentrations 60 minutes after consuming a high protein pelleted meal during 5 days of consecutive NSAID administration in mature Thorougbred horses.

| Day | -NSAIDs treatment group | | | | | +NSAIDs treatment group | | | | | Pooled SEM | p-Value | | |
|----------------------|-------------------------|-------------------|--------------------|-------------------|-------------------|-------------------------|-------------------|-------------------|-------------------|------------------|------------|---------|-----------|-------------|
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | | Day | Treatment | Interaction |
| Glucose ¹ | 5.6 ^a | 5.9 ^b | 5.9 ^b | 5.7 ^{ab} | 5.7 ^{ab} | 6.1 ^a | 6.0 ^a | 5.5 ^{ab} | 5.8 ^{ab} | 5.4 ^b | 0.23 | 0.06 | 0.98 | 0.05 |
| Insulin ² | 36.2 | 49.7 | 38.7 | 52.3 | 47.5 | 43.7 | 43.3 | 34.2 | 36.5 | 40.0 | 11 | 0.72 | 0.67 | 0.44 |
| Ala ³ | 187 | 200 | 197 | 173 | 170 | 234 ^a | 210 ^{ab} | 197 ^b | 179 ^b | 192 ^b | 17 | 0.05 | 0.37 | 0.41 |
| Arg | 89 | 113 | 135 | 113 | 110 | 128 | 127 | 125 | 118 | 119 | 14 | 0.26 | 0.49 | 0.23 |
| Asn | 59 | 69 | 76 | 64 | 63 | 83 | 74 | 73 | 67 | 74 | 6.9 | 0.39 | 0.31 | 0.13 |
| Asp | 2.9 ^a | 4.2 ^{bc} | 3.4 ^{abc} | 4.5 ^c | 3.1 ^{ab} | 5.1 ^{**a} | 4.3 ^{ab} | 3.5 ^b | 3.6 ^b | 3.8 ^b | 0.5 | 0.32 | 0.26 | 0.04 |
| Cit | 85 | 89 | 95 | 78 | 78 | 73 | 80 | 84 | 84 | 82 | 8.6 | 0.13 | 0.69 | 0.10 |
| Glu | 12 | 15 | 12 | 12 | 11 | 14 | 13 | 12 | 12 | 13 | 1.3 | 0.55 | 0.77 | 0.25 |
| Gln | 826 | 883 | 999 | 835 | 836 | 931 | 928 | 935 | 942 | 933 | 72 | 0.24 | 0.52 | 0.24 |
| Gly | 467 | 443 | 520 | 433 | 431 | 462 | 460 | 476 | 474 | 484 | 40 | 0.32 | 0.79 | 0.36 |
| His | 56 | 62 | 71 | 62 | 58 | 68 | 65 | 68 | 66 | 66 | 6.7 | 0.28 | 0.56 | 0.46 |

Table 4.1 continued: Plasma metabolite concentrations 60 minutes after consuming a high protein pelleted meal during 5 days of consecutive NSAID administration in mature Thoroughbred horses.

| | | | | | | | | | | | | | | |
|-----|------------------|-------------------|------------------|-------------------|------------------|------------------|-------------------|------------------|------------------|------------------|-----|------|-------|------|
| Ile | 72 | 81 | 87 | 78 | 76 | 114* | 108* | 102 | 90 | 96 | 8.9 | 0.48 | 0.04 | 0.28 |
| Leu | 128 | 149 | 158 | 146 | 140 | 94 | 182 | 169 | 160 | 167 | 16 | 0.80 | 0.10 | 0.26 |
| Lys | 148 | 178 | 210 | 188 | 172 | 217 | 207 | 199 | 179 | 186 | 23 | 0.55 | 0.50 | 0.19 |
| Met | 34 | 33 | 38 | 32 | 31 | 44 | 40 | 38 | 33 | 36 | 3.5 | 0.13 | 0.27 | 0.36 |
| Orn | 42 | 45 | 52 | 48 | 43 | 60 | 56 | 55 | 52 | 54 | 4.5 | 0.65 | 0.09 | 0.19 |
| Phe | 56 | 59 | 68 | 61 | 59 | 74 | 68 | 67 | 61 | 66 | 5.6 | 0.52 | 0.28 | 0.17 |
| Pro | 105 | 117 | 130 | 107 | 112 | 166* | 154* | 159 | 134 | 148* | 11 | 0.14 | 0.007 | 0.44 |
| Ser | 215 | 225 | 248 | 211 | 210 | 261 | 236 | 220 | 214 | 218 | 22 | 0.22 | 0.77 | 0.15 |
| Tau | 25 ^a | 29 ^{ab} | 32 ^b | 28 ^{ab} | 25 ^a | 31 | 33 | 35 | 30 | 31 | 3.4 | 0.04 | 0.39 | 0.78 |
| Thr | 133 ^a | 124 ^{ab} | 135 ^a | 122 ^{ab} | 109 ^b | 147 ^a | 136 ^{ab} | 124 ^t | 122 ^b | 116 ^b | 12 | 0.05 | 0.77 | 0.49 |
| Trp | 17 | 17 | 18 | 30 | 16 | 9.7 | 11 | 11 | 9.5 | 10 | 7.3 | 0.34 | 0.33 | 0.23 |
| Tyr | 62 | 66 | 76 | 72 | 70 | 81 | 79 | 79 | 68 | 72 | 6.9 | 0.47 | 0.43 | 0.12 |
| Val | 220 | 240 | 263 | 227 | 229 | 289 | 278 | 274 | 259 | 266 | 19 | 0.40 | 0.10 | 0.43 |

^{a,b}Within a treatment, values with different superscript letters are significantly ($P < 0.05$) different.

Table 4.1 continued: Plasma metabolite concentrations 60 minutes after consuming a high protein pelleted meal during 5 days of consecutive NSAID administration in mature Thoroughbred horses.

*Within a day, values are significantly ($P < 0.05$) different from the –NSAID group.

¹Glucose values are reported as least square means in mmol/L.

²Insulin values are reported as least square means in $\mu\text{IU/mL}$

³Amino acids values are reported as least square means in $\mu\text{mol/L}$

Table 4.2: Relative quantities of gluteal muscle inflammatory cytokine mRNA of mature Thoroughbred mares during 5 days of consecutive NSAID administration.

| Cytokine | -NSAIDs treatment group | | | | | +NSAIDs treatment group | | | | | Pooled SEM |
|---------------|-------------------------|-------------------|--------------------|--------------------|--------------------|-------------------------|------|------|------|--------|------------|
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 day | |
| IFN- γ | 1.34 ^a | 2.08 ^a | 0.56 ^b | 0.47 ^b | 0.52 ^b | 0.95 | 1.17 | 0.58 | 0.73 | 0.57 | 0.28 |
| TNF- α | 1.29 | 1.59 | 1.04 | 1.13 | 7.88 | 1.39 | 1.07 | 0.84 | 1.73 | 1.03 | 0.86 |
| IL-6 | 1.20 | 1.26 | 1.53 | 0.87 | 4.84 | 1.04 | 2.37 | 1.13 | 1.43 | 0.61 | 0.74 |
| IL-1 β | 0.43 ^a | 3.23 ^a | 12.30 ^a | 12.47 ^a | 82.50 ^b | 1.43 | 3.56 | 3.19 | 2.97 | 10.34* | 15.3 |
| IL-10 | 0.96 | 0.68 | 0.64 | 0.77 | 1.62 | 1.25 | 0.79 | 0.53 | 1.17 | 0.55 | 0.31 |

^{a,b} Within a treatment, values with different superscript letters are different ($P < 0.05$).

* Within a day, values differ ($P < 0.05$) from the -NSAID group.

4.6. FIGURES

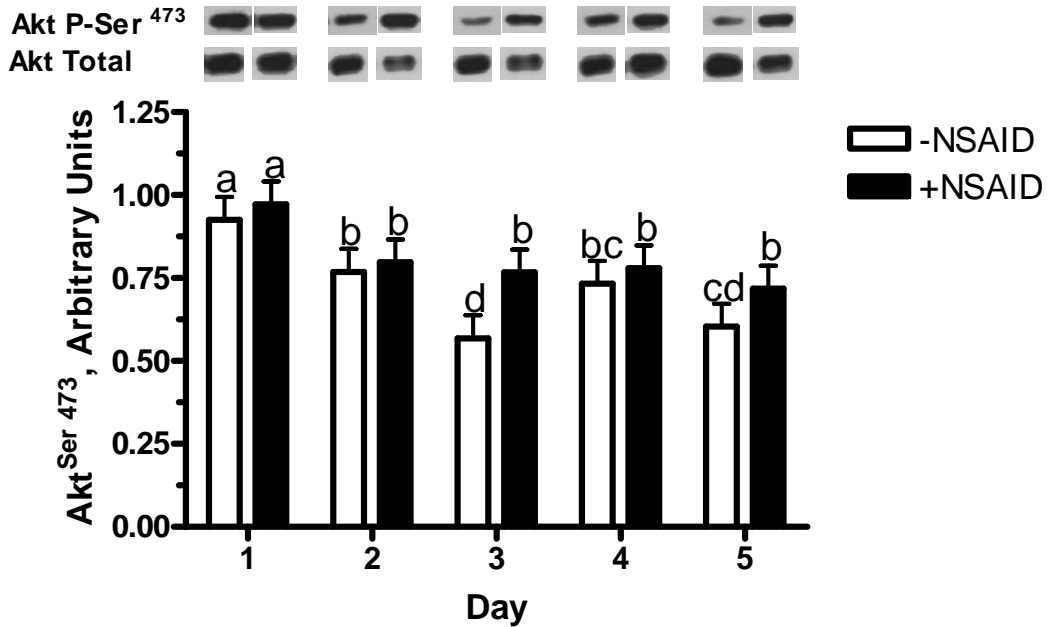


Figure 4.1. Gluteal muscle phosphorylation of Akt at Ser⁴⁷³ in mature Thoroughbred horses either receiving NSAID administration (1g/12 hours; +NSAID) or not receiving NSAID administration (-NSAID) for 5 days 60 minutes following consumption of a 3g/kg of a high protein pelleted meal. Muscle biopsies were taken at 60 min after t = 0 min. Akt phosphorylation at Ser⁴⁷³ was corrected by total Akt abundance, with the value for the left side at day 1 set at 1.0 AU. Values are pooled least square means \pm pooled SE of both gluteal muscle sides because side was not significant ($P > 0.05$), $n=6$ per treatment group. Representative images of the immunoblots are shown above.

^{abcd}Different letters indicate a significant day effect ($P < 0.05$) within a treatment group.

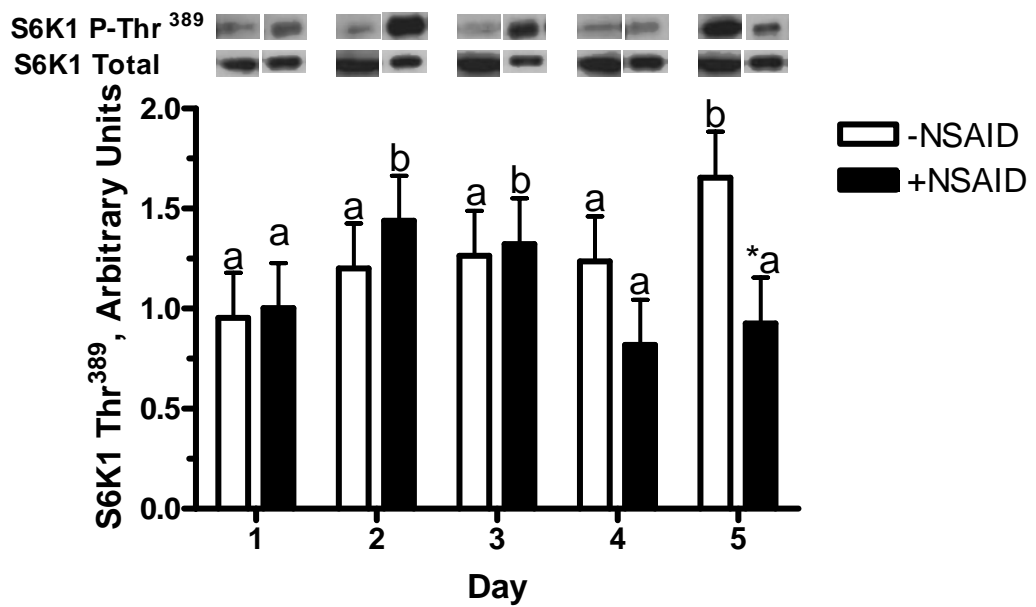


Figure 4.2. Gluteal muscle phosphorylation of S6K1 at Thr³⁸⁹ Ser⁴⁷³ in mature Thoroughbred horses either receiving NSAID administration (1g/12 hours; +NSAID) or not receiving NSAID administration (-NSAID) for 5 days 60 minutes following consumption of a 3g/kg of a high protein pelleted meal. Muscle biopsies were taken at 60 min after t = 0 min. S6K1 phosphorylation at Thr³⁸⁹ was corrected by total S6K1 abundance, with the value for the left side at day 1 set at 1.0 AU. Values are pooled least square means \pm pooled SE of both gluteal muscle sides because side was not significant ($P > 0.05$), $n=6$ per treatment group. Representative images of the immunoblots are shown above.

*Indicates that, within a day, the value is significantly different ($P < 0.05$) from the -NSAID group value.

^{ab}Different letters indicate a significant day effect ($P < 0.05$) within a treatment group.

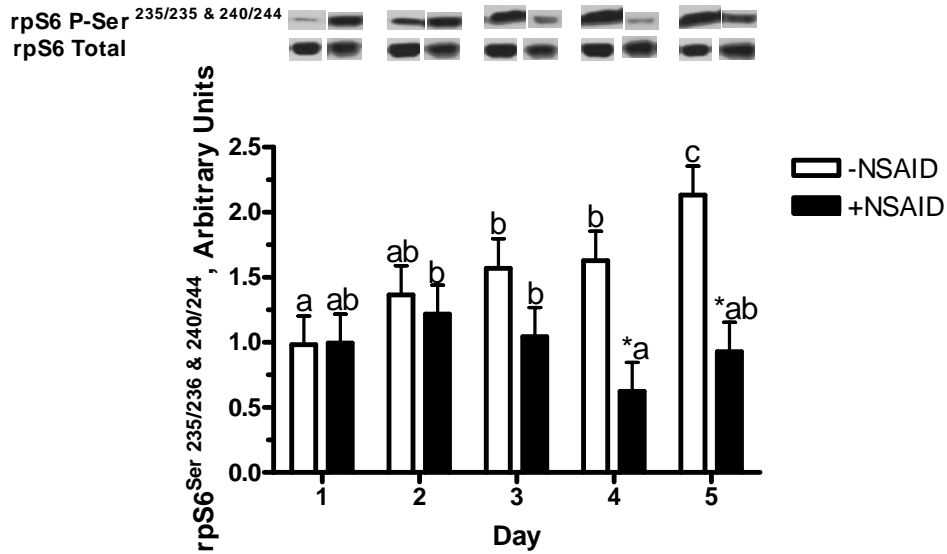


Figure 4.3. Gluteal muscle phosphorylation of rpS6 at Ser^{235/236 & 240/244} in mature Thoroughbred horses either receiving NSAID administration (1g/12 hours; +NSAID) or not receiving NSAID administration (-NSAID) for 5 days 60 minutes following consumption of a 3g/kg of a high protein pelleted meal. Muscle biopsies were taken at 60 min after t = 0 min. rpS6 phosphorylation at Ser^{235/236 & 240/244} was corrected by total rpS6 abundance, with the value for the left side at day 1 set at 1.0 AU. Values are pooled least square means \pm pooled SE of both gluteal muscle sides because side was not significant ($P > 0.05$), $n=6$ per treatment group. Representative images of the immunoblots are shown above.

*Indicates that, within a day, the value is significantly different ($P < 0.05$) from the – NSAID group value.

^{abc} Different letters indicate a significant day effect ($P < 0.05$) within a treatment group.

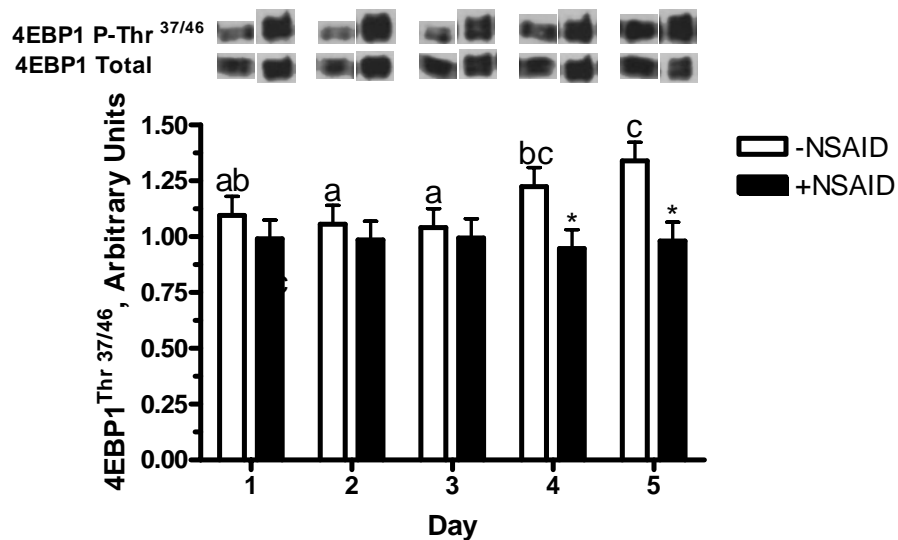


Figure 4.4. Gluteal muscle phosphorylation of 4EBP1 at Thr^{36/47} in mature Thoroughbred horses either receiving NSAID administration (1g/12 hours; +NSAID) or not receiving NSAID administration (-NSAID) for 5 days 60 minutes following consumption of a 3g/kg of a high protein pelleted meal. Muscle biopsies were taken at 60 min after t = 0 min. 4EBP1 phosphorylation at Thr^{36/47} was corrected by total rpS6 abundance, with the value for the left side at day 1 set at 1.0 AU. Values are pooled least square means ± pooled SE of both gluteal muscle sides because side was not significant ($P > 0.05$), n=6 per treatment group. Representative images of the immunoblots are shown above.

*Indicates that, within a day, the value is significantly different ($P < 0.05$) from the – NSAID group value.

^{abc} Different letters indicate a significant day effect ($P < 0.05$) within a treatment group.

Chapter V¹

Developmental regulation of the activation of translation initiation factors in response to feeding in the skeletal muscle of horses

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5.1. INTRODUCTION

The neonatal period is characterized by the highest growth rate during the lifespan with skeletal muscle showing the largest increase in mass (125). Elevated growth rate in the neonate is a result of protein synthesis rates being higher than protein degradation rates. However, with post-natal maturation, protein synthesis rates gradually decrease (125-127) until both synthesis and degradation are equal in non-growing adult muscle. To date, the life stage following the neonatal period, but before maturity, which is often characterized by slow growth and is often referred to as the adolescent phase in human development, has not been investigated with regards to protein synthesis.

Protein synthesis is limited by both the abundance and efficiency of ribosomes to translate mRNA into protein and the availability of amino acids to form a protein (128). The skeletal muscle of neonates has been reported to have a higher concentration of ribosomes which decreases with age (127, 147); this may also contribute to the elevated protein synthetic rates. The efficiency of ribosomes to translate mRNA into a protein is modulated by a series of intracellular signaling cascades that are associated with the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway regulates translation initiation and has been the subject of numerous recent reviews (21, 146, 488, 496). Briefly, insulin and insulin like growth factor 1 bind to their respective receptors

activating protein kinase B (PKB or Akt) through the activation of several intermediate signaling proteins (16, 18, 19, 21). Akt activation occurs when both the Thr³⁰⁸ and Ser⁴⁷³ sites are phosphorylated, and both sites are activated in association with insulin receptor signaling (18, 19, 21). Activation of Akt inactivates the mTOR inhibitor tuberous sclerosis complex 2 through phosphorylation (19, 21); thus, allowing activation of mTOR through phosphorylation. Amino acids, specifically Leu, have also been demonstrated to phosphorylate mTOR through the activation of several signaling proteins (45, 46, 497). mTOR is recognized in skeletal muscle as the key regulator of translation initiation and subsequent protein synthesis through the phosphorylation of two downstream signaling proteins: ribosomal S6 Kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein-1 (4EBP1). Phosphorylation of S6K1 results in the activation of ribosomal protein S6 (rpS6), a component of the 40S ribosomal subunit (498). Phosphorylation of rpS6 and 4EBP1 result in the activation of the translational equipment; thus, allowing the synthesis of a protein (146, 496).

Examination of protein synthesis in response to anabolic stimuli during the neonatal period has been demonstrated using both translation initiation factor activation and isotope infusion techniques to measure protein synthesis (125, 126, 520). Anabolic stimuli such as feeding a meal (521), amino acid administration (520, 522), or insulin infusion (522) increases the activation of translation initiation factors in the mTOR signaling pathway and fractional muscle protein synthesis rates (133, 140). As with protein synthesis, the responsiveness of the activation of translation initiation factors to anabolic stimuli is blunted with post-natal age (133, 140), and is lowered in the adult (149); however, the adolescent period of slowed growth has yet to be examined.

Furthermore, the activation of translation initiation factors and subsequent protein synthesis in response to an anabolic stimuli has been demonstrated to decline from the mature to aged individual in rodents (133, 140) and humans (149, 155). The activation of translation initiation factors has been examined in equine skeletal muscle tissue in only a single study (2), where the response to meal feeding increased the activation of translation initiation factors in the mature horse, but age-related effects have not been studied.

The objective of this study was to determine the effects of feeding following an 18 hour period of feed withholding on the activation of translation initiation factors, specifically Akt, S6K1, rpS6, and 4EBP1 in yearling, two year old and mature horses. We chose an adolescent age group, consisting of yearlings and two year olds, because to the best of our knowledge, there has not yet been a single study in any species to examine the activation of translation initiation factors in response to an anabolic stimulus in adolescents. The translation initiation factors studied were chosen as a representation of both up- and down-stream factors in the mTOR signaling pathway.

5.2. MATERIALS AND METHODS

5.2.1. Animals and housing.

The University of Kentucky Institutional Animal Care and Use Committee approved all procedures used in this study (2009-0442). Fifteen Thoroughbred mares, two Thoroughbred/Quarter Horse cross mares, and one Quarter Horse mare were obtained from the University of Kentucky Animal and Food Sciences' Maine Chance Farm. Of these 18 mares, 6 were yearlings (15.8±0.8 mo old; 395±12 kg BW), 6 were 2

years olds (27.4 ± 1.0 mo old; 484 ± 28 kg BW), 6 were mature (13.8 ± 2.9 years old; 549 ± 59 kg BW) mares. All horses were of moderate body condition for their age group (body condition score: yearlings: 4-5, two year olds and mature horses: 5-7; scale 1-9 (489)), and clinically healthy. The group of mature mares were selected from a candidate pool of mares which were no longer growing, but had not yet been defined as chronologically old (ie. greater than 20 years) (523). For the duration of the study, horses were housed in 3.7×3.7 m stalls bedded with pine shavings overnight, with daily turn out into drylots, and ad libitum access to water and salt at all times. Horses were adapted to diet and housing procedures for 2 weeks prior to any experimental or sampling procedures. During the 18 hour period of feed withholding, and during the sampling procedures, horses remained in the stalls bedded with pine shavings.

5.2.2. Feeding procedures.

All animals were individually fed in stalls two meals daily (at 0800 and 1500). Diets were designed to meet the National Research Council (336) requirements for mature horses with average maintenance needs and a BW of 600 kg, two year old horses with a targeted average daily gain (ADG) of 0.22 kg/d, and yearling horses with a targeted ADG of 0.54 kg/d. The meals consisted of alfalfa cubes, a ration balancer, and a protein/mineral supplement. The alfalfa cubes [mean \pm SD; 0.49 ± 0.01 Mcal/kg digestible energy (DE), $16.95 \pm 0.24\%$ crude protein (CP), $35.2 \pm 0.82\%$ acid detergent fiber (ADF), $44.88 \pm 1.31\%$ neutral detergent fiber (NDF), $2.28 \pm 0.05\%$ crude fat, and $9.5 \pm 0.22\%$ ash] were fed at a rate of 1.75% of body weight per day to all age groups. The ration balancer pellet (mean \pm SD; 0.48 ± 0.01 Mcal/kg DE, $14.93 \pm 0.15\%$ CP, $22.53 \pm 0.81\%$ ADF, $43.2 \pm 0.36\%$ NDF, $3.57 \pm 0.06\%$ crude fat, and $12.9 \pm 0.1\%$ ash) was fed at a rate of 0.15, 0.25,

and 0.40% of body weight per day to mature, 2 years old, and yearlings, respectively. The protein/mineral supplement (mean \pm SD; 0.57 \pm 0.01Mcal/kg DE, 36.3 \pm 0.72% CP, 6.77 \pm 0.25% ADF, 13.8 \pm 0.8% NDF, 3.87 \pm 0.06% crude fat, and 17.2 \pm 0.3% ash) was fed at a rate of 0, 0.15, and 0.20% of body weight per day to mature, 2 year olds, and yearlings, respectively. Samples of the alfalfa cubes, ration balancer pellet, and protein/mineral supplement were collected throughout the experimental period and sent to Dairy One Forage Laboratory (Ithaca, NY) for nutrient analysis.

5.2.3. Experimental design and procedures.

This study was conducted as a 3 \times 2-factorial crossover design with age and feeding state (post-absorptive versus postprandial) as the fixed effects, with each horse being studied in both feeding states. To facilitate sampling procedures, the eighteen horses were divided into three blocks, with 2 horses from each age group in each block. Each horse was studied under both feeding states in a randomly determined order, such that within each block there were an equal number of horses ($n = 3$) receiving each treatment during each period. There was a minimum of 1 week between the sampling periods for each horse.

The experimental procedures used in this study were similar to those previously reported (2). Briefly, on the day prior to sampling procedures, horses were weighed and a jugular vein catheter (14 gauge X 14.0 cm, Abbocath; Abbott Laboratories, North Chicago, IL) was placed. The gluteus medius muscle was ultrasounded to determine subcutaneous fat and gluteal muscle depth (524). This allowed for the accurate determination of 50% of gluteal muscle depth, in order to standardize biopsy collection

depth between the different ages of horses; which was determined to be 6.0 ± 0.4 , 7.4 ± 0.3 , and 8.1 ± 0.3 cm for yearlings, two year olds, and mature horses, respectively (Appendix 6). Horses were then placed into individual stalls and provided a high crude protein pelleted feed meal (Table 5.1), at 2 g/kg BW on an as-fed basis. The purpose of this meal was to standardize the duration of feed withholding prior to the sampling procedures for all horses. Horses were kept in stalls overnight (18 hour), with ad libitum access to water, but feed and hay were withheld.

At $t = 0$ minute, 18 hours following the previous meal, horses either remained in the post-absorptive state for an additional 90 minutes (post-absorptive treatment) or received a 2 g/kg BW meal of the aforementioned pelleted feed at $t = 0$ minute and again at $t = 30$ minutes, for a total targeted intake of 4 g/kg BW of pelleted feed during the postprandial period (postprandial treatment). This feeding regimen has been previously shown to result in differences in plasma glucose, insulin and amino acid concentration and in the activation of mTOR-related signaling factors in the gluteal muscle in mature horses (2). Feed intake was monitored and any feed refusals were removed at the end of the postprandial period and weighed.

Two baseline blood samples (10 mL), separated by a minimum of 15 minutes, were collected prior to $t = 0$ minute. Subsequent blood samples were taken every 10 minutes until $t = 80$ minutes. All samples were collected into evacuated tubes containing sodium heparin (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ) and were immediately centrifuged at $3,000 \times g$ for 10 minutes at 4°C . Aliquots of the plasma samples were frozen at -20°C until the time of analysis.

At t = 80 minutes, horses were placed in equine stocks (HS Equine Stocks; Priefert® Rodeo & Ranch Equipment, Mount Pleasant, TX), and lightly sedated with xylazine hydrochloride (0.3 mg/kg, intravenous [100 mg/mL]). The area over the middle gluteal muscle (~100 cm²) was shaved, aseptically prepared, and desensitized with a local anesthetic (12 mL of 2% lidocaine). At t = 90 minutes, approximately 500 mg of muscle biopsy specimen was obtained at a standardized site and depth (50% of gluteal muscle depth) in the middle gluteal muscle by use of the percutaneous needle biopsy technique (411). Approximately 400 mg of the sample was flash frozen in liquid nitrogen, and stored at -80°C until analysis. The remaining 100 mg was processed in preparation for Western blot analysis, as described below. Phenylbutazone was administered after the biopsy (1g every 12 hours for 48 hours post-biopsy) to alleviate any potential discomfort associated with the procedure.

During the second treatment period, horses underwent the same experimental procedures as during the first period, but received the alternate treatment. In the second treatment period, the jugular vein catheter was inserted into the vein not used for the previous period and the muscle biopsy specimen was taken from the contralateral side. Following the second muscle biopsy, horses returned to drylots during the day and were stalled overnight and were then returned to the Department of Animal and Food Sciences' Maine Chance Farm Research herd.

5.2.4. Sample analysis procedures.

5.2.4.1. Plasma Glucose and Insulin: Plasma glucose concentrations were assayed enzymatically using a glucose analyzer (YSI 2300 STAT Plus™ Glucose and Lactate

Analyzer, YSI Inc., Life Sciences, Yellow Springs, OH). Plasma insulin concentrations were determined using a commercially available kit (Coat-A-Coat RIA®kitSiemens, Healthcare Diagnostics Inc., Deerfield, IL).

5.2.4.2. Amino Acids: The plasma free amino acid concentrations and feed amino acid content were measured using reverse-phase HPLC (3.9 x 300 mm PICO-TAG reverse phase column; Waters, Milford, MA) of phenylisothiocyanate derivatives as previously described (2).

5.2.4.3. Western blot analysis of muscle samples: Electrophoresis of the gluteal muscle homogenates was followed by Western blotting techniques to determine the abundance of the total and phosphorylated forms of Akt, S6K1, rpS6, and 4E-BP1 (Chapter III).

5.2.5. *Statistical analysis.*

All data were analyzed using the mixed procedure of SAS Version 9.1 (SAS Institute Inc., Cary NC), and data were considered statistically significant if $P < 0.05$, and considered a statistical trend if $0.05 < P < 0.10$. When the fixed effects were significant, pre-planned comparisons of least squares means were made using the pdiff test.

The dependent variables plasma glucose, insulin, and amino acid concentrations were analyzed using a repeated measures analysis, with age, treatment, time, age*treatment, time*treatment, age*time, age*time*treatment interaction as the fixed effects and horse nested in treatment and block as the random effects. The two baseline sample values were averaged for each of the dependent variables, and the resulting baseline concentration was included in the model as a covariate if its effect was determined to be significant ($P < 0.05$). Baseline was significant ($P < 0.05$) for plasma

glucose, insulin, and each amino acid and was therefore included in each of the respective models. Each of the translation initiation factors were analyzed using a repeated measures analysis, with treatment, age, treatment*age interaction, treatment period, and block as the fixed effects and horse nested with in age*block as the subject. For all repeated measures analysis, the variance-covariance matrix was chosen for each analysis based on the lowest value for Schwarz's Bayesian Criterion. Data are presented as means \pm pooled standard error unless otherwise noted.

5.3. RESULTS

All horses remained healthy and maintained normal growth rates according to the recommendations of the NRC (336) throughout the course of the experimental period. The yearlings and two year olds maintained an ADG of 0.94 ± 0.67 and 0.18 ± 0.38 kg/d, respectively. The mature mares did not gain weight over the course of the experimental period with an ADG of 0.00 ± 0.25 kg/d.

5.3.1. Plasma Glucose

There was a significant effect of treatment ($P = 0.0002$), time ($P < 0.0001$), and treatment*time interaction ($P < 0.0001$) on plasma glucose concentration (Figure 5.1); where, the yearlings had higher ($P < 0.05$) plasma glucose concentrations at 30 minutes following the meal stimulus than the two year old and mature horses. Regardless of age, the horses in the postprandial state had higher glucose concentrations at 60 and 80 minutes than those in the post-absorptive state. During the postprandial phase, plasma glucose concentration increased from baseline with time in all age groups. There was also a trend for decreased glucose concentrations with age ($P = 0.08$). However, there

was no effect of treatment*age interaction ($P = 0.53$), age*time interaction ($P = 0.26$), or treatment*age*time interaction ($P = 0.36$) on plasma glucose concentration.

5.3.2. *Plasma Insulin*

Plasma insulin was significantly affected by treatment ($P < 0.0001$), time ($P < 0.0001$), treatment*time ($P < 0.0001$), treatment*age ($P = 0.003$), and treatment*age*time interaction ($P = 0.032$; Figure 5.2). Circulating insulin was higher in the postprandial phase ($P < 0.05$) compared to the post-absorptive phase beginning at 30 minutes in the mature horses. However, the yearlings and the two year olds did not show differences between the postprandial and post-absorptive phase until 80 minutes when insulin was greater in the postprandial phase. During the postprandial phase, insulin concentrations increased with time across all age groups. At both 0 and 30 minutes following a meal stimulus there was no difference ($P > 0.05$) between the age groups for circulating insulin in the postprandial group. However, at both 60 and 80 minutes post consumption of a high protein pelleted meal the mature horses had significantly greater ($P < 0.05$) circulating insulin than either the yearlings or two year olds. There was no effect of age ($P = 0.53$), but there was an age*time interaction ($P = 0.07$) on plasma insulin concentration.

5.3.3. *Plasma Amino Acids*

There was a significant effect of treatment, time, and treatment*time interaction ($P < 0.05$; Table 5.2) on all plasma amino acids examined, except glycine, where its concentration was only affected by time ($P < 0.05$). For the essential amino acids, plasma concentrations were increased at $t = 80$ minutes by an average of 48% in the postprandial

phase, while the plasma amino acid concentrations in the post-absorptive phase remained unchanged ($P < 0.05$) compared to the baseline values. With the exception of glutamate and aspartate, there was no effect of age on any plasma amino acid concentrations ($P > 0.05$; Table 5.2). There was no interaction of treatment*time*age ($P > 0.05$) for any of the plasma amino acids examined.

5.3.4. Muscle Translation Initiation Factors

The phosphorylation of Akt at Ser⁴⁷³ was significantly greater in the postprandial period ($P < 0.0001$; Figure 5.3) in all ages of horses; however, there was no effect of age ($P = 0.24$) or the treatment*age interaction ($P = 0.51$). The abundance of Akt phosphorylation at Thr³⁰⁸ trended to increase in the postprandial period compared to the post-absorptive period. However, there was no effect of age ($P = 0.90$) or treatment*age interaction ($P = 0.48$) on Akt phosphorylation at Thr³⁰⁸. There was a significantly greater phosphorylation of S6K1 at Thr³⁸⁹ from the post-absorptive to postprandial period ($P = 0.0001$; Figure 5). There was also a significantly higher phosphorylation of S6K1 at Thr³⁸⁹ with age ($P = 0.0079$), and a significant treatment*age interaction ($P = 0.0027$). Postprandial phosphorylation of S6K1 at Thr³⁸⁹ was greater ($P < 0.05$) in the mature horses than the yearlings or two year olds, and the yearlings had a higher ($P = 0.008$) abundance than the two year olds. The phosphorylation of rpS6 at Ser^{235/236} was significantly greater in the postprandial period compared to the post-absorptive period ($P < 0.0001$; Figure 6), and a significant treatment*age interaction ($P = 0.03$) was present; where, the yearlings ($P = 0.009$; 3.3 ± 0.3 arbitrary units) and mature horses ($P = 0.002$; 3.6 ± 0.3 arbitrary units) were greater in the postprandial phase compared to the two year olds (2.0 ± 0.3 arbitrary units). There was no difference in the postprandial phase between

the yearlings and mature horses ($P = 0.42$). However, there was a trend for the main effect of age ($P = 0.07$). There was a significantly higher phosphorylation of rpS6 at Ser^{240/244} rpS6 in the postprandial period ($P < 0.0001$; Figure 7); however, there was no effect of age ($P = 0.35$) or treatment*age interaction ($P = 0.27$). The phosphorylation of 4EBP1 at Thr^{37/46} was significantly greater in the postprandial period ($P = 0.003$; Figure 8), and a significant treatment*age interaction ($P = 0.04$) was present. The yearlings had an increase ($P = 0.0007$) in the postprandial phase compared to the post-absorptive, with no effects of feeding seen in the other ages of horses. There was no effect of age ($P = 0.68$) on the phosphorylation of 4EBP1 at Thr^{36/47}.

5.4. DISCUSSION

To the best of our knowledge, this is the first study to examine S6K1 in the skeletal muscle of horses of any age, and the first study to examine the activation of translation initiation factors in response to anabolic stimuli in the skeletal muscle of adolescent animals of any species. There was a marked increase in the phosphorylation of both up and downstream mTOR signaling factors during the postprandial state compared to the post-absorptive state in the gluteal muscle of yearling, two year old and mature horses, with the higher degree of sensitivity to anabolic stimuli exhibited by the yearlings.

Following mTOR activation, S6K1 and subsequently rpS6 are phosphorylated (21). Previous work in our lab (2) has shown that the protein abundance of the phosphorylated form of rpS6 was higher in the postprandial state in mature horses. This is in agreement with the current study, where protein abundance of the phosphorylated

forms of S6K1 and rpS6 were elevated in the postprandial state. There is an absence of comparable literature on the adolescent, therefore, we examined previous work in the swine neonate (521, 525) to determine if similar developmental trends continued during adolescence. In the present study, we have demonstrated that there is a decrease in the activation of translation initiation factors to a meal stimulus with adolescent development which is a trend that appears to continue from neonatal development (521, 525). There was a 158, -7, and 109% percent change in the phosphorylation of S6K1 from the post-absorptive to postprandial states in yearlings, two year olds and mature horses, respectively. Not only was there a difference in the abundance of S6K1 P-Thr³⁸⁹ in the postprandial state between the age groups, but there were also differences in the post-absorptive state where the yearling had a lower expression than either the mature or two year olds, allowing for a more pronounced postprandial response in the younger horses. Research in developmental changes of skeletal muscle protein abundance of S6K1-Thr³⁸⁹ during the post-absorptive state in the neonatal pig vary with reports of no difference (521, 525) and increases (526) from 7 to 26d of age, respectively. Although we examined adolescent horses, we demonstrated a similar phenomenon as previously described in the swine neonate (526), indicating the horse is not unique in the activation of translation initiation in skeletal muscle during development. Additionally, there were increases in the protein abundance of rpS6 phosphorylation at Ser^{235/236} and Ser^{240/244}. rpS6 P-Ser^{235/236} increased 147, 73, and 228% in yearlings, two year olds, and mature horses, respectively, from the post-absorptive to postprandial periods. Increases of 144, 76, and 167% in the protein abundance of rpS6 P-Ser^{240/244} from the post-absorptive to postprandial state were exhibited in yearlings, two year olds and mature horses,

respectively. Thus, the S6K1 portion of the signaling pathway following mTOR phosphorylation was activated in all age groups in the postprandial period indicating a potential increase in translation initiation. Our results of the downstream effectors indicate that there is a decrease in the response to a meal-feeding stimuli in the adolescent horse from year one to year two of life, which may be continuation of the development decline demonstrated in the neonate (521, 522, 525). However, further research is warranted to elucidate this in the equine neonate.

The other side of the downstream pathway following mTOR activation is the phosphorylation of 4EBP1 at Thr^{37/46}. Our laboratory has previously shown (2) that protein abundance of the phosphorylated form of 4EBP1 was higher in the postprandial state in mature horses; however, in the current study, the yearlings were the only age group that responded to the anabolic stimuli with a higher protein abundance of the phosphorylated form of 4EBP1 in the postprandial state. This discrepancy may be at least partially attributable to the differences in the antibodies used during the Western blotting procedure. In the previous study (2), the 4EBP1 antibody was specific for all forms of 4EBP1 (α , β and γ) and then expressed as the percentage of γ ; however, in this study two antibodies were used: one specific for phosphorylated Thr^{37/46} 4EBP1 and the other recognized total 4EBP1. Activation of 4EBP1 during the postprandial state was 50, 4, and 15% greater in yearlings, two year olds, and mature horses, respectively, than the post-absorptive state; with only a significant activation over post-absorptive abundance in the yearlings. This may indicate that the yearlings were more sensitive to the anabolic stimuli of a meal than the two year olds or mature horses, which is in agreement with the

previous work in the swine neonate (521, 525) demonstrating a decrease in the activation of translation initiation factors to a meal stimulus with development.

Based on previous research we hypothesized that the anabolic stimuli of consuming a meal would cause a greater activation of translation initiation factors in the yearlings than the two year olds, who we expected to be more responsive than the mature horses; however, this was not observed. In the adolescent age groups examined in the current study the downstream effectors were more responsive to a meal stimulus in the yearling group of horses in comparison to the two year olds. This is comparable to the results of previous studies performed in the swine neonate which show a decrease in the responsiveness of downstream effectors in the 26 compared to the 7 days old pig to a meal stimulus (521, 525, 526), amino acid supplementation (520, 522) or insulin infusion (522). However, postprandial protein abundance of the phosphorylated forms of rpS6 and 4EBP1 were not different between the yearlings and the mature horses, with the lowest abundance seen in the two year old horses. This may be partially attributed to the feeding protocol utilized. In the current study we fed all age groups a total of 4 g/kg of feed in the postprandial treatment in an effort to standardize the protein intake between the age groups. This standardization was intended to meet the protein requirements of a mature sedentary horse with an average body weight of 600 kg; as a result, this meal did not meet the requirements of the adolescent horses. This meal only supplied 54 and 80% of the daily requirements of the yearlings and two year olds, respectively. The increased responsiveness seen in the yearlings, who were consuming only 54% of their daily protein intake, indicates that if this study were repeated standardizing the meal by meeting 100% of daily protein requirements, would likely be sufficient anabolic stimuli

to elucidate a potential step wise decrease in the activation of translation initiation factors with age.

Although we saw increases in the downstream effectors following a meal stimulus across the age groups, future research is needed to examine if this indeed lead to increased protein synthesis using isotopic techniques. It is also necessary to determine if there are age related differences in the adolescent equine in whole body and muscle fractional protein synthetic rates following a meal stimulus. Accretion of skeletal muscle occurs when protein synthesis is greater than protein degradation. In mature sedentary animals, there should be a balance between protein synthesis and degradation resulting in maintenance of skeletal muscle, but no accretion. Similar to the mTOR signaling pathway for protein synthesis, there are signaling pathways in skeletal muscle that are associated with protein degradation which include the ubiquitin and proteosomal pathways. In the present study, we did not measure any molecular indicators of protein breakdown such as muscle-RING-finger protein 1 or forkhead box proteins. Previous research has demonstrated that there is an increase in the abundance of the factors associated with protein degradation with aging in other mammals (179, 180, 296) and in the post-absorptive versus postprandial state (180). Although we saw lower activation of the downstream mTOR signaling effectors following a meal stimulus in the two year old horses in comparison to the mature horses, we expect that the mature horses would also have elevated rates of muscle protein breakdown, as is seen in other mature species (179, 180, 296); therefore, it is likely that the 2 year old horses were accreting more muscle protein than the mature horses, despite having a lower activation of the mTOR related signaling factors.

Exercise, amino acid, and insulin dependent mechanisms can all stimulate mTOR phosphorylation (21, 521, 522). In the current study, mTOR may have been phosphorylated in an insulin- and amino acid-dependent manner because both up- and down- stream effectors were activated. Although Akt phosphorylation at Thr³⁰⁸ only showed a trend to increase between the post-absorptive and postprandial states, Akt phosphorylation at Ser⁴⁷³ was elevated approximately 86% across the age groups with no difference seen between the age groups. It has been shown that although Akt activation requires both sites to be phosphorylated, the contribution of each site is not equal (18, 19). Akt P-Thr³⁰⁸ can sufficiently stimulate Akt activation without the phosphorylation of Ser⁴⁷³; however, the reverse is not true (18, 19). Our results may indicate that Akt may have already been sufficiently phosphorylated at Thr³⁰⁸ in the post-absorptive state resulting in a lack of difference at the postprandial state. In agreement with the present study, Akt P-Ser⁴⁷³ in human skeletal muscle is more responsive to postprandial insulin concentrations than phosphorylation at the Thr³⁰⁸ site (527); however, further research is needed to confirm this in horses using a hyperinsulinemic-euglycemic isoaminoacidemic clamp technique. The use of the hyperinsulinemic-euglycemic isoaminoacidemic clamp technique would allow for the determination of the responsiveness of skeletal muscle Akt to administered insulin concentrations independent of other changes that occur in response to a meal. The phosphorylation of Ser⁴⁷³ Akt results in the mature horses during the postprandial period are in disagreement with previous results from our lab (2), where there was no difference from the post-absorptive to postprandial state in the gluteal muscle of mature horses. The same feed and feeding practices were used in mares of a similar age in both studies; however, a different population of horses was used. Despite

the similar postprandial insulin concentrations at the time of biopsy in both groups, the horses in the previous study (2) may have been more insulin resistant; explaining the absent change in Akt P-Ser⁴⁷³ with feeding. In future studies, all horses should be screened for insulin sensitivity prior to being included in the study population.

The absence of an age effect on Akt may be attributed to differences in insulin sensitivity between the three ages of horses studied. Postprandial insulin concentrations were higher in the mature horses than the yearlings or two year olds at the time of biopsy; therefore, we expected the phosphorylation of Akt to be greater in the mature horses than the yearlings or two year olds, but this was not the case. This may indicate that the younger age groups had higher insulin sensitivity than the mature horses, which corresponds with epidemiological studies on the prevalence of obesity and hyperinsulinemia (528). Furthermore, the majority of obese and hyperinsulinemic horses are between the ages of 5 and 15 years (528), which is substantially older than a yearling or a two year old, and similar to the mature horses used in the current study whose average age was 13.8 ± 2.9 years old. The response of Akt phosphorylation may have also been maximized at the lower insulin concentrations in the yearlings and two year olds; however, this requires further investigation.

Insulin alone cannot sufficiently simulate protein synthesis in adults (529, 530); and because the downstream effectors were significantly elevated in the postprandial state, it is more likely that the increase in translation initiation was the result of a combination of increased amino acids and insulin following the high crude protein pellet. There was no difference in the majority of the circulating plasma indispensable amino acids at the time of biopsy across the ages; however, the mature horses had an elevated

S6K1 response compared to the yearlings or two year olds. This may indicate an increase in amino acid sensitivity with age. However, amino acid sensitivity decreases with neonatal development in pigs (531), and has been shown to decline from maturity to old age in humans (155). Therefore, further research is needed using amino acid clamp techniques, which would allow for a controlled dosage of amino acids, in the horse to determine if the mature horse truly has increased amino acid sensitivity.

The current study revealed that feeding a high protein diet after an 18 hours period of feed withholding resulted in an increase in the activation of translation initiation factors, Akt, S6K1, rpS6 and 4EBP1 in mature, two year old, and yearling horses. This would suggest that regardless of age there is a postprandial increase in rates of muscle protein synthesis. The effect of age on the activation of the downstream effectors with a greater postprandial increase in the yearlings compared to the two year olds may indicate developmental differences in the responsiveness of muscle protein synthesis to insulin and amino acids. The mature horses appeared to have a greater postprandial responsiveness to amino acids and were less sensitive to insulin than the yearlings or two year old in the activation of translation initiation factors. This finding warrants further investigation, and it may have implications for designing feeding and management strategies specific to young adolescent versus mature horses.

5.5. TABLES

Table 5.1: As-fed nutrient composition of a high crude protein pelleted diet and nutrient intake from the pelleted diet during the postprandial period in horses that had feed withheld for 18 hours (mean \pm SD values).

| Nutrient | Pellet composition * | Nutrient intake[†] (mg/kg of bodyweight) |
|----------------------|-----------------------------|---|
| Moisture | 9.07 \pm 0.31% | |
| Crude protein | 33.0 \pm 0.53% | 1,320 \pm 21 |
| Alanine | 1.05 \pm 0.01% | 42 \pm 1 |
| Arginine | 1.79 \pm 0.06% | 71 \pm 2 |
| Aspartate+asparagine | 1.76 \pm 0.08% | 71 \pm 3 |
| Glutamate+glutamine | 4.32 \pm 0.07% | 173 \pm 3 |
| Glycine | 0.91 \pm 0.01% | 37 \pm 1 |
| Histidine | 0.58 \pm 0.02% | 23 \pm 1 |
| Isoleucine | 0.90 \pm 0.03% | 36 \pm 2 |
| Leucine | 1.96 \pm 0.15% | 78 \pm 6 |
| Lysine | 1.36 \pm 0.01% | 54 \pm 1 |
| Methionine | 0.32 \pm 0.01% | 13 \pm 1 |
| Proline | 1.93 \pm 0.09% | 77 \pm 4 |
| Phenylalanine | 1.22 \pm 0.01% | 49 \pm 1 |
| Serine | 1.28 \pm 0.02% | 51 \pm 1 |

Table 5.1 continued: As-fed nutrient composition of a high crude protein pelleted diet and nutrient intake from the pelleted diet during the postprandial period in horses that had feed withheld for 18 hours (mean \pm SD values).

| | | |
|-----------------------------|---------------------|-----------------|
| Threonine | 0.93 \pm 0.04% | 37 \pm 2 |
| Tyrosine | 0.85 \pm 0.10% | 34 \pm 1 |
| Valine | 0.92 \pm 0.01% | 37 \pm 2 |
| Acid detergent fiber | 6.17 \pm 0.25% | 247 \pm 10 |
| Neutral detergent fiber | 12.56 \pm 0.75% | 503 \pm 30 |
| Non-fiber carbohydrates | 26.2 \pm 0.36% | 1048 \pm 14 |
| Starch | 4.67 \pm 0.06% | 191 \pm 2 |
| Water-soluble carbohydrates | 8.43 \pm 0.15% | 337 \pm 6 |
| Crude fat | 3.47 \pm 0.07% | 139 \pm 2 |
| Ash | 15.6 \pm 0.26% | 624 \pm 11 |
| Calcium | 3.34 \pm 0.13% | 134 \pm 5 |
| Phosphorus | 2.05 \pm 0.09% | 82 \pm 4 |
| Potassium | 1.44 \pm 0.01% | 58 \pm 0.2 |
| Sodium | 0.682 \pm 0.01% | 27 \pm 0.4 |
| Iron | 1263 \pm 32 mg/kg | 5.05 \pm 0.13 |
| Zinc | 548 \pm 40 mg/kg | 2.19 \pm 0.16 |

Table 5.1 continued: As-fed nutrient composition of a high crude protein pelleted diet and nutrient intake from the pelleted diet during the postprandial period in horses that had feed withheld for 18 hours (mean \pm SD values).

*Values represent the amount of the nutrient present as a portion of the total diet on an as-fed basis. †During the postprandial period, following an 18-hour feed withholding period, horses were given 2 equal meals of the pelleted feed (targeted total intake of 4 g of feed/kg of bodyweight), separated by 30 minutes .

Table 5.2: The effect of an 18 hour feed withholding period followed by either continued feed withholding (post-absorptive) or subsequent re-feeding of a high protein pellet (postprandial) on plasma amino acid concentrations at time of biopsy (80 minutes) in yearling, two year old and mature horses¹

| Amino Acids | Post-absorptive | | | Postprandial | | | Pooled SE |
|---------------|------------------|-------------------|------------------|-------------------|-------------------|------------------|-----------|
| | Yearling | Two Year Old | Mature | Yearling | Two Year Old | Mature | |
| Alanine | 157 | 163 | 163 | 245* | 259* | 236* | 12.3 |
| Arginine | 73 | 70 | 71 | 156* | 143* | 153* | 8.3 |
| Asparagine | 47 | 43 | 43 | 80* | 70* | 73* | 5.4 |
| Aspartate | 4.2 ^a | 3.8 ^{ab} | 3.6 ^b | 5.7 ^{a*} | 5.7 ^{a*} | 4.8 ^b | 0.2 |
| Citrulline | 70 | 65 | 66 | 83* | 89* | 94* | 3.8 |
| Glutamate | 22 ^{ab} | 24 ^a | 19 ^b | 26 ^{ab*} | 28 ^{a*} | 23 ^{b*} | 1.5 |
| Glutamine | 442 | 443 | 420 | 580* | 540* | 531* | 26 |
| Glycine | 579 | 569 | 562 | 610 | 629 | 555 | 23 |
| Histidine | 60 | 57 | 56 | 72* | 68* | 68* | 3.2 |
| Isoleucine | 67 | 69 | 61 | 97* | 102* | 88* | 4.3 |
| Leucine | 120 | 124 | 117 | 161* | 170* | 152* | 6.7 |
| Lysine | 107 | 108 | 102 | 197* | 200* | 196* | 11 |
| Methionine | 27 | 28 | 29 | 34* | 40* | 37* | 2.5 |
| Ornithine | 35 | 35 | 33 | 56* | 56* | 57* | 2.7 |
| Phenylalanine | 56 | 56 | 52 | 71* | 72* | 62* | 2.8 |
| Proline | 74 | 70 | 61 | 121* | 112* | 102* | 5.2 |
| Serine | 186 | 184 | 171 | 245* | 234* | 231* | 11 |

Table 5.2 continued: The effect of an 18 hour feed withholding period followed by either continued feed withholding (post-absorptive) or subsequent re-feeding of a high protein pellet (postprandial) on plasma amino acid concentrations at time of biopsy (80 minutes) in yearling, two year old and mature horses¹

| | | | | | | | |
|------------|-----|-----|-----|------|------|------|-----|
| Taurine | 34 | 35 | 34 | 48* | 46* | 39* | 2.1 |
| Threonine | 102 | 102 | 94 | 140* | 139* | 132* | 6.7 |
| Tryptophan | 10 | 9.3 | 9.1 | 14* | 12 | 11 | 1.3 |
| Tyrosine | 56 | 49 | 51 | 90* | 78* | 77* | 4.4 |
| Valine | 205 | 204 | 196 | 258* | 259* | 251* | 8.3 |

¹ Plasma amino acid concentrations are presented as least squares mean values in $\mu\text{mol/L}$.

^{a,b} Within a treatment, values with different superscript letters are significantly ($P < 0.05$) different.

*Within an age, value is significantly ($P < 0.05$) different from the value post-absorptive value.

5.6. FIGURES

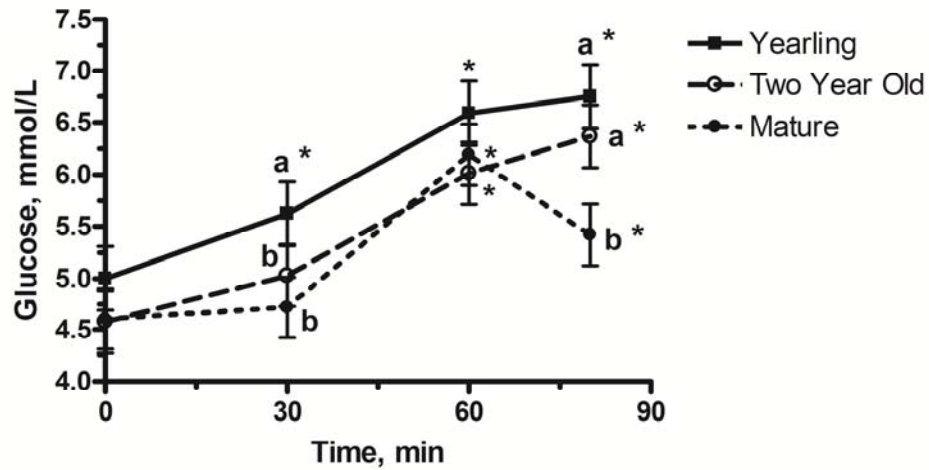


Figure 5.1. Plasma glucose concentrations (mmol/L) in yearling, two year old, and mature horses that were fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes following an 18 hour period of feed withholding. Values are least square means \pm pooled SE, n=6 per age group.

*Indicates that, within an age group, the value is significantly different ($P < 0.05$) from the baseline value.

^{ab}Different letters indicate a significant age effect ($P < 0.05$) within a time period.

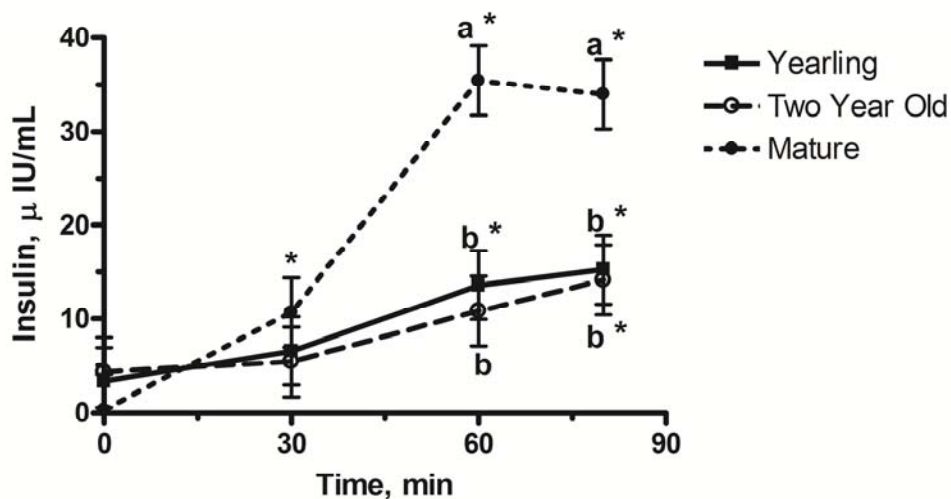


Figure 5.2. Plasma insulin concentrations (mIU/L) in yearling, two year old, and mature horses that were fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes following an 18 hour period of feed withholding. Values are least square means \pm pooled SE, n=6 per age group.

*Indicates that, within an age group, the value is significantly different ($P < 0.05$) from the baseline value.

^{ab}Different letters indicate a significant age effect ($P < 0.05$) within a time period.

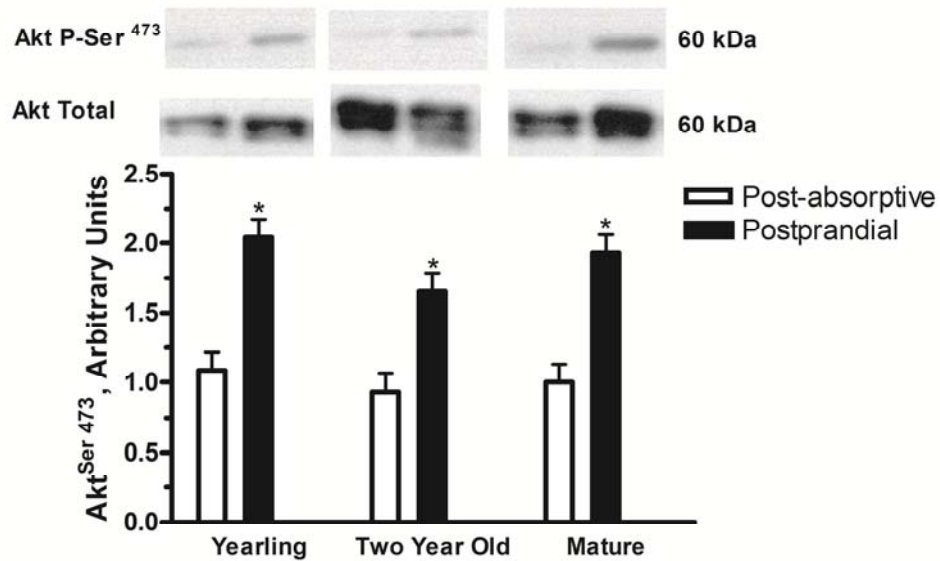


Figure 5.3. Gluteal muscle phosphorylation of Akt at Ser⁴⁷³ in yearling, two year old, and mature horses that were either fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes (postprandial) or had feed withheld for an additional 90 minutes (post-absorptive) following an 18 hour period of feed withholding. Muscle biopsies were taken at 90 minutes after t = 0 minutes. Akt phosphorylation at Ser⁴⁷³ was corrected by total Akt abundance, with the value for the post-absorptive mature horse set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per age group. Representative images of the immunoblots are shown above.

*Indicates that, within an age group, the value is significantly different (P < 0.05) from the postprandial value.

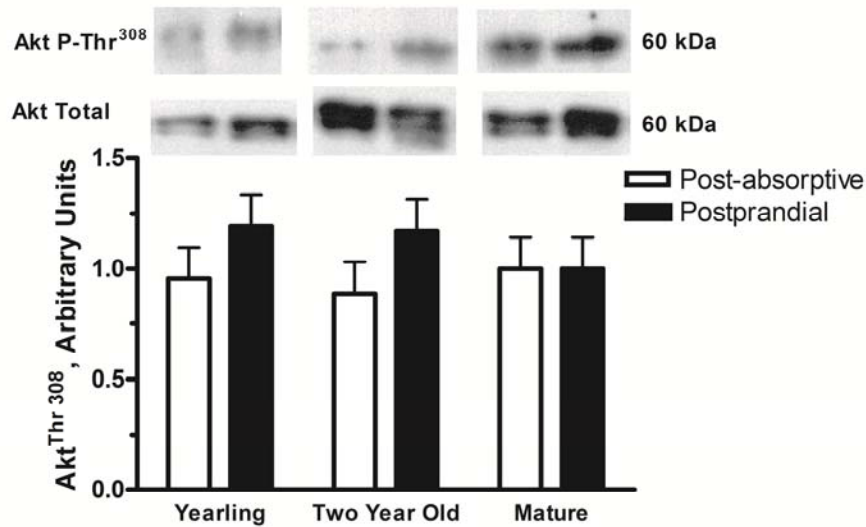


Figure 5.4. Gluteal muscle phosphorylation of Akt at Thr³⁰⁸ in yearling, two year old, and mature horses that were either fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes (postprandial) or had feed withheld for an additional 90 minutes (post-absorptive) following an 18 hour period of feed withholding. Muscle biopsies were taken at 90 minutes after t = 0 minutes. Akt phosphorylation at Thr³⁰⁸ was corrected by total Akt abundance, with the value for the post-absorptive mature horse set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per age group. Representative images of the immunoblots are shown above.

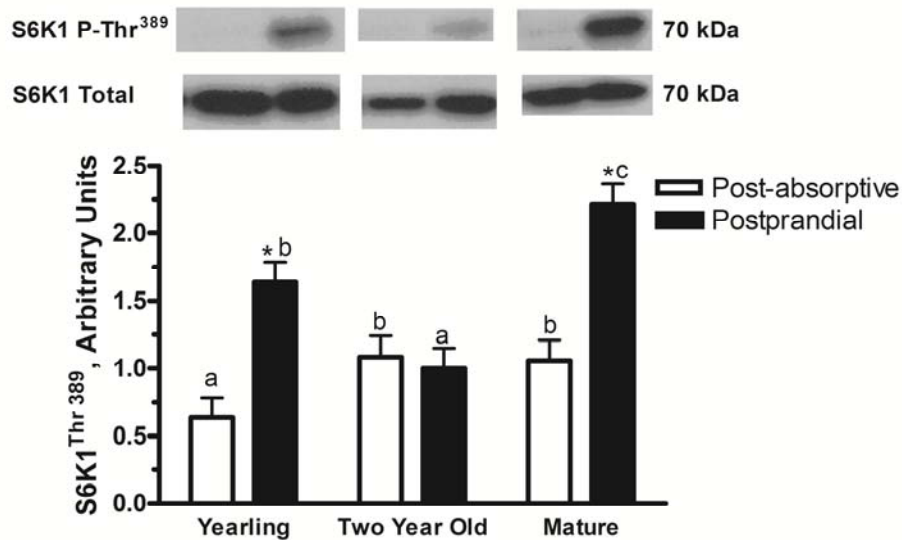


Figure 5.5. Gluteal muscle phosphorylation of S6K1 at Thr³⁸⁹ in yearling, two year old, and mature horses that were either fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes (postprandial) or had feed withheld for an additional 90 minutes (post-absorptive) following an 18 hour period of feed withholding. Muscle biopsies were taken at 90 minutes after t = 0 minutes. S6K1 phosphorylation at Thr³⁸⁹ was corrected by total S6K1 abundance, with the value for the post-absorptive mature horse set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per age group.

Representative images of the immunoblots are shown above.

*Indicates that, within an age group, the value is significantly different ($P < 0.05$) from the postprandial value.

^{abc}Different letters indicate a significant age effect ($P < 0.05$) within a treatment group.

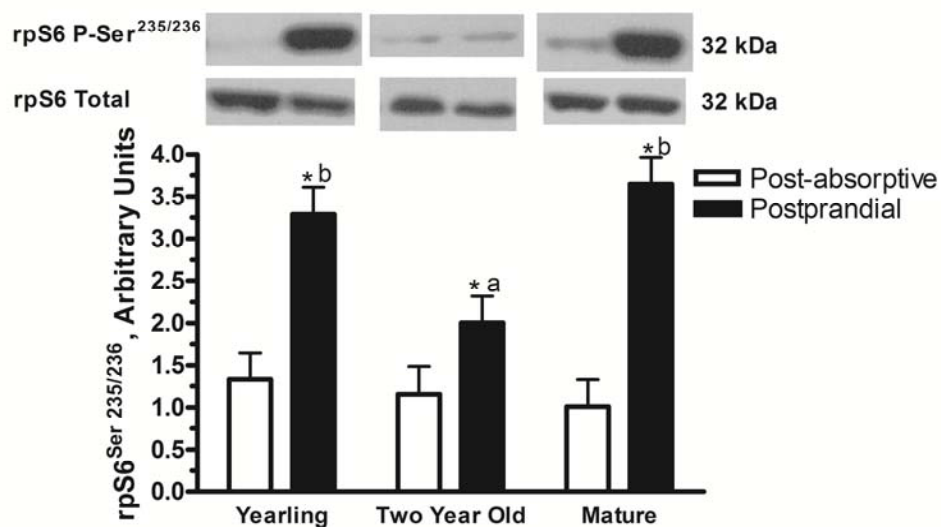


Figure 5.6. Gluteal muscle phosphorylation of rpS6 at Ser^{235/236} in yearling, two year old, and mature horses that were either fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes (postprandial) or had feed withheld for an additional 90 minutes (post-absorptive) following an 18 hour period of feed withholding. Muscle biopsies were taken at 90 minutes after t = 0 minutes. rpS6 phosphorylation at Ser^{235/236} was corrected by total rpS6 abundance, with the value for the post-absorptive mature horse set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per age group.

Representative images of the immunoblots are shown above.

*Indicates that, within an age group, the value is significantly different ($P < 0.05$) from the postprandial value.

^{abc}Different letters indicate a significant age effect ($P < 0.05$) within a treatment group.

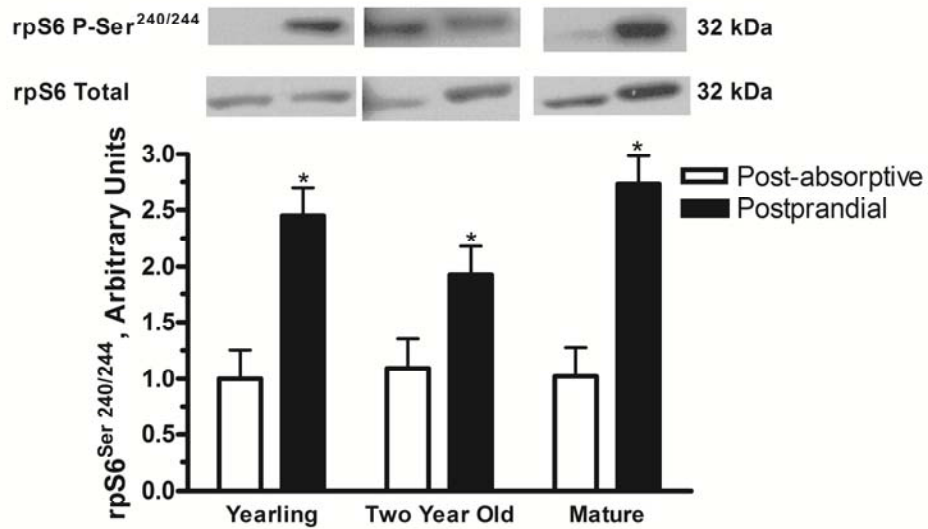


Figure 5.7. Gluteal muscle phosphorylation of rpS6 at Ser^{240/244} in yearling, two year old, and mature horses that were either fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes (postprandial) or had feed withheld for an additional 90 minutes (post-absorptive) following an 18 hour period of feed withholding. Muscle biopsies were taken at 90 minutes after t = 0 minutes. rpS6 phosphorylation at Ser^{240/244} was corrected by total rpS6 abundance, with the value for the post-absorptive mature horse set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per age group. Representative images of the immunoblots are shown above.

*Indicates that, within an age group, the value is significantly different ($P < 0.05$) from the postprandial value.

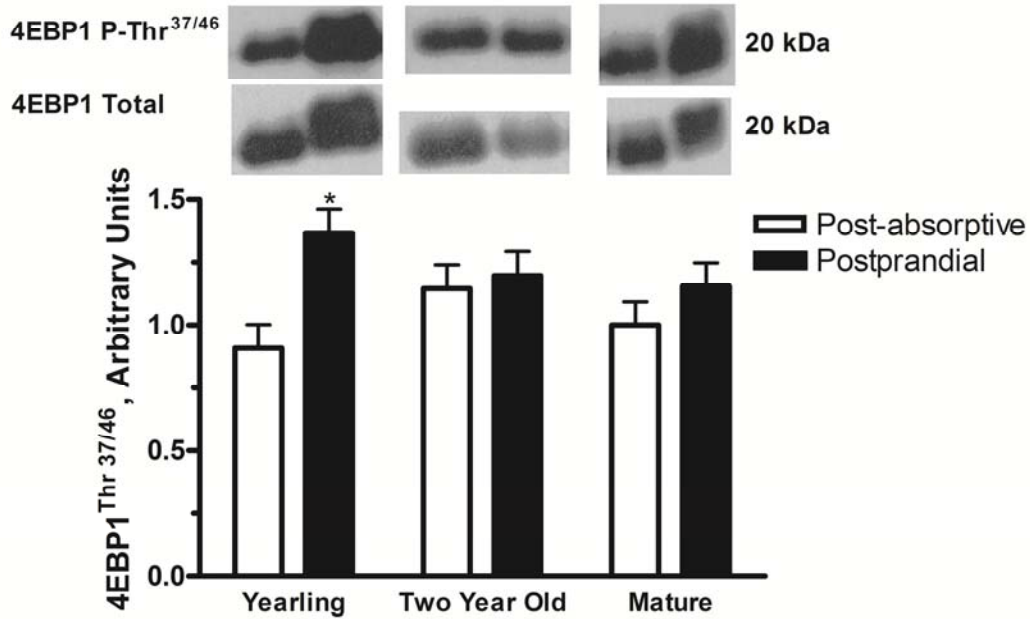


Figure 5.8. Gluteal muscle phosphorylation of 4EBP1 at Thr^{36/47} in yearling, two year old, and mature horses that were either fed 2g/kg of a high protein pellet at t = 0 and t = 30 minutes (postprandial) or had feed withheld for an additional 90 minutes (post-absorptive) following an 18 hour period of feed withholding. Muscle biopsies were taken at 90 minutes after t = 0 minutes. 4EBP1 phosphorylation at Thr^{36/47} was corrected by total rpS6 abundance, with the value for the post-absorptive mature horse set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per age group. Representative images of the immunoblots are shown above.

*Indicates that, within an age group, the value is significantly different (P < 0.05) from the postprandial value.

Chapter VI

Whole body protein metabolism is not different between healthy aging and older mature horses

6.1. INTRODUCTION

Sarcopenia is the involuntary loss of muscle mass and strength, and has been partially attributed to a multitude of factors which include a decline in physical activity and a less than optimal diet in the aging human population (148-150). Similar to aging humans, horses have also shown a decrease in muscle mass (532). Furthermore, the number of horses over the age of 20 years old is approximately 7.6% of the equine population and approaches 20% in some areas (523, 533). Horses over the age of 20 years old typically exhibit visible signs of aging (533). Although there is anecdotal evidence to suggest that the aging equine population exhibits a decline in physical activity similar to what is seen in aging humans, there has been limited research regarding protein requirements. From the few studies that have examined protein nutrition in the aging equine population, it appears that old horses may have lower protein digestibility than their mature counterparts (534); however, when studies were repeated with a different cohort of old horses the results were conflicting (535). As a result, it is necessary to determine if protein metabolism of the aging horse varies from the mature counterparts which would be the first step in determining if protein requirements differ between these groups of horses.

Sarcopenia is a result of an imbalance of protein synthesis and breakdown. The increase in muscle protein fractional synthesis rates in response to anabolic stimuli such as exercise (149, 151, 152), amino acids (149, 153), insulin (154), or meal consumption

(152, 153) is decreased in the aged compared to the younger adult. Additionally, differences in whole-body protein metabolism suggest that a greater protein requirement is necessary for the aging human population (487). Systemic inflammation associated with aging has been associated with lowered rates of muscle protein synthesis (493). Although the effects of aging on whole-body or muscle protein metabolism have not been elucidated in the horse, there has been a single study to determine whole-body protein synthesis rates using stable isotope infusion techniques in the mature horses (Urschel et al., University of Kentucky, *submitted*). The most effective way to measure whole-body protein synthesis is through the use of isotope infusion and stochastic analysis to calculate parameters of whole-body protein synthesis and breakdown and these methods have been extensively used in a variety of physiological states in humans (151, 487, 536). The use of these methodologies may be the first step in determining if the aging equine has different protein requirements than their mature counterparts.

Protein synthesis is modulated by a series of intracellular signaling cascades that are associated with the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway regulates translation initiation and has been the subject of numerous recent reviews (21, 488, 537). In short, insulin activates Akt (also referred to as PKB) through the activation of several intermediate signaling proteins (16, 18, 19, 21). Akt activation occurs when both the Thr³⁰⁸ and Ser⁴⁷³ sites are phosphorylated, and both sites are activated in association with insulin receptor signaling (18, 19, 21). Activation of Akt inactivates the mTOR inhibitor tuberous sclerosis complex 2 through phosphorylation (19, 21); thus, allowing activation of mTOR through phosphorylation. Amino acids (45, 46, 497) and exercise (149, 488, 538) also phosphorylate mTOR through Akt-

independent mechanisms. The activation of mTOR allows for the phosphorylation of two downstream signaling proteins: ribosomal S6 Kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein-1 (4EBP1). Phosphorylation of S6K1 results in the activation of ribosomal protein S6 (rpS6) (498), a component of the 40S ribosomal subunit (498). The phosphorylation of rpS6 and 4EBP1 result in the activation of the translational equipment and subsequently protein synthesis (21, 537). The activation of translation initiation factors in the mTOR signaling pathway in response to anabolic stimuli is reduced with aging (155-157). Although the activation of translation initiation factors in response to a meal have been examined in adolescent (Chapter V) and mature (2) equine skeletal muscle, this has not been studied in the aging equine population. The objective of this study was to determine the effects of aging on the activation of translation initiation factors, specifically Akt, S6K1, rpS6, and 4EBP1, and whole-body protein synthesis in mature and aged horses.

6.2. MATERIALS AND METHODS

6.2.1. Animals and housing

The University of Kentucky Institutional Animal Care and Use Committee (2009-0562) approved all procedures used in this study. Twelve mixed breed horses were obtained from the University of Kentucky Veterinary Sciences' Farm: 6 aged (23.5 ± 2.6 y old; 3 geldings and 3 mares) and 6 mature (11 ± 2.6 y old; 3 geldings and 3 mares) horses. To facilitate sampling procedures, the twelve horses were divided into three blocks, with 2 horses from each age group (1 mare and 1 gelding) in each block. All horses were of a moderate body condition (body condition score 5-7, scale 1-9 (489)),

were clinically healthy with all of their teeth and were able to live outdoors in a group housing environment, and on a regular farrier, anthelmintic, dental, and vaccination regimen. Additionally, the pool of candidate horses were screened for plasma α -melanocyte stimulating hormone (α -MSH) concentration, a marker of pituitary pars intermedia dysfunction (PPID; Equine Cushing's Disease) (539). Selected horses were well below the normal cut-off value of < 35 pmol/L (539). Horses were group housed on dry lots with *ad libitum* access to salt and water, and grass hay (mean \pm SD; 0.91 ± 0.02 Mcal/kg DE, $8.2 \pm 0.8\%$ CP; $48.3 \pm 0.3\%$ ADF; $76.5 \pm 1.4\%$ NDF; $2.0 \pm 0.5\%$ crude fat; and $7.0 \pm 0.5\%$ ash) was provided to the group at 2% of body weight per day. Horses were brought into 3.7 \times 3.7 m stalls and individually fed the concentrate ration twice daily at 0800 and 1500. The concentrate ration consisted of a 50:50 mixture of a ration balancer pelleted feed (Table 6.1) and oats (Table 6.1) at 0.2% of body weight per day. Horses were adapted to diet and housing protocols for 2 weeks prior to the initiation of experimental procedures. During the isotope infusion procedures horses were housed in 3.7 \times 3.7 m stalls bedded with pine shavings with *ad libitum* access to water and salt.

6.2.2. *Experimental design and procedures*

Following the 2 week adaptation period, horses were removed from the group housed dry lot at 0800 on day 0, weighed on a portable electronic scale (model 700, Tru Test Inc., Mineral Wells, TX), put into individual stalls and fed the morning concentrate meal of 0.1% of body weight and their individual portion of hay at 2% of body weight per day. Following consumption of the morning concentrate meal, a blood sample was collected into PAXgene™ Blood RNA tubes (Quiagen, Inc., Santa Clarita, CA) via jugular vein venipuncture to measure circulating inflammatory cytokines gene

expression. At 1500, horses were fed their afternoon concentrate meal. Horses remained in their individual stall during day 1 and were fed in the same manner as on day 0.

On day 2, following consumption of the morning concentrate meal which was given as previously described, and 2 indwelling jugular vein catheters (14 gauge X 14.0 cm, Abbocath; Abbott Laboratories, North Chicago IL) were placed using aseptic techniques as previously described (2): one for isotope infusion and one for blood sampling. Fat thickness on the croup near the site of biopsy was ultrasounded in order to calculate percent body fat (481). After these procedures were completed, horses were returned to their stalls and fed their afternoon meal as previously described.

On day 3, the whole-body phenylalanine kinetics were measured using primed, constant stable isotope infusions. During the course of the whole-body phenylalanine kinetic measurements, horses were fed the morning concentrate divided into 24 equal portions, with 1 portion fed every 30 minutes for 7.5 h, with the initial portion was provided 1.5 h prior to the start of isotope administration. This feeding regimen was used in order to bring all of the horses to a steady state, which is necessary when using steady-state isotope kinetics. In order to accurately quantify feed intake during the isotope infusions, hay was removed from the stall during these procedures. Each horse received a 14.4 $\mu\text{mol/kg}$ priming dose of [^{13}C] sodium bicarbonate solution (IsotecTM, Miamisburg, OH), followed by a 2-h constant infusion at 12 $\mu\text{mol/kg/h}$ in order to determine total CO_2 production(540). This was followed by a 4-h primed (8.4 $\mu\text{mol/kg}$), constant (6 $\mu\text{mol/kg/h}$) infusion of [$1\text{-}^{13}\text{C}$]phenylalanine solution (IsotecTM, Miamisburg, OH) to measure whole-body phenylalanine oxidation and flux(541). The primed to

constant ratio for both [^{13}C] sodium bicarbonate (542) and [1- ^{13}C]phenylalanine were previously determined (Urschel et al., University of Kentucky, *submitted*). The [^{13}C] sodium bicarbonate and [1- ^{13}C]phenylalanine solutions were individually prepared by dissolving the isotope into 0.9% sterile saline (Butler Animal Health Supply, Dublin, OH) and filtering the solution through 0.22 μm sterile filters (Millipore, Billerica, MA) into empty sterile ethylene vinyl acetate bags (Baxter Healthcare Corporation, Deerfield, IL). The isotope filled ethylene vinyl acetate bags were attached to a surcingle and connected to the catheter using a primary IV set (Baxter Healthcare Corporation, Deerfield, IL). Isotope was delivered into the catheter using pressure sensitive, cordless intravenous infusion pumps (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc., Loveland, CO) which was also attached to the surcingle on each horse. This cordless pump-surcingle system permitted the horses to remain in individual stalls during the infusion period without restraint.

Two baseline breath samples were collected prior to the [^{13}C] sodium bicarbonate infusion (-30 min and 0 min), and subsequent breath samples were collected every 30 min throughout the infusion procedures of both [^{13}C] sodium bicarbonate and [1- ^{13}C]phenylalanine. Breath samples were collected using a modified Equine Aeromask® (Trudell Medical International, London ON, Canada) enabling the collection of air through a 1-way valve into impermeable gas bags (Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany). During breath collection, horses wore the Aeromask® for approximately 1 minute to allow time for the air in the mask to equilibrate, and then bags were attached to the 1-way valve and remained there until full (approximately 1 min). Immediately following each sample collection, another 1/24 meal was provided. Blood

sampling commenced at $t = 90$ min into the [^{13}C] sodium bicarbonate infusion, and continued every 30 min until the end of the [$1\text{-}^{13}\text{C}$]phenylalanine infusion. All blood samples were collected into evacuated vacutainers (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) containing sodium heparin. The 90 min [^{13}C] sodium bicarbonate and 0 min [$1\text{-}^{13}\text{C}$]phenylalanine blood samples provided 2 baseline samples to measure the background enrichments of [$1\text{-}^{13}\text{C}$] phenylalanine in the blood prior to isotope infusion.

At the end of the [$1\text{-}^{13}\text{C}$]phenylalanine infusion, the infusion pumps were turned off and the horses were led to a set of equine stocks. Horses were lightly sedated with xylazine hydrochloride (0.3mg/kg, IV [100mg/mL]; Butler Animal Health Supply, Dublin, OH), and a gluteal muscle biopsy was collected as previously described (2). Briefly, the area over the middle gluteal muscle ($\sim 100\text{-cm}^2$) was shaved, aseptically prepared, and desensitized with a local anesthetic (12 mL of 2% lidocaine; Butler Animal Health Supply, Dublin, OH). Approximately 500 mg of muscle biopsy specimen was collected at approximately 50% (~ 8 cm; Chapter III; ChapterV) depth of the middle gluteal muscle using the percutaneous needle biopsy technique(411). Of this, ~ 100 mg was processed in preparation for Western blot analysis, as described below, and ~ 80 mg was stored in *RNAlater* (Quiagen, Inc., Santa Clarita, CA) for qRT-PCR analysis of inflammatory cytokines as described below. The remainder was flash frozen in liquid nitrogen, and stored at -80°C until analysis. Catheters were removed following muscle biopsy collection.

At the end of the sampling period, horses were given the remaining portions of concentrate and their daily allotment of hay as a single meal, and then returned to the

drylot. Samples of the grass hay, ration balancer pellet, and oats were collected throughout the experimental period and sent to Dairy One Forage Laboratory (Ithaca, NY) for nutrient analysis. For the next 3 days, 2g/d of phenylbutazone was administered to alleviate any discomfort associated with the sampling procedures.

6.2.3. Sample analysis procedures

6.2.3.1. Blood sample collection and storage: Blood samples collected for qRT-PCR in PAXgene™ Blood RNA tubes (Quiagen, Inc., Santa Clarita, CA) were gradually frozen to -80°C per manufacturer instructions and remained at -80°C until analysis. The remaining blood samples were immediately centrifuged at 1,500 x g for 10 minutes at 4°C, and aliquots of plasma samples were frozen at -20°C until the time of analysis.

6.2.3.2. Plasma glucose and insulin: Plasma glucose concentrations were assayed enzymatically using a YSI 2700 SELECT™ Biochemistry Analyzer (YSI Inc., Life Sciences, Yellow Springs, Ohio). Plasma insulin concentrations were determined using Coat-A-Coat RIA®kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL).

6.2.3.3. Amino acids: The plasma free amino acid concentrations were measured using reverse-phase HPLC (3.9 x 300 mm PICO-TAG reverse phase column; Waters, Milford MA) of phenylisothiocyanate derivatives as previously described (2). Total amino acid concentrations of the concentrate, oats, and grass hay were also measured using reverse-phase HPLC (3.9 x 300 mm PICO-TAG reverse phase column; Waters, Milford MA) of phenylisothiocyanate derivatives as previously described (2), following 24 hour acid hydrolysis in 6N HCl at 110°C.

6.2.3.4. Plasma phenylalanine enrichment: The isotopic enrichment of phenylalanine (the amount of [1-¹³C]phenylalanine relative to unlabeled phenylalanine) in the plasma samples collected on day 3 was determined by Metabolic Solutions, Inc. (Nashua, NH) using a previously described method (543) which was modified by Matthews, Persola and Campbell (544). Briefly, the isotopic enrichment of plasma samples were determined by negative chemical ionization GC-MS analysis of a heptafluorobutyric, n-propyl derivative. 1-¹³C-phenylalanine enrichment was measured using methane negative chemical ionization GC-MS (Agilent 5973 EI/CI MSD with a Agilent 6890 GC). A Phenomenex ZB-1MS capillary column was used to separate the derivative of phenylalanine. Selected ion chromatograms were obtained by monitoring ions *m/z* 383 and 384 for L-phenylalanine and L-[1-¹³C]phenylalanine, respectively.

6.2.3.5. Muscle phenylalanine enrichment: Approximately 25-30 mg of flash frozen muscle tissue was sent to Metabolic Solutions, Inc. (Nashua, NH) for the determination of mixed-muscle protein and intracellular 1-¹³C-phenylalanine enrichment (545). Muscle was homogenized twice in 10% trichloroacetic acid. The protein-free solution was treated as described above for plasma 1-¹³C-phenylalanine. The muscle proteins were hydrolyzed in 6N HCl for 24 h at 110°C. Amino acids were isolated using cation-exchange chromatography (50W-x8 resin, Sigma-Aldrich®, St. Louis, MO). Amino acids were eluted from the resin using 2 ml of 4N NH₄OH. Eluates were evaporated to dryness with nitrogen gas. The heptafluorobutyric, n-propyl derivative of phenylalanine was prepared as described above.

6.2.3.6. Breath sample analysis: The ratio of ¹³CO₂:¹²CO₂ in the breath samples (Appendix 7) was determined using an isotope selective non-dispersive infrared

absorption (NDIR) analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany).

6.2.3.7. Western blot analysis of muscle samples: The abundance of the total and phosphorylated forms of Akt, S6K1, rpS6, and 4E-BP1 in the gluteal muscle homogenates were determined using electrophoresis followed by Western blotting techniques (Chapter III)

6.2.3.8. RNA isolation: Total RNA was isolated from PAXgene™ tubes using the PAXgene™ RNA Blood Kit (Quiagen, Inc., Santa Clarita, CA). Muscle samples that had been stored in RNAlater (Quiagen, Inc., Santa Clarita, CA) were homogenized using the bead beating technique (510) into the RNA-Stat60 solution (Tel- Test, Friendswood, TX). Total RNA was then isolated and quantified from both types of samples as previously described (Chapter IV). Reverse transcription reactions were performed as previously described (Chapter IV).

6.2.3.9. Real time polymerase chain reaction: The mRNA expressions of pro-inflammatory cytokines, IFN- γ , IL-1 β , IL-6 and TNF α , and the anti-inflammatory cytokine IL-10 were measured in cDNA samples using equine specific intron-spanning primer/probe sets (Chapter IV). Differences in RNA isolation and cDNA construction between samples were corrected with the use of an internal control β -glucorindase for each sample ((244, 484); ChapterIV).

6.2.4. Calculations and statistical analysis

6.2.4.1. Fat free mass: Body fat percentage was determined using the following equation:

$$\text{Fat (\%)} = [5.4 \times \text{ultrasonic fat depth in cm}] + 2.47 \text{ (546).}$$

Once determined, body fat percentage was used to calculate fat mass from total body mass:

$$\text{Fat mass (kg)} = \text{Fat (\%)} \times \text{body mass (kg)}.$$

Fat-free mass was determined by difference of body mass and fat mass.

6.2.4.2. Plasma phenylalanine enrichment: Isotope enrichment in mol percent excess was calculated from peak area ratios at isotopic steady state and baseline. The final value for all determinations was corrected using an enrichment calibration curve (Appendix 7).

6.2.4.3. Breath CO₂ enrichment: The δ enrichment value obtained from the NDIR analyzer for each sample was converted to a % enrichment:

$$\text{Enrichment (\%)} = [0.0112372\delta / (0.0112372\delta + 1000)] \times 100\% \text{ ((547); Urschel et al., University of Kentucky, submitted).}$$

Then, total CO₂ production (FCO₂) was calculated based on breath CO₂ enrichment using the following equation:

$$\text{FCO}_2 = i \times [(E_i/E_b) - 1] \text{ (Appendix 7).}$$

Where, i was the rate of isotope administration in $\mu\text{mol/kg/min}$, E_i was the enrichment of the isotope in the solution and E_b was the enrichment of the breath samples at plateau, corrected for baseline enrichment ((540, 542, 547); Urschel et al., University of Kentucky, *submitted*)

6.2.4.4. Whole-body phenylalanine kinetics: The average plasma enrichment at isotopic steady state (plateau) was used to calculate whole-body phenylalanine kinetics. This plateau in phenylalanine enrichment included at least 3 values and was defined as not having a slope statistically different from 0 ($P > 0.05$), determined single linear regression analysis (GraphPad Prism 4; GraphPad Software, La Jolla CA). These plateau enrichment values were then used to calculate the whole-body phenylalanine flux:

$$\text{Flux (Q; } \mu\text{mol/kg/h)} = i \times [(E_i/E_p) - 1] \text{ (Appendix 7),}$$

where I was the rate of isotope infusion (in $\mu\text{mol/kg/h}$), E_i was the enrichment of infused isotope, and E_p was the plateau plasma enrichment. Flux includes the amount of amino acids entering the pool through dietary intake (I), *de novo* synthesis (N), and protein breakdown (B), or leaving the pool through protein synthesis (Z), oxidation (E), or the conversion to other metabolites:

$$Q = I + N B = Z + E + M.$$

In order to account for the ~50% of dietary phenylalanine that is extracted during first-pass splanchnic metabolism in other monogastric species ((548, 549); Urschel et al., University of Kentucky, *submitted*), the amount of phenylalanine entering the plasma free amino acid pool from dietary intake was estimated by multiplying dietary intake by 0.5.

De novo synthesis of phenylalanine does not occur in horses because it is a dietary indispensable amino acid; therefore, phenylalanine released into the free amino acid pool as a result of protein breakdown can be estimated using the following equation:

$$B = Q - I,$$

where I is phenylalanine intake corrected for first-pass splanchnic extraction.

The rate of [1-¹³C]phenylalanine oxidation to ¹³CO₂ (F¹³CO₂) was calculated:

$$F^{13}\text{CO}_2 = \text{FCO}_2 \times E_{\text{CO}_2} \text{ ((550); Urschel et al., University of Kentucky, submitted)}$$

(Appendix 7),

where E_{CO₂} was the average enrichment of the breath samples at isotopic steady state (minimum of 3 points, with a slope not different from zero), corrected for baseline enrichment, during the [1-¹³C]phenylalanine infusion. Then, whole-body phenylalanine oxidation was calculated using the following equation:

$$O = F^{13}\text{CO}_2 \times (1/E_p - 1/E_i) \times 100 \text{ ((550); Urschel et al., University of Kentucky, submitted) (Appendix 7).}$$

Non-oxidative phenylalanine metabolism can be calculated through the difference between phenylalanine flux and oxidation, and this can be used as an indicator of whole-body phenylalanine use for protein synthesis. The major non-CO₂ product of phenylalanine is tyrosine, and since all horses were consuming the same diet, and tyrosine is not generally considered to be a limiting amino acid in any other monogastric species, it was assumed that phenylalanine conversion to tyrosine was minimal.

Therefore, any change in non-oxidative phenylalanine disposal was assumed to signify changes in phenylalanine use for whole-body protein synthesis.

6.2.4.5. Relative quantity of inflammatory cytokines: Changes in mRNA expression were calculated using the $\Delta\Delta\text{CT}$ method (484), with the mean ΔCT averaged for mature horses set as the calibrator for each individual cytokine. Results are expressed as relative quantity (RQ) calculated as $2^{-\Delta\Delta\text{CT}}$ (Chapter IV).

6.2.4.6. Statistics: All data were analyzed using the mixed procedure of SAS Version 9.2 (SAS Institute Inc., Cary, NC), with statistical significance and trends considered if $P < 0.05$ and $0.05 < P < 0.10$, respectively. When the fixed effects were significant, pre-planned comparisons of least squares means were made using the pdiff test. All of the dependent variables were analyzed using an ANOVA with the fixed effect of age and horse nested in age and block as the random subject. Data are presented as means \pm pooled standard error, unless otherwise noted.

6.3. RESULTS

6.3.1. Equine demographics

The two age groups were only statistically different in age. There was no effect of age ($P > 0.05$) on body weight (mature: 469 ± 30 kg; aged 499 ± 30 kg) or fat free mass (mature: 421 ± 26 kg; aged 448 ± 26 kg). The measurement of fat free mass indicates that the mature and aged animals used in this study were moderately lean. α -MSH concentrations were well below (mature: 10.1 ± 1.8 pmol/L; aged: 9.5 ± 1.8 pmol/L) the < 35 pmol/L cut-off value to exclude potentially PPID horses(539).

6.3.2. Plasma insulin, glucose, and amino acids

Plasma insulin, glucose, and amino acid concentrations were measured in the final blood sample of the [1-¹³C] phenylalanine infusion, immediately prior to biopsy. Plasma insulin (P = 0.53; Table 6.2) and glucose (P = 0.43; Table 6.2) concentrations at the time of biopsy were not affected by age. However, there was a significant effect of age on the plasma concentrations of isoleucine (P = 0.05; Table 6.2) and lysine (P = 0.04; Table 6.2), where both were elevated in the aged compared to the mature group. Additionally, there was a trend for elevated plasma concentrations of asparagine (P = 0.08; Table 6.2) and phenylalanine (P = 0.08; Table 6.2) in the aged horses. The remainder of the amino acids were not affected by age (P > 0.05; Table 6.2).

6.3.3. Inflammatory cytokines

Age did not affect mRNA expression of the circulating inflammatory cytokines IL-1 β (P = 0.92; Table 6.3), IL-6 (P = 0.90; Table 6.3), IL-10 (P = 0.20; Table 6.3), IFN γ (P = 0.30; Table 6.3), and TNF α (P = 0.38; Table 6.3). Additionally, there was no effect of age on the mRNA expression of muscle IL-1 β (P = 0.53; Table 6.3), IL-10 (P = 0.23; Table 6.3), IFN γ (P = 0.49; Table 6.3), and TNF α (P = 0.58; Table 6.3). There was limited detection of muscle IL-6 mRNA expression where expression was detected in only 3 mature horses and a single aged animal.

6.3.4. Activation of translation initiation factors

There was no effect of age on the activation of Akt at Ser⁴⁷³ (P = 0.33; Figure 6.1) and Thr³⁰⁸ (P = 0.83; Figure 6.1), rpS6 at Ser^{235/236 & 240/244} (P = 0.48; Figure 6.1), or

4EBP1 at Thr^{37/46} (P = 0.13; Figure 6.1). However, the activation of S6K1 at Thr³⁸⁹ was significantly lower (P = 0.03; Figure 6.1) in the aged compared to the mature horses.

6.3.5. *Whole-body protein synthesis*

There was no effect of age on whole-body CO₂ production (P = 0.51; Table 6.4), phenylalanine flux (P = 0.84; Table 6.4), phenylalanine oxidation (P = 0.15; Table 6.4), phenylalanine release from protein breakdown (P = 0.99; Table 6.4) or non-oxidative phenylalanine disposal (P = 0.48; Table 6.4).

6.3.6. *Muscle phenylalanine enrichments*

There was no effect of age on the free (P = 0.53; mature: 11.1±0.8%; aged: 10.3±0.8%) or protein bound (P = 0.62; mature: 0.3±0.1%; aged: 0.4±0.1%) enrichment of [1-¹³C] phenylalanine in the gluteal muscle. Because we only collected a single biopsy at the end of the isotope infusion period, we could not calculate the change in the protein bound phenylalanine enrichment, which is necessary to calculate muscle protein fractional synthesis rates. However, if we assume that there were no initial differences in muscle bound enrichments of [1-¹³C] phenylalanine between the age groups, the lack of a difference between the ages in both free and protein bound muscle enrichment of [1-¹³C] phenylalanine indicates no difference in muscle protein fractional synthesis rates between the age groups.

6.4. DISCUSSION

To the best of the authors' knowledge this is the first time that whole-body protein kinetics and mTOR signaling have been examined in the aging horse. There were no differences in the whole-body protein kinetics of the mature and aged horses; however the phosphorylation of the downstream mTOR signaling factor, S6K1, was lower in the gluteal muscle of the aged compared to the mature horses following 7.5 hours of steady state feeding.

Numerous studies of the effects of aging in humans have studied aging populations with different demographics than the mature population. Body fat mass and weight were elevated in the aging populations of these previous studies (156, 185, 204); however, in the present study there were no differences in the aged compared to the mature group for any measurement of body mass. Additionally, all of the aged horses in the current study were clinically normal with no signs of PPID, as plasma α -MSH concentrations were within the clinically normal reference range (539). Although aging is typically characterized by low grade chronic inflammation, specifically elevations in the mRNA expression of circulating IL-1, IL-6, and TNF α (483, 493, 495), there were no differences in the mRNA expression of circulating or muscle inflammatory cytokines between the mature and aged horses in the current study. Previous studies (483) in horses have demonstrated age related differences in circulating inflammatory cytokine expression; however the mature population in the previous study (483) was younger (4.5 years old) than the mature group in the current study (11 years old). Furthermore, the aged horses were mobile, had all of their teeth, and were thriving in an outdoor group

housing environment, indicating that the aging population in the current study was in a healthy aging state.

The similar demographics and health status of the mature and aged groups used in the present study may explain the unexpected result of no significant difference in any of the markers of whole-body protein kinetics. It has been previously demonstrated that up to 87% of the variance seen in whole-body protein kinetics can be attributed to fat free mass(151). Further, muscle protein fractional synthesis rates in lean young adult (20 years old) and aged lean men (75 years old) following the co-ingestion of a protein and leucine supplement are not different (209). Leucine oxidation and fractional synthesis rates of sarcoplasmic proteins do not differ between middle aged (52 years old) and aged (77 years old) humans (200). The results from previous studies (200, 201) support the present findings that the narrow age range, and similar physical characteristics of the subjects were likely a cause of no significant difference in phenylalanine kinetics.

Of all of the translation initiation factors studied, S6K1 appears to be the most influenced by aging. The phosphorylation of S6K1 at Thr³⁸⁹ was reduced by 42% in the aged horses compared to the mature horses, which has also been demonstrated in aged (30 mo) and very aged (36 mo) male rodent soleus muscle (551). Additionally, the phosphorylation of S6K1 in response to amino acids and insulin does not increase from the basal state in aging humans (156), or following a meal in aged rats with and without low grade chronic inflammation (493). The significant reduction in the abundance of S6K1 P-Thr³⁸⁹ without an increase in inflammatory cytokines in the aged horse may indicate that the effect of aging on S6K1 occurs through some other mechanism; however, this requires further elucidation.

The effect of aging on S6K1 phosphorylation in the absence of an effect on whole-body protein synthesis may indicate that examining protein synthesis at the whole body level masked the possible changes occurring in skeletal muscle. Although skeletal muscle comprises approximately 50% of body weight, there is only about a 25% contribution to whole-body protein synthesis (198, 199); therefore, without drastic changes at the skeletal muscle level there will not be alterations at the whole-body level or if another tissue decreases its rate of protein synthesis and the muscle increases its rate of protein synthesis then there would be no net effect at the whole-body level. The significant reduction in the abundance of S6K1 P-Thr³⁸⁹, without similar indications of change in muscle fractional synthesis rates or whole-body protein synthesis, requires further elucidation.

No age differences were detected in the activation of Akt, rpS6, and 4EBP1. The lack of an effect of age on Akt, rpS6, and 4EBP1 may be attributed to the narrow age range between the mature and aged horses in the current study, which has also been demonstrated in middle aged and aged rodents (551). Additionally, the lack of elevated circulating and muscle inflammatory cytokines in the aged group compared to the mature group may also explain the absence of an age effect on the activation of Akt, rpS6, and 4EBP1 because inflammatory cytokines stimulate NF- κ B activation which inhibits the phosphorylation of Akt (517). Although NF- κ B was not examined in this study, the lack of an age effect on inflammatory cytokines may explain the absence of an age effect on Akt, rpS6, and 4EBP1 activation.

The experimental protocol used in the current study may have concealed any differences in the activation of translation initiation factors and whole-body protein

synthesis that were possibly present. For 7.5 hours prior to biopsy collection, horses received half of their daily concentrate allocation (0.1% BW) divided into 1/24 portions every 30 minutes. Because the whole diet was primarily hay based (2% BW), each of these small concentrate meals only met 0.5% and 0.37% of the daily CP and digestible energy requirement, respectively. Feeding small meals throughout the isotope infusion procedures may at least partially explain the lack of an age effect on the phosphorylation of Akt, rpS6, and 4EBP1, or whole-body protein synthesis rates as it may not have been a strong enough anabolic stimulus. In mature horses, the consumption of a larger high protein meal (4g/kg) did result in the activation of translation initiation factors in the skeletal muscle of mature horses (2) and it is unknown whether the magnitude of this activation may have been lower in the old horses of the present study. This phenomenon is seen during development when yearlings and two year olds consumed 4 g/kg of a high protein meal the activation of translation initiation factors was greater in the yearling horses compared to two year olds (Chapter V). The plasma glucose (mature: 5.5 ± 0.28 mmol/L, aged: 5.3 ± 0.28 mmol/L), insulin (mature: 5.8 ± 0.8 μ IU/mL; aged: 6.1 ± 0.8 μ IU/mL), and total indispensable amino acid (mature: 543 ± 40 μ mol/L; aged: 670 ± 40 μ mol/L) concentrations at the time of biopsy more closely resembled concentrations of mature horses in the post-absorptive state (glucose: 5.5 mmol/L; insulin: 5 mU/L; total indispensable amino acids: 898 μ mol/L) (2) rather than the postprandial state (glucose: 6.5 mmol/L; insulin: 36 mU/L; total indispensable amino acids: 1245 μ mol/L) (2). Additionally, non-oxidative disposal, the indirect measure of protein synthesis, was less than phenylalanine release from protein breakdown which is characteristic of the post-absorptive state (185). Because the horses were in a physiological state more similar to

the post-absorptive state, then our results are consistent with previous findings that there are no age-related differences in the activation of translation initiation factors or protein synthesis during the post-absorptive state (156, 157, 204). Further research is necessary to determine if age related changes in the activation of translation initiation factors or whole-body protein synthesis exist in horses during a true postprandial state.

Although we did not see differences in whole-body protein synthesis or breakdown, there may have been differences in the molecular markers of muscle protein degradation, which we did not measure. Muscle loss traditionally associated with aging occurs when there is an imbalance in muscle protein synthesis and breakdown, and because there was no difference in the activation of translation initiation factors with the exception of S6K1, which may be enough to reduce skeletal muscle protein synthesis, differences in the molecular markers of muscle protein degradation (muscle-RING-finger protein 1 or forkhead box proteins) may also contribute for the loss of muscle seen with aging. Aging rodents have elevated proteasome content and activity and free ubiquitin and ubiquitylated protein concentrations in skeletal muscle than mature rodents (179). The aged also have higher abundance of forkhead box proteins than the mature counterparts (35). Additionally, ubiquitination rate is greater in the post-absorptive state than the postprandial state in the aged rodent whereas the mature rodents are not affected by physiological state (180). Because our horses were in a state more similar to the post-absorptive state it is possible that our aging may have had elevated molecular markers of muscle protein breakdown. The lack of a difference in body composition between the mature and aging horses in the current study may indicate that over the course of an entire day protein synthesis is equal to protein degradation because our horses were

clinically healthy; however, this requires further investigation. Additionally, further investigation is warranted to determine if aging horses have increased skeletal muscle proteolysis which may account for reduced muscle mass traditionally seen in this population.

The implications of the current findings are that clinically healthy aging horses may not require different management practices than their mature counterparts. Previous research (534) has indicated that aged horses have lowered protein digestibility than mature horses. However, when the same research group repeated the study 12 years later, there was no difference in protein digestibility between the two age groups (535). The authors attributed the differences in the results to the fact that the aged horses in the initial study may have endured intestinal damage during their youth, growing up prior to the use of regular anthelmintics. As a result, the most recent edition of the NRC (336) does not indicate differing requirements between mature and aged horses. Our results show that there are no differences in whole-body protein metabolism, which may imply that the protein requirements do not differ between healthy aging and mature horses; however, further research is needed to specifically determine individual amino acid requirements during aging.

The clinically healthy aging horses in the current study were similar to their mature counterparts; however, the growing aging equine population contains horses with PPID at a rate of 15 to 30% (552). PPID horses are typically insulin resistant, hyperglycemic, have abnormal fat deposits, are susceptible to laminitis, and have elevated cortisol levels and muscle atrophy (552). To the best of the authors' knowledge, there is currently no literature pertaining to the activation of translation initiation factors

or protein synthesis in this population. Although clinically healthy aging horses did not differ from mature horses and thus may not require different management practices, symptoms of the aging horse with PPID may affect Akt and mTOR signaling and whole-body protein metabolism. Protein metabolism in the less healthy aged horse is an area where additional research is warranted.

Aging appears to affect equine skeletal muscle similar to both humans and rodents in that S6K1 activation is lowered, which could impair muscle protein synthesis, although there was not evidence of this in the present study. Additionally, healthy aging horses do not have differences in circulating or muscle inflammatory cytokine mRNA expression compared to older mature counterparts (~13 years old) which may have resulted in similar whole-body protein synthesis rates. Although additional research is needed to determine age-related differences in muscle protein metabolism in response to a larger anabolic stimuli, these findings indicate that healthy older horses have similar whole-body protein metabolism and requirements to the mature horse.

6.5. TABLES

Table 6.1: As-fed nutrient composition of ration balancer pellet and oat diet fed to the horses during the 7.5 hours of steady state feeding prior to muscle biopsy collection

| Nutrient | Ration balancer pellet composition,¹ (mean ± SD) | Oat composition,¹ (mean ± SD values) |
|-----------------------------------|--|--|
| Moisture ^a | 9.5 ± 0.2% | 11.2 ± 0.3% |
| Digestible energy ^a | 2.76 ± 0.01 Mcal/kg | 3.15 ± 0.06 Mcal/kg |
| Crude protein ^a | 17.0 ± 0.3% | 13.4 ± 1.9% |
| Alanine ^b | 0.51 ± 0.03% | 0.35 ± 0.04% |
| Arginine ^b | 0.56 ± 0.03% | 0.49 ± 0.07% |
| Aspartate+asparagine ^b | 0.81 ± 0.04% | 0.58 ± 0.04% |
| Glutamate+glutamine ^b | 1.50 ± 0.06% | 1.43 ± 0.14% |
| Glycine ^b | 0.44 ± 0.02% | 0.35 ± 0.05% |
| Histidine ^b | 0.24 ± 0.01% | 0.16 ± 0.03% |
| Isoleucine ^b | 0.37 ± 0.02% | 0.25 ± 0.04% |
| Leucine ^b | 0.75 ± 0.02% | 0.54 ± 0.08% |
| Lysine ^b | 0.41 ± 0.02% | 0.27 ± 0.04% |
| Methionine ^b | 0.09 ± 0.01% | 0.09 ± 0.01% |

Table 6.1 continued: As-fed nutrient composition of ration balancer pellet and oat diet fed to the horses during the 7.5 hours of steady state feeding prior to muscle biopsy collection

| | | |
|--|--------------|--------------|
| Proline ^b | 0.71 ± 0.04% | 0.40 ± 0.06% |
| Phenylalanine ^b | 0.47 ± 0.02% | 0.37 ± 0.06% |
| Serine ^b | 0.47 ± 0.01% | 0.37 ± 0.05% |
| Threonine ^b | 0.35 ± 0.02% | 0.23 ± 0.05% |
| Tyrosine ^b | 0.29 ± 0.02% | 0.21 ± 0.04% |
| Valine ^b | 0.47 ± 0.03% | 0.32 ± 0.05% |
| Acid detergent fiber ^a | 18.4 ± 1.1% | 11.0 ± 0.9% |
| Neutral detergent fiber ^a | 32.5 ± 2.8% | 23.3 ± 2.2% |
| Non-fiber carbohydrates ^a | 45.1 ± 2.4% | 63.8 ± 2.7% |
| Starch ^a | 29.7 ± 3.0% | 56.0 ± 3.8% |
| Water-soluble carbohydrates ^a | 7.1 ± 0.5% | 4.2 ± 0.8% |
| Crude fat ^a | 6.2 ± 0.2% | 8.4 ± 0.7% |
| Ash ^a | 9.6 ± 0.1% | 3.6 ± 0.9% |
| Calcium ^a | 1.15 ± 0.03% | 0.24 ± 0.20% |
| Phosphorus ^a | 0.64 ± 0.04% | 0.32 ± 0.06% |
| Potassium ^a | 1.81 ± 0.01% | 0.62 ± 0.06% |
| Sodium ^a | 0.33 ± 0.04% | 0.12 ± 0.15% |

Table 6.1 continued: As-fed nutrient composition of ration balancer pellet and oat diet fed to the horses during the 7.5 hours of steady state feeding prior to muscle biopsy collection

| | | |
|-------------------|----------------|----------------|
| Iron ^a | 509 ± 13 mg/kg | 157 ± 47 mg/kg |
| Zinc ^a | 62 ± 5 mg/kg | 35 ± 5 mg/kg |

¹Values represent the amount of the nutrient present as a portion of the total diet on an as-fed basis. Horses received 0.1% of body weight/day of the ration balancer pellet and the oats, individually, which resulted in a total of 0.2% of body weight/day.

^aProximate analysis of the feed was conducted by Dairy One Forage Laboratory (Ithaca, NY).

^bAmino acid content of the feed was determined using high performance liquid chromatography analysis following an acid hydrolysis of the feed sample, as described in Materials and Methods.

Table 6.2: Plasma metabolite concentration at the time of biopsy in mature and aged horses

| Metabolite | Mature | Aged | Pooled SEM |
|----------------------|---------------|-----------------|-------------------|
| Insulin ¹ | 5.8 | 6.1 | 0.8 |
| Glucose ² | 5.5 | 5.3 | 0.28 |
| Alanine ³ | 203 | 218 | 24 |
| Arginine | 69 | 74 | 5.8 |
| Asparagine | 38 | 52 [†] | 4.9 |
| Aspartate | 19 | 14 | 2.4 |
| Citrulline | 83 | 91 | 8.3 |
| Glutamate | 54 | 49 | 6.3 |
| Glutamine | 273 | 306 | 27 |
| Glycine | 352 | 441 | 39 |
| Histidine | 58 | 64 | 2.8 |
| Isoleucine | 33 | 46* | 3.9 |
| Leucine | 68 | 87 | 7.4 |
| Lysine | 69 | 92* | 6.9 |
| Methionine | 26 | 32 | 2.8 |
| Ornithine | 34 | 44 | 4.8 |
| Phenylalanine | 68 | 83 [†] | 5.4 |
| Proline | 90 | 97 | 7.6 |
| Serine | 232 | 288 | 23 |
| Taurine | 21 | 28 | 3.4 |

Table 6.2 continued: Plasma metabolite concentration at the time of biopsy in mature and aged horses

| | | | |
|------------|-----|-----|-----|
| Threonine | 94 | 112 | 11 |
| Tryptophan | 4.7 | 5.3 | 0.4 |
| Tyrosine | 50 | 55 | 5.0 |
| Valine | 121 | 148 | 12 |

¹ Plasma insulin concentrations are reported as least square means in $\mu\text{IU/mL}$.

² Plasma glucose concentrations are reported as least square means in mmol/L .

³ Plasma amino acids concentrations are reported as least square means in $\mu\text{mol/L}$.

*Indicates the aged horse value is significantly ($P < 0.05$) different from the mature horse value.

†Indicates the values in the aged horses showed a trend ($P < 0.10$) to be different from the mature horse values.

Table 6.3: Relative quantities of circulating and gluteal muscle inflammatory cytokines of mature and aged horses¹

| Inflammatory Cytokine | Mature | Aged | Pooled SEM |
|------------------------------|---------------|-------------|-------------------|
| <i>Circulation</i> | | | |
| IFN γ | 1.23 | 1.58 | 0.22 |
| TNF α | 1.11 | 1.35 | 0.18 |
| IL-6 | 1.63 | 1.71 | 0.77 |
| IL-1 β | 1.16 | 1.14 | 0.13 |
| IL-10 | 1.21 | 0.72 | 0.22 |
| <i>Muscle</i> | | | |
| IFN γ | 1.02 | 1.5 | 0.43 |
| TNF α | 0.43 | 0.64 | 0.23 |
| IL-1 β | 2.61 | 5.06 | 2.53 |
| IL-10 | 0.24 | 0.52 | 0.17 |

¹Values are least squares means \pm SEM.

Table 6.4: Whole-body phenylalanine kinetics in mature and aged horses¹.

| | Mature | Aged | Pooled SEM |
|---|---------------|-------------|-------------------|
| <hr/> | | | |
| <i>Phenylalanine flux [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]</i> | | | |
| | 41.5 | 42.2 | 2.3 |
| <i>Carbon dioxide production [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]</i> | | | |
| | 16977 | 15080 | 1410 |
| <i>Phenylalanine entering the free phenylalanine pool [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]²</i> | | | |
| Phenylalanine from dietary intake | 4.0 | 3.9 | 0.08 |
| Phenylalanine from protein breakdown | 37.5 | 37.5 | 2.2 |
| <i>Phenylalanine leaving the free phenylalanine pool [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]²</i> | | | |
| Phenylalanine oxidation | 13.2 | 16.7 | 1.6 |
| Non-oxidative phenylalanine disposal | 28.3 | 25.5 | 2.7 |

¹Values are least squares means \pm SEM.

²The following stochastic model of phenylalanine kinetics was used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal.

6.6. FIGURES

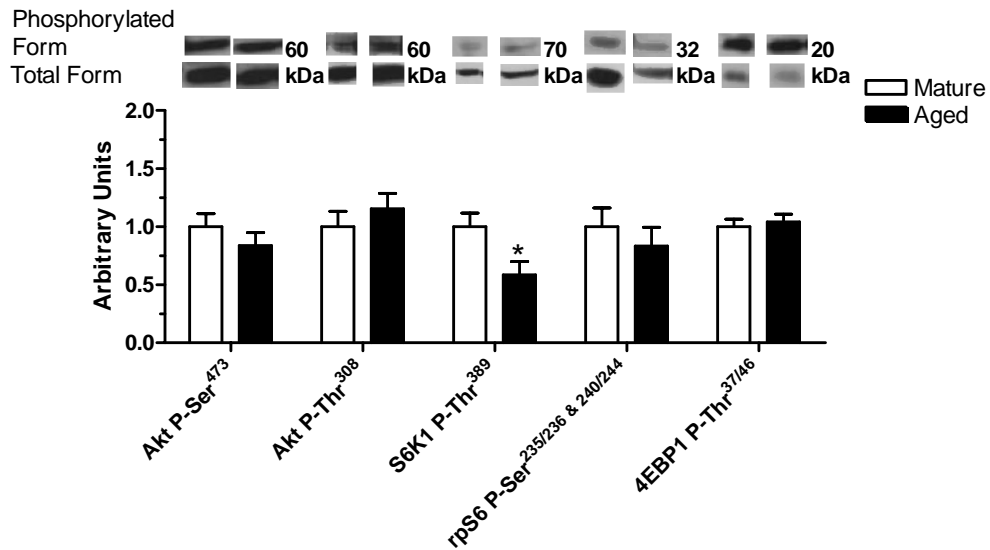


Figure 6.1. Gluteal muscle phosphorylation of Akt at Ser⁴⁷³, Akt at Thr³⁰⁸, S6K1 at Thr³⁸⁹, rpS6 at Ser^{235/236 & 240/244}, and 4EBP1 at Thr^{37/46} in mature and aged horses following 7.5-hours of steady state feeding. The phosphorylated forms of the translation initiation factors was corrected by the respective total form abundance, with the value for mature horses set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per age group. Representative images of the immunoblots are shown above.

*Indicates that, within a translation initiation factor, the value is significantly different ($P < 0.05$) from the mature horse value.

Chapter VII

Whole body protein synthesis is improved following non-steroidal anti-inflammatory drug administration to aging horses

7.1. INTRODUCTION

Aging has been associated with low grade chronic inflammation in humans (495), rodents (493), and horses (483) and is referred to as “inflamm-aging”. Chronic inflammation is characterized by elevated levels of circulating inflammatory cytokines, which have been linked with decreased physical performance and increased incidence of injury (553, 554), as well as with the development of sarcopenia in the elderly (553) and in aged horses (532). Additionally, the population of aging horses has been estimated at 7.6% and in some regions over 20% of all horses (523, 533). Older horses may demonstrate visible signs of aging such as loss of muscle mass and are usually horses over the age of 20 years old (523). Sarcopenia is the involuntary loss of muscle mass and strength, and has been partially attributed to a multitude of factors which include a decline in physical activity and a less than optimal diet (149, 150, 553), and results from a shift in the balance between protein synthesis and breakdown. Sarcopenia has been partly attributed to reduced protein synthesis and activation of translation initiation factors in response to anabolic stimuli such as amino acid administration (149, 155), insulin (165), or exercise (149, 538). Further, systemic inflammation in the aged has also been associated with decreased activation of translation initiation factors in response to various anabolic stimuli (155) and with lower rates of muscle protein synthesis (493);

however, the effects of aging on muscle protein metabolism have not been fully elucidated in the horse.

Protein synthesis is limited by both the abundance and efficiency of ribosomes to translate mRNA into protein and the availability of amino acids to form a protein (128), which is regulated by a series of intracellular signaling cascades associated with the mammalian target of rapamycin (mTOR) pathway. Insulin and insulin like growth factor phosphorylate Akt through the activation of several intermediate signaling proteins (16, 18, 19, 21). Activation of Akt inactivates the mTOR inhibitor tuberous sclerosis complex 2 through phosphorylation (19, 21), allowing for the phosphorylation of mTOR. The phosphorylation of mTOR can also be stimulated by amino acids (45, 46, 497) and exercise (149, 488, 538), through separate Akt-independent mechanisms. Phosphorylated mTOR phosphorylates and activates two downstream signaling proteins: ribosomal S6 Kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein-1 (4EBP1). Phosphorylation of S6K1 results in the activation of ribosomal protein S6 (rpS6) (498), a component of the 40S ribosomal subunit (498). Therefore, the phosphorylation of rpS6 and 4EBP1 result in the activation of the translational equipment, allowing protein synthesis (21, 537).

The decline in the responsiveness of translation initiation factors and subsequent protein synthesis to various anabolic stimuli with aging in rodents (538) and humans (149, 155) has been associated with elevated systemic inflammation (155, 493). In aged rodents with systemic inflammation, the administration of a NSAID was able to increase muscle protein synthesis (335), but to the best of our knowledge, this has not been studied at the whole-body level in any species. We hypothesized that daily NSAID

administration would reduce systemic and muscle inflammation in aged horses and increase whole-body and muscle protein synthesis. The objective of this study was to determine the effects of NSAID administration over 4 weeks on the activation of mTOR signaling and whole-body protein synthesis in aged horses.

7.2. MATERIALS AND METHODS

7.2.1. Animals, housing, and feeding

The University of Kentucky Institutional Animal Care and Use Committee (2009-0562) approved all procedures used in this study. Six mixed breed aged (23.5 ± 2.6 y old; 3 geldings and 3 mares) horses were obtained from the University of Kentucky Veterinary Sciences' Maine Chance Farm. Prior to selection, the pool of candidate horses were screened for plasma α -melanocyte stimulating hormone concentration, a marker of pituitary pars intermedia dysfunction (PPID; Equine Cushing's Disease) (539, 555), and horses below the normal cut-off value of < 35 pmol/L were selected (539, 555). Selected horses were of a moderate body condition (body condition score 5-7, scale 1-9 (489)), were clinically healthy with all of their teeth and the ability to live outdoors in a group housing environment, and on a regular farrier, anthelmintic, dental, and vaccination regiment. Horses were group housed on dry lots with *ad libitum* access to salt and water. Grass hay (mean \pm SD; 0.91 ± 0.02 Mcal/kg DE, $8.2 \pm 0.8\%$ CP; $48.3 \pm 0.3\%$ ADF; $76.5 \pm 1.4\%$ NDF; $2.0 \pm 0.5\%$ crude fat; and $7.0 \pm 0.5\%$ ash) was provided to the group at 2% of body weight per day and omeprazole (Gastroguard® Merial Limited, Duluth, GA) was administered at preventative dose rate of 1.2 mg/horse/d. Horses were brought into 3.7 \times 3.7 m stalls and individually fed the concentrate ration twice daily at

0800 and 1500. The concentrate ration consisted of a 50:50 mixture of a ration balancer pelleted feed (Chapter VI) and oats (Chapter VI) at 0.2% of body weight per day. Horses were adapted to diet, omeprazole administration and housing protocols for 2 weeks prior to the initiation of experimental procedures. During the isotope infusion procedures horses were housed in 3.7×3.7 m stalls bedded with pine shavings with *ad libitum* access to water and salt.

7.2.2. *Experimental procedures*

There were 3 sampling periods before NSAID administration commenced (0 week of NSAID administration), and then following 2 and 4 weeks of NSAID administration. The following sampling procedures which were described in detail (Chapter VI) were repeated during each of the sampling periods. Briefly, at 0800 on day 0, following the two week adaptation, horses were weighed fed the morning concentrate meal at 0.1% of body weight and their individual portion of hay at 2% of body weight per day in individual stalls. Horses remained in stalls throughout the sampling period. At this time a blood sample was collected into PAXgene™ Blood RNA tubes (Quiagen, Inc., Santa Clarita, CA) via jugular vein venipuncture to measure circulating inflammatory cytokines via qRT-PCR analysis, described below. Horses were fed their afternoon concentrate meal at 1500. On d1, horses remained in the individual stalls and received feed as described above.

On day 2, horses were individually fed the morning concentrate meal as described above. Following morning meal consumption, two indwelling jugular vein catheters (14 gauge X 14.0 cm, Abbocath; Abbott Laboratories, North Chicago IL) were placed using

aseptic techniques as previously described (2): one for isotope infusion and one for blood sampling. At this time, fat thickness on the croup near the site of biopsy was ultrasounded in order to calculate percent body fat (481). At 1100 all forage, water and salt were removed, and 2 hours later a baseline blood sample (10 mL) was collected into an evacuated vacutainer (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) containing sodium heparin prior to the bolus intravenous administration of deuterium oxide (Sigma-Aldrich, St. Louis, MO) at 0.15 g/kg BW. Three hours following deuterium oxide infusion a second blood sample was collected, in order to determine changes in total body water, as an indicator of body composition alterations, during the 4 weeks of NSAID administration. Deuterium oxide infusion measurements were performed during the 0 and 4 week sampling period. Salt, water and forage were returned at 1630 with the addition of the evening concentrate meal. The removal of all feed and water was necessary for the measurement of body water using deuterium oxide isotope techniques.

On day 3, whole-body phenylalanine kinetics were measured using primed, constant stable isotope infusions as previously described (Chapter VI). During the whole-body phenylalanine kinetic measurements, horses were fed half of the daily concentrate allocation divided into 24 equal portions, with 1 portion fed every 30 minutes for 7.5 h, with the initial portion provided 1.5 h prior to the start of isotope administration. Each horse received a 14.4 $\mu\text{mol/kg}$ primed dose of [^{13}C] sodium bicarbonate solution (IsotecTM, Miamisburg, OH), followed by a 2-h constant infusion at 12 $\mu\text{mol/kg/h}$ in order to measure total CO_2 production (540). This was followed by a 4-h primed (8.4 $\mu\text{mol/kg}$), constant (6 $\mu\text{mol/kg/h}$) infusion of [$1\text{-}^{13}\text{C}$]phenylalanine solution (IsotecTM, Miamisburg, OH) in order to measure whole-body phenylalanine oxidation and

flux (541). The isotope filled ethylene vinyl acetate bags were attached to a surcingle and connected to the catheter using a primary IV set (Baxter Healthcare Corporation, Deerfield, IL). Isotope was delivered into the catheter using a pressure sensitive, cordless intravenous infusion pump (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc., Loveland, CO) which was also attached to the surcingle on each horse.

Two baseline breath samples were collected prior to the [^{13}C] sodium bicarbonate infusion (-30 min and 0 min), and subsequent breath samples were collected every 30 min throughout the infusion procedures of both [^{13}C] sodium bicarbonate and [1- ^{13}C]phenylalanine for a total of 6.5 h. Breath samples were collected using a modified Equine Aeromask® (Trudell Medical International, London ON, Canada) enabling the collection of air through a 1-way valve into gas impermeable bags (Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany). Immediately following each sample collection, another 1/24 meal was provided. Blood sampling commenced at 90 min into the [^{13}C]sodium bicarbonate infusion, and continued every 30 min until the end of the [1- ^{13}C]phenylalanine infusion. All blood samples were collected into evacuated vacutainers (Vacutainer; Becton-Dickinson, Franklin Lakes NJ) containing sodium heparin. The $t=$ 90 min [^{13}C]sodium bicarbonate and $t=$ 0 min [1- ^{13}C]phenylalanine blood samples were used to measure the background amounts of [1- ^{13}C] phenylalanine in the blood, prior to isotope infusion.

At the end of the [1- ^{13}C]phenylalanine infusion, the infusion pumps were turned off and the horses were led to a set of equine stocks. Horses were lightly sedated with xylazine hydrochloride (0.3mg/kg, IV [100mg/mL]; Butler Animal Health Supply, Dublin, OH), and a gluteal muscle biopsy was collected as previously described (2).

Briefly, the area over the middle gluteal muscle (~100-cm²) was shaved, aseptically prepared, and desensitized with a local anesthetic (12 mL of 2% lidocaine; Butler Animal Health Supply, Dublin, OH). Approximately 500 mg of muscle biopsy specimen was obtained at a depth of approximately 50% (~8 cm; Chapter III; Chapter V) of the depth of the middle gluteal muscle, by use of the percutaneous needle biopsy technique (411). Of this, ~100 mg was processed in preparation for Western blot analysis, as described below, and ~80 mg was stored in *RNAlater* (Quiagen, Inc., Santa Clarita, CA) for qRT-PCR analysis of inflammatory cytokines as described below. The remainder was flash frozen in liquid nitrogen, and stored at -80°C until analysis. Catheters were removed and 1 g of phenylbutazone was administered following muscle biopsy collection. At this time, horses were given the remaining portions of concentrate and their daily allotment of hay as a single meal. Following consumption of the evening meal, horses were returned to the drylot. For the next 4 weeks, 2g/d of phenylbutazone paste (Butler Animal Health Supply, Dublin, OH) was administered to the horses; 1g was given orally prior to each meal. The experimental procedures were repeated following 2 weeks and 4 weeks of phenylbutazone administration. Samples of the grass hay, ration balancer pellet, and oats were collected throughout the experimental period and sent to Dairy One Forage Laboratory (Ithaca, NY) for nutrient analysis.

7.2.3. Sample analysis procedures

7.2.3.1. Blood sample collection and storage: Blood samples collected for qRT-PCR in PAXgene™ Blood RNA tubes (Quiagen, Inc., Santa Clarita, CA) were gradually frozen to -80°C per manufacturer instructions and remained at -80°C until analysis. The

remaining blood samples were immediately centrifuged at 1,500 x g for 10 minutes at 4°C, and aliquots of plasma samples were frozen at -20°C until the time of analysis.

7.2.3.2. Plasma glucose and insulin: Plasma glucose concentrations were assayed enzymatically using a YSI 2700 SELECT™ Biochemistry Analyzer (YSI Inc., Life Sciences, Yellow Springs, Ohio). Plasma insulin concentrations were determined using Coat-A-Coat RIA®kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL).

7.2.3.3. Amino acids: Samples of grass hay, pelleted concentrate, and oats were hydrolyzed for 24 hours at 110°C in 6N HCl. Total amino acid concentrations of the hydrolysates were then measured using reverse-phase HPLC (3.9 x 300 mm PICO-TAG reverse phase column; Waters, Milford MA) of phenylisothiocyanate derivatives as previously described (2). The plasma free amino acid concentrations were also measured using reverse-phase HPLC (3.9 x 300 mm PICO-TAG reverse phase column; Waters, Milford MA) of phenylisothiocyanate derivatives as previously described (2).

7.2.3.4. Plasma phenylalanine enrichment: The isotopic enrichment of phenylalanine (the amount of [1-¹³C]phenylalanine relative to unlabeled phenylalanine) in the plasma samples collected on day 3 was determined by Metabolic Solutions, Inc. (Nashua, NH) using a previously described method (543) which was modified by Matthews, Persola and Campbell (544) (Chapter VI).

7.2.3.5. Muscle phenylalanine enrichment: Approximately 25-30 mg of flash frozen muscle tissue was sent to Metabolic Solutions, Inc. (Nashua, NH) for the determination of mixed-muscle protein and intracellular 1-¹³C-phenylalanine enrichment ((545); Chapter VI).

7.2.3.6. Breath sample analysis: The ratio of $^{13}\text{CO}_2$: $^{12}\text{CO}_2$ in the breath samples was determined using an isotope selective non-dispersive infrared absorption (NDIR) analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany) (Chapter VI).

7.2.3.7. Western blot analysis of muscle samples: The abundance of the total and phosphorylated forms of Akt, S6K1, rpS6, and 4E-BP1 in the gluteal muscle homogenates were determined using electrophoresis followed by Western blotting techniques (Chapter III).

7.2.3.8. Real time polymerase chain reaction. Pro-inflammatory cytokines, IFN- γ , IL-1 β , IL-6 and TNF α , and the anti-inflammatory cytokine IL-10 mRNA expressions were measured in cDNA samples using equine specific intron-spanning primer/probe sets ((244, 484); Chapter IV).

7.2.4. *Calculations and statistical analysis*

7.2.4.1. Fat free mass. Fat free mass was calculated from ultrasonic fat depth as previously described (Chapter VI).

7.2.4.2. Plasma phenylalanine enrichment. Isotope enrichment in mol % excess was calculated from peak area ratios at isotopic steady state and baseline (Chapter VI). The final value for all determinations was corrected using an enrichment calibration curve (Chapter VI).

7.2.4.3. Breath CO₂ enrichment. The equation used to convert the δ enrichment value obtained from the NDIR analyzer for each sample to a % enrichment was described in Chapter VI.

7.2.4.4. Whole-body phenylalanine kinetics. The average plasma enrichment at isotopic steady state (plateau) was used to calculate whole-body phenylalanine kinetics, and was described in Chapter VI.

7.2.4.5. Relative quantity of inflammatory cytokines. Changes in mRNA expression were calculated using the $\Delta\Delta CT$ method ((244, 484); Chapter IV), with the mean ΔCT averaged for all horses during the week 0 sampling period set as the calibrator for each individual cytokine. Results are expressed as relative quantity (RQ) calculated as $2^{-\Delta\Delta CT}$ (Chapter IV).

7.2.4.6. Statistics. All data were analyzed using in the PROC MIXED procedure of SAS Version 9.2 (SAS Institute Inc., Cary, NC), with statistical significance and trends considered if $P < 0.05$ and $0.05 < P < 0.10$, respectively. When the fixed effect was significant, pre-planned comparisons of least squares means were made using the pdiff test. All of the dependent variables were analyzed using repeated measures with the fixed effect of time on NSAID and horse nested in block as the random subject. For all repeated measures analysis, the variance-covariance matrix was chosen for each analysis based on the lowest value for Schwarz's Bayesian Criterion. Data are presented as means \pm pooled standard error unless otherwise noted.

7.3. RESULTS

7.3.1. Equine demographics

All horses remained healthy and maintained their body weight ($P = 0.87$; 0 weeks on NSAID: 499 ± 35 kg; 4 weeks on NSAID: 500 ± 35 kg) throughout the experimental period. Additionally, there was no change in fat free mass calculated from ultrasounded rump fat thickness (481) ($P = 0.75$; 0 weeks on NSAID: 448 ± 30 kg; 4 weeks on NSAID: 451 ± 30 kg). The measurements of fat free mass that these horses maintained approximately 10% body fat throughout the study procedures.

7.3.2. Plasma metabolites

There was no effect of time on NSAID on the plasma insulin ($P = 0.37$; Table 7.1) or glucose ($P = 0.12$; Table 7.1) concentrations at the time of biopsy. Plasma concentrations of Ala, Asp, Glu, Ile, Leu, Lys, and Thr decreased with time on NSAID ($P < 0.05$; Table 7.1) at the time of biopsy. Arg, Gln, and Gly plasma concentrations at the time of biopsy increased with time on NSAID ($P < 0.05$; Table 7.1). Plasma concentrations of Phe and Trp at the time of biopsy decreased at week 2 of NSAID administration, and then returned to baseline concentrations by week 4 of NSAID administration ($P < 0.05$; Table 7.1). There was no effect of time on NSAID ($P > 0.05$) at the time of biopsy on plasma concentrations of Asn, His, Met, Pro, Ser, Tyr, and Val.

7.3.3. Inflammatory cytokines

Time on NSAID did not affect mRNA expression of the circulating inflammatory cytokines: IL-1 β ($P = 0.22$; Figure 7.1), IL-6 ($P = 0.20$; Figure 7.1); IL-10 ($P = 0.42$;

Figure 7.1); IFN γ (P = 0.40; Figure 7.1), and TNF α (P = 0.72; Figure 7.1). The muscle mRNA expression of IL-1 β decreased with time on NSAID (P = 0.02; Figure 7.2).

However, there was no effect of time on NSAID on the mRNA expression of muscle IL-10 (P = 0.86; Figure 7.2), IFN γ (P = 0.15; Figure 7.2), or TNF α (P = 0.15; Figure 7.2).

There was limited detection of muscle IL-6 mRNA expression; where expression was only detected in 1 horse prior to NSAID administration, 2 horses following 2 weeks of NSAID administration, and 1 horse following 4 weeks of NSAID administration.

7.3.4. *Activation of translation initiation factors*

There was no effect of time on NSAID on the activation of Akt at Ser⁴⁷³ (P = 0.14; Figure 7.3) and Thr³⁰⁸ (P = 0.52; Figure 7.3), rpS6 at Ser^{235/236 & 240/244} (P = 0.14; Figure 7.3), or 4EBP1 at Thr^{37/46} (P = 0.53; Figure 7.3). However, the activation of S6K1 at Thr³⁸⁹ showed a trend to increase following 2 weeks of NSAID administration and then returned to baseline following 4 weeks of NSAID administration (P = 0.09; Figure 7.3).

7.3.5. *Whole-body protein synthesis*

Although there was no effect of time on NSAID on whole-body CO₂ production (P = 0.41; Table 7.2), phenylalanine flux (P = 0.55; Table 7.2), phenylalanine oxidation (P = 0.28; Table 7.2), or phenylalanine release from protein breakdown (P = 0.53; Table 7.2), there was an increase in non-oxidative phenylalanine disposal (P = 0.02; Table 7.2). An increase in non-oxidative phenylalanine disposal with time on NSAID is indicative of an increase in phenylalanine used for whole-body protein synthesis with time on NSAID.

7.3.6. *Muscle phenylalanine enrichment*

There was no effect of time on NSAID on free ($P = 0.64$; Table 7.3) or protein bound ($P = 0.18$; Table 7.3) enrichment of $[1-^{13}\text{C}]$ phenylalanine in the gluteal muscle. Because we only collected a single biopsy at the end of the isotope infusion period, we could not measure the changes in $[1-^{13}\text{C}]$ phenylalanine incorporation into protein over time and could not calculate muscle fractional synthesis rates. However, if the assumption is made that there were no initial differences in muscle bound enrichments of $[1-^{13}\text{C}]$ phenylalanine on each sampling day, then no change with time on NSAID at the end of the isotope infusion periods in both free and protein bound fractional muscle enrichment of $[1-^{13}\text{C}]$ phenylalanine implies no effect of NSAID administration on muscle protein fractional synthesis rates.

7.4. DISCUSSION

This is the first time whole-body protein kinetics and mTOR signaling have been examined in the aging horse during NSAID administration. The oral administration of a NSAID, phenylbutazone, at a rate of 2g/day reduced the mRNA expression of IL-1 β in skeletal muscle, increased whole-body protein synthesis, and tended to increase the phosphorylation of S6K1 following 7.5 hours of steady state feeding. The results of the present study indicate that NSAID administration in aged horses may ameliorate the blunted response of protein synthesis to anabolic stimuli that is seen in aging mammals.

The horses used in this study were also used in the companion study (Chapter VI), and were characterized as healthy aging horses. We previously (Chapter VI) determined this group of aging horses to be similar to mature horses in body fat mass and weight,

circulating and muscle inflammatory cytokines, and to be clinically normal with no signs of PPID, as plasma α -MSH concentrations were within the clinically normal reference range (539, 555).

The reduction in the muscle mRNA expression of IL-1 β indicates the effectiveness of the phenylbutazone administration of 2 g per day at reducing inflammation in the current study. We have previously (Chapter IV) demonstrated that this dose of phenylbutazone was effective at preventing the increase in the mRNA expression of IL-1 β in skeletal muscle following the collection of 5 days of repeated biopsies. Collectively, these studies may indicate that IL-1 β mRNA expression in skeletal muscle is responsive to phenylbutazone treatment. However, there was no effect of phenylbutazone administration on the mRNA expression of IFN γ , IL-6, TNF α , and IL-10 in skeletal muscle or IL-1 β , IFN γ , IL-6, TNF α , and IL-10 in circulation in the present study. The lack of an effect of NSAID administration on circulating inflammatory cytokines may be due to the fact that the aged horses (23.5 ± 2.6 y old) used in the present study did not have elevated levels of circulating inflammatory cytokines compared to older (11 ± 2.6 y old) mature horses (Chapter VI). Additionally, this study was designed to determine differences in protein metabolism, where a sample size of 6 is large enough to detect statistical differences (Urschel et al., University of Kentucky, *submitted*; Chapter VI); however, previous studies used a minimum of 8 horses (484) when studying changes in circulating inflammatory cytokines in response to experimental treatments. As a result, there may not have been enough statistical power to detect differences in inflammatory cytokine production in response to NSAID treatment. The extended use (5 months) of oral NSAID administration has been shown to reduce

circulating inflammatory cytokines (IL-1 β , and IL-6), in the aged rodent (335). The reduction in circulating inflammatory cytokines may occur after prolonged NSAID administration, which would explain the lack of an effect of a short term (4 weeks) of oral NSAID administration on circulating inflammatory cytokines in the present study. The studies examining the effect of NSAID administration on muscle inflammatory cytokines in the aged are limited; however, there is an abundance of literature examining the reduction of post-exercise skeletal muscle inflammation by NSAID, where NSAID administration reduced post-exercise inflammatory cytokines (332, 333). It is important to recognize that post-exercise inflammation is an acute inflammatory response, whereas aging is characterized as low grade chronic inflammation; therefore, further research is needed to examine the effects of oral NSAID administration on skeletal muscle inflammatory cytokine production during chronic inflammation. Although phenylbutazone administration has been implicated with the development of gastric ulcers in horses (323, 556) we do not believe there were any adverse effects in the horses in the present study. All horses maintained an appetite, body weight and lean mass throughout the four week study period, which may be partly attributed to the administration of omeprazole for the prevention of gastric ulcers (557). Ultimately, we believe the dose of phenylbutazone in the present study was effective at reducing some of the chronic inflammation associated with aging, because of the reduction of muscle IL-1 β .

Variations in whole-body protein metabolism between mature and aged humans, suggest that the aged population may have a higher protein requirement (202). Within the aged population, presence of low grade inflammation in circulation may be partially

responsible for to the reduced muscle protein fractional synthesis rates in the postprandial state (493). However, both whole-body protein synthesis and muscle protein fractional synthesis do not vary between these age groups during the post-absorptive state (186, 493). In the present study, there was a 77% increase in whole-body non-oxidative phenylalanine disposal, which is an indirect measure of protein synthesis, with the administration of oral NSAID for 4 weeks. This is consistent with the results of a previous study (335), where oral administration of ibuprofen reduced in circulating levels of inflammatory cytokines and increased muscle fractional synthesis rates in aged rodents. Additionally, the present study demonstrated a reduction in the plasma concentrations of the indispensable amino acids, Ile, Leu, Lys, Phe, Thr, and Trp with time on NSAID, which is suggestive of an increase in the incorporation of these amino acids to support the synthesis of new proteins. Together the decreased plasma amino acids and increased whole-body protein synthesis indicates that oral administration of a NSAID may reduce inflammation and increase whole-body protein synthesis. Studying whole-body protein metabolism is a useful tool to examine the subject in its entirety; however, it does not allow researchers to isolate the tissues that are responsible for the alterations seen at the whole-body level. Because skeletal muscle comprises approximately 25% of whole-body protein synthesis (198, 199), it is possible that alterations seen at the whole-body level are due to changes in protein metabolism in skeletal muscle, although additional research is necessary to identify the specific tissues most affected by NSAID administration.

Our original hypothesis was that protein synthesis in the skeletal muscle would increase following NSAID treatment; however, there was only limited evidence that

muscle protein metabolism was altered by NSAID treatment. Of the translation initiation factors studied, S6K1 appears to be the most affected by aging (Chapter VI) and NSAID administration. The phosphorylation of S6K1 at Thr³⁸⁹ tended to be elevated 118% following 2 weeks of NSAID administration and then returned to baseline following 4 weeks of NSAID administration. The increase in the phosphorylation of S6K1 following 2 weeks of NSAID administration coincided with a 59% reduction in skeletal muscle IL-1 β mRNA expression which remained reduced by week 4, and no change in whole-body protein synthesis or breakdown. Oral administration of ibuprofen for 5 months to rodents resulted in a postprandial elevation in muscle protein fractional synthesis rates and reduced circulating inflammatory cytokines, but there were no differences in the activation of any of the mTOR signaling factors studied (335), which is in agreement with the results from the present study. However, there were no differences in muscle protein fractional synthesis rates or mTOR signaling with 5 months of oral administration of ibuprofen to aging rodents in the post-absorptive state (335).

One of the aims of the experimental protocol was to measure whole-body protein kinetics, which necessitated keeping all of the horses in a steady state during the infusion procedures. This was achieved through the consumption of small meals every 30 minutes which consisted of half of their daily concentrate allocation (0.1% BW) divided into 1/24 portions. This feeding protocol placed plasma glucose (0 week: 5.3 ± 0.2 mmol/L; 2 weeks: 5.5 ± 0.2 mmol/L; 4 weeks: 5.1 ± 0.2 mmol/L), insulin (0 week: 6.1 ± 0.8 μ IU/mL; 2 weeks: 10.1 ± 0.8 μ IU/mL; 4 weeks: 7.9 ± 0.8 μ IU/mL), and total indispensable amino acid (0 week: 670 ± 40 μ mol/L; 2 weeks: 607 ± 40 μ mol/L; 4 weeks: 571 ± 40 μ mol/L) concentrations of these horses at the time of biopsy in a

physiological state that more closely resembled a post-absorptive state (glucose: 5.5 mmol/L; insulin: 5 mU/L; total indispensable amino acids: 898 μ mol/L) (2) rather than the postprandial state (glucose: 6.5 mmol/L; insulin: 36 mU/L; total indispensable amino acids: 1245 μ mol/L) (2). In addition, the whole-body protein metabolism data indicated that these horses were in a post-absorptive state, because the indirect measure of protein synthesis, non-oxidative disposal, was less than phenylalanine from protein breakdown (185). Since our horses were in a post-absorptive state, the lack of an effect of NSAID administration on mTOR signaling factors is consistent with previous findings (335).

Because this is the first study to examine the effects of oral administration of a NSAID with the aim of reducing inflammation and examining whole-body protein metabolism, there is ample room for additional research. In the present study, horses received a short course (4 weeks) of oral NSAID, which proved to reduce muscle inflammation and increase whole-body protein synthesis; however, administration of oral NSAID over a longer course may be necessary to examine mechanistic changes in skeletal muscle. Additionally, in the present study horses were examined in a post-absorptive state, and although we saw an increase in whole-body protein synthesis, examining horses in the postprandial state during oral NSAID administration will be useful in determining if these alterations seen at the whole-body level will translate to skeletal muscle.

The use of daily administration of a NSAID for 4 weeks to horses in the present study was a tool to aid in the elucidation of the mechanism behind reductions in protein synthesis with aging. Although the results of the present study show promise, that with reduced inflammation there was an increase in whole-body protein synthesis, it would not

be practical or acceptable to horse owner to advocate the daily administration of an NSAID for an extended period of time to aging horses. Rather, the objectives of the present study were to examine from a mechanistic standpoint whether the reduction in chronic inflammation would have beneficial effects on whole-body and muscle protein metabolism. We chose to use phenylbutazone as our anti-inflammatory agent because of its known anti-inflammatory properties (321) and because it is structurally related to ibuprofen, which has previously been shown to increase rates of muscle protein synthesis in aged rodents (335). This study provides initial insight the potential impact of chronic inflammation on protein metabolism in the aged horse and is the first step towards investigating other means of reducing inflammation in the aged population through alterations in the diet or by other strategies. For example, resveratrol supplemented to the diet has showed promise in reducing inflammation in horses through inhibiting the formation of reactive oxygen species (558). Ultimately, there is a need to examine the diet of the aging equine population and discover practical methods of reducing inflammation which will allow for an increase in whole-body, and hopefully muscle, protein synthesis.

NSAID administration affects aging equine skeletal muscle by tending to elevate S6K1 activation after 2 weeks, which coincided with a reduction in mRNA expression of IL-1 β , and was followed by an increase in whole-body protein synthesis. Although additional research is needed to further elucidate the mechanistic effect that inflammation has on both whole-body and muscle protein fractional synthesis, these findings indicate that management strategies targeted at reducing inflammation result in elevations in whole-body protein synthesis.

7.5. TABLES

Table 7.1: Plasma metabolite concentrations at the time of biopsy before NSAID administration and following 2 and 4 weeks of NSAID administration in aging horses.

| Metabolite | 0 weeks on NSAID | 2 weeks on NSAID | 4 weeks on NSAID | Pooled SEM | p-Value |
|----------------------|---------------------------------|---------------------------------|---------------------------------|-----------------------|----------------|
| Insulin ¹ | 6.1 | 10.1 | 7.9 | 2.0 | 0.37 |
| Glucose ² | 5.3 | 5.5 | 5.1 | 0.2 | 0.12 |
| Alanine ³ | 218 ^a | 170 ^b | 185 ^b | 12 | 0.05 |
| Arginine | 74 ^b | 90 ^a | 88 ^a | 4.9 | 0.007 |
| Asparagine | 52 | 54 | 52 | 5.8 | 0.83 |
| Aspartate | 15 ^a | 11 ^b | 6.7 ^c | 1.2 | 0.002 |
| Glutamate | 49 ^a | 35 ^b | 28 ^c | 2.4 | 0.0001 |
| Glutamine | 306 ^b | 358 ^a | 349 ^a | 9.3 | 0.004 |
| Glycine | 441 ^b | 467 ^b | 520 ^a | 54 | 0.02 |
| Histidine | 64 | 63 | 65 | 3.1 | 0.75 |
| Isoleucine | 46 ^a | 45 ^a | 39 ^b | 2.6 | 0.05 |
| Leucine | 87 ^a | 79 ^{ab} | 70 ^b | 4.3 | 0.01 |
| Lysine | 92 ^a | 86 ^a | 69 ^b | 5.3 | 0.01 |
| Methionine | 32 | 30 | 29 | 1.8 | 0.63 |
| Phenylalanine | 83 ^a | 75 ^b | 80 ^{ab} | 2.2 | 0.05 |
| Proline | 97 | 91 | 86 | 5.7 | 0.24 |
| Serine | 288 | 277 | 281 | 8.3 | 0.66 |

Table 7.1 continued: Plasma metabolite concentration at the time of biopsy before NSAID administration and following 2 and 4 weeks of NSAID administration in aging horses.

| | | | | | |
|------------|-------------------|------------------|------------------|-----|--------|
| Threonine | 112 ^a | 87 ^b | 75 ^c | 5.3 | 0.0001 |
| Tryptophan | 5.3 ^{ab} | 4.3 ^b | 6.2 ^a | 0.4 | 0.03 |
| Tyrosine | 55 | 53 | 53 | 2.1 | 0.63 |
| Valine | 148 | 138 | 138 | 7.1 | 0.16 |

¹ Plasma insulin concentrations are reported as least square means in $\mu\text{IU/mL}$.

² Plasma glucose concentrations are reported as least square means in mmol/L .

³ Plasma amino acids concentrations are reported as least square means in $\mu\text{mol/L}$.

^{abc} Differing letters indicate that values are significantly ($P < 0.05$) different from each other.

Table 7.2: Whole-body phenylalanine kinetics in aged horses over 4 weeks of NSAID administration¹.

| | 0 weeks of NSAID administration | 2 weeks of NSAID administration | 4 weeks of NSAID administration | Pooled SEM | p- value |
|---|--|--|--|-----------------------|---------------------|
| <i>Phenylalanine flux [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]</i> | 42.1 | 47.7 | 44.0 | 3.5 | 0.55 |
| <i>Carbon dioxide production [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]</i> | 15080 | 16658 | 14164 | 1387 | 0.41 |
| <i>Phenylalanine entering the free phenylalanine pool [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]²</i> | | | | | |
| Phenylalanine from dietary intake | 3.9 | 3.9 | 3.8 | 0.06 | 0.53 |
| Phenylalanine from protein breakdown | 37.5 | 43.2 | 39.4 | 3.5 | 0.53 |
| <i>Phenylalanine leaving the free phenylalanine pool [$\mu\text{mol}/(\text{kg}\cdot\text{h})$]²</i> | | | | | |
| Phenylalanine oxidation | 16.7 | 17.3 | 9.4 | 3.9 | 0.28 |
| Non-oxidative phenylalanine disposal | 25.5 ^a | 19.6 ^a | 34.6 ^b | 3.0 | 0.02 |

¹Values are least squares means \pm SEM.

²The following stochastic model of phenylalanine kinetics was used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal

^{ab}Values with different superscript letters are significantly ($P < 0.05$) different.

Table 7.3: Muscle phenylalanine enrichments in aged horses over 4 weeks of NSAID administration¹.

| | 0 weeks of NSAID administration | 2 weeks of NSAID administration | 4 weeks of NSAID administration | Pooled SEM | p-value |
|---|--|--|--|-----------------------|----------------|
| <hr/> | | | | | |
| <i>Muscle free enrichments (%)</i> | 10.1 | 10.4 | 11.2 | 0.8 | 0.64 |
| <i>Muscle protein bound enrichments (%)</i> | 0.41 | 0.56 | 0.50 | 0.08 | 0.18 |

¹Values are least squares means \pm SEM.

7.6. FIGURES

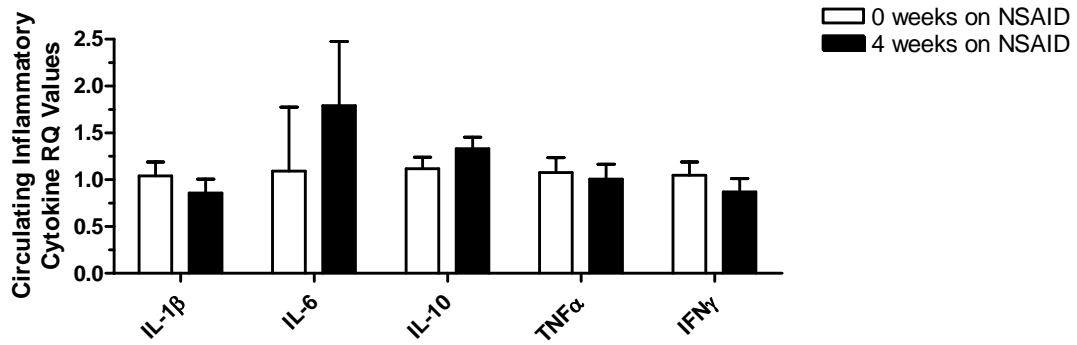


Figure 7.1. Circulating inflammatory cytokines (RQ) in aged horses following 0 weeks, 2 weeks, and 4 weeks of NSAID administration. Values are least square means \pm pooled SE, n=6 per treatment group.

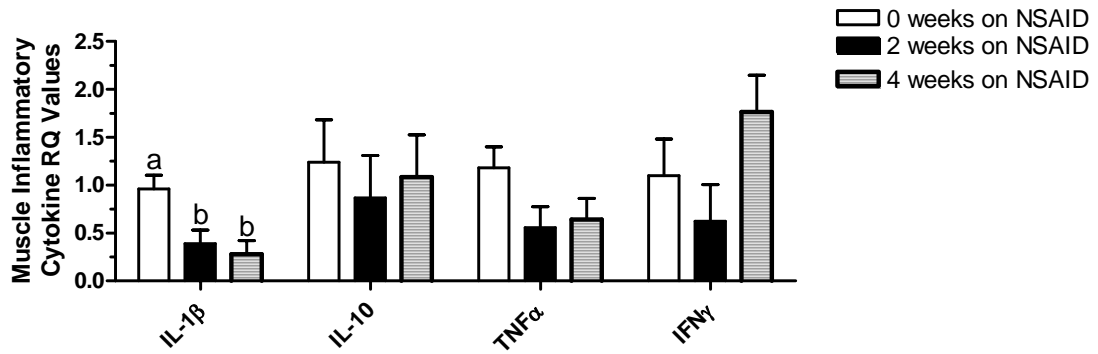


Figure 7.2. Muscle inflammatory cytokines (RQ) in aged horses following 0 weeks, 2 weeks, and 4 weeks of NSAID administration. Values are least square means \pm pooled SE, n=6 per treatment group.

^{ab}Differing letters indicate, within an inflammatory cytokine, the values are significantly different ($P < 0.05$).

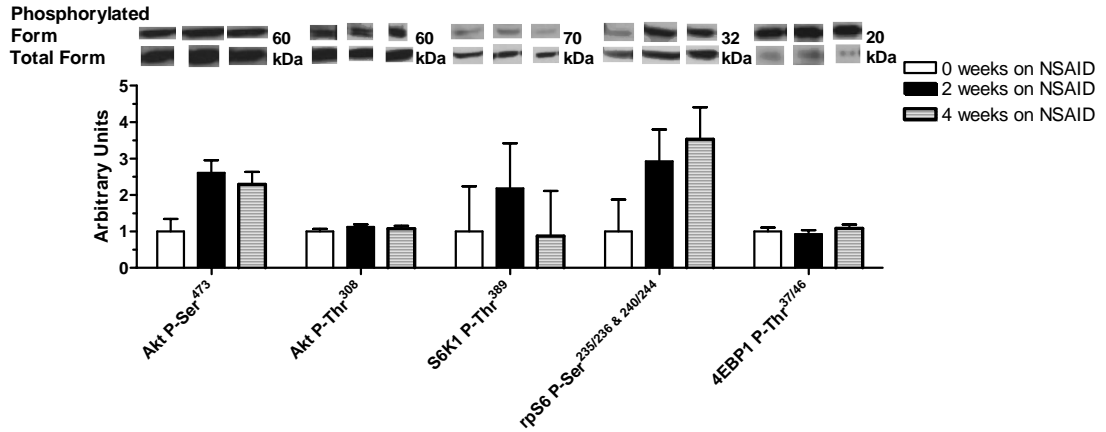


Figure 7.3. Gluteal muscle phosphorylation of Akt at Ser⁴⁷³, Akt at Thr³⁰⁸, S6K1 at Thr³⁸⁹, rpS6 at Ser^{235/236 & 240/244}, and 4EBP1 at Thr^{37/46} in aged horses following 0 weeks, 2 weeks, and 4 weeks of NSAID administration after 7.5-hour of steady state feeding. The phosphorylated forms of the translation initiation factors was corrected by the respective total form abundance, with the value for mature horses set at 1.0 AU. Values are least square means \pm pooled SE, n=6 per treatment group. Representative images of the immunoblots are shown above.

Chapter VIII

Summary, General Discussion and Future Directions

8.1. OPTIMIZING METHODOLOGICAL PROCEDURES FOR ASSESSING mTOR SIGNALING IN THE HORSE

One of the primary objectives of this dissertation was to determine the optimum methodological procedures for assessing mTOR signaling in the horse. Because there has only been a single previous study examining mTOR signaling in horses (2), the optimum methodological procedures have not been assessed. The first study in this dissertation (Chapter III) examined the effect of sampling depth in the gluteus medius muscle on the activation of mTOR signaling in response to feeding and showed that there was no effect of sampling depth (6, 8 or 10 cm below the surface of the skin) on the activation of any of the mTOR signaling factors in response to feeding in mature horses. The focus of the second study in this dissertation (Chapter IV) was to determine the effects of conducting percutaneous gluteal muscle biopsy procedures every 24 hours for 5 days on mTOR signaling in response to feeding in mature horses, both with and without NSAID administration. These results showed that in the absence of NSAID administration, obtaining gluteal muscle biopsies every 24 hours for 5 days resulted in the decreased activation of Akt and increased the activation of the downstream signaling factors (S6K1, rpS6, and 4EBP1) in response to feeding. On the other hand, there was no effect of sampling day on the activation of the downstream mTOR signaling factors in the presence of NSAID administration. A secondary objective this study (Chapter IV) was to determine whether there would be differences in mTOR signaling in response to feeding

in samples collected from the right and left gluteal muscles at the same time. The results showed that there was no difference in mTOR signaling based on the side of gluteal muscle sample. These initial studies in this dissertation were designed to determine optimum sampling methods for assessing mTOR signaling in the horse.

The results of the first two studies in this dissertation suggest that:

1. The activation of mTOR signaling in response to feeding in mature horses is not affected by biopsy sampling depth as long as samples are collected from 6 to 10 cm below the surface of the skin (Chapter III).
2. NSAID administration was able to prevent changes in the activation of downstream mTOR signaling in response to feeding during 5 days of repeated biopsy collection. Therefore, if study design requires repeated biopsy procedures over the course of 24 hours to up to 5 days, NSAID administration is recommended to prevent sampling-related changes in mTOR activation (Chapter IV).
3. mTOR signaling in response to feeding from biopsies collected at the same time from the same site in the right and left side of the gluteal muscle can be compared (Chapter IV).

These studies were only an initial attempt to determine the optimum methodological procedures for assessing mTOR signaling in horses and led to several further directions for method development. Although there was no alteration in mTOR signaling in response to feeding in mature horses when biopsies were collected at depths ranging from 6 to 10 cm below the surface of

the skin (Chapter III), there may have been alterations in mTOR signaling if a wider range of depths had been examined, for example, if gluteal muscle depth ranged from 2 to 12 cm. The rodent data has shown that the phosphorylation of mTOR (491) and S6K1 (559) in response to contractile activity appears greater in Type II than Type I fibers. The proportion of the different fiber types in the gluteal muscle in horses changes with muscle depth, and there is an increase in Type I fibers with gluteal muscle depth in the horse (560). However, the greatest changes in fiber type proportions seem to occur between 2 and 6 cm depth, with a 140% increase in Type I fibers, whereas, changes from 6 to 10 cm in depth resulted in no change in Type I fibers (Chapter III). Therefore, there may be a greater activation of downstream mTOR signaling in response to anabolic stimuli at a shallower depth of 2 cm than at a deeper depth of 12 cm. Not only does mTOR signaling need to be examined at a wider range of depths, but the different types of anabolic stimulus used to activate mTOR signaling must also be examined. Because the anabolic stimuli used in the rodent studies (491, 559) was contraction as opposed to feeding, there may have been differences in the activation of downstream signaling in response to sampling depth if exercise had been used as the anabolic stimuli. Although contraction and feeding both stimulate mTOR signaling, these stimuli act through different upstream factors (Section 1.2). Additionally, in all of the studies in this dissertation muscle biopsies were collected from the gluteus medius muscle due to the role of this muscle in both locomotion and posture and because external landmarks for identifying this muscle have been well described (411). However, differences in

the mitogen activated protein kinase (MAPK) family, a pathway upstream of mTOR signaling (Section 1.2), in response to exercise in mature horses were determined when biopsies were obtained from the vastus lateralis and pectoralis descendens muscles (470). The authors chose the vastus lateralis because of the involvement of this hind limb muscle in forward movement and the pectoralis descendens because of the role in posture, and the MAPK family signaling in response to exercise was greater in the vastus lateralis than the pectoralis descendens (470). Additionally, the response of mTOR signaling to contraction has been reported to vary due to muscle group in rodents, with greatest activity in the tibialis anterior followed by the plantaris and then the soleus (491). The fiber type composition of these muscles in the rodent is tibialis anterior: 3% Type I, 61% Type IIA, 36% Type IIB; plantaris: 7% Type I, 52% Type IIA, 41% Type IIB; and soleus: 83% Type I, 17% Type IIA, 0% Type IIB (561). Therefore, it is necessary to determine the response of mTOR signaling in various muscle groups to both feeding and exercise because the effect of exercise will likely be greater in muscle groups used primarily for locomotion rather than for posture.

Another important factor to consider in optimizing procedures to study mTOR signaling is determining the best time following the anabolic stimuli to obtain the muscle biopsy samples. Previous research (562) has demonstrated that in the gastrocnemius and soleus of neonatal piglets 60 and 90 minutes following feeding, there was no difference in the abundance of the phosphorylated forms of mTOR, 4EBP1, and S6K1. As a result, in the first study to examine mTOR signaling in horses, Urschel and colleagues (2) chose to collect gluteal muscle biopsies 90 minutes following feeding in horses. Their study (2)

determined that there was no change in plasma amino acids, insulin and glucose concentrations from 60 to 80 minutes post-feeding, indicating that any feed induced changes in mTOR signaling due to increased plasma metabolites should not differ between 60 and 80 minutes. Thus, muscle biopsy samples were obtained 60 – 90 minutes following the consumption of a high protein meal in the research described in this dissertation (Chapters III, IV and V). However, in order to confirm that the muscle samples were obtained at the peak of post-stimuli mTOR signaling, it is necessary to determine the time course changes in mTOR signaling in response to feeding in horses.

The second study of this dissertation (Chapter IV) looked at the effects of repeated gluteal muscle biopsies, with biopsies collected every 24 hours for 5 days. This study did not characterize the acute time course changes in mTOR signaling for the first 24 hours following the initial biopsy. Although muscle protein fractional synthesis rates do not change with multiple muscle biopsies collected between 60, 210 and 240 minutes of a 4 hour period (507) or during hourly biopsies over a 6 hour period (508), there has not been a single study to examine the effects of repeated multiple biopsy collection within a 24 hour period on mTOR signaling. However, others (248, 563) have examined the activation of stress related signaling factors including ERK1/2 and MAPK, which influence mTOR signaling (Section 1.2), in response to multiple biopsies. There is an increase in ERK1/2 and MAPK with repeated biopsies collected 30 and 60 minutes following the initial biopsy from the same incision site (564). However, there were no alterations in MAPK signaling when repeated biopsies were collected within 126 minute period from separate incision sites (248). Unfortunately, there has not been a study to examine the time course changes in MAPK signaling following the collection of a muscle

biopsy; however the previous studies (248, 565) have demonstrated that repeated biopsies if collected within 2 hours of the initial biopsy from separate incision sites do not alter stress related MAPK signaling. Thus, mTOR signaling should not be altered through this mechanism; however, additional research is necessary to elucidate this. Additional research is also necessary to examine the collection of repeated biopsies over a 24-72 hour period, because the results of the Chapter IV show a greater increase in the activation of mTOR signaling in response to feeding 24 hours after the collection of the initial biopsy. The first two studies of this dissertation led to many other areas where the knowledge base is lacking and the methods may be improved.

Although the goal of the last three studies (Chapters V – VII) in this dissertation was not to further methodological development, there are methodological lessons that are learned from every study. The goal of the third study in this dissertation (Chapter V) was to determine to activation of mTOR signaling in response to feeding in yearlings, two year olds and mature horses. Although the first study in this dissertation (Chapter III) showed that sampling depth (6 to 10 cm below the surface of the skin) did not alter mTOR signaling in response to feeding in mature horses, there was no way to be sure this would hold true in yearlings and two years olds. Therefore, the decision was made to ultrasound the rump fat and gluteal muscle to determine their depths, so that muscle biopsy collection could be standardized to 50% of the gluteal muscle depth (Chapter V). In this study, we determined that there was considerable variation in subcutaneous fat within and across the age groups (mature horses: 2.07 ± 0.30 cm, 1.7-2.7 cm range; two year olds: 1.29 ± 0.49 cm, 0.9-2.1 cm range; yearlings: 1.05 ± 0.18 cm, 0.9 – 1.4 cm range), which led to 2 additional considerations. First, in the initial study (Chapter III)

biopsies were collected 6, 8, and 10 cm below the surface of the skin; however, if subcutaneous fat in mature horses can vary by a cm, then biopsies were actually being collected 4-5 cm, 6-7 cm, and 8-9 cm within the gluteal muscle for the 6, 8 and 10 cm depths below the surface of the skin, respectively. So this variation in collection sites may have led to the absence of alterations in mTOR related signaling because there is a 170% increase in Type I fibers from 4 to 8 cm (560). The second consideration was that because there are alterations in fiber type with development in horses (410, 446-450), it is unknown whether 50% of the gluteal muscle is optimum depth for collecting muscle biopsies for assessment of differences in mTOR signaling across different ages of horses. Therefore, it is also necessary to study mTOR signaling in response to various anabolic stimuli at a wide range of gluteal muscle biopsy depths throughout development.

Another methodological consideration in this study (Chapter V), is that regardless of age group, all horses were fed 4g/kg of body weight of high protein pelleted feed following an 18 hour period of feed withholding in order to standardize protein intake. This standardization met the protein requirement of a mature sedentary horse; however, the protein requirements of the yearlings and two year olds differ greatly from that of a mature sedentary horse (336). As a result, this meal only supplied 50 and 72% of the daily requirements of the yearlings and two year olds, respectively, and so there was a different magnitude of anabolic stimuli used in each of the three ages of horses studied. This difference in anabolic stimuli may partly explain why we did not see the hypothesized decrease in responsiveness to anabolic stimuli between the adolescent and mature horses (Chapter V). Perhaps rather than examining the response of mTOR signaling to a particular protein intake, it may be more valuable for comparison purposes

to focus on the response of mTOR signaling to a percentage of protein requirements. Because the effects of amino acid concentration on the activation of mTOR signaling is dose dependent (101), a potentially graded response of mTOR signaling may exist with graded protein requirements (50, 75, 100, 150% of protein requirements) and this should be examined in the horse. Overall, from a methodological perspective Chapter V suggests that:

1. When examining mTOR signaling response to feeding across various age groups to standardize feeding by a percentage of protein requirements may be more appropriate than standardizing by absolute protein intake.

Because the samples from the studies described in Chapters VI and VII were analyzed at the same time, the methodological lessons for the assessment of mTOR signaling in the horse are the same for both studies. In these studies mTOR signaling was examined from gluteal muscle biopsies collected following whole-body protein kinetics measurements. In order to maintain study horses at steady state for the measurement of whole-body protein metabolism, horses received half of the daily allocation of concentrate divided into 1/24 portions served every 30 minutes for 7.5 hours. Because the whole diet was primarily hay based (2% body weight), each of these small concentrate meals only met 0.5% and 0.37% of the daily CP and digestible energy requirement, respectively. The feeding protocol used in this study did not result in differences between mature and aged horses in the activation of Akt, rpS6, and 4EBP1, and it is unknown whether this was because there were no differences in mTOR signaling between these ages of horses or because the small meals did not provide sufficient anabolic stimuli to elicit a postprandial mTOR signaling response. This raises an

additional question: at what level of intake (or percent of protein requirements) does mTOR signaling increase from the post-absorptive level? Additionally, it is necessary to determine if mTOR signaling is altered by the consumption of a large meal to a similar extent as same percent of protein requirements offered as small over a period of time in the horse, because feeding a large meal activates mTOR signaling to a greater extent than the consumption of the same amount of nutrients through small meals over a period of time in neonatal pigs (566). Therefore, if feeding is to be used as the anabolic stimulus for activating mTOR signaling then it may be necessary to administer it as a large meal in order for it to be a large enough anabolic stimulus to elicit alterations in mTOR signaling.

Overall, from a methodological perspective Chapters VI and VII suggest that:

1. When examining mTOR signaling response to feeding, the feeding stimuli must be strong enough to elicit changes in mTOR signaling.

The focus of the studies of this dissertation were to examine mTOR signaling; however, in Chapters VI and VII isotopes were infused with the intention of characterizing whole-body protein synthesis and the end point of mTOR signaling, muscle protein synthesis. Although muscle samples were collected following the isotope infusion procedures in the studies described in Chapter VI and VII, because only a single muscle biopsy was obtained, it was not possible to measure how the amount of isotope incorporated in the muscle protein changed over time and so we could not calculate the fractional synthesis rates of muscle protein in the horses studied. Future studies will need to collect muscle biopsies during and following isotope infusions in order to calculate muscle protein fractional synthesis rates.

8.1.1. Summary of the optimum methodological procedures for assessing mTOR signaling in the horse

The studies in this dissertation have 5 lessons that should be considered by future researchers intending to study mTOR signaling in the horse. In mature horses, mTOR signaling in response to feeding from the right or left side of the gluteal muscle biopsies from a depth of 6 to 10 cm below the surface of the skin can be compared; however if these biopsies are repeated then a NSAID should be administered in order to avoid the confounding effect of biopsy. Additionally, when examining mTOR signaling in response to feeding, the meal should be standardized by a percentage of the animal's protein or amino acid requirement and the researcher must ensure that the level of feeding provides a strong enough anabolic stimuli to elicit alterations in mTOR signaling compared to the post-absorptive state.

8.2. ALTERATIONS IN mTOR SIGNALING THROUGHOUT THE LIFESPAN IN HORSES

The second major objective of this dissertation was to examine the alterations in the activation of mTOR signaling throughout the lifespan in the horse. mTOR signaling in response to feeding in various life stages, including the neonatal period, adulthood, and post-adulthood aging, has been characterized in other species. However, there are no studies in any species examining mTOR signaling in the slower growing pre- and post-pubertal adolescent and relatively few studies examining mTOR signaling and protein synthesis in healthy aging animals or humans. Furthermore, the only studies to examine mTOR signaling (2) and protein synthesis (3) in response to anabolic stimuli in the horse

have been in mature horses. As a result, the third study in this dissertation (Chapter V) examined developmental changes in the activation of mTOR signaling in response to feeding, and the fourth and fifth studies (Chapter VI and VII) examined mTOR signaling during post-adulthood aging. The research described in Chapter V showed that there was an increase in mTOR signaling during the postprandial state, compared to the post-absorptive state, in the gluteal muscle of yearling, two year old and mature horses, and the yearlings exhibited a higher degree of sensitivity to anabolic stimuli than the other two ages of horses studied. Specifically, the activation of mTOR signaling factors in yearlings appeared to be more sensitive to insulin than for the two year olds and mature horses, because there was no difference in the activation of Akt between the age groups even though there was an increase in insulin in circulation with development. The results of Chapter VI demonstrated that whole-body protein synthesis and the activation of Akt, rpS6 and 4EBP1 did not differ between mature (11 ± 2.6 year old) and healthy aging (23.5 ± 2.6 year old) horses. If taken together, the results of Chapters V and VI may suggest that post-natal development alters mTOR signaling to a greater extent than aging; however, the magnitude of anabolic stimulus was different in the two studies and therefore an additional study examining all of these age groups in response to the same anabolic stimuli is necessary.

The previous literature examining mTOR signaling throughout the lifespan of other mammalian species has focused on alterations in mTOR signaling during the postprandial state; however, as discussed earlier (Section 8.1), a true postprandial state was not obtained in the horses used for Chapter VI, and therefore, comments on the postprandial changes in mTOR signaling throughout lifespan cannot be made.

Alternatively, the changes in mTOR signaling during the post-absorptive period throughout the lifespan can be commented on. From the results in the post-absorptive state, it appears that mTOR signaling is altered to a greater extent by aging than during development. The evidence of this was shown by the lowered abundance of the phosphorylated form of S6K1 in the aging horses compared to the mature horses in Chapter VI and no difference in the abundance of the phosphorylated form of S6K1 during the post-absorptive state between yearlings, two year olds and mature horses in Chapter V. Examination of mTOR signaling during the post-absorptive period in the neonatal piglet demonstrated no change in mTOR signaling with neonatal development in skeletal muscle (129). Additionally, in humans there is no change in mTOR signaling during the post-absorptive period during post-adulthood aging (157). Muscle protein fractional synthesis rates are also not altered by development (157) or aging (157) during the post-absorptive state. Thus, the results of our studies uniquely indicate that aging in horses may have a greater influence on mTOR signaling during the post-absorptive period than it does during adolescent development. This requires further examination during the post-absorptive state in the equine neonate to determine if mTOR signaling is regulated more during aging than during any other life stage in the horse. Although the results of these studies Chapter V and VI indicate the post-absorptive alteration in mTOR signaling throughout the lifespan, even more valuable information could be obtained by studying the equine neonate, mature and aging horse using similar methodologies to that used in Chapter V in order to study all horses in a postprandial state. Not only is there more available literature to compare postprandial results to, but in the literature the

postprandial state shows differences in the increase in mTOR signaling and subsequently protein synthesis across different ages of research subjects.

As mentioned in the (Section 8.1), in order to accurately compare mTOR signaling in skeletal muscle in response to feeding in different age groups, the meal should meet an equal percentage of protein requirements for these animals (i.e. 100% for each animal). However, during the research described in Chapter V, horses consumed an equal protein intake which met 100, 72, and 50% of the daily requirements of the mature horses, two year olds and yearlings, respectively. Regardless of the varied requirements, the yearlings, who were consuming only 50% of their daily protein intake, had an increase in mTOR signaling. This indicates that if this study were repeated standardizing the meal by meeting 100% of daily protein requirements, the anabolic stimuli may likely be strong enough to elucidate a potential step wise decrease in mTOR signaling with age, with mTOR signaling being the greatest in the yearlings, followed by the two year olds, then the mature horses, and possibly the aged horse. This pattern has been described in neonatal piglets and mature and aging humans (129, 157).

The possible mechanisms of amino acids on mTOR signaling were discussed in detail in Section 1.2.2.3. Briefly, amino acids cause the phosphorylation of S6K1 through mechanisms that are both dependent and independent on mTOR activation, although in both cases amino acids phosphorylate S6K1 independently of Akt. Of all of the amino acids, leucine has the greatest influence of mTOR signaling. Because the greatest postprandial rise in the phosphorylated form of S6K1 occurred in the yearlings compared to the two year olds or the mature horses (Chapter V), the yearlings may have a greater sensitivity to amino acids than the two year olds or mature horses. Therefore, the

results of this study may indicate that younger horses are more sensitive to amino acids, which could be confirmed through examining the responsiveness of mTOR signaling in growing, mature and aging horses to different levels of amino acid, or specifically leucine, intake.

The research described in Chapters V and VI found no developmental (during the postprandial and post-absorptive states) or aging (during the post-absorptive state) effects on the activation of Akt, regardless of the fact that postprandial circulating insulin concentrations increased with age in Chapter V. As previously mentioned, the mature and aging horses in studied in Chapter VI were in a post-absorptive state, and there were no alterations in circulating insulin concentrations due to age during this physiological state. However, insulin sensitivity in regards to carbohydrate metabolism has been demonstrated to decrease with the age of the horse (481). The effects of insulin sensitivity on protein metabolism has been examined in humans, where young humans had a greater increase in muscle protein fractional synthesis rates at the same dose of insulin than older humans (161), and only supraphysiological doses of insulin were able to improve muscle protein fractional synthesis rates and the activation of Akt and mTOR signaling in the aged (159). Thus, the lack of an increase in Akt activation with increased concentration of insulin in the mature horse in Chapter V may be a result of a greater degree of insulin resistance in the mature horses. Therefore, the equal activation of Akt in adolescent horses and mature horses (regardless of physiological state; Chapter V) and mature horses and aging horses (in the post-absorptive state; Chapter VI) indicates that Akt activation is more responsive to insulin in the adolescent horses, or that the Akt activation response is maximized at the lowest plasma concentrations of insulin (yearling

horses). However, the latter is unlikely, because unpublished data by Urschel and colleagues found that mature horses subjected to a wide range of insulin infusion rates (0, 1.2, 3, 6 mU/kg/min), resulting in plasma insulin concentrations ranging from 10 – 1000 mU/L, had a dose dependent increase in Akt activation. This requires further examination across the lifespan with controlled levels of insulin using a hyperinsulinemic-euglycemic isoaminoacidemic clamp technique, which would allow for the determination of the responsiveness of skeletal muscle Akt to administered insulin concentrations, independent of other changes that occur in response to a meal.

In addition to advancing the physiological understanding of how stage of development and aging alter mTOR signaling in response to feeding, the research described Chapters V and VI of this dissertation also have several implications specific to the horse. Based on the results discussed in Chapter V, the mTOR signaling in the muscle of young growing horses was more sensitive to feeding stimuli than 2 year old horses, which coincides with the fact that the greatest rate of average daily gain occurs in the yearlings compared to the 2 year olds (336). This also indicates that the muscle of this age of horse may be more sensitive to other anabolic stimuli such as exercise, although this requires confirmation. A related implication is that if the skeletal muscle of younger growing horses is more responsive than that of older growing horses to anabolic stimuli, then if some factor, such as illness or poor nutrition, impairs growth and muscle accretion during the more responsive younger stages of development, could have lasting effects on muscle mass throughout the rest of the lifespan. Additionally, prolonged periods of inactivity or stall rest may be more deleterious in the mature horse compared to the growing horse, because the muscle lost during this period would be more difficult

to recover due to decreased responsiveness to anabolic stimuli. In order to slow the loss of muscle mass associated with post-adulthood aging, it may be necessary to gain a better understanding of the factors regulating muscle protein metabolism the mature horse first, especially in response to exercise. Reducing both insulin resistance and inflammation will improve protein synthesis in response to feeding; this may reduce the loss of muscle mass associated with post-adulthood aging. A related implication is that if the skeletal muscle of aging horses is less responsive than that of mature horses to anabolic stimuli, then if some factor, such as illness or poor nutrition, increases age associated muscle loss, then muscle recovery will be extremely difficult. Ultimately, more research is needed but the results of the studies in this dissertation provide insight to alternatives in management practices that may improve skeletal muscle accretion and maintenance throughout the lifespan.

8.2.1. Summary of the alterations in mTOR signaling throughout the lifespan in horses

A substantial amount of work is still required to examine the effects of aging throughout the lifespan on mTOR signaling in the skeletal muscle of horses; however, it is also imperative to examine the physiological endpoint of the mTOR signaling pathway which is protein synthesis in the muscle. The studies in this dissertation have provided the initial insight to the alterations in mTOR signaling throughout the lifespan in horses and are the first to examine changes in mTOR signaling during the adolescent phase in any species. These works indicate that in the post-absorptive state, in post-adulthood aging may cause the reduction in mTOR signaling, where there were no differences in post-absorptive mTOR signaling during growth. However, during the postprandial state, it appears that mTOR signaling is more responsive in the faster growing young animals.

These studies open up many possible future directions for additional research, and imply that muscle accretion is more responsive to feeding in growing compared to mature horses.

8.3. THE INFLUENCE OF INFLAMMATION ON mTOR SIGNALING IN HORSES

The final key objective of this dissertation was to examine the influence of inflammation on mTOR signaling horses. Models of acute (Chapter IV) and chronic (Chapter VI and VII) inflammation were studied through the collection of repeated muscle biopsies and aging, respectively. In both series of studies, the administration of a NSAID, phenylbutazone, was administered to determine whether the reduction in inflammation would result in changes in the responsiveness of mTOR signaling. Acute inflammation reduced the activation of upstream mTOR signaling and increased the activation of downstream mTOR signaling in response to meal consumption. However, when a NSAID was administered, inflammation was reduced and the increase in mTOR signaling due to acute inflammation was mediated. However, during chronic inflammation in a relatively post-absorptive state, only the activation of S6K1 was reduced. Because both states of inflammation were studied under different physiological states they cannot be compared in the sense of which type of inflammation alters mTOR signaling to a greater extent, although they can be discussed from a mechanistic perspective. However, future research is warranted to examine the effects of both types of inflammation (acute and chronic) on mTOR signaling under equal physiological states (both postprandial and post-absorptive). For example, aging horses with confirmed low grade chronic inflammation could be studied in an experimental design similar to that of

Chapter IV: this would allow for the examination of the effect of acute inflammatory stimuli in a state of chronic inflammation during the postprandial state on mTOR signaling.

The activation of NF- κ B by inflammatory cytokines was discussed in the inflammatory signaling Section 1.5.1. Briefly, inflammatory cytokines activate I κ KB which phosphorylates NF- κ B. Once activated, NF- κ B inhibits the activation of Akt (Section 1.3). Therefore the reduction in the activation of Akt in response to a meal during acute inflammation may be a result of the increase in inflammatory cytokines stimulating NF- κ B activation. However, the administration of NSAID did not ameliorate this reduction in Akt activation, even though there was a decrease in the mRNA expression of IL-1 β . Because the mRNA expression of TNF- α was not altered in skeletal muscle due to NSAID administration and TNF- α has been demonstrated to influence I κ KB activation to a greater extent than any other inflammatory cytokine, then it is possible that NSAID administration did not alter NF- κ B activity which would explain the absence of an effect of NSAID on Akt activation. Either the NSAID used (phenylbutazone) did not target TNF- α expression or was not a large enough dose (1g every 12 hours) to reduce TNF- α mRNA expression in skeletal muscle. Therefore, the activation of I κ KB may not have been altered and subsequently neither was NF- κ B phosphorylation. Unfortunately, the activation of I κ KB and NF- κ B were not examined, which is needed in order to have a clearer perspective of this pathway. However, acute inflammation increased the activation of downstream signaling factors in response to meal consumption. In horses, MAPK signaling in skeletal muscle (470) and

inflammatory cytokines in circulation and skeletal muscle (244) are increased following exercise. MAPK signaling reduces the phosphorylation of the TSC1/2 complex independent of Akt (Section 1.2), and would be expected to increase mTOR signaling in cases of acute inflammation. The MAPK pathways may be an additional mechanism in which inflammatory cytokines alter mTOR signaling.

During the post-absorptive state, low grade chronic inflammation has not been shown to alter mTOR signaling in humans. However, Chapter VII showed that reducing low grade chronic inflammation by administering 2g/day of phenylbutazone increased the activation of S6K1. This may be due to an increase in MAPK signaling as mentioned above; however, further research is warranted to fully elucidate this mechanism during both the post-absorptive and postprandial states, and during both acute and chronic inflammation.

The effect of NSAID administration on whole-body protein synthesis during low grade chronic inflammation was also examined in Chapter VII. The results demonstrated an increase in non-oxidative phenylalanine disposal, which is an indicator of protein synthesis (Section 1.4.1), following 4 weeks of NSAID administration (2g/day of phenylbutazone). The increase in protein synthesis coincided with a decrease in skeletal muscle mRNA expression of IL-1 β . The examination of the effects of NSAID in reducing low grade chronic inflammation on protein synthesis are limited; however, it has been demonstrated that 5 months of ibuprofen administration to aged rodents reduced IL-6 and -1 β and increased muscle protein fractional synthesis rates during the postprandial state, with no change in muscle protein fractional synthesis rates during the post-absorptive state (567). Thus, if age-related sarcopenia is to be controlled, a

reduction in low grade chronic inflammation may be necessary. It is not practical or healthy to recommend the daily administration of an NSAID, such as phenylbutazone, to horses because of the possible damage to the gastrointestinal tract (323). Therefore, investigations in reducing low grade chronic inflammation through dietary alterations are necessary. Resveratrol may be a possible alternative because supplementation has showed promise in reducing inflammation in horses through inhibiting the formation of reactive oxygen species (558). Ultimately, there is a need to examine the diet of horses with low grade chronic inflammation, most commonly the aged, in order to discover practical methods of reducing inflammation which may allow for increased whole-body and muscle protein synthesis and support the maintenance of muscle mass with advancing age.

The studies in this dissertation did not examine the effects of altered mTOR signaling on satellite cell proliferation during acute and chronic inflammation. This is an area that requires examination because acute inflammation may be beneficial during post-exercise or biopsy recovery. In humans, the reports of the effects of NSAID administration during post-exercise recovery on protein synthesis are mixed due to the NSAID drug administered, the dose of the drug administered, the method of administration, the intensity of the exercise and the time post-exercise that muscle protein fractional synthesis rates were examined. Reports indicate that oral NSAID administration inhibits (568) the increase in muscle protein fractional synthesis rates from pre- to post exercise, and localized NSAID administration does not alter (333) post-exercise increase in the NSAID leg compared to the opposite leg. The increase in inflammatory cytokines following exercise may be necessary for muscle repair post-

exercise (334) because inflammatory cytokines are regulators of satellite cell proliferation (312, 313). However, local NSAID administration reduces satellite cell proliferation (316) without altering muscle protein fractional synthesis rates (333) following exercise. Therefore, it remains to be determined if a connection between mTOR signaling and subsequent protein synthesis and satellite cell proliferation following exercise exists. Additionally, low grade chronic inflammation has been demonstrated to increase apoptosis of satellite cells (174, 281) which may influence the loss of muscle mass seen with aging; however, further research is required to examine this.

In addition to advancing the physiological understanding of how acute and chronic inflammation alter mTOR signaling in response to feeding, the research described in Chapters IV and VII of this dissertation also have several implications specific to the horse. First, the use of NSAID following acute inflammation in the muscle may impair tissue regeneration and protein synthesis, and should be avoided in cases where muscle growth or regeneration is desired. For example, horses in active training, where the goal is to develop muscle needed for a particular discipline, the beneficial effects of the exercise on muscle growth may be counteracted by NSAID administration. The use of NSAID in reducing low grade chronic inflammation in the aged showed that from mechanistic standpoint, the reduction of inflammation may have beneficial effects on whole-body and muscle protein metabolism in horses. However, daily NSAID administration would not likely be a practical part of a management plan for the older horses, rather alternative strategies should be studied in an attempt to mitigate the chronic inflammation that occurs in old horses (483, 484) and control muscle loss in the aged.

8.3.1. Summary of the influence of inflammation on mTOR signaling in horses

Although a substantial amount of work is required to elucidate the mechanisms through which inflammatory cytokines alter mTOR signaling, the studies in this dissertation provide insight into the effects of both acute and chronic inflammation on mTOR signaling. Acute and chronic inflammation increased and decreased downstream mTOR signaling, respectively, under the experimental conditions used in this research. The use of phenylbutazone provided a mechanistic tool to reduce acute and chronic inflammation and indicated that inflammatory cytokines may alter mTOR signaling through Akt dependent and independent mechanisms. Ultimately, acute inflammation may be beneficial for tissue repair, whereas chronic inflammation reduces mTOR signaling and subsequent protein synthesis, likely resulting in a loss of skeletal muscle mass over time.

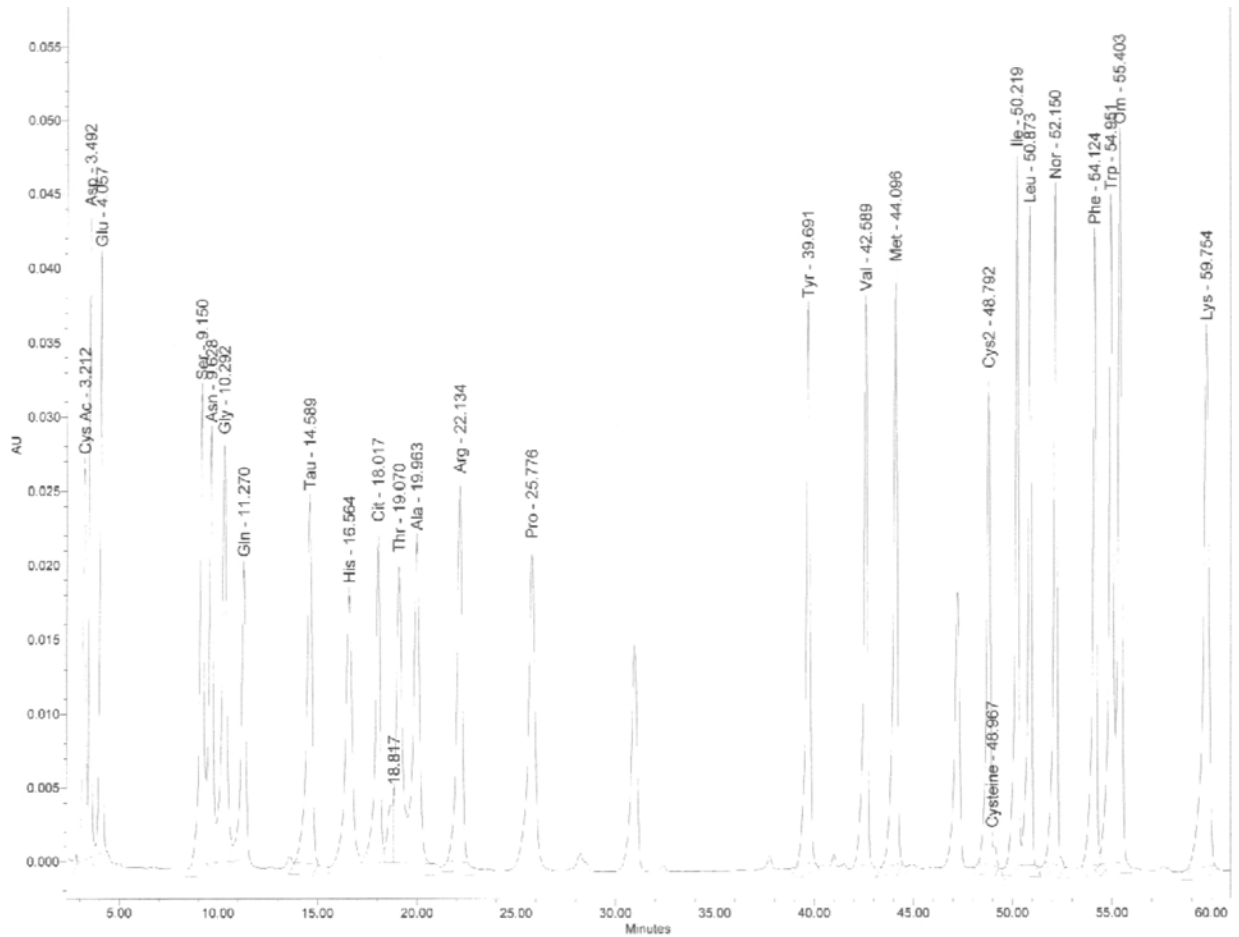
8.4. CONCLUSION

In conclusion, the series of studies described in this dissertation have provided initial knowledge in mTOR signaling in the adolescent and during acute inflammation resulting from repeated biopsies. These studies were also the first examination of mTOR signaling and whole-body protein synthesis in the aged horse. Methodologically, the examination of mTOR signaling in mature horses can be compared from right and left gluteal muscles at a depth of 6 to 10 cm below the surface of the skin, but if biopsies are repeated administration of a NSAID is suggested in order to avoid the confounding effect of biopsy. When examining mTOR signaling in response to feeding stimuli in different ages of horses, the protein intake should be standardized by a percentage of the animal's protein requirement and the researcher must ensure that this is a strong enough anabolic

stimuli to elicit alterations in mTOR signaling. Aging appeared to have a greater influence on mTOR signaling during the post-absorptive state compared to any other life stage; however, mTOR signaling during development appeared to be more responsive to anabolic stimuli than any other life stage. Acute and chronic inflammation altered mTOR signaling differently, where acute inflammation may be increase to mTOR signaling and aid with muscle recovery from the inflammatory challenge. The results from this dissertation are an important contribution to our knowledge of mTOR signaling, regardless of the species studied, but particularly in the horse where the current knowledge about muscle protein metabolism is extremely limited. This dissertation also revealed the many potential areas for future research with regards to mTOR signaling in horses.

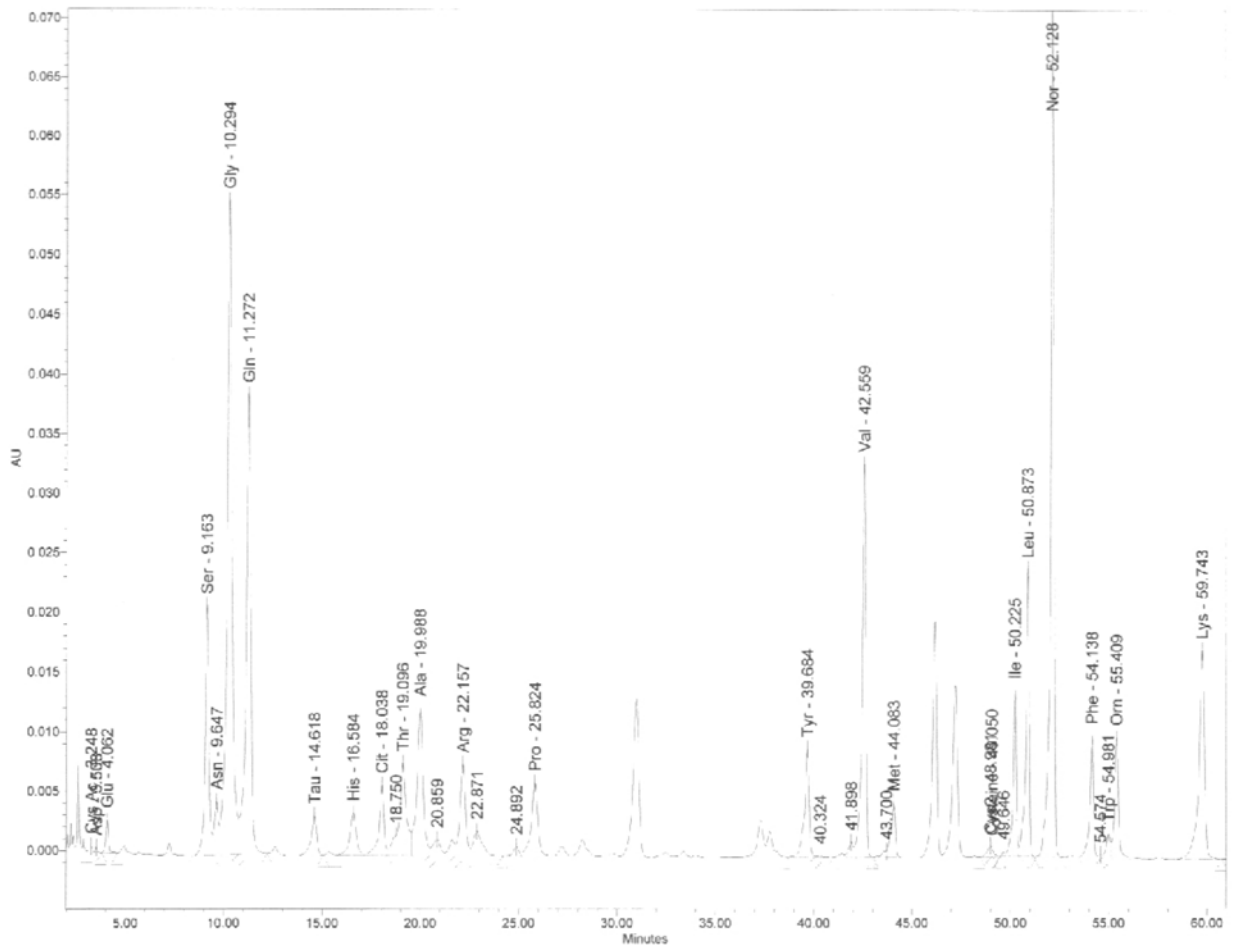
Appendix

A.1. HPLC CHROMATOGRAM EXAMPLES



A.1.1. An example of a physiological free amino acid standard chromatogram. The free amino acid standard was used in the calculations of free plasma amino acid samples and free muscle amino acid samples. The injected standard had a concentration of 0.5 nmol/ μ L of each amino acid and 500 pmol of each amino acid was injected onto the column. Since all of the amino acids had the same concentration, the purpose of running the standard was to determine amino acid differences in peak area relative to Norleucine (the internal standard run with every sample and standard) to correct peak area to correct

sample amino acid peak areas. The interassay C.V. expressed as standard AA relative to Norleucine ranged from as low as $0.07 \pm 0.08\%$ for Asp and as high as $1.42 \pm 0.10\%$ for Lys.

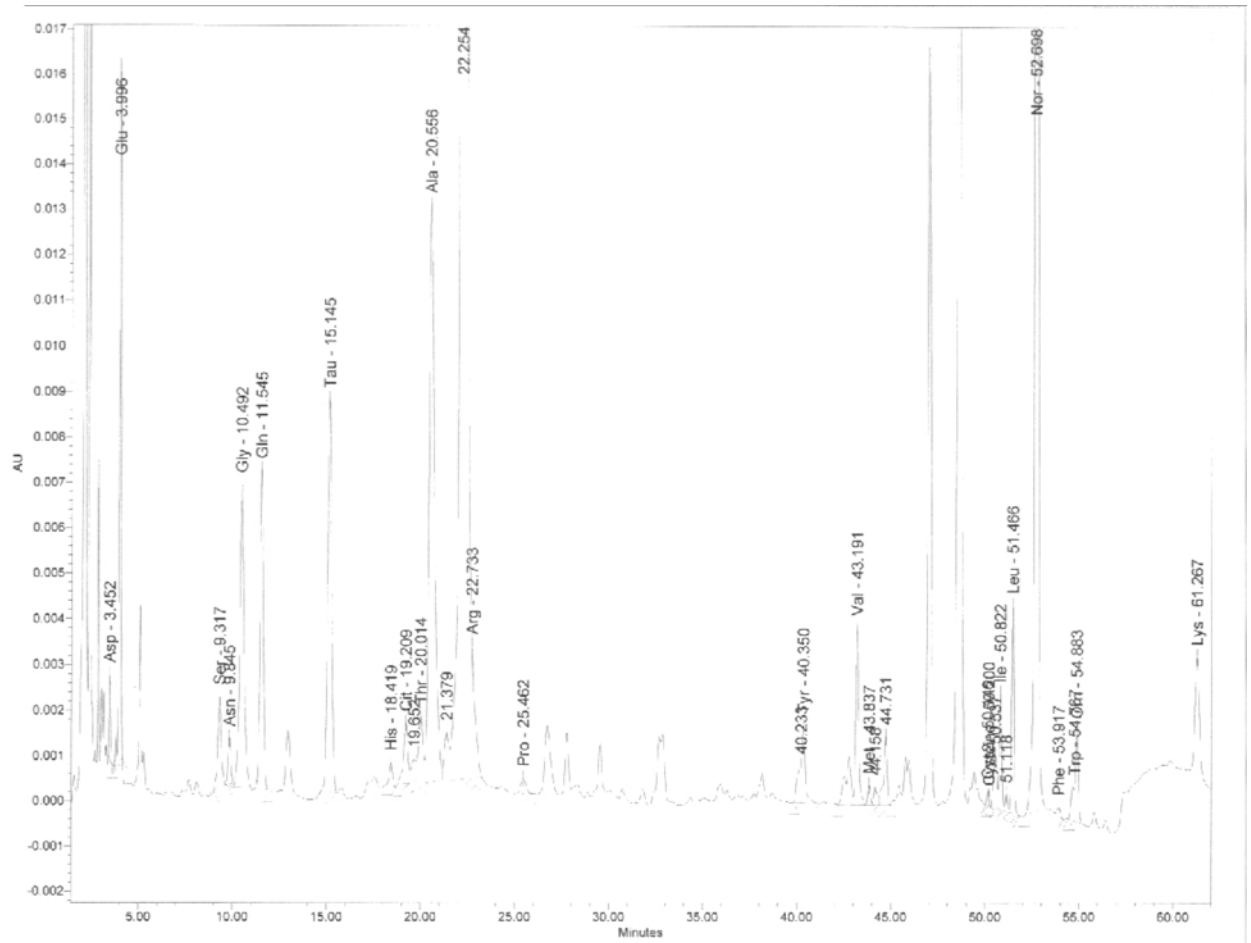


A.1.2. An example of a plasma free amino acid chromatogram. This is an example of a horse from Chapter V at the 15 minute sampling period of the post-absorptive state. The calculation used for determining free plasma amino acid concentrations is as follows:

$$\text{pmol} = [(\text{sample AA peak area})/(\text{standard AA peak area}/\text{standard internal standard peak area})] * (\text{pmol of internal standard injected with sample}/\text{peak area of internal standard in sample})$$

Then pmol must be converted to $\mu\text{mol/L}$ plasma using the following equation:

$\mu\text{mol/L plasma} = (\text{pmol of AA} / \mu\text{L of reconstituted sample injected}) * (\mu\text{L of diluents used to reconstitute derivatized sample} / \mu\text{L of ultrafiltrate that was derivatized}) * \text{dilution of plasma prior to ultrafiltration} * ((10^6 \mu\text{L/L}) / (10^9 \text{ pmol}/\mu\text{mol}))$.



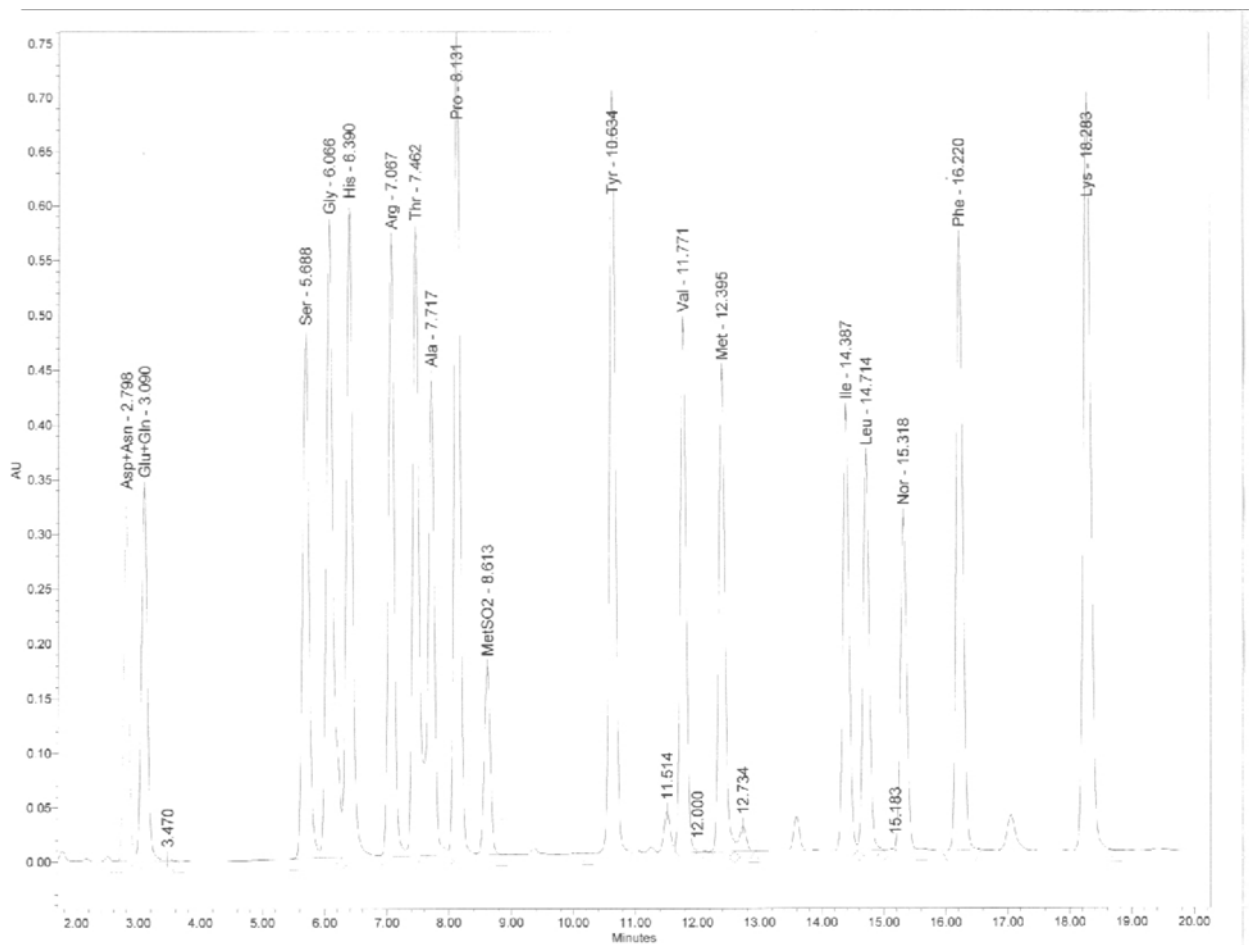
A.1.3. An example of a muscle free amino acid chromatogram. This is an example of

a horse from Chapter III at a sampling depth of 6 cm below the surface of the skin. The calculation used for determining free muscle amino acid concentrations is as follows:

$$\text{pmol} = [(\text{sample AA peak area})/(\text{standard AA peak area}/\text{standard internal standard peak area})] * (\text{pmol of internal standard injected with sample}/\text{peak area of internal standard in sample})$$

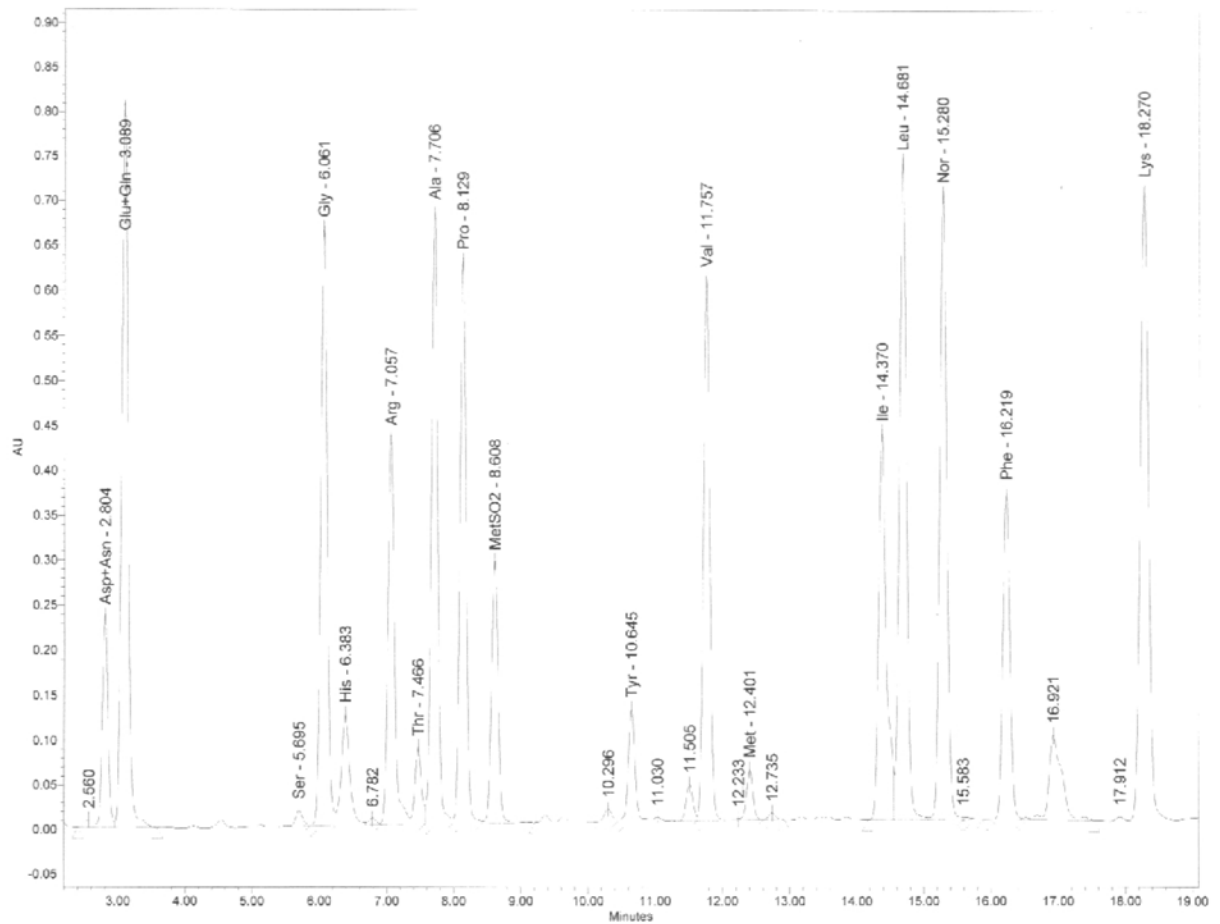
Then pmol must be converted to $\mu\text{mol/g}$ of wet weight muscle using the following equation:

nmol/g of wet weight muscle = (pmol of AA/ μ L of reconstituted sample injected)*(μ L of diluents used to reconstitute derivatized sample/ μ L of ultrafiltrate that was derivatized)* (extraction volume/tissue extracted)*(10^{-9} μ mol/pmol).



A.1.4. An example of a total hydralysate amino acid standard chromatogram. The total hydralysate amino acid standard was used in the calculations of total hydralysate amino acid samples for example feed samples. The injected standard had a concentration of 6.25 nmol/ μ L, and 6250 pmol of each amino acid was injected onto the column. Since all of the amino acids had the same concentration, the purpose of running the standard was to determine amino acid differences in peak area relative to Norleucine (the internal standard run with every sample and standard) to correct peak area to correct sample amino acid peak areas. The interassay C.V. expressed as standard AA relative to

Norleucine ranged from as low as $0.86 \pm 0.05\%$ for Thr and as high as $1.76 \pm 0.08\%$ for Lys.



A.1.5. An example of a total hydrolyzate amino acid chromatogram from a feed sample. This is an example of the total hydrolyzate amino acid chromatogram from the high protein pellet fed in Chapters III – V. The calculation used for determining total hydrolyzate amino acid concentrations is as follows:

$$\text{pmol} = \text{pmol of AA in injected from the standard} * \left[\frac{\text{AA peak area in sample}}{\text{internal standard peak area in the sample} / \text{internal standard peak area in the standard}} \right] / \text{AA peak area in the standard}.$$

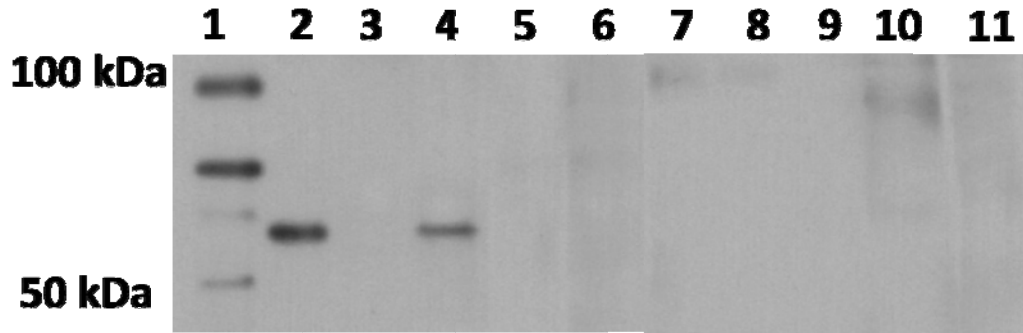
Then pmol must be converted to pmol/g of feed using the following equation:

pmol/g of feed = (pmol of AA in the injected sample/injection volume, μL)*(volume of diluent to reconstitute sample, μL /amount of HCl filtrate derivatized, μL) * (amount of HCl used, mL/feed sample weight, g) * (10^{-3}).

Then to convert pmol of AA/g of feed to g of AA/100g of feed:

g of AA/100g of feed = (pmol of AA/g feed)* ($1 \text{ mol}/10^{12} \text{ pmol}$) * (MW of AA residue in g/mol) *100.

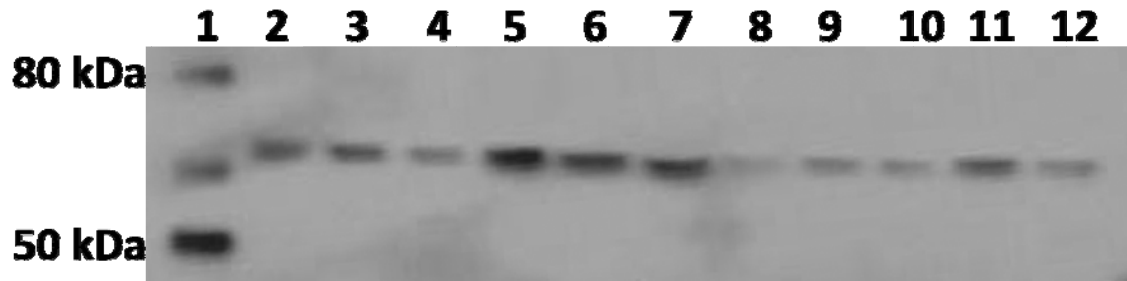
A.2. ANTIBODY VALIDATIONS



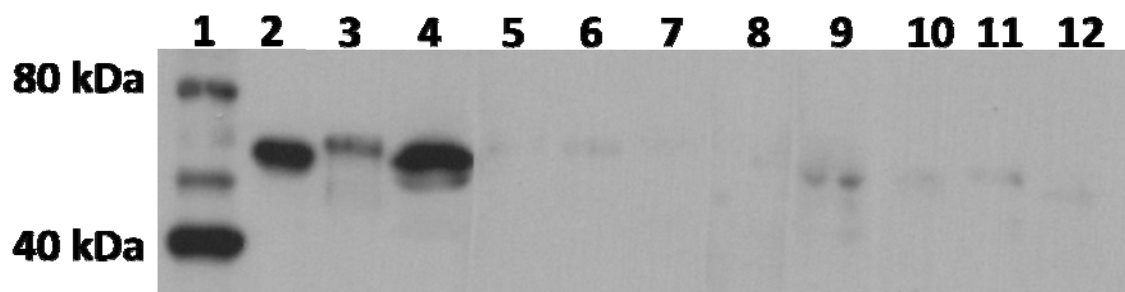
A.2.1a. An example of the P- Akt Ser⁴⁷³ Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the 60 kDa P-Akt Ser⁴⁷³, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 5 and 6, 6 and 7, 9 and 10 and 10 and 11, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit polyclonal antibody that recognized P-Akt Ser⁴⁷³ (Cell Signaling Technology®, Inc., Boston, MA) and prepared as a 1:2000 dilution in 5% fat-free milk solution; the “0% primary antibody” was a 5% fat-free milk solution; the “1:1/16 antibody to antigen mixture” contained 0.00004 µg of antigen; the “1:1/4 antibody to antigen mixture”

contained 0.002 μg of antigen; the “1:1 antibody to antigen mixture” contained 0.2 μg of antigen; and the “1:4 antibody to antigen mixture contained 2 μg of antigen. These mixtures were allowed to incubate at room temperature for 30 minutes, which was the manufacturer’s suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA) (2) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (Akt Control Cell Extracts from Jurkat cells treated with LY294002, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) mature equine gluteal muscle in the postprandial state, (6) mature equine gluteal muscle in the postprandial state, (7) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (8) a negative control (Akt Control Cell Extracts from Jurkat cells treated with LY294002, Cell Signaling Technology ®, Inc., Boston, MA), (9) mature equine gluteal muscle in the postprandial state, (10) mature equine gluteal muscle in the

postprandial state, and (11) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Well 5 was treated with 0% primary antibody. Well 6 was treated with a primary antibody to antigen (P-Akt Ser⁴⁷³ Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1/16. Wells 7-9 were treated with a primary antibody to antigen ratio of 1:1/4. Well 10 was treated with a primary antibody to antigen ratio of 1:1, and well 11 was treated with a primary antibody to antigen ratio of 1:4. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. Additionally, the “0% primary antibody” validated the use of this secondary antibody, because in the absence of a primary antibody there was total inhibition of binding.



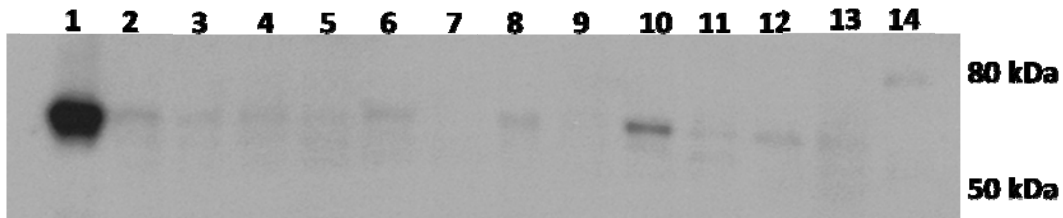
A.2.1b. An example of a P- Akt Ser⁴⁷³ x-ray film from Chapter IV. This is an example of a horse on –NSAID treatment. In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (3) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (12) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right side.



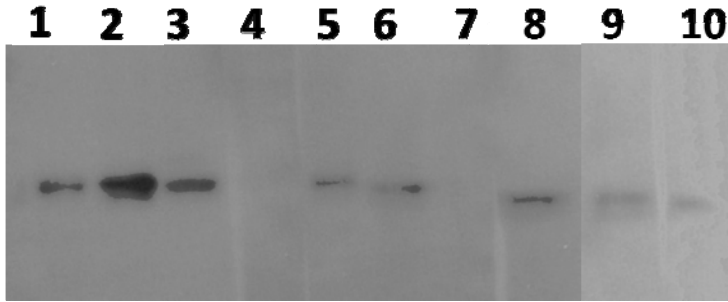
A.2.2a. An example of the P-Akt Thr³⁰⁸ Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the 60 kDa P-Akt Thr³⁰⁸, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9, 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit polyclonal antibody that recognized P-Akt Thr³⁰⁸ (Cell Signaling Technology®, Inc., Boston, MA) and prepared as a 1:2000 dilution in 5% fat-free milk solution; the “1:1/16 antibody to antigen mixture” contained 0.0000000004 µg antigen; the “1:1/4 antibody to antigen mixture” contained 0.000001 µg antigen; the “1:1 antibody to antigen mixture” contained 0.005 µg antigen; and the “1:4 antibody to antigen mixture” contained 5 µg of antigen. These mixtures were allowed to incubate at room temperature for 30 minutes, which was

the manufacturer's suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA) (2) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (Akt Control Cell Extracts from Jurkat cells treated with LY294002, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (Akt Control Cell Extracts from Jurkat cells treated with LY294002, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative control (Akt Control Cell Extracts from Jurkat cells treated with LY294002, Cell Signaling Technology ®, Inc., Boston, MA), and (12) mature

equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Well 5-7 were treated with a primary antibody to antigen (P-Akt Thr³⁰⁸ Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:4. Well 8 was treated with a primary antibody to antigen ratio of 1:1. Well 9 was treated with a primary antibody to antigen ratio of 1:1/4, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/16. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state.



A.2.2b. An example of a P- Akt Thr³⁰⁸ x-ray film from Chapter V. . In the wells of the above figure: (1) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (2) a gluteal muscle biopsy collected from a two year old in the postprandial state, (3) a gluteal muscle biopsy collected from a two year old in the post-absorptive state, (4) a gluteal muscle biopsy collected from a two year old in the postprandial state, (5) a gluteal muscle biopsy collected from a two year old in the post-absorptive state, (6) a gluteal muscle biopsy collected from a two year old in the postprandial state, (7) a gluteal muscle biopsy collected from a two year old in the post-absorptive state, (8) a gluteal muscle biopsy collected from a mature horse in the postprandial state, (9) a gluteal muscle biopsy collected from a mature horse in the post-absorptive state, (10) a gluteal muscle biopsy collected from a mature horse in the postprandial state, (11) a gluteal muscle biopsy collected from a mature horse in the post-absorptive state, (12) a gluteal muscle biopsy collected from a yearling horse in the postprandial state, (13)) a gluteal muscle biopsy collected from a yearling horse in the post-absorptive state, (14) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA).

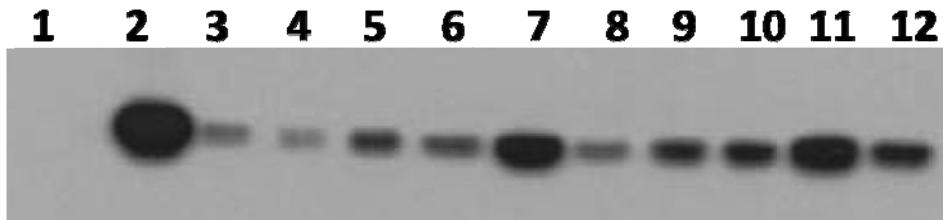


A.2.3a. An example of the Akt Total Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the 60 kDa Akt Total, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 3 and 4, 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit polyclonal antibody that recognized Akt Total (Cell Signaling Technology®, Inc., Boston, MA) and prepared as a 1:2000 dilution in 5% fat-free milk solution; the “0% primary antibody” was a 5% fat-free milk solution; the “1:1/16 antibody to antigen mixture” contained 0.0000000004 μg antigen; the “1:1/4 antibody to antigen mixture” contained 0.000001 μg antigen; the “1:1 antibody to antigen mixture” contained 0.005 μg

antigen; and the “1:4 antibody to antigen mixture” contained 5 μ g of antigen. These mixtures were allowed to incubate at room temperature for 30 minutes, which was the manufacturer’s suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a negative control (Akt Control Cell Extracts from Jurkat cells treated with LY294002, Cell Signaling Technology ®, Inc., Boston, MA), (2) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (3) mature equine gluteal muscle in the postprandial state, (4) mature equine gluteal muscle in the postprandial state, (5) mature equine gluteal muscle in the postprandial state, (6) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology ®, Inc., Boston, MA), (7) a negative control (Akt Control Cell Extracts from Jurkat cells treated with LY294002, Cell Signaling Technology ®, Inc., Boston, MA), (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, and (10) mature equine gluteal muscle in the postprandial state. Wells 1-3 were treated with 100% primary antibody. Well 4 was

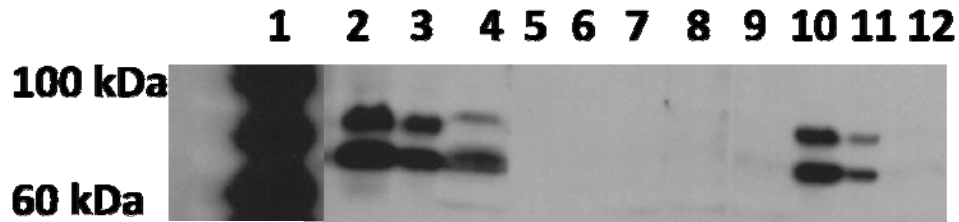
treated with 0% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (Akt pan Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:4. Well 8 was treated with a primary antibody to antigen ratio of 1:1. Well 9 was treated with a primary antibody to antigen ratio of 1:1/4, and well 10 was treated with a primary antibody to antigen ratio of 1:1/16. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state.

However, it is important to note that the presence of Akt total in the negative control was expected. Because this protein is ubiquitous, the negative controls serve a greater purpose in the validation of the phosphorylated forms. Additionally, the “0% primary antibody” validated the use of this secondary antibody, because in the absence of a primary antibody there was total inhibition of binding.



A.2.3b. An example of an Akt Total x-ray film from Chapter IV. This is an x-ray of the same membrane that is shown in A.2.1b. after it has been stripped and re-probed with Akt Total primary antibody. In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (Akt Control Cell Extracts from Jurkat cells treated with Calyculin A, Cell Signaling Technology®, Inc., Boston, MA), (3) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (12) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right

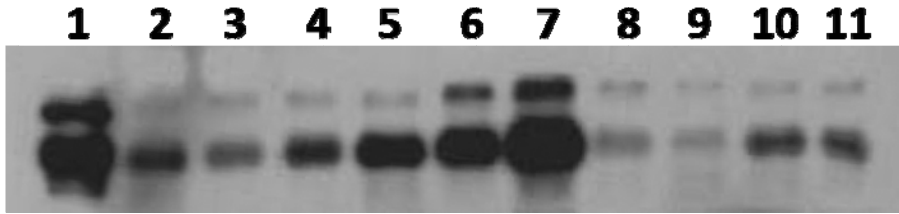
side. The abundance of this protein is reported as a ratio of the phosphorylated to total form. The positive control was used to account for interassay variability. The C.V. of the phosphorylated to total ratio of the positive control for P-Akt Ser⁴⁷³ and P-Akt Thr³⁰⁸ are 0.22 ± 0.13 and 0.12 ± 0.12 , respectively. The right to left side phosphorylated to total ratio of for P-Akt Ser⁴⁷³ was 0.16 ± 0.12 . P-Akt Thr³⁰⁸ was not examined in Chapter IV. These were from Chapter IV for P-Akt Ser⁴⁷³ and Chapter V for P-Akt Thr³⁰⁸. In Chapter IV side was tested in the statistical model and found to not be significant ($P < 0.05$).



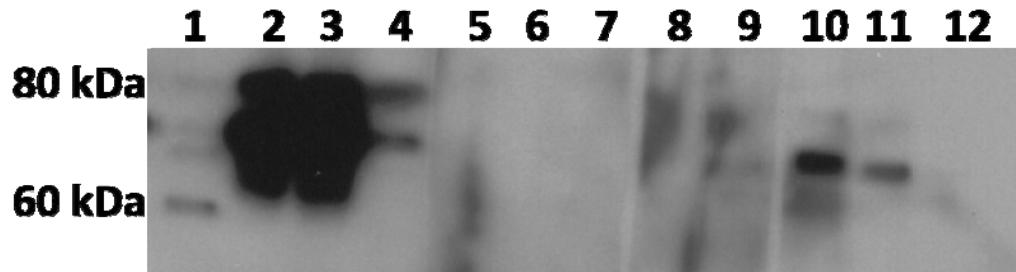
A.2.4a. An example of the P-S6K1 Thr³⁸⁹ Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the P-p70 kDa S6K1 Thr³⁸⁹, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit polyclonal antibody that recognized P-S6K1 Thr³⁸⁹, which is present in two forms the 85 kDa and 70 kDa our interest is in the 70 kDa S6K1 (Cell Signaling Technology®, Inc., Boston, MA), and prepared as a 1:500 dilution in 5% fat-free milk solution; the “1:1/32 antibody to antigen mixture” contained 9.77×10^{-22} μg antigen; the “1:1/16 antibody to antigen mixture” contained 3.9×10^{-18} μg antigen; the “1:1/4 antibody to antigen mixture” contained 1.56×10^{-14} μg antigen; and the “1:1 antibody to antigen mixture” contained

0.002 µg of antigen. These mixtures were allowed to incubate at room temperature for 30 minutes, which was the manufacturer's suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative

control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), and (12) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (P-S6K1 Thr³⁸⁹ Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1. Well 8 was treated with a primary antibody to antigen ratio of 1:1/4. Well 9 was treated with a primary antibody to antigen ratio of 1:1/16, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/32. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. However, it is important to note that the presence of both the 85 kDa and 70 kDa S6K1 total in the negative and positive controls was expected. The use of the molecular weight marker allowed us to determine the 70 kDa from the 85 kDa S6K1 bands, our interest was in the 70 kDa S6K1. Because S6K1 is phosphorylated following mTORC1 activation, which can be activated by both insulin and amino acids, the negative controls were untreated with insulin but remained in a media that contained amino acids resulting in a diminished response than the positive controls.



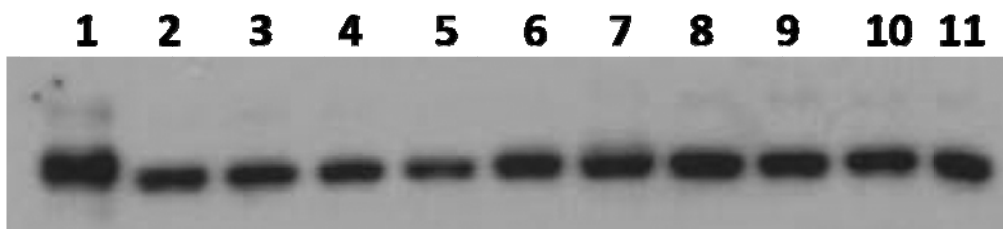
A.2.4b. An example of a P-S6K1 Thr³⁸⁹ x-ray film from Chapter IV. This is an example of a horse on –NSAID treatment. In the wells of the above figure: (1) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (2) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (3) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right side.



A.2.5a. An example of the S6K1 Total Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the 70 kDa S6K1 Total, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit polyclonal antibody that recognized S6K1 Total, which is present in two forms the 85 kDa and 70 kDa our interest is in the 70 kDa S6K1 (Cell Signaling Technology®, Inc., Boston, MA), and prepared as a 1:1,000 dilution in 5% fat-free milk solution; the “1:1/32 antibody to antigen mixture” contained 9.77×10^{-22} μg antigen; the “1:1/16 antibody to antigen mixture” contained 3.9×10^{-18} μg antigen; the “1:1/4 antibody to antigen mixture” contained 1.56×10^{-14} μg antigen; and the “1:1 antibody to antigen mixture” contained

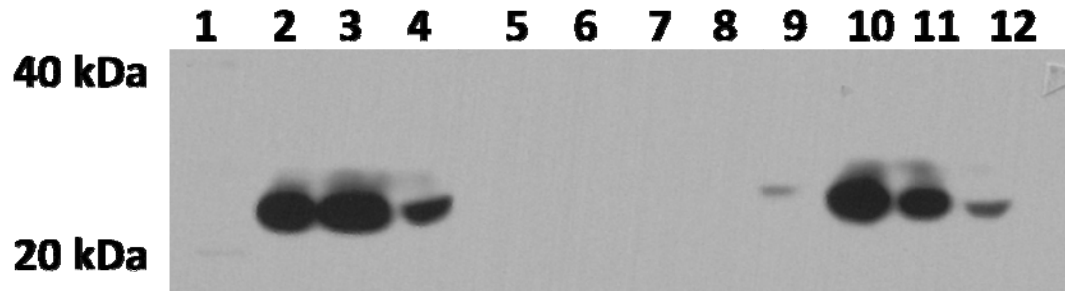
0.002 µg of antigen. These mixtures were allowed to incubate at room temperature for 30 minutes, which was the manufacturer's suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative

control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), and (12) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (S6K1 Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1. Well 8 was treated with a primary antibody to antigen ratio of 1:1/4. Well 9 was treated with a primary antibody to antigen ratio of 1:1/16, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/32. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. However, it is important to note that the presence of both the 85 kDa and 70 kDa S6K1 total in the negative and positive controls was expected. The use of the molecular weight marker allowed us to determine the 70 kDa from the 85 kDa S6K1 bands, our interest was in the 70 kDa S6K1. Because this protein is ubiquitous, the negative controls serve a greater purpose in the validation of the phosphorylated forms.



A.2.5b. An example of an S6K1 Total x-ray film from Chapter IV. This is an x-ray of the same membrane that is shown in A.2.4b. after it has been stripped and re-probed with S6K1 Total primary antibody. In the wells of the above figure: (1) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology®, Inc., Boston, MA), (2) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (3) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right side. The abundance of this protein is reported as a

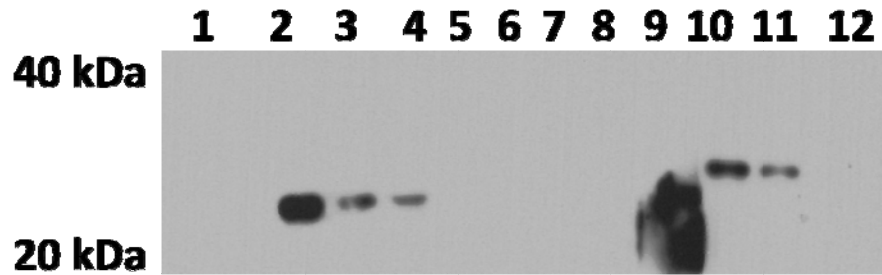
ratio of the phosphorylated to total form. The positive control was used to account for interassay variability. The C.V. of the phosphorylated to total ratio of the positive control for P-S6K1 Thr³⁸⁹ was 0.14 ± 0.13 . The right to left side phosphorylated to total ratio of for P-S6K1 Thr³⁸⁹ was 0.18 ± 0.16 . These were obtained from Chapter IV. In Chapter IV side was tested in the statistical model and found to not be significant ($P < 0.05$).



A.2.6a. An example of the P-rpS6 Ser^{235/236} Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the P-rpS6 Ser^{235/236}, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit polyclonal antibody that recognized P-rpS6 Ser^{235/236} (Cell Signaling Technology®, Inc., Boston, MA), and prepared as a 1:2,000 dilution in 5% fat-free milk solution; the “1:1/32 antibody to antigen mixture” contained 4.88×10^{-22} μg antigen; the “1:1/16 antibody to antigen mixture” contained 1.95×10^{-18} μg antigen; the “1:1/4 antibody to antigen mixture” contained 7.8×10^{-15} μg antigen; and the “1:1 antibody to antigen mixture” contained 0.001 μg of antigen. These mixtures were allowed to incubate at room

temperature for 30 minutes, which was the manufacturer's suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling

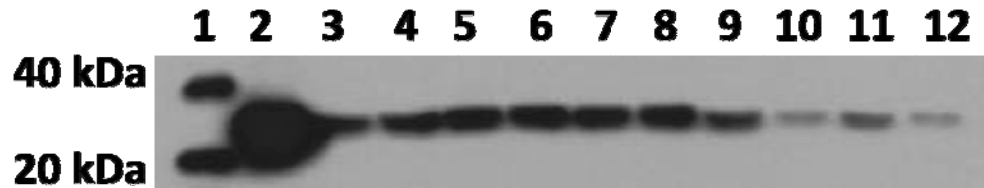
Technology ®, Inc., Boston, MA), and (12) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (P-rpS6 Ser^{235/236} Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1. Well 8 was treated with a primary antibody to antigen ratio of 1:1/4. Well 9 was treated with a primary antibody to antigen ratio of 1:1/16, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/32. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. However, it is important to note that the presence P-rpS6-Ser^{235/236} in the negative and positive controls was expected, because rpS6 is phosphorylated following S6K1 phosphorylation which is activated by the active form of mTORC1. mTORC1 is activated due to both insulin and amino acids, the negative controls were untreated with insulin and the positive controls were treated with insulin, but both were in media containing amino acids. As a result, P-rpS6 Ser^{235/236} is present in the negative control, but diminished in comparison to the positive control.



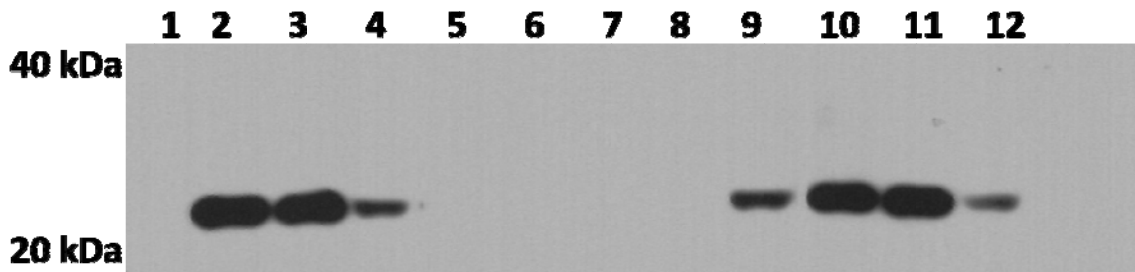
A.2.6b. An example of the P-rpS6 Ser^{240/244} Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the P-rpS6 Ser^{240/244}, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit polyclonal antibody that recognized P-rpS6 Ser^{244/240} (Cell Signaling Technology®, Inc., Boston, MA), and prepared as a 1:2,000 dilution in 5% fat-free milk solution; the “1:1/32 antibody to antigen mixture” contained 4.88×10^{-22} μg antigen; the “1:1/16 antibody to antigen mixture” contained 1.95×10^{-18} μg antigen; the “1:1/4 antibody to antigen mixture” contained 7.8×10^{-15} μg antigen; and the “1:1 antibody to antigen mixture” contained 0.001 μg of antigen. These mixtures were allowed to incubate at room

temperature for 30 minutes, which was the manufacturer's suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling

Technology ®, Inc., Boston, MA), and (12) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (P-rpS6 Ser^{240/244} Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1. Well 8 was treated with a primary antibody to antigen ratio of 1:1/4. Well 9 was treated with a primary antibody to antigen ratio of 1:1/16, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/32. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. However, it is important to note that the presence P-rpS6-Ser^{240/244} in the negative and positive controls was expected, because rpS6 is phosphorylated following S6K1 phosphorylation which is activated by the active form of mTORC1. mTORC1 is activated due to both insulin and amino acids, the negative controls were untreated with insulin and the positive controls were treated with insulin, but both were in media containing amino acids. As a result, P-rpS6 Ser^{240/244} is present in the negative control, but diminished in comparison to the positive control.



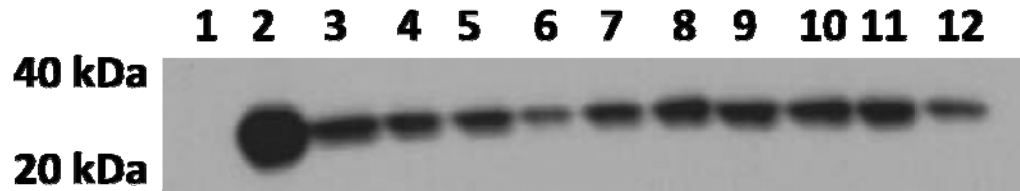
A.2.6c. An example of a P-rpS6 Ser^{235/236 & 240/244} x-ray film from Chapter IV. This is an example of a horse on +NSAID treatment. In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (12) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right side.



A.2.7a. An example of the rpS6 Total Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of rpS6 Total, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit monoclonal antibody that recognized rpS6 Total (Cell Signaling Technology®, Inc., Boston, MA), and prepared as a 1:10,000 dilution in 5% fat-free milk solution; the “1:1/32 antibody to antigen mixture” contained 2.44×10^{-26} μg antigen; the “1:1/16 antibody to antigen mixture” contained 3.9×10^{-19} μg antigen; the “1:1/4 antibody to

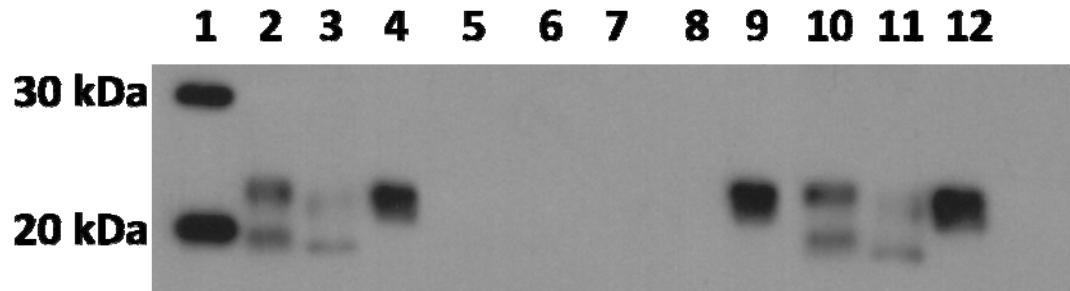
antigen mixture” contained 6.3×10^{-12} μg antigen; and the “1:1 antibody to antigen mixture” contained 0.0002 μg of antigen. These mixtures were allowed to incubate at room temperature for 30 minutes, which was the manufacturer’s suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells

treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), and (12) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (rpS6 Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1. Well 8 was treated with a primary antibody to antigen ratio of 1:1/4. Well 9 was treated with a primary antibody to antigen ratio of 1:1/16, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/32. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. However, it is important to note that the presence of rpS6 Total in both positive and negative controls was expected. Because this protein is ubiquitous, the negative controls serve a greater purpose in the validation of the phosphorylated forms.



A.2.7b. An example of a rpS6 Total x-ray film from Chapter IV. This is an x-ray of the same membrane that is shown in A.2.6c. after it has been stripped and re-probed with rpS6 Total primary antibody. In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology®, Inc., Boston, MA), (3) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (12) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right side. The abundance of this protein is reported as a ratio of the phosphorylated to total

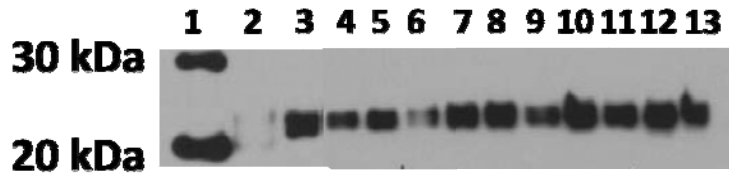
form. The positive control was used to account for interassay variability. The C.V. of the phosphorylated to total ratio of the positive control for P-rpS6 Ser^{235/236 & 240/244} was 0.14 ± 0.14 . The right to left side phosphorylated to total ratio of for P-rpS6^{235/236 & 240/244} was 0.19 ± 0.13 . These were obtained from Chapter IV. In Chapter IV side was tested in the statistical model and found to not be significant ($P < 0.05$).



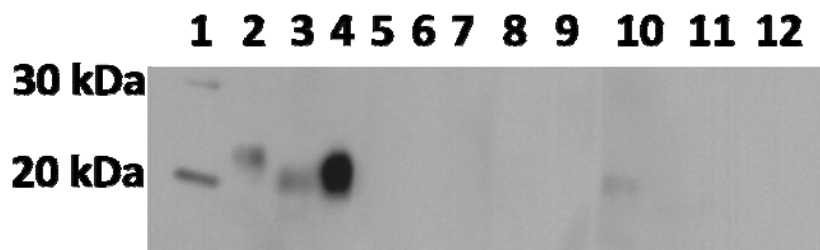
A.2.8a. An example of the P-4EBP1 Thr^{37/46} Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the P-4EBP1 Thr^{37/46}, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit monoclonal antibody that recognized P-4EBP1 Thr^{37/46} (Cell Signaling Technology®, Inc., Boston, MA), and prepared as a 1:1,000 dilution in 5% fat-free milk solution; the “1:1/32 antibody to antigen mixture” contained 2.44×10^{-25} μg antigen; the “1:1/16 antibody to antigen mixture” contained 3.9×10^{-18} μg antigen; the “1:1/4 antibody to antigen mixture” contained 6.3×10^{-11} μg antigen; and the “1:1 antibody to antigen mixture” contained 0.002 μg of antigen. These mixtures were allowed to incubate at

room temperature for 30 minutes, which was the manufacturer's suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 18 hours at 4°C. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling

Technology ®, Inc., Boston, MA), and (12) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (P-4EBP1 Thr^{37/46} Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1. Well 8 was treated with a primary antibody to antigen ratio of 1:1/4. Well 9 was treated with a primary antibody to antigen ratio of 1:1/16, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/32. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. However, it is important to note that the presence P-4EBP1 Thr^{37/46} in the negative and positive controls was expected, because 4EBP1 is phosphorylated following activation of mTORC1. mTORC1 is activated due to both insulin and amino acids, the negative controls were untreated with insulin and the positive controls were treated with insulin, but both were in media containing amino acids. As a result, P-4EBP1 Thr^{37/46} is present in the negative control, but diminished in comparison to the positive control. Also, 4EBP1 has a large band that represents the three isoforms (α , β , and γ).



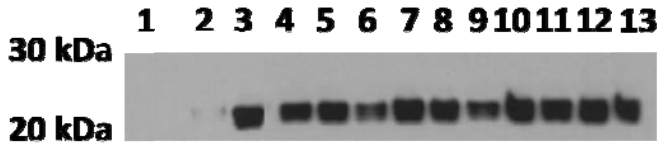
A.2.8b. An example of a P-4EBP1 Thr^{37/46} x-ray film from Chapter IV. This is an example of a horse on +NSAID treatment. In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology®, Inc., Boston, MA), (3) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology®, Inc., Boston, MA), (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (12) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (13) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right side.



A.2.9a. An example of the 4EBP1 Total Antibody Validation. In order to determine cross-species reactivity of the primary and secondary antibodies used for the determination of the abundance of the 4EBP1 Total, pooled equine gluteal muscle samples from horses in the postprandial state underwent electrophoresis on polyacrylamide gels and then were transferred to a PVDF membrane (additional detail regarding electrophoresis and transfer can be found in Section 3.4.1.3). The membrane was then stained with Fast Green Stain (1% fast green, 50% methanol, 1% glacial acetic acid), which stains the protein bound to the membrane. Next the membrane was cut between wells 4 and 5, 7 and 8, 8 and 9 and 9 and 10, and placed in individual containers. The Fast Green Stain was removed with Destain (50% methanol, 1% glacial acetic acid), and the membranes were washed and blocked with 5% fat-free milk solution for 1 hour at room temperature. Meanwhile, the primary antibody and primary antibody and antigen mixtures were prepared as follows: the “100% primary antibody” was a rabbit monoclonal antibody that recognized 4EBP1 Total (Cell Signaling Technology®, Inc., Boston, MA), and prepared as a 1:1,000 dilution in 5% fat-free milk solution; the “1:1/32 antibody to antigen mixture” contained 2.44×10^{-25} μg antigen; the “1:1/16 antibody to antigen mixture” contained 3.9×10^{-18} μg antigen; the “1:1/4 antibody to antigen mixture” contained 6.3×10^{-11} μg antigen; and the “1:1 antibody to antigen mixture” contained 0.002 μg of antigen. These mixtures were allowed to incubate at room temperature for

30 minutes, which was the manufacturer's suggested time allotment for the reaction between the antibody and antigen to occur. The mixtures were then placed on the membrane and allowed to react with the proteins bound to the membrane for 1 hour at room temperature. Then, all sections of the membrane were washed and treated with secondary antibody which was a goat anti-rabbit IgG (H+L) with conjugated horseradish peroxidase (1:10,000 dilution in 5% fat-free milk solution; BioRad, Hercules, CA) for 1 hour at room temperature. Membranes were developed using a chemiluminescence kit (Amersham ECL Plus Western Blotting Detection Reagents; GE Healthcare, Piscataway, NJ) and visualized on x-ray film using a film processor (Kodak X-OMAT film processor, Kodak Health Imaging Division, Rochester, NY). In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (3) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (4) mature equine gluteal muscle in the postprandial state, (5) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (6) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (7) mature equine gluteal muscle in the postprandial state, (8) mature equine gluteal muscle in the postprandial state, (9) mature equine gluteal muscle in the postprandial state, (10) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (11) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling

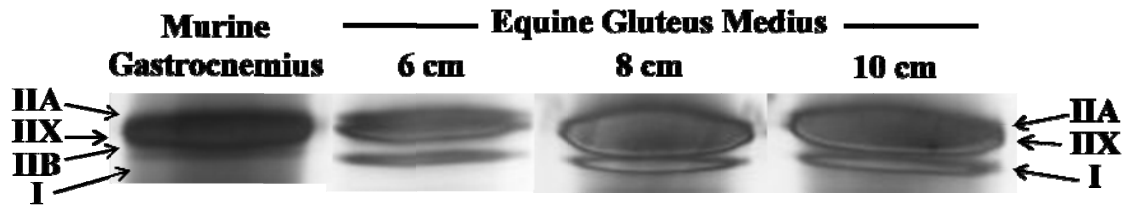
Technology ®, Inc., Boston, MA), and (12) mature equine gluteal muscle in the postprandial state. Wells 1-4 were treated with 100% primary antibody. Wells 5-7 were treated with a primary antibody to antigen (4EBP1 Total Blocking Peptide, Cell Signaling Technology ®, Inc., Boston, MA) at a ratio of 1:1. Well 8 was treated with a primary antibody to antigen ratio of 1:1/4. Well 9 was treated with a primary antibody to antigen ratio of 1:1/16, and wells 10-12 were treated with a primary antibody to antigen ratio of 1:1/32. This validated the use of this primary antibody in the horse, because when an antigen was applied there was inhibition of the antibody to bind with the mature equine gluteal muscle in the postprandial state. However, it is important to note that the presence P-4EBP1 Thr^{37/46} in the negative and positive controls was expected, because 4EBP1 is a ubiquitous protein. The negative control has a greater role in phosphorylated forms. Also, 4EBP1 has a large band that represents the three isoforms (α , β , and γ).



A.2.9b. An example of a 4EBP1 Total x-ray film from Chapter IV. This is an x-ray of the same membrane that is shown in A.2.8b. after it has been stripped and re-probed with rpS6 Total primary antibody. In the wells of the above figure: (1) a molecular marker (MagicMark™, Invitrogen, Carlsbad, CA), (2) a negative control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells untreated, Cell Signaling Technology ®, Inc., Boston, MA), (3) a positive control (p70 S6 Kinase Control Cell Extracts from MCF-7 cells treated with insulin, Cell Signaling Technology ®, Inc., Boston, MA), (4) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the left side, (5) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 1 from the right side, (6) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the left side, (7) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 2 from the right side, (8) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the left side, (9) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 3 from the right side, (10) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the left side, (11) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 4 from the right side, (12) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the left side, (13) a gluteal muscle biopsy collected 60 minutes following consuming a high protein pellet on Day 5 from the right side. The abundance of this

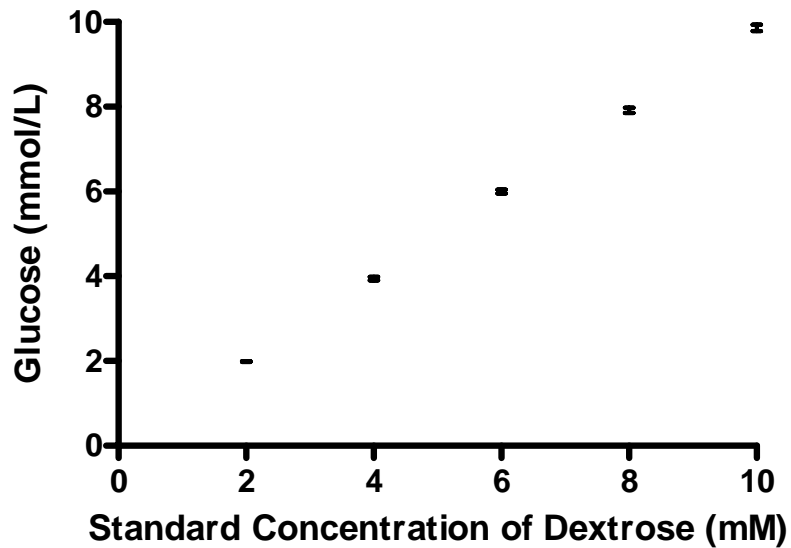
protein is reported as a ratio of the phosphorylated to total form. The positive control was used to account for interassay variability. The C.V. of the phosphorylated to total ratio of the positive control for P-4EBP1 Thr^{37/46} was 0.10 ± 0.07 . The right to left side phosphorylated to total ratio of for P-4EBP1 Thr^{37/46} was 0.13 ± 0.10 . These were obtained from Chapter IV. In Chapter IV side was tested in the statistical model and found to not be significant ($P < 0.05$).

A.3. MYOSIN HEAVY CHAIN ISOFORM SEPERATION



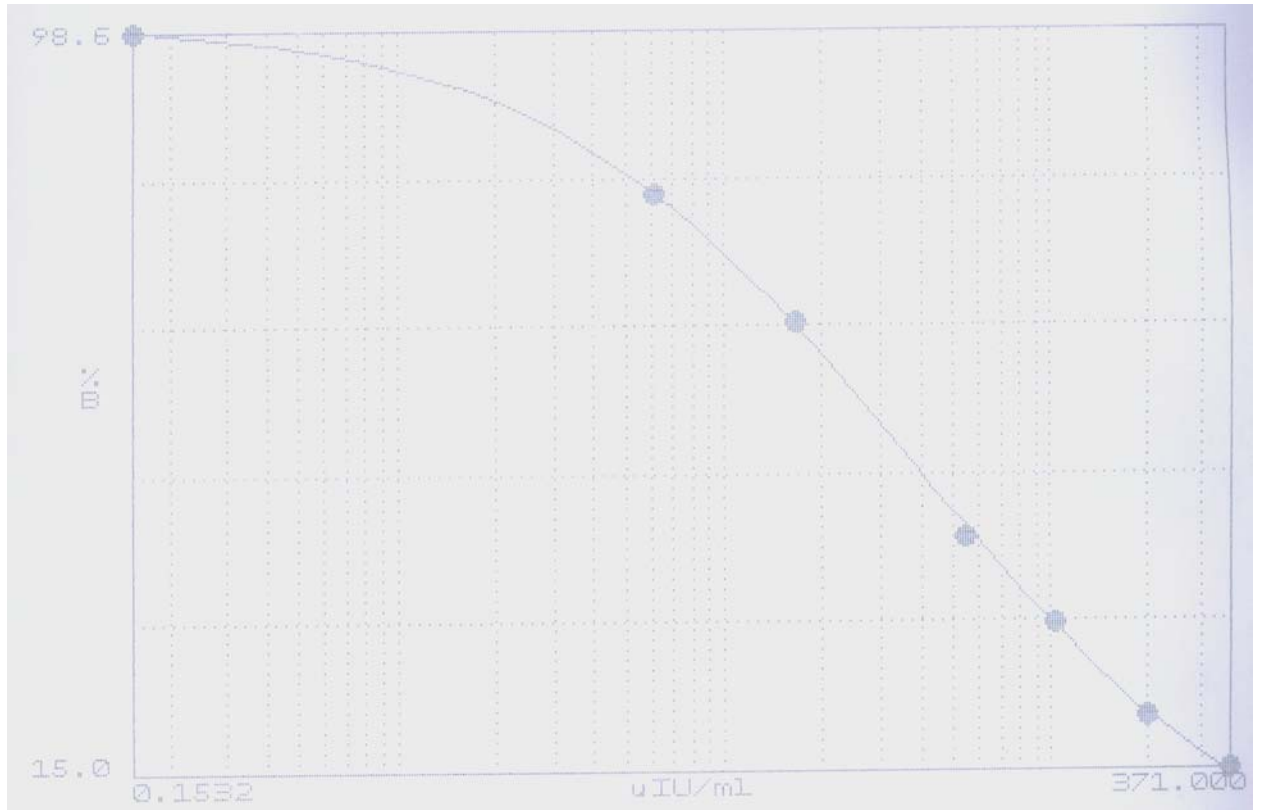
A.3.1 An example of the MHC Isoform gel separation. This is an example of a horse in Chapter III at the various sampling depths and murine gastrocnemius which served as a control

A.4. STANDARD CURVES



A.4.1. An example of a glucose standard curve used in the YSI 2700 SELECT™

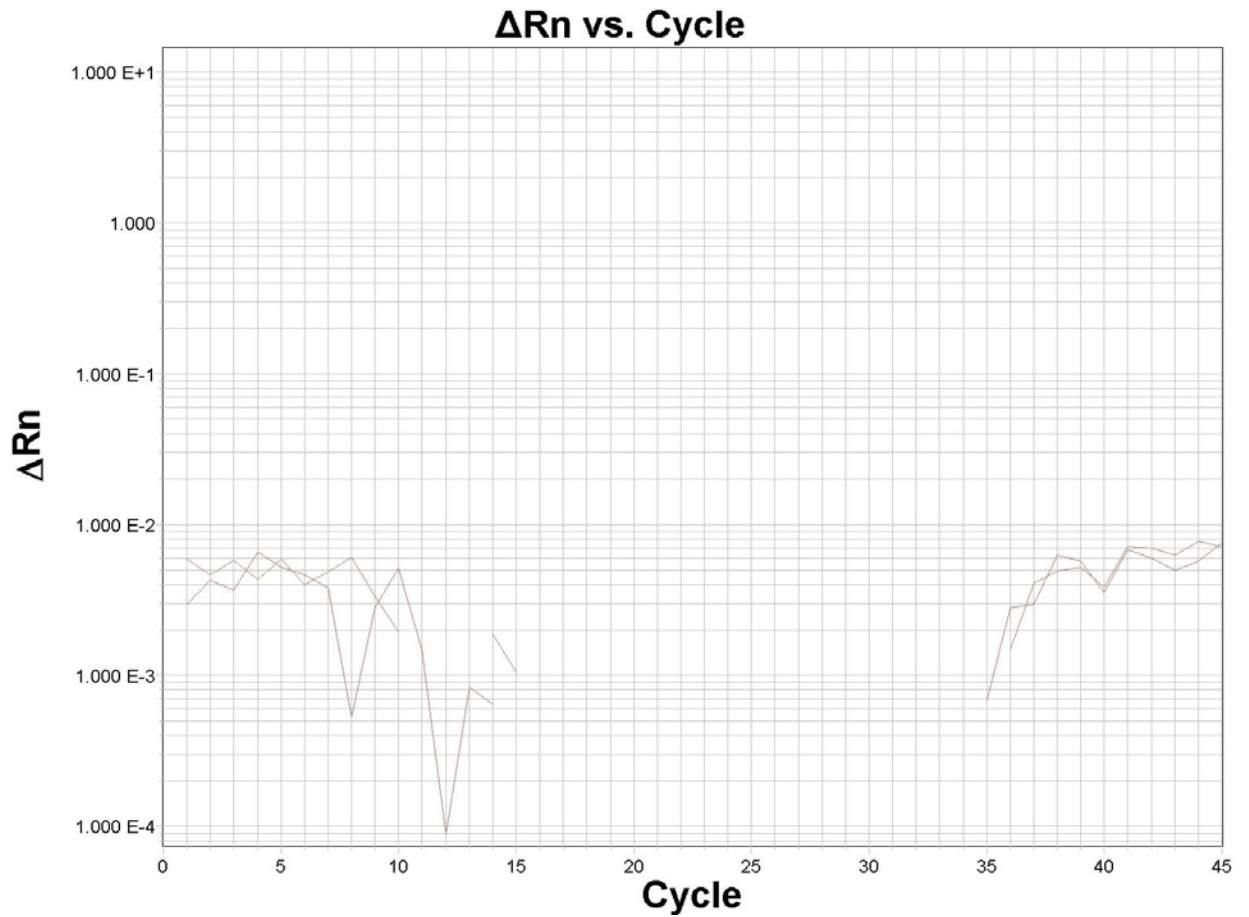
Biochemistry Analyzer. The YSI 2700 SELECT™ Biochemistry Analyzer determines glucose concentration enzymatically. This is an example of the standard curve of glucose from Chapter VI. The r^2 is 0.99 and the standard C.V. is 0.004 ± 0.003 .



A.4.2. An example of the standard curve used in the Coat-A-Count RIA® kit.

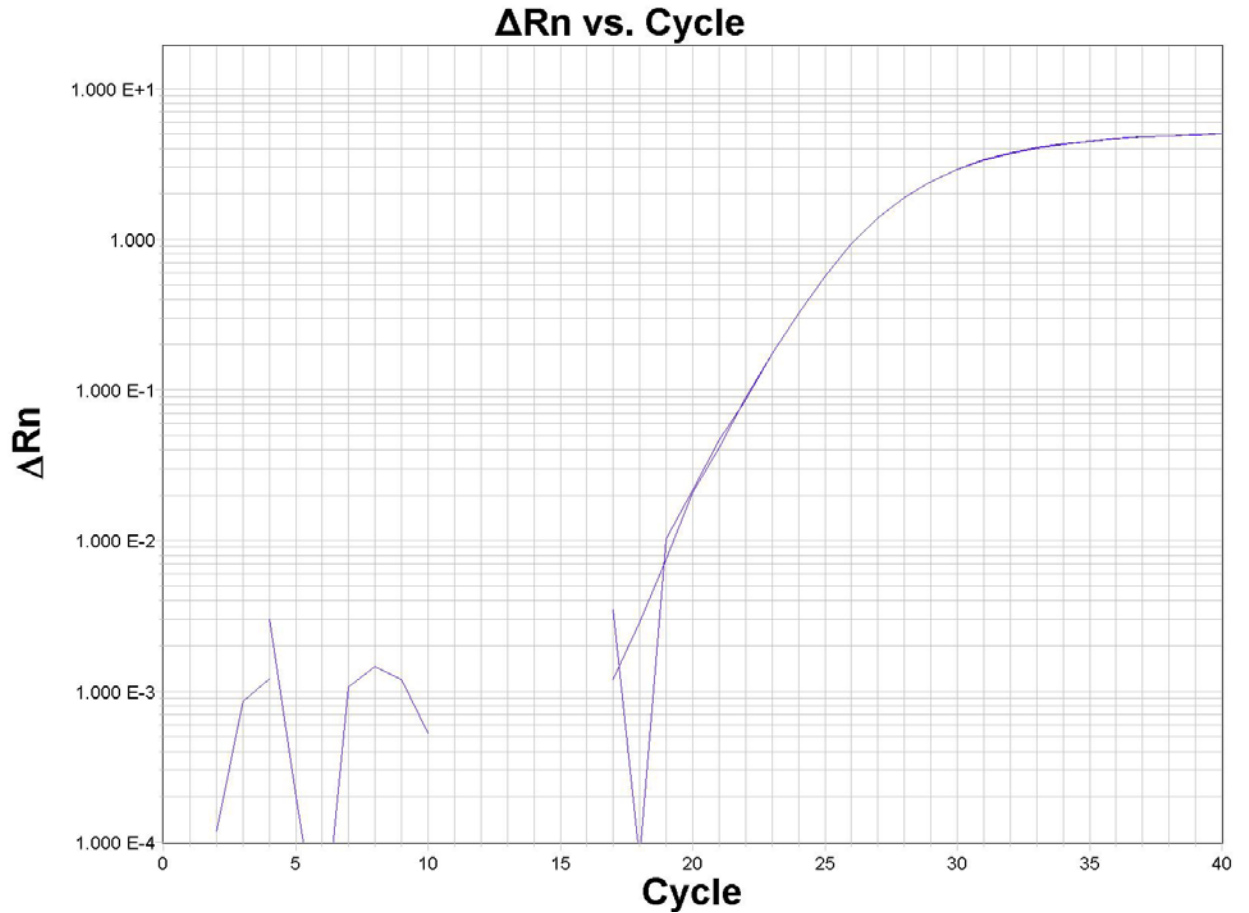
Along the y axis is %B: percent of radioactive binding, and along the x axis is the concentration of insulin in μIU/mL. The linear range of this graph is from 6 μIU/mL to 103.6 μIU/mL with a %B of 80.17 and 31.6%, respectively.

A.5. qRT-PCR AMPLIFICATION PLOTS



A.5.1. An example of β -glucuronidase amplification plot from a negative control.

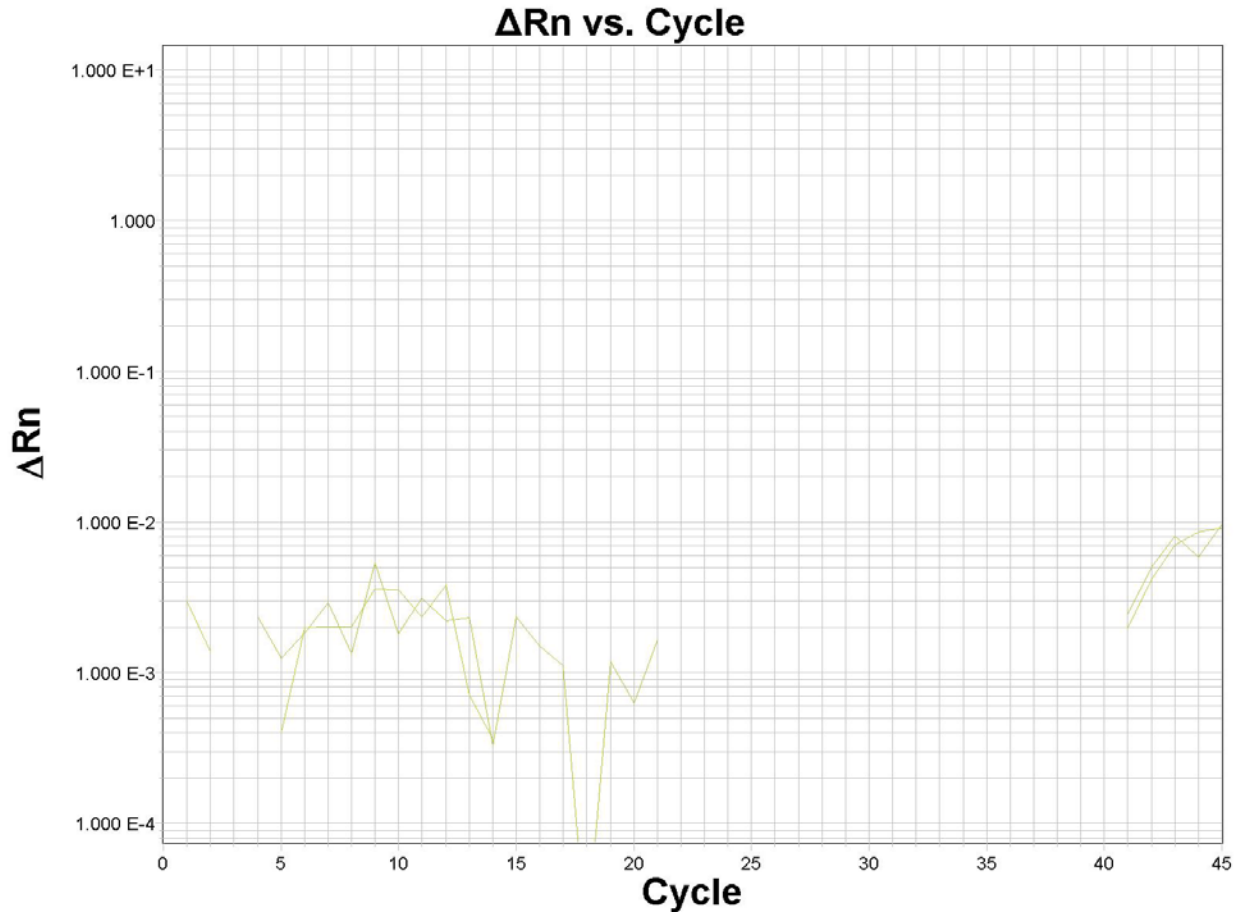
This is an example of an amplification plot of the housekeeping gene, β -glucuronidase, from a negative control (RNA free water) in Chapter IV. As expected, there was a lack of amplification.



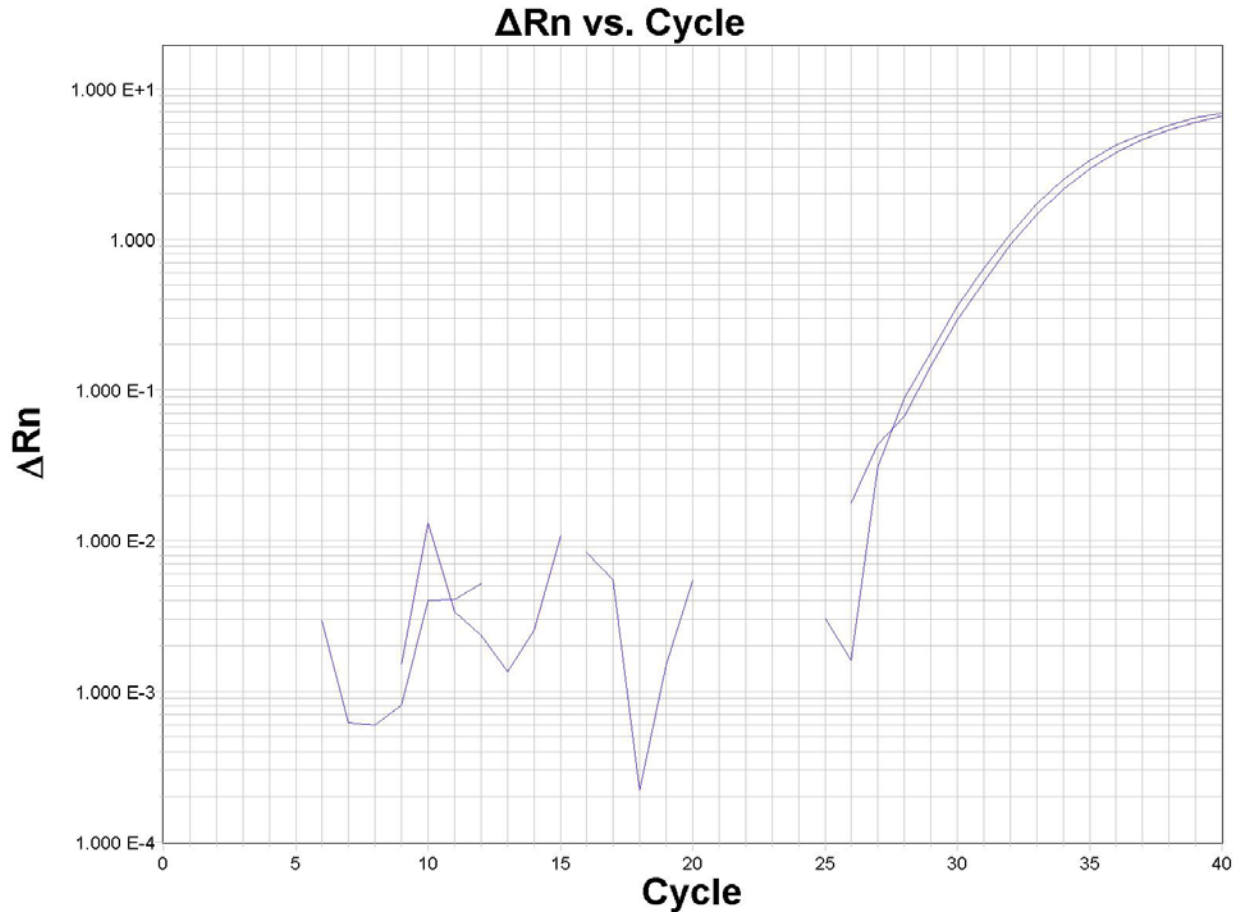
A.5.2. An example of β -glucuronidase amplification plot from a positive control.

This is an example of an amplification plot of the housekeeping gene, β -glucuronidase, from a positive control (pooled equine tissues post infection challenge) in Chapter IV.

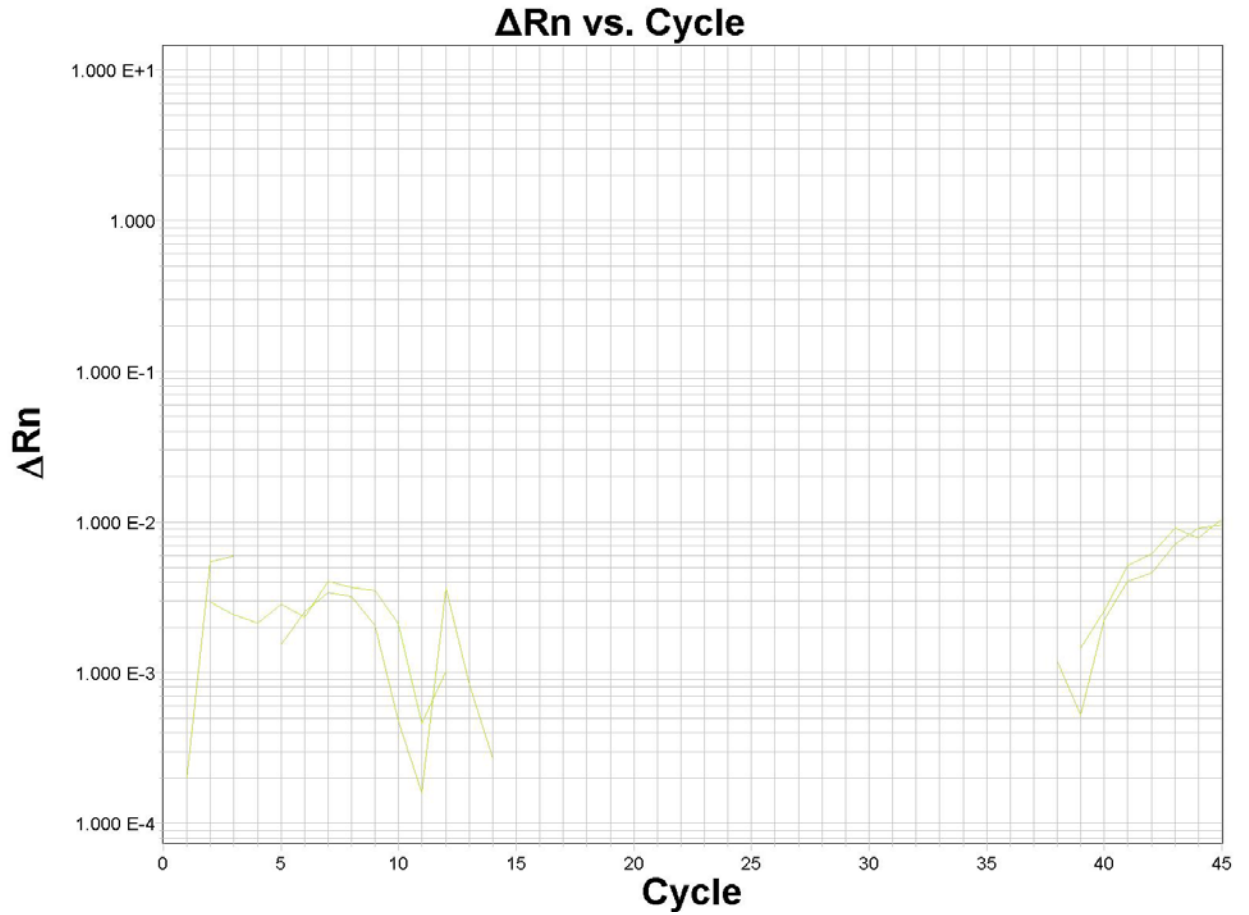
The linear portion of the amplification curve begins following 20 cycles and ends at 26 cycles. The linear portion has an r^2 of no less than 0.95, and the amplification efficiency must be between 0.8 and 1.



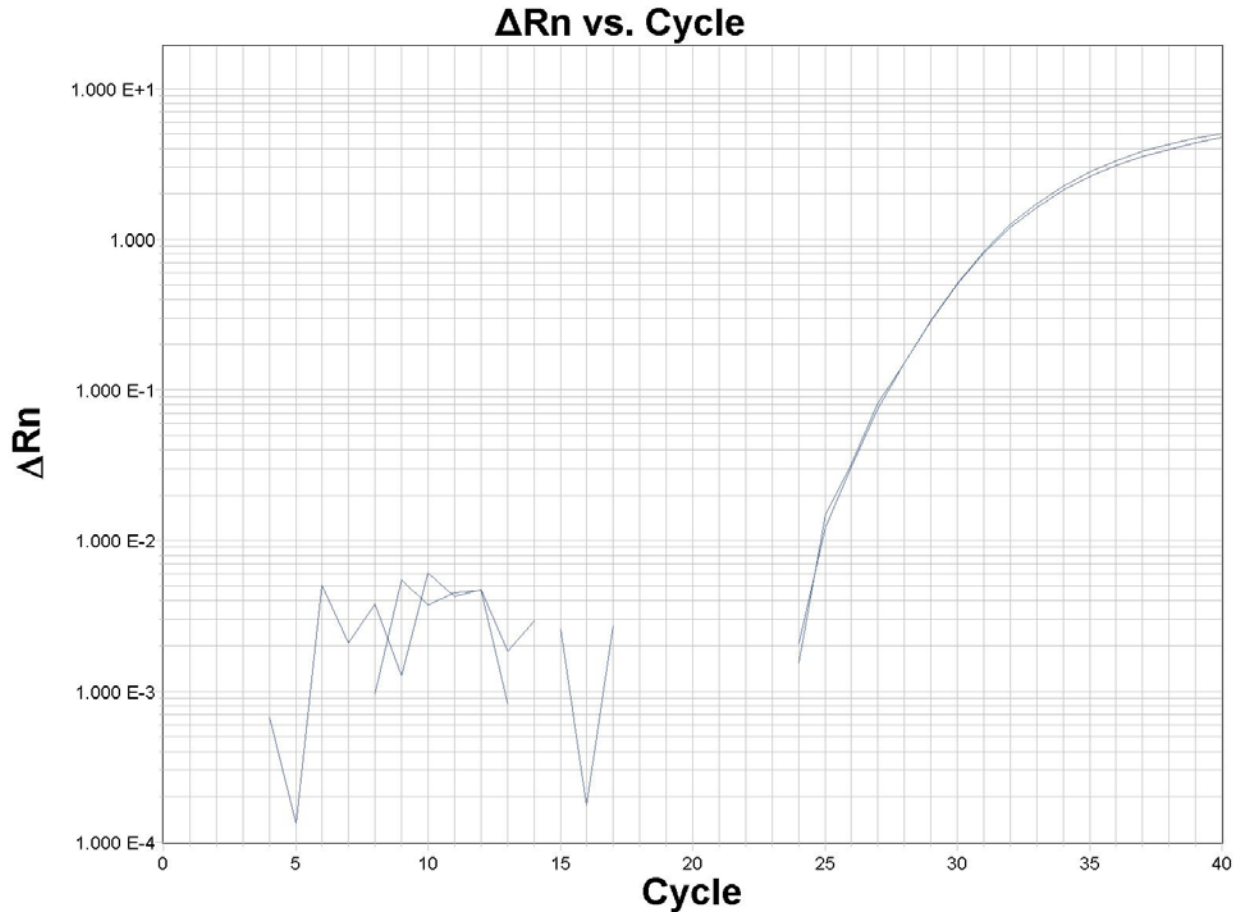
A.5.3. An example of an IFN- γ amplification plot from a negative control. This is an example of an amplification plot of the pro-inflammatory cytokine, IFN- γ , from a negative control (RNA free water) in Chapter IV. As expected, there was a lack of amplification.



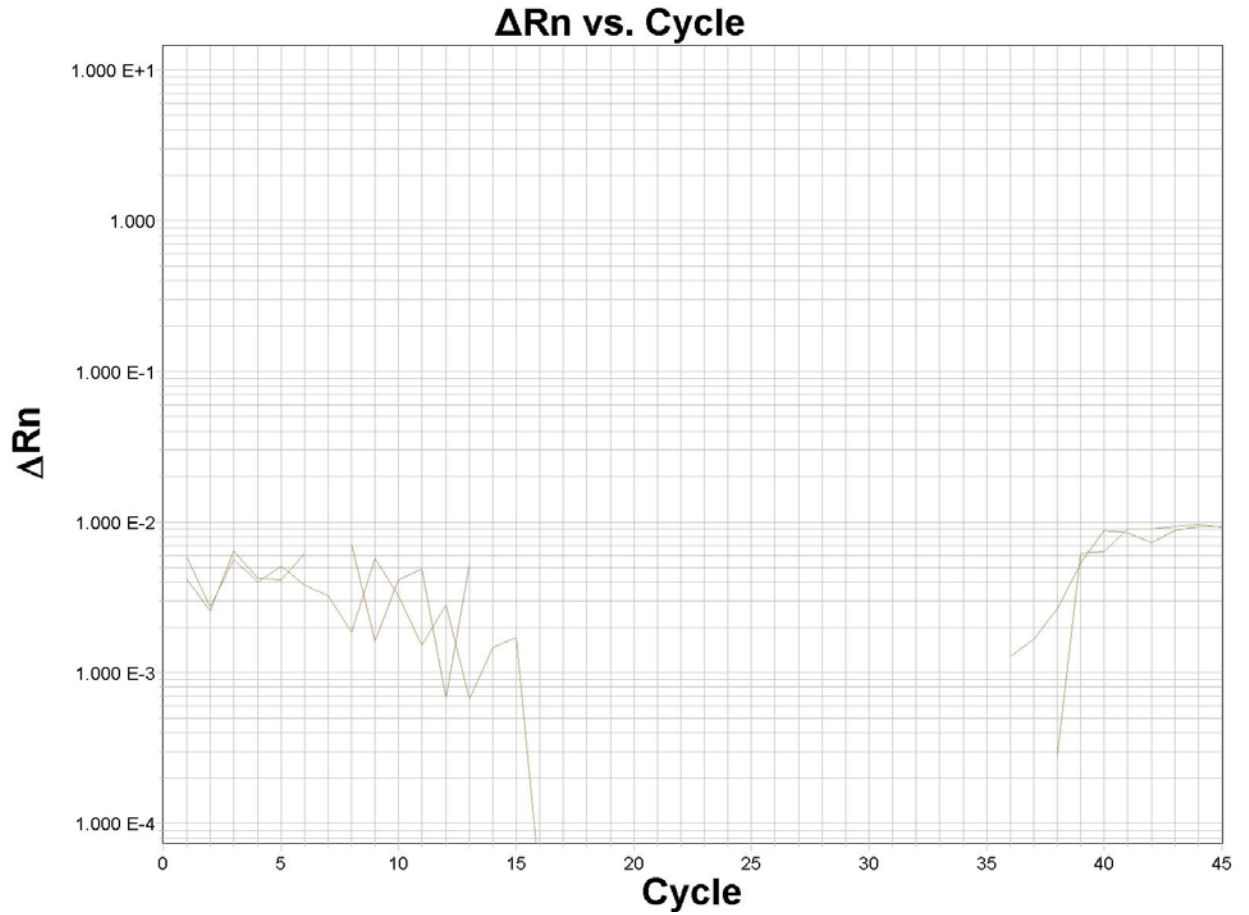
A.5.4. An example of an IFN- γ amplification plot from a positive control. This is an example of an amplification plot of the pro-inflammatory cytokine, IFN- γ , from a positive control (pooled equine tissues post infection challenge) in Chapter IV. The linear portion of the amplification curve begins following 27 cycles and ends at 33 cycles. The linear portion has an r^2 of no less than 0.95, and the amplification efficiency must be between 0.8 and 1.



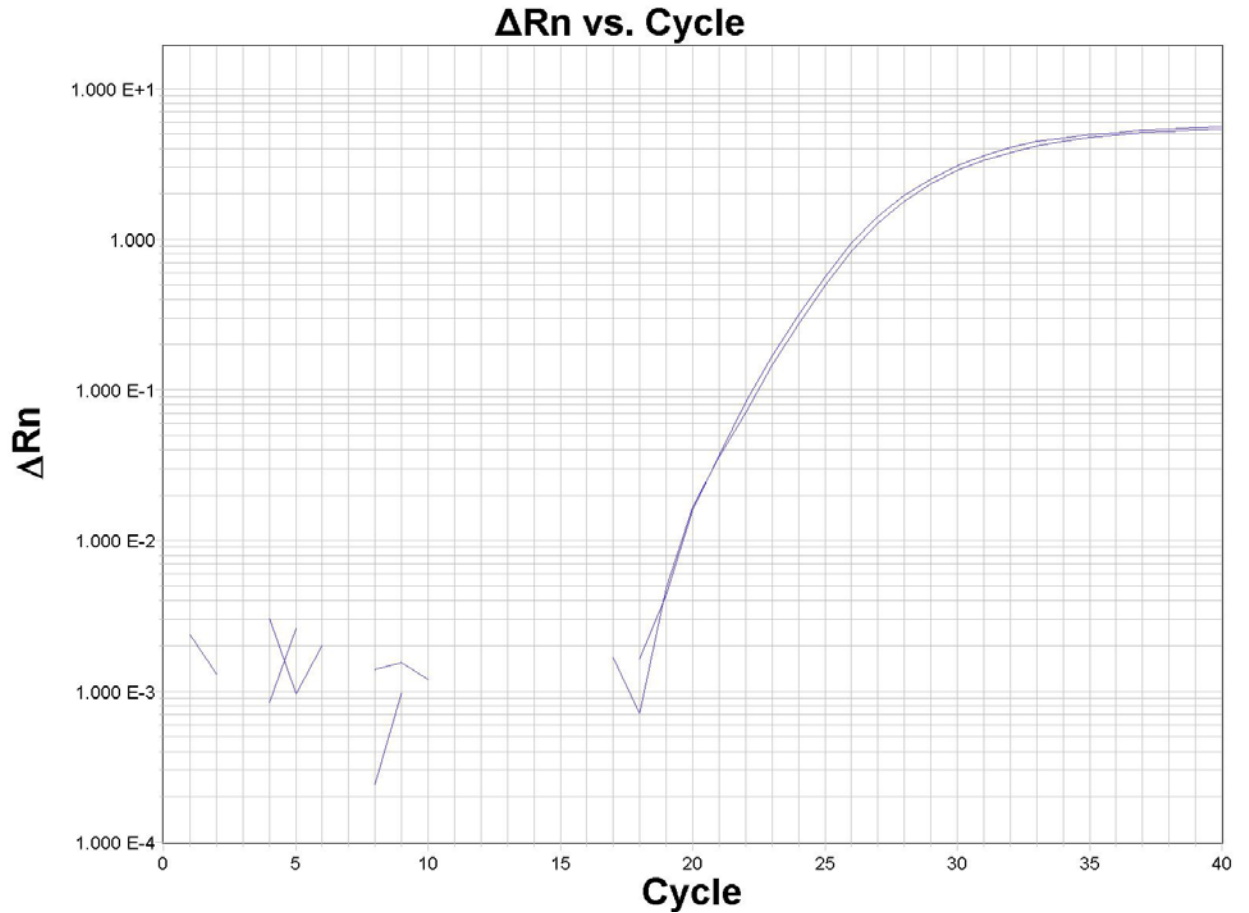
A.5.5. An example of a TNF- α amplification plot from a negative control. This is an example of an amplification plot of the pro-inflammatory cytokine, TNF- α , from a negative control (RNA free water) in Chapter IV. As expected, there was a lack of amplification.



A.5.6. An example of a TNF- α amplification plot from a positive control. This is an example of an amplification plot of the pro-inflammatory cytokine, TNF- α , from a positive control (pooled equine tissues post infection challenge) in Chapter IV. The linear portion of the amplification curve begins following 25 cycles and ends at 29 cycles. The linear portion has an r^2 of no less than 0.95, and the amplification efficiency must be between 0.8 and 1.



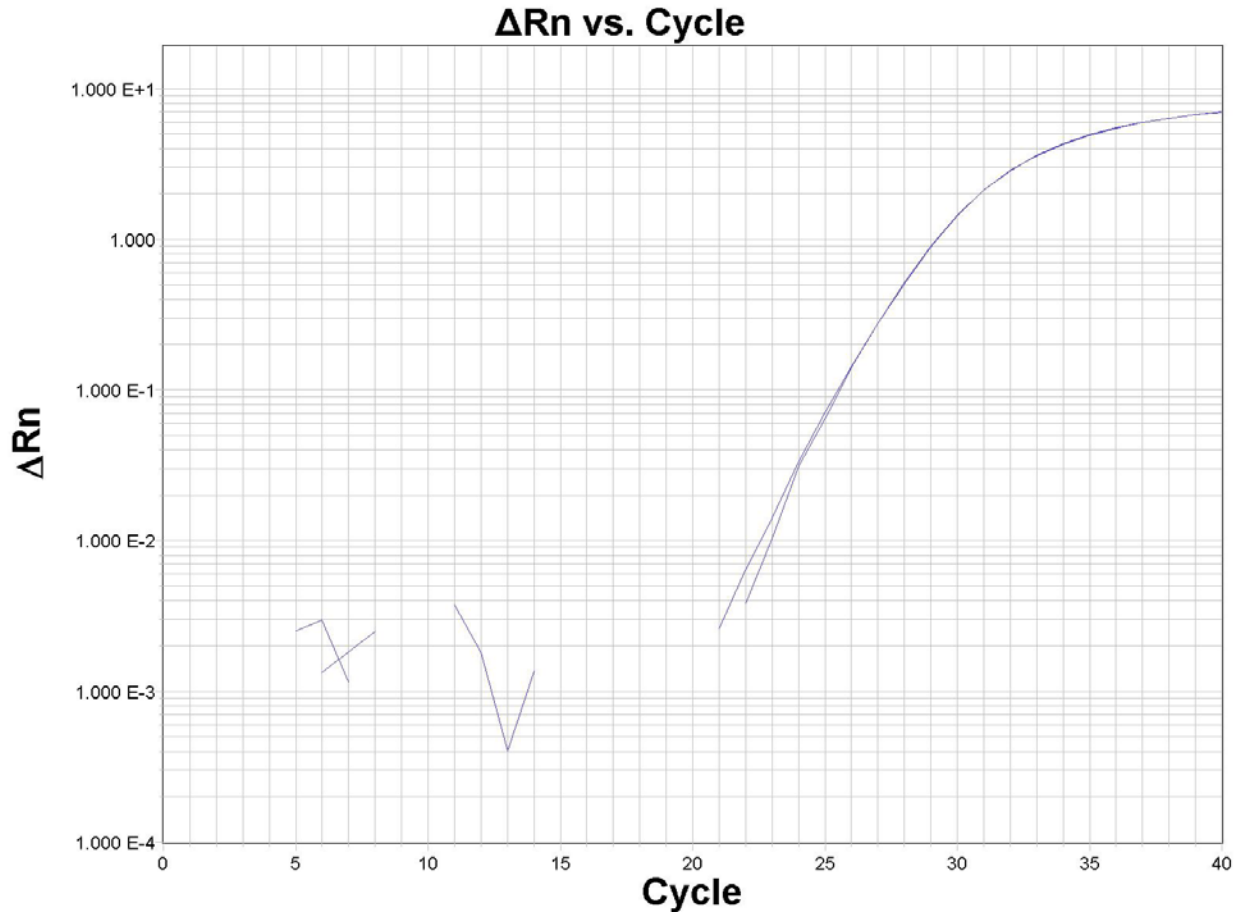
A.5.7. An example of an IL-1 β amplification plot from a negative control. This is an example of an amplification plot of the pro-inflammatory cytokine, IL-1 β , from a negative control (RNA free water) in Chapter IV. As expected, there was a lack of amplification.



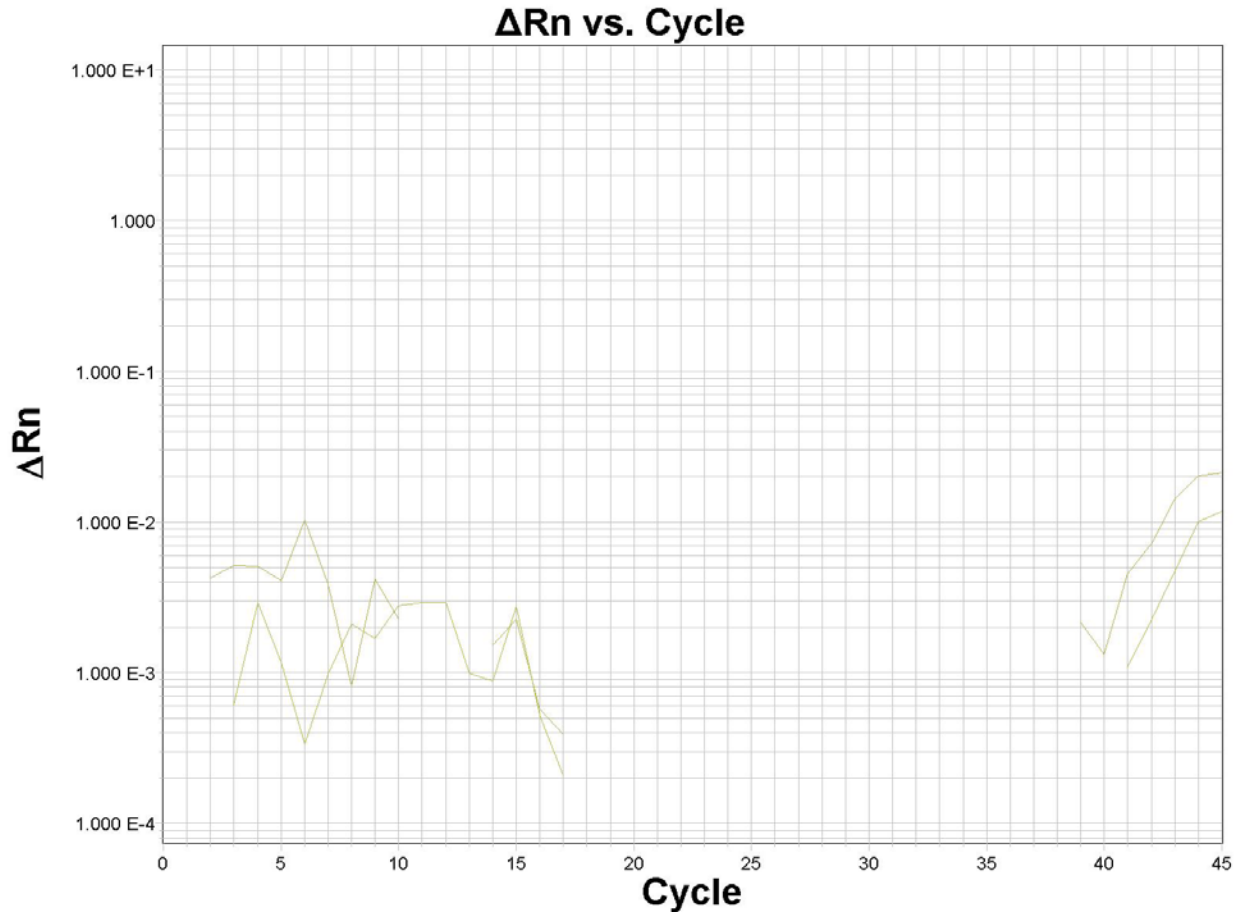
A.5.8. An example of an IL-1 β amplification plot from a positive control. This is an example of an amplification plot of the pro-inflammatory cytokine, IL-1 β , from a positive control (pooled equine tissues post infection challenge) in Chapter IV. The linear portion of the amplification curve begins following 20 cycles and ends at 26 cycles. The linear portion has an r^2 of no less than 0.95, and the amplification efficiency must be between 0.8 and 1.



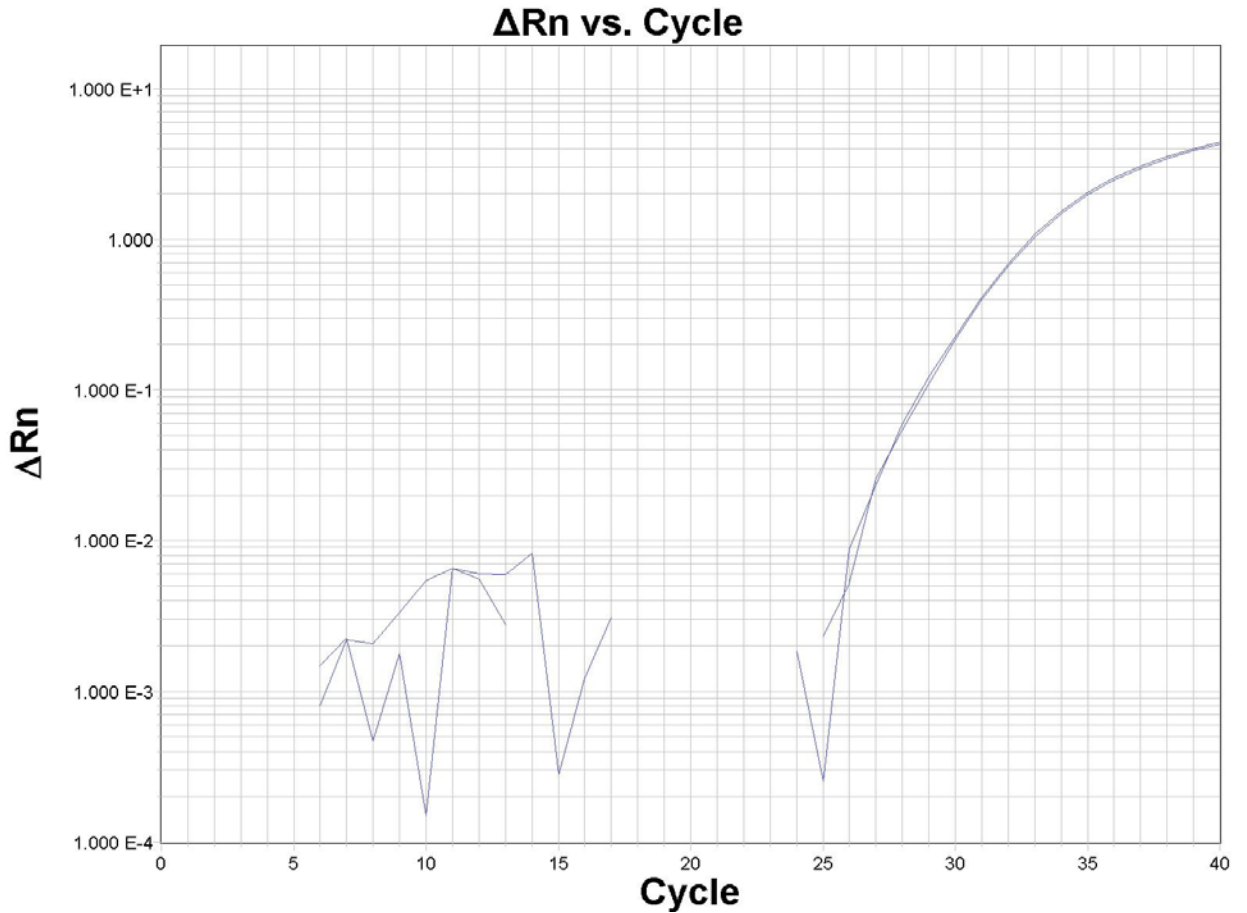
A.5.9. An example of an IL-6 amplification plot from a negative control. This is an example of an amplification plot of the pro-inflammatory cytokine, IL-6, from a negative control (RNA free water) in Chapter IV. As expected, there was a lack of amplification.



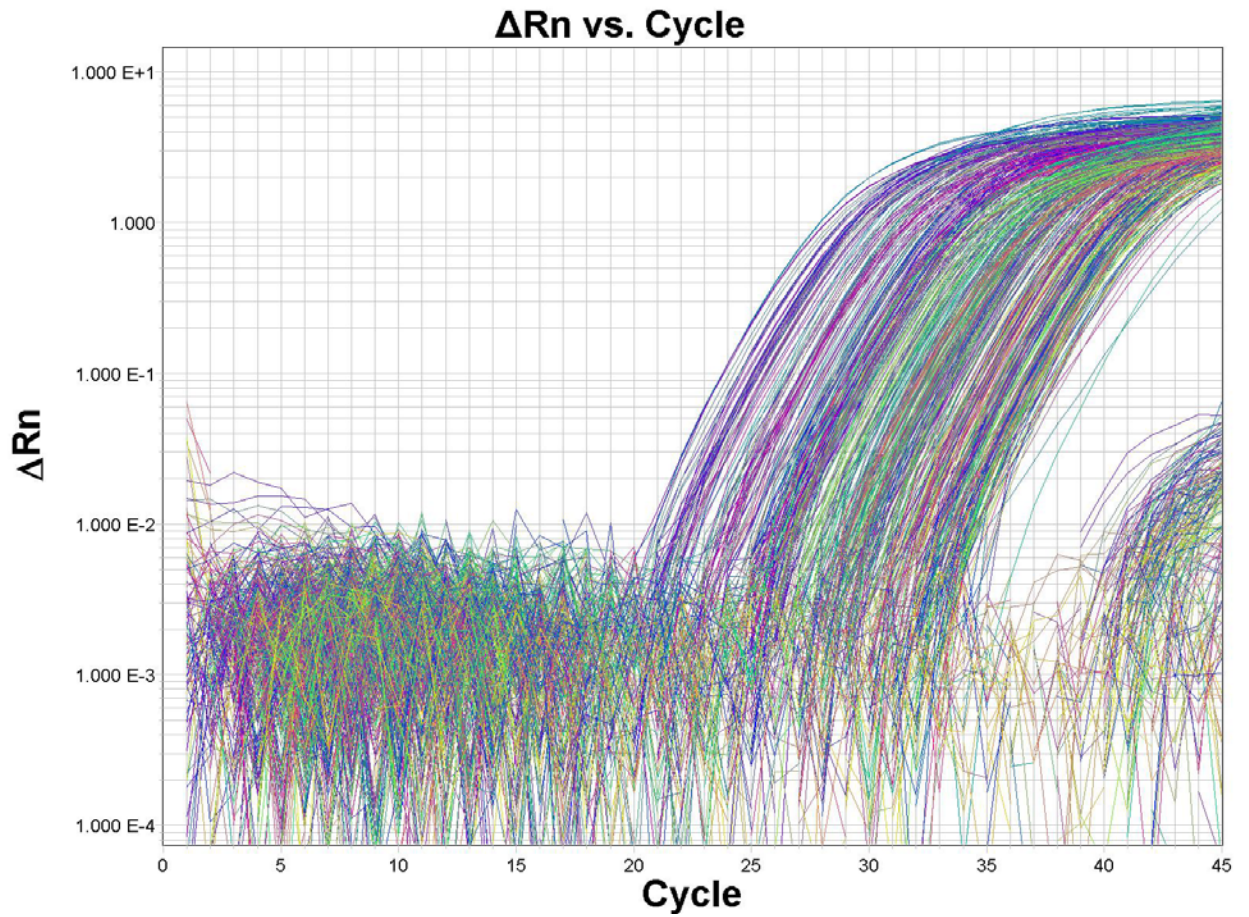
A.5.10. An example of an IL-6 amplification plot from a positive control. This is an example of an amplification plot of the pro-inflammatory cytokine, IL-6, from a positive control (pooled equine tissues post infection challenge) in Chapter IV. The linear portion of the amplification curve begins following 24 cycles and ends at 30 cycles. The linear portion has an r^2 of no less than 0.95, and the amplification efficiency must be between 0.8 and 1.



A.5.11. An example of an IL-10 amplification plot from a negative control. This is an example of an amplification plot of the anti-inflammatory cytokine, IL-10, from a negative control (RNA free water) in Chapter IV. As expected, there was a lack of amplification.

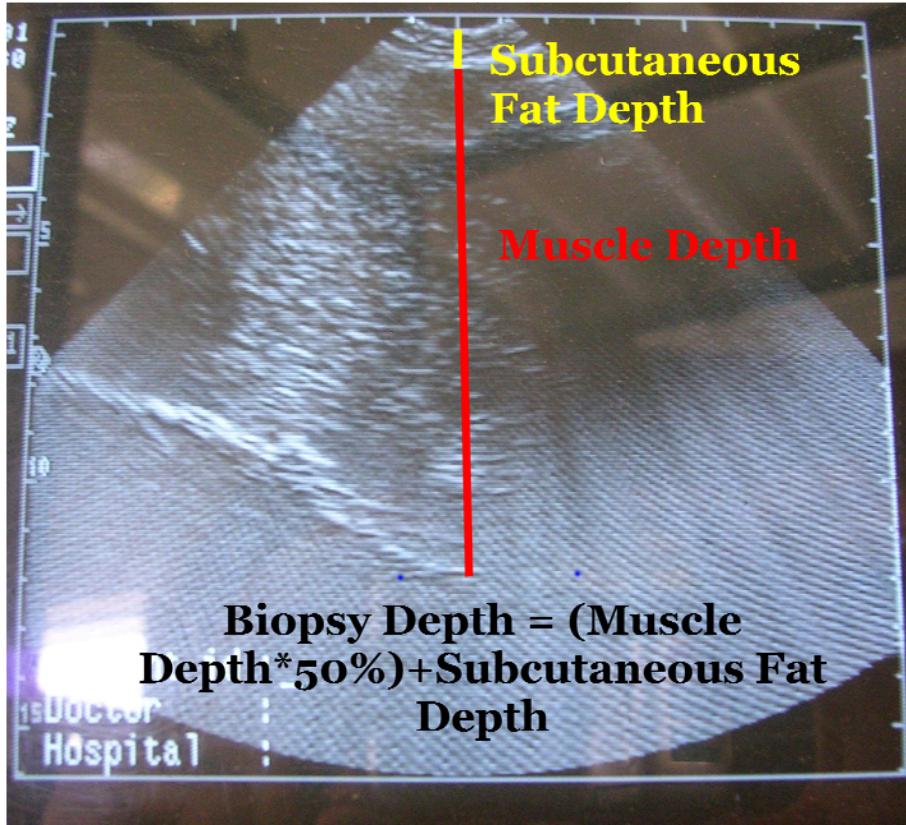


A.5.12. An example of an IL-10 amplification plot from a positive control. This is an example of an amplification plot of the anti-inflammatory cytokine, IL-10, from a positive control (pooled equine tissues post infection challenge) in Chapter IV. The linear portion of the amplification curve begins following 27 cycles and ends at 32 cycles. The linear portion has an r^2 of no less than 0.95, and the amplification efficiency must be between 0.8 and 1.

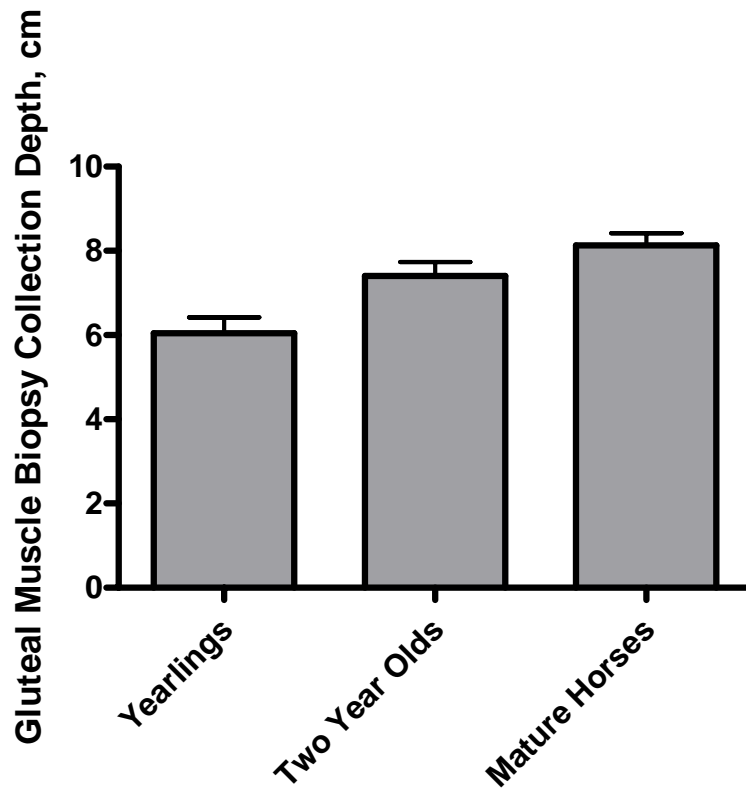


A.5.13. The amplification plots for β -gus, IFN- γ , TNF- α , IL-1 β , IL-6, and IL-10 in various equine gluteal muscle samples collected in Chapter IV.

A.6. ULTRASOUNDING GLUTEAL MUSCLE DEPTH

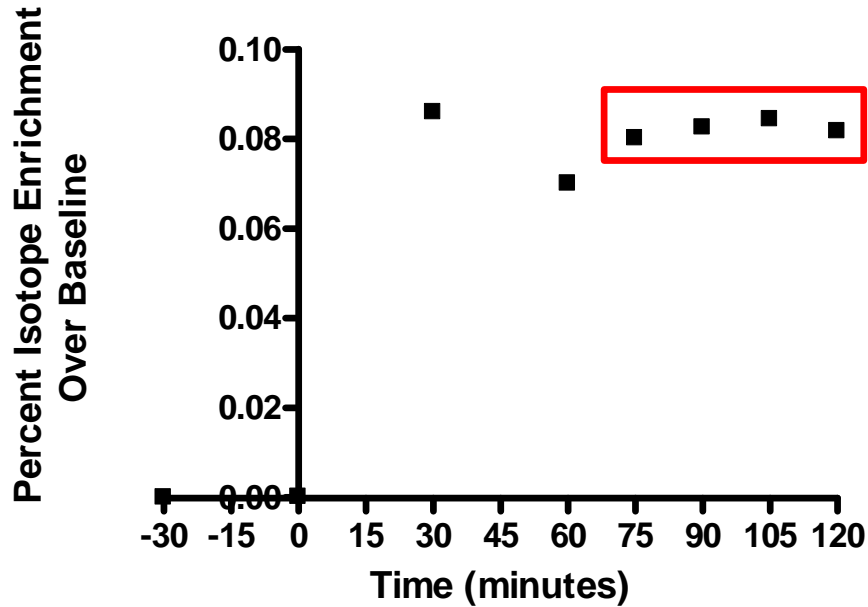


A.6.1. An ultrasound image of gluteal muscle depth and subcutaneous fat from a horse in Chapter V. Rump fat thickness and gluteal muscle depth was determined at the site of biopsy, which is the place where two imaginary lines cross when drawn from dorsocaudally from the tuber coxae and ventrally from the tuber sacral. The rump fat thickness and gluteal muscle depth were used to determine the depth of biopsy: (muscle depth*50%) + subcutaneous fat depth.



A.6.2. Gluteal muscle biopsy collection depths from yearlings, two year olds and mature horses in Chapter V. The gluteal muscle biopsy collection depths were determined following the performance of ultrasound measurements on gluteal muscle depth and subcutaneous fat. Gluteal muscle biopsy collection depth was determined as (50% of the gluteal muscle depth) + subcutaneous fat depth.

A.7. WHOLE-BODY PROTEIN METABOLISM

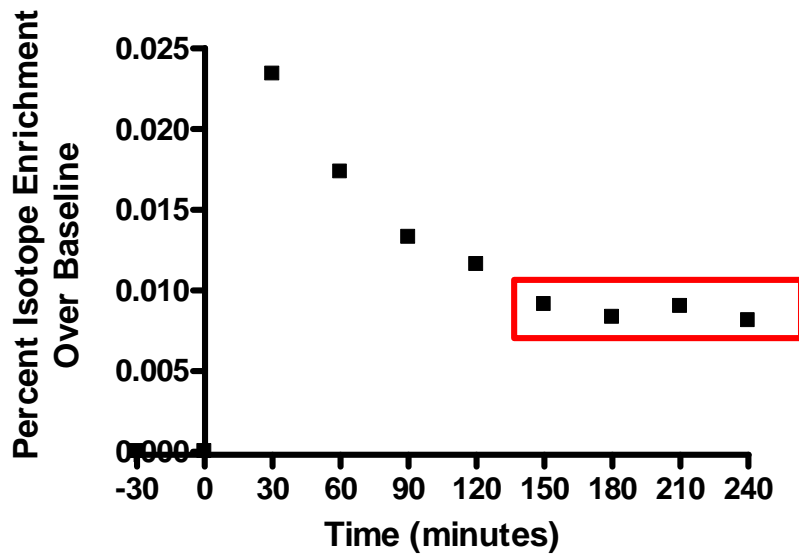


A.7.1. An example of the percent isotope enrichment over baseline in the breath from a mature horse during the [¹³C] sodium bicarbonate infusion period in Chapter VI.

The ratio of ¹³CO₂:¹²CO₂ in the breath samples was determined using an isotope selective non-dispersive infrared absorption (NDIR) analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany). This ratio is given in δ units, which is the ratio in the sample – the average ratio at baseline. This δ enrichment value can then be converted into a percent enrichment using the following equation:

Enrichment (%) = $[0.0112372\delta / (0.0112372\delta + 1000)] \times 100\%$. The red box indicates a plateau in the percent enrichment over baseline, which can be used to calculate total CO₂ production. The average number of points used to determine plateau were 3.2 ± 0.5 . The average C.V. of the plateau percent enrichment was $2.4 \pm 1.8\%$. Total production of CO₂ can be calculated using the following equation:

$FCO_2 = i \times [(E_i/E_b) - 1]$. Where, i is the rate of isotope administration in $\mu\text{mol/kg/min}$, E_i is the enrichment of the isotope in the solution and E_b is the enrichment of the breath samples at plateau, corrected for baseline enrichment (average of the red box points).



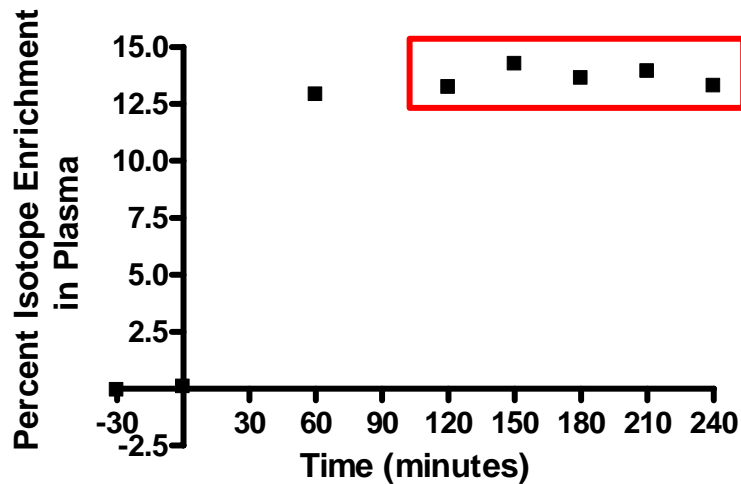
A.7.2. An example of the percent isotope enrichment over baseline in the breath from an aged horse during the [1-¹³C] phenylalanine infusion period in Chapter VII.

The ratio of ¹³CO₂:¹²CO₂ in the breath samples was determined using an isotope selective non-dispersive infrared absorption (NDIR) analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany). This ratio is given in δ units, which is the ratio in the sample – the average ratio at baseline. This δ enrichment value can then be converted into a percent enrichment using the following equation:

$$\text{Enrichment (\%)} = [0.0112372\delta / (0.0112372\delta + 1000)] \times 100\%.$$

The red box indicates a plateau in the percent enrichment over baseline. This plateau can be used to calculate the rate of [1-¹³C]phenylalanine oxidation to ¹³CO₂ (F¹³CO₂): F¹³CO₂ = FCO₂ × E_{CO₂}, where E_{CO₂} is the average enrichment of the breath samples at isotopic steady state (average of the 3 points in the red box), corrected for baseline enrichment, during the [1-¹³C]phenylalanine infusion. The average number of points used for the

plateau were 3.1 ± 0.5 . The average C.V. for the plateau percent enrichments was $1.1 \pm 1.3\%$.



A.7.3. An example of the percent isotope enrichment in the plasma from an aged horse during the [1-¹³C] phenylalanine infusion period in Chapter VII. This is the percent enrichment corrected for the baseline enrichment. The red box indicates a plateau in the percent enrichment over baseline. The average number of points used in the plateau were 4.8 ± 1 , and the average C.V. of the enrichment values used in the plateau was $3.7 \pm 1.3\%$. This plateau can be used to calculate whole-body phenylalanine flux:

Flux (Q ; $\mu\text{mol/kg/h}$) = $i \times [(E_i/E_p) - 1]$, where i is the rate of isotope infusion (in $\mu\text{mol/kg/h}$), E_i is the enrichment of infused isotope, and E_p is the plateau plasma enrichment (average of the red box points). Flux includes the amount of amino acids entering the pool through dietary intake (I), *de novo* synthesis (N), and protein breakdown (B), or leaving the pool through protein synthesis (Z), oxidation (E), or the conversion to other metabolites: $Q = I + N$ $B = Z + E + M$. Because phenylalanine is a dietary indispensable amino acid, N is negligible; thus $B = Q - I$. Then, whole-body phenylalanine oxidation can be calculated using the following equation: $O = F^{13}\text{CO}_2 \times$

$(1/E_p - 1/E_i) \times 100$, where E_i is the enrichment of infused isotope, and E_p is the plateau plasma enrichment (average of 3 points in the red box). If tyrosine is balanced in the diet and is not generally considered to be a limiting amino acid in nonruminant, the assumption can be made that phenylalanine conversion to tyrosine is minimal. As a result, non-oxidative phenylalanine metabolism can be calculated through the difference of $Q - O$, which can be used to indicate changes in phenylalanine used for whole-body protein synthesis.

A.8. SAS EXAMPLES

A.8.1. Repeated measures input example.

```
data rpS6_Density;
input horse depth$ block$ PrpS6;
cards;
    1  6cm      A      1.027141
    1  8cm      A      1.048161
    1 10cm      A      1.068832
    2  6cm      B      1.034807
    2  8cm      B      1.172738
    2 10cm      B      0.942196
    3  6cm      A      .
    3  8cm      A      1.285698
    3 10cm      A      1.122919
    4  6cm      B      0.938052
    4  8cm      B      0.958085
    4 10cm      B      1.121907
    5  6cm      B      0.520908
    5  8cm      B      0.958247
    5 10cm      B      0.867442
    6  6cm      A      1.479092
    6  8cm      A      1.48873
    6 10cm      A      1.571769
;
run;
PROC mixed data= rpS6_Density;
class horse depth block;
model PrpS6 = depth block/ddfm=kr;
random horse (block);
repeated depth/type=un r rcorr sub=horse(block);
lsmeans depth/pdiff;
run;
PROC mixed data= rpS6_Density;
class horse depth block;
model PrpS6 = depth block/ddfm=kr;
random horse (block);
repeated depth/type=ante(1) r rcorr
sub=horse(block);
lsmeans depth/pdiff;
run;
PROC mixed data= rpS6_Density;
```



```

class horse depth block;
model PrpS6 = depth block/ddfm=kr;
random horse (block);
repeated depth/type=simple r rcorr
sub=horse(block);
lsmeans depth/pdiff;
run;
PROC mixed data= rpS6_Density;
class horse depth block;
model PrpS6 = depth block/ddfm=kr;
random horse (block);
repeated depth/type=toep r rcorr sub=horse(block);
lsmeans depth/pdiff;
run;
PROC mixed data= rpS6_Density;
class horse depth block;
model PrpS6 = depth block/ddfm=kr;
random horse (block);
repeated depth/type=arh(1) r rcorr
sub=horse(block);
lsmeans depth/pdiff;
run;
PROC mixed data= rpS6_Density;
class horse depth block;
model PrpS6 = depth block/ddfm=kr;
random horse (block);
repeated depth/type=ar(1) r rcorr sub=horse(block);
lsmeans depth/pdiff;
run;

```

A.8.2. *Repeated measures output example.*

The variance-covariance matrix was chosen for each analysis based on the lowest value for Schwarz's Bayesian Criterion, which is highlighted in this example.

The Mixed Procedure

Model Information

| | |
|---------------------------|--------------------------------------|
| Data Set | WORK.RPS6_DENSITY |
| Dependent Variable | PrpS6 |
| Covariance Structures | Variance Components, Unstructured |
| Subject Effect | horse(block) |
| Estimation Method | REML |
| Residual Variance Method | None |
| Fixed Effects SE Method | Kenward-Roger |
| Degrees of Freedom Method | Kenward-Roger |

Fit Statistics

| | |
|--------------------------|------|
| -2 Res Log Likelihood | -5.6 |
| AIC (smaller is better) | 8.4 |
| AICC (smaller is better) | 30.8 |
| BIC (smaller is better) | 6.9 |

Type 3 Tests of Fixed Effects

| Effect | Num DF | Den DF | F Value | Pr > F |
|--------|-----------|-----------|---------|--------|
| depth | 2 | 3.65 | 1.19 | 0.4005 |
| block | 1 | 4.08 | 1.45 | 0.2932 |

The Mixed Procedure

Model Information

| | |
|---------------------------|---|
| Data Set | WORK.RPS6_DENSITY |
| Dependent Variable | PrpS6 |
| Covariance Structures | Variance Components, Ante-dependence |
| Subject Effect | horse(block) |
| Estimation Method | REML |
| Residual Variance Method | None |
| Fixed Effects SE Method | Kenward-Roger |
| Degrees of Freedom Method | Kenward-Roger |

Fit Statistics

| | |
|--------------------------|------|
| -2 Res Log Likelihood | -5.6 |
| AIC (smaller is better) | 6.4 |
| AICC (smaller is better) | 20.4 |
| BIC (smaller is better) | 5.1 |

Type 3 Tests of Fixed Effects

| Effect | Num DF | Den DF | F Value | Pr > F |
|--------|-----------|-----------|---------|--------|
| depth | 2 | 3.65 | 0.83 | 0.5035 |
| block | 1 | 4.08 | 1.49 | 0.2873 |

The Mixed Procedure

Model Information

| | |
|---------------------------|---------------------|
| Data Set | WORK.RPS6_DENSITY |
| Dependent Variable | PrpS6 |
| Covariance Structure | Variance Components |
| Subject Effect | horse(block) |
| Estimation Method | REML |
| Residual Variance Method | Parameter |
| Fixed Effects SE Method | Kenward-Roger |
| Degrees of Freedom Method | Kenward-Roger |

Fit Statistics

| | |
|--------------------------------|-------------|
| -2 Res Log Likelihood | -4.1 |
| AIC (smaller is better) | -0.1 |
| AICC (smaller is better) | 1.1 |
| BIC (smaller is better) | -0.5 |

Type 3 Tests of Fixed Effects

| Effect | Num DF | Den DF | F Value | Pr > F |
|--------|-----------|-----------|---------|--------|
| depth | 2 | 9.13 | 1.85 | 0.2117 |
| block | 1 | 4.06 | 3.29 | 0.1426 |

The Mixed Procedure

Model Information

| | |
|----------|-------------------|
| Data Set | WORK.RPS6_DENSITY |
|----------|-------------------|

| | |
|---------------------------|----------------------------------|
| Dependent Variable | PrpS6 |
| Covariance Structures | Variance Components, Toeplitz |
| Subject Effect | horse(block) |
| Estimation Method | REML |
| Residual Variance Method | Profile |
| Fixed Effects SE Method | Kenward-Roger |
| Degrees of Freedom Method | Kenward-Roger |

Fit Statistics

| | |
|--------------------------|------|
| -2 Res Log Likelihood | -4.1 |
| AIC (smaller is better) | 1.9 |
| AICC (smaller is better) | 4.6 |
| BIC (smaller is better) | 1.3 |

Type 3 Tests of Fixed Effects

| Effect | Num DF | Den DF | F Value | Pr > F |
|--------|-----------|-----------|---------|--------|
| depth | 2 | 4.98 | 1.51 | 0.3073 |
| block | 1 | 3.93 | 3.22 | 0.1485 |

The Mixed Procedure

Model Information

| | |
|---------------------------|---|
| Data Set | WORK.RPS6_DENSITY |
| Dependent Variable | PrpS6 |
| Covariance Structures | Variance Components, Heterogeneous Autoregressive |
| Subject Effect | horse(block) |
| Estimation Method | REML |
| Residual Variance Method | None |
| Fixed Effects SE Method | Kenward-Roger |
| Degrees of Freedom Method | Kenward-Roger |

Fit Statistics

| | |
|--------------------------|------|
| -2 Res Log Likelihood | -5.6 |
| AIC (smaller is better) | 4.4 |
| AICC (smaller is better) | 13.0 |
| BIC (smaller is better) | 3.4 |

Type 3 Tests of Fixed Effects

| Effect | Num DF | Den DF | F Value | Pr > F |
|--------|-----------|-----------|---------|--------|
|--------|-----------|-----------|---------|--------|

| | | | | |
|-------|---|------|------|--------|
| depth | 2 | 5.03 | 1.09 | 0.4034 |
| block | 1 | 4.07 | 1.44 | 0.2949 |

The Mixed Procedure

Model Information

| | |
|---------------------------|--|
| Data Set | WORK.RPS6_DENSITY |
| Dependent Variable | PrpS6 |
| Covariance Structures | Variance Components, Autoregressive |
| Subject Effect | horse(block) |
| Estimation Method | REML |
| Residual Variance Method | Profile |
| Fixed Effects SE Method | Kenward-Roger |
| Degrees of Freedom Method | Kenward-Roger |

Fit Statistics

| | |
|--------------------------|------|
| -2 Res Log Likelihood | -4.1 |
| AIC (smaller is better) | 1.9 |
| AICC (smaller is better) | 4.6 |
| BIC (smaller is better) | 1.3 |

Type 3 Tests of Fixed Effects

| Effect | Num DF | Den DF | F Value | Pr > F |
|--------|-----------|-----------|---------|--------|
| depth | 2 | 4.98 | 1.25 | 0.3625 |
| block | 1 | 3.93 | 3.46 | 0.1377 |

A.8.3. *One Way ANOVA input example.*

```
data Akt_Density;
input horse age$ block$ PserAkt;
cards;
    2 Mature A 0.808913
    4 Mature A 1.191087
    8 Old A 0.775572
   10 Old A 0.987907
    3 Old B 0.670911
    6 Mature B 1.082008
   11 Old B 0.271198
   12 Mature B 0.917992
    1 Mature C 1.288538
    5 Mature C 0.711462
    7 Old C 1.165816
    9 Old C 1.15654
;
run;
PROC mixed data=
Akt_Density;
class horse age block;
model PserAkt = age block/ddfm=kr;
random horse (age block);
lsmeans age/pdiff;
run;
```

A.8.4. One Way ANOVA output example.

The Mixed Procedure

Model Information

| | |
|---------------------------|---------------------|
| Data Set | WORK.AKT_DENSITY |
| Dependent Variable | PSerAkt |
| Covariance Structure | Variance Components |
| Estimation Method | REML |
| Residual Variance Method | Profile |
| Fixed Effects SE Method | Kenward-Roger |
| Degrees of Freedom Method | Kenward-Roger |

Type 3 Tests of Fixed Effects

| Effect | Num DF | Den DF | F Value | Pr > F |
|--------|-----------|-----------|---------|--------|
| age | 1 | 8 | 1.06 | 0.3342 |
| block | 2 | 8 | 1.62 | 0.2573 |

Least Squares Means

| Effect | age | Estimate | Standard Error | DF | t Value | Pr > t |
|--------|--------|----------|-------------------|----|---------|---------|
| age | Mature | 1.0000 | 0.1115 | 8 | 8.97 | <.0001 |
| age | Old | 0.8380 | 0.1115 | 8 | 7.52 | <.0001 |

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Vita

Ashley Leigh Wagner was born in Virginia Beach, VA on November 28, 1985. She grew up in Cheriton, VA, which is located on the Eastern Shore of VA. After graduating from Northampton County High School in 2003, she moved to Blacksburg, VA to attend Virginia Polytechnic Institute and State University (Virginia Tech). She received a B.S. in Animal and Poultry Sciences from Virginia Tech in 2006. She stayed there to pursue a M.S. under the guidance of Jeffery Escobar, Ph.D. in Animal and Poultry Sciences with an emphasis in non-ruminant nutrition. Her M.S. thesis was titled *Impacts of phytase on various nonstarch polysaccharidase activities in distillers dried grains with solubles*. After receiving her M.S. in 2008, Ashley moved to Lexington, KY to pursue a Ph.D. under the guidance of Kristine L. Urschel, Ph.D. in Animal and Food Sciences at the University of Kentucky. During her Ph.D., Ashley was awarded the 2011 American Society for Nutrition Nutritional Sciences Council Graduate Student Research Award for the Wagner, A.L., R.B. Ennis, A.A. Adams, D.W. Horohov, and K.L. Urschel. 2011. Non-steroidal anti-inflammatory drug (NSAID) administration to mature and old horses influences the activation of translation initiation factors. 2011 EB Annual Meeting, Washington, D.C. 1920. Her dissertation is titled *Factors affecting skeletal muscle protein synthesis in the horse*. A version of Chapter V. Developmental regulation of the activation of translation initiation factors in response to feeding in the skeletal muscle of horses has been accepted for publication in the American Journal of Veterinary Science. A version of Chapter III. Gluteal muscle sampling depth does not affect mTOR signaling in response to feeding in mature Thoroughbred mares was under review for publication in the Equine Veterinary Journal at the time of her defense. Ashley Wagner defended her Ph.D. dissertation on November 21, 2011, and upon completing her Ph.D. will begin as a Postdoctoral Scholar under the guidance of Karyn Esser, Ph.D. at the University of Kentucky, Department of Physiology, Center for Muscle Biology.