The neonatal period is characterized by the highest growth rate during the life of an animal, with skeletal muscle undergoing the largest increase in mass. Elevated growth rate in neonates is a result of protein synthesis rates being higher than protein degradation rates. However, with postnatal maturation, protein synthesis rates gradually decrease until synthesis and degradation are equal in adult muscle. 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 regard to protein synthesis.

Protein synthesis is limited by the abundance and efficiency of ribosomes to translate mRNA into protein and the availability of amino acids to form protein. The skeletal muscle of neonates has a high concentration of ribosomes, which decreases with age; this may also contribute to the elevated rates of protein synthesis. The efficiency of ribosomes to translate mRNA into protein is modulated by a series of intracellular signaling cascades that are associated with the mTOR pathway. The mTOR pathway regulates translation initiation and has been the subject of recent reviews.

### Objective
To determine whether feeding-induced activation of translation initiation factors, specifically protein kinase B, ribosomal protein S6 kinase (S6K1), ribosomal protein S6 (rpS6), and eukaryotic initiation factor 4E binding protein 1, in horses is affected by age.

### Animals
6 yearlings, six 2-year-old horses, and 6 mature horses.

### Procedures
After an 18-hour period of feed withholding, horses consumed a high-protein meal (2 g/kg) at time 0 and 30 minutes (postprandial state) or continued to have feed withheld (postabsorptive state). Blood samples were collected for the duration of the experimental procedures and used to determine plasma concentrations of glucose, insulin, and amino acids. At 90 minutes, biopsy specimens were collected from a gluteal muscle and used to measure phosphorylation of translation initiation factors.

### Results
Plasma glucose, insulin, and amino acid concentrations were elevated for the postprandial state, compared with results for the postabsorptive state, regardless of age. Phosphorylation of protein kinase B, S6K1, rpS6, and eukaryotic initiation factor 4E binding protein 1 was increased for the postprandial state. There was an effect of age with increased phosphorylation of S6K1 at Thr389 and rpS6 at Ser235/236 in the yearlings and mature horses, compared with results for the 2-year-old horses.

### Conclusions and Clinical Relevance
Food consumption resulted in an increase in the activation of translation initiation factors, with the highest degree of responsiveness in the yearlings. This indicated that increased muscle accretion seen during growth could be a result of increased rates of muscle protein synthesis in response to a meal stimulus. (Am J Vet Res 2012;73:1241–1251)
activates Akt through the activation of several intermedi-ate signaling proteins. Akt is activated when the Thr and Ser sites are both phosphorylated, and both sites are activated in association with insulin-receptor signaling.

Activation of Akt inactivates the mTOR inhibitor tuberous sclerosis complex 2 through phosphorylation, which allows activation of mTOR through phosphorylation. Amino acids, specifically leucine, have also been found to phosphorylate mTOR through the activation of several signaling proteins. In skeletal muscle, mTOR is recognized as the key regulator of translation initiation and subsequent protein synthesis through the phosphorylation of 2 downstream signaling proteins (S6K1 and 4EBP1). Phosphorylation of S6K1 results in the activation of rpS6, a component of the 40S ribosomal subunit, which allows activation of the translational equipment, which allows synthesis of protein.

Protein synthesis in response to anabolic stimuli during the neonatal period has been examined by use of translation initiation factor activation and iso- tope infusion techniques to measure protein synthesis. Anabolic stimuli, such as consumption of a meal, administration of amino acids, or administration of insulin, increases the activation of translation initiation factors in the mTOR signaling pathway and increases isoto- topically measured rates of muscle protein synthesis. Similar to protein synthesis, the responsiveness of the activation of translation initiation factors to anabolic stimuli is blunted with postnatal age and is lower in adults; however, the adolescent period of slowed growth has not been examined. Furthermore, the activation of translation initiation factors and subsequent protein synthesis in response to anabolic stimuli have been found to further decrease between ma- ture and aged rodents and humans. The activation of translation initiation factors has been examined in equine skeletal muscle tissue in only a single study, in which the response to consumption of a meal increased the activation of translation initiation factors in mature horses. However, to our knowledge, age-related effects have not been evaluated.

The purpose of the study reported here was to determine the effects of an 18-hour period of feed withholding followed by consumption of a meal on the activation of translation initiation factors, specifically Akt, S6K1, rpS6, and 4EBP1, in yearlings, 2-year-old horses, and mature horses. Our hypothesis was that the anabolic stimulus of consuming a meal would cause greater activation of translation initiation factors in yearlings than in the 2-year-old horses, which we expected to be more responsive than the mature horses. We chose an adolescent age group that consisted of yearlings and 2-year-old horses because, to our knowledge, no study has been conducted in any species to examine the activation of translation initiation factors in response to an anabolic stimulus in adolescents. The translation initiation factors evaluated were chosen as a representation of both upstream and downstream factors in the mTOR signaling pathway.

**Materials and Methods**

**Animals**—Eighteen horses (15 Thoroughbred mares, 2 Thoroughbred–Quarter Horse crossbred mares, and 1 Quarter Horse mare) were obtained from the University of Kentucky Animal and Food Sciences’ Maine Chance Farm. Of these 18 mares, 6 were yearlings (mean ± SD age, 13.8 ± 0.8 months; mean body weight, 393 ± 12 kg), 6 were 2 years old (mean age, 27.5 ± 7.0 months; mean body weight, 484 ± 28 kg), and 6 were mature (mean age, 13.8 ± 2.9 years; mean body weight, 549 ± 50 kg). All horses were of moderate body condition (body condition score, 5 to 7 on a scale of 1 to 9) and healthy. The group of mature mares was selected from a candidate pool of mares that were no longer growing but that had not yet been defined as chronologically old (ie, > 20 years). For the duration of the present study, horses were housed each night in 3.7 × 3.7-m stalls bedded with pine shavings, with daily turn out into dry lots. Horses had ad libitum access to water and salt at all times. Horses were allowed to accustom to diet and housing procedures for 2 weeks prior to any experimental procedure. The University of Kentucky Institutional Animal Care and Use Committee approved all procedures used in the study.

**Feeding procedures**—All horses were fed individually in stalls. Each horse received 2 meals daily (8:00 AM and 3:00 PM). Diets were designed to meet the National Research Council requirements for mature horses with standard maintenance needs and a body weight of 600 kg, 2-year-old horses with an estimated ADG of 0.22 kg/d, and yearlings with an estimated ADG of 0.54 kg/d. Meals consisted of alfalfa cubes, ration balancer pellets, and a protein and mineral dietary product. Alfalfa cubes (mean ± SD values for crude protein, 16.95 ± 0.24%; acid detergent fiber, 35.2 ± 0.82%; neutral detergent fiber, 44.88 ± 1.31%; crude fat, 2.28 ± 0.05%; and ash, 9.5 ± 0.22%) were fed at a rate of 1.75% of body weight/d to all age groups. Ration balancer pellets (crude protein, 14.93 ± 0.15%; acid detergent fiber, 22.53 ± 0.81%; neutral detergent fiber, 43.2 ± 0.36%; crude fat, 3.57 ± 0.06%; and ash, 12.9 ± 0.1%) were fed at a rate of 0.19%, 0.29%, and 0.40% of body weight/d to mature, 2-year-old, and yearling horses, respectively. Samples of the alfalfa cubes, ration balancer pellets, and protein and mineral dietary product were collected throughout the experimental period and submitted to a commercial laboratory for nutrient analysis.

**Experimental design and procedures**—The study was conducted as a 3 × 2-factorial crossover design with age and feeding state (postabsorptive vs postprandial) as the fixed effects, with each horse included for both feeding states. To facilitate sampling procedures, the 18 horses were allocated into 3 blocks, with 2 horses from each age group in each block. Each horse was included in both feeding states (order determined via a random number generator), such that within each block there was an equal number of horses (n = 3) receiving each treatment during each period. There was a minimum of 1 week between subsequent treatment periods for each horse.
The experimental procedures were similar to those reported for another study. Briefly, on the day before sampling procedures, horses were weighed and a catheter was placed in a jugular vein of each horse. Ultrasonographic examination of the gluteus medius muscle was performed to determine thickness of the subcutaneous fat and gluteal muscle. This allowed for the accurate determination of 50% of the gluteal muscle thickness, which enabled us to standardize depth at which biopsy specimens were collected from the various age groups of horses, which was determined to be a mean ± SD of 6.0 ± 0.4 cm, 7.4 ± 0.3 cm, and 8.1 ± 0.3 cm for yearlings, 2-year-old horses, and mature horses, respectively. Horses were then placed into individual stalls and fed a meal that was high in crude protein. Each meal was fed at 2 g of feed/kg of body weight on an as-fed basis. The purpose of this meal was to standardize the duration of feed withholding prior to sampling procedures for all horses. After horses consumed the meal, they were housed individually in pine-bedded stalls overnight (18 hours); horses had ad libitum access to water, but feed and hay were withheld.

The following morning (18 hours after the previous meal), horses remained in the postabsorptive state for an additional 90 minutes (postabsorptive treatment) or were fed a 2 g/kg meal of the aforementioned pelleted feed (meal high in crude protein) at 0 minutes and again at 30 minutes, for a total targeted intake of 4 g of pelleted feed/kg during the postprandial period (postprandial treatment). This feeding regimen has been found to result in differences in plasma glucose, insulin, and amino acid concentrations and in the activation of mTOR-related signaling factors in the gluteal muscle of mature horses. Feed intake was monitored, and any uneaten feed was removed at the end of the postprandial period and weighed.

Two baseline blood samples (10 mL) were collected prior to 0 minutes; the minimum interval between collection of these blood samples was 15 minutes. Subsequent blood samples were obtained at 10-minute intervals for 80 minutes. All samples were collected into evacuated tubes containing sodium heparin and were centrifuged at 1,500 g for 10 minutes at 4°C. Plasma was harvested, and aliquots of the plasma were immediately centrifuged at 10,000 g for 10 minutes at 4°C, and the supernatant was divided into aliquots and then stored at −80°C until further analysis. After protein concentrations were determined by use of a Bradford assay kit modified for a 96-well plate, 50 μL of supernatant was added to 25 μL of a 3X Laemmli buffer (125 mM tris(hydroxymethyl)aminomethane hydrochloride [pH, 6.8], 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 100 mM dithiothreitol, and 0.01% [wt/vol] bromophenol blue). Then, various amounts of 1X Laemmli buffer were added to each sample and Laemmli buffer mixture to reach a final protein concentration of 2 μg/μL. The sample and Laemmli buffer mixture was boiled for 5 minutes, then immediately placed on ice prior to gel electrophoresis.

**Western blot analysis of muscle specimens**—The abundance and phosphorylation of 4EBP1, Akt, S6K1, and rpS6 in the gluteal muscle specimens were determined via western blot analysis, similar to procedures described elsewhere. In the muscle specimens processed and stored in Laemmli buffer were separated in polyacrylamide gels via electrophoresis and then transferred to 0.45-μm polyvinylidene fluoride membranes. Samples were standardized on the basis of the amount of protein loaded per well for electrophoresis (20 μg of protein was loaded for Akt, rpS6, and 4EBP1; 30 μg of protein was loaded for S6K1). Membranes were blocked by incubation in a 5% fat-free milk solution and then incubated with the appropriate primary antibodies for 16 hours at 4°C (phosphorylated and total forms of Akt, S6K1, and rpS6 and phosphorylated form of 4EBP1) or 1 hour at room temperature (approx 20°C; total form of 4EBP1).

Individual rabbit polyclonal antibodies were used that recognized total, Ser[73], and Thr[368] Akt (1:2,000 dilution); total and Thr[80] S6K1 (1:1,000 and 1:500 dilution).
dilution, respectively); and Ser235/236 and Ser240/244 rpS6 (1:2,000 dilution). Rabbit monoclonal antibodies specific for total and Thr39/41 4EBP1 (1:1,000 dilution) and total rpS6 (1:10,000 dilution) were also used. After membranes were washed, they were incubated with a goat anti-rabbit IgG (heavy and light chain) with conjugated horseradish peroxidase (1:10,000 dilution) for 1 hour at room temperature. Membranes were developed with a chemiluminescence kit and developed on radiographic film with a film processor. Band densities were quantified via a photograph-editing software program as the mean times the number of pixels.

Gels were assayed in duplicate. To probe for the total and phosphorylated forms with different primary antibodies, the polyvinylidene fluoride membranes were first used to evaluate phosphorylated protein abundance and then were stripped with a solution (62.5 mM tris[hydroxymethyl]aminomethane hydrochloride, 2% [wt/vol] SDS, and 0.1M β-mercaptoethanol) and reprobed to evaluate total protein abundance. Membrane stripping and reprobing served to minimize the interassay error that would have resulted if the total and phosphorylated protein abundances had been quantified on different membranes.

Total Akt and Akt at Ser473 as well as total, Ser235/236, and Ser240/244 rpS6 have been evaluated in equine skeletal muscle by use of the rabbit antibodies; however, to the authors’ knowledge, the other antibodies used in the study have not been used in horse samples. There-to, the authors’ knowledge, the other antibodies used in the study have not been used in horse samples. Therefore, positive control samples with known reactivity for the antibodies used were included with all gels. In addition, prior to performing the western blot analysis for the samples in the study, we confirmed that all antibodies were cross-species specific against these proteins in horses through the use of blocking peptides. Primary antibody was mixed with blocking peptide in decreasing ratios of 1:4, 1:1, 1:0.25, and 1:0.0625 (ratio of antibody to blocking peptide) 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 reacted with the equine forms of the proteins of interest because there was a reduction in the chemiluminescent signal for the protein of interest as we increased the amount of the blocking peptide added to the reaction mixture.

Statistical analysis—All data were analyzed with statistical software. Values of P < 0.05 were considered significant. When the fixed effects were significant (P < 0.05), we conducted preplanned comparisons of least squares means.

The dependent variables plasma glucose, insulin, and amino acid concentrations were analyzed with a repeated-measures ANOVA, with age, treatment, time, age X treatment, time X treatment, age X time, and age X time X treatment as the fixed effects and horse nested within treatment and block as the random effects. The mean value for the 2 baseline samples was calculated for each of the dependent variables, and the resulting baseline concentration was included in the model as a covariate if its effect was found to be significant (P < 0.05). The baseline value was a significant covariate for plasma concentrations of glucose, insulin, and each amino acid and was therefore included in each of the respective models. Each of the translation initiation factors was analyzed via a repeated-measures ANOVA, with treatment, age, treatment X age, treatment period, and block as the fixed effects and horse nested within age X block. For all repeated-measures analyses, the variance-covariance matrix was chosen for each analysis on the basis of the lowest value for the Schwarz Bayesian criterion. Data were reported as mean ± pooled SE unless otherwise indicated.

Results

Animals and diet—All horses remained healthy and maintained normal growth rates, in accordance with recommendations of the NRC. Throughout the experimental period, the yearlings and 2-year-old horses maintained a mean ± SD ADG of 0.94 ± 0.67 kg/d and 0.18 ± 0.38 kg/d, respectively. The mature mares did not gain weight during the course of the experimental period, with a mean ADG of 0 ± 0.25 kg/d. Values for the various components of the pelleted diet that was high in crude protein were determined (Table 1). Plasma glucose concentration—We detected a significant (P < 0.001) effect of treatment, time, and treat-

Table 1—Mean ± SD values for the nutrient composition on an as-fed basis of a pelleted diet that was high in crude protein and the nutrient intake for the pelleted diet during the postprandial period in 18 Thoroughbred mares.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Pellet composition (%)</th>
<th>Nutrient intake (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>9.07 ± 0.31</td>
<td>−</td>
</tr>
<tr>
<td>Crude protein</td>
<td>33.0 ± 0.53</td>
<td>1,320 ± 21</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.05 ± 0.01</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.79 ± 0.06</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>Aspartate and asparagine</td>
<td>1.76 ± 0.08</td>
<td>71 ± 3</td>
</tr>
<tr>
<td>Glutamate and glutamine</td>
<td>4.32 ± 0.07</td>
<td>173 ± 3</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.91 ± 0.01</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.58 ± 0.02</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.90 ± 0.03</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.98 ± 0.15</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.36 ± 0.01</td>
<td>54 ± 1</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.32 ± 0.01</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Proline</td>
<td>1.93 ± 0.09</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.22 ± 0.01</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>Serine</td>
<td>1.28 ± 0.02</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.93 ± 0.04</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.85 ± 0.10</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>Valine</td>
<td>0.92 ± 0.01</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>6.17 ± 0.25</td>
<td>247 ± 10</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>12.56 ± 0.75</td>
<td>503 ± 30</td>
</tr>
<tr>
<td>Nonfiber carbohydrates</td>
<td>26.2 ± 0.36</td>
<td>1,048 ± 14</td>
</tr>
<tr>
<td>Starch</td>
<td>4.67 ± 0.06</td>
<td>191 ± 2</td>
</tr>
<tr>
<td>Water-soluble carbohydrates</td>
<td>8.43 ± 0.15</td>
<td>337 ± 6</td>
</tr>
<tr>
<td>Crude fat</td>
<td>3.47 ± 0.07</td>
<td>139 ± 2</td>
</tr>
<tr>
<td>Ash</td>
<td>15.6 ± 0.26</td>
<td>624 ± 11</td>
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<tr>
<td>Calcium</td>
<td>3.34 ± 0.13</td>
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<tr>
<td>Phosphorus</td>
<td>2.05 ± 0.09</td>
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<tr>
<td>Potassium</td>
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<tr>
<td>Sodium</td>
<td>0.68 ± 0.01</td>
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</tr>
<tr>
<td>Iron</td>
<td>1.263 ± 0.32</td>
<td>5.05 ± 0.13</td>
</tr>
<tr>
<td>Zinc</td>
<td>5.48 ± 0.10</td>
<td>2.19 ± 0.16</td>
</tr>
</tbody>
</table>

*Values represent the amount of the nutrient as a portion of the total diet on an as-fed basis. †Food was withheld from horses for 18 hours; horses then were fed a high-protein diet (12 mg/kg of body weight) at 0 and 30 minutes (postprandial state). ‡Values are reported as mg/kg of body weight.

— = Not applicable.
ment X time on plasma glucose concentration (Figure 1). The yearlings had significantly higher plasma glucose concentrations 30 minutes after the meal stimulus than did the 2-year-old and mature horses. Regardless of age, horses had higher glucose concentrations at 60 and 80 minutes in the postprandial state than in the postabsorptive state. During the postprandial state, plasma glucose concentration increased from the baseline value over time in all age groups. However, there was not a significant effect of age (P = 0.08), treatment X age (P = 0.53), age X time (P = 0.26), or treatment X age X time (P = 0.36) on plasma glucose concentration.

Plasma insulin concentration—The plasma insulin concentration was significantly affected by treatment (P < 0.001), time (P < 0.001), treatment X time (P < 0.001), treatment X age (P = 0.003), and treatment X age X time (P = 0.032; Figure 2). The plasma insulin concentration was significantly higher for the mature horses in the postprandial state, compared with the concentration in the postabsorptive state, beginning

![Figure 1](image1.png)

**Figure 1**—Least squares mean ± pooled SE plasma glucose concentrations in 6 yearling (black squares), six 2-year-old (white circles), and 6 mature (black circles) Thoroughbred mares from which feed was withheld for an 18-hour period and that were then fed a high-protein pelleted meal (2 g/kg) at 0 and 30 minutes. Two blood samples were collected prior to 0 minutes (minimum interval between sample collections, 15 minutes) and used to determine the baseline concentration. *Within an age group, the value differs significantly (P < 0.05) from the baseline value. a,bWithin a time period, values with different letters differ significantly (P < 0.05) among age groups.

![Figure 2](image2.png)

**Figure 2**—Least squares mean ± pooled SE plasma insulin concentrations in the same horses as in Figure 1. See Figure 1 for key.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Yearling</th>
<th>2-year-old</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>157</td>
<td>163</td>
<td>163</td>
</tr>
<tr>
<td>Arginine</td>
<td>73</td>
<td>70</td>
<td>71</td>
</tr>
<tr>
<td>Asparagine</td>
<td>47</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Aspartate</td>
<td>4.2*</td>
<td>3.8**</td>
<td>3.8*</td>
</tr>
<tr>
<td>Citrulline</td>
<td>70</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>Glutamate</td>
<td>224</td>
<td>24*</td>
<td>19*</td>
</tr>
<tr>
<td>Glutamine</td>
<td>442</td>
<td>443</td>
<td>420</td>
</tr>
<tr>
<td>Glycine</td>
<td>579</td>
<td>569</td>
<td>562</td>
</tr>
<tr>
<td>Histidine</td>
<td>60</td>
<td>57</td>
<td>56</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>67</td>
<td>69</td>
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</tr>
<tr>
<td>Leucine</td>
<td>120</td>
<td>124</td>
<td>117</td>
</tr>
<tr>
<td>Lysine</td>
<td>107</td>
<td>108</td>
<td>102</td>
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<td>Methionine</td>
<td>27</td>
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<td>Ornithine</td>
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<td>Tryptophan</td>
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<td>Tyrosine</td>
<td>56</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>Valine</td>
<td>205</td>
<td>204</td>
<td>196</td>
</tr>
</tbody>
</table>

Plasma amino acid concentrations were measured in blood samples collected at 80 minutes; values reported are least squares mean (µmol/L).

*Within an age group, value differs significantly (P < 0.05) from the value for the postabsorptive state.

**Table 2**—Mean plasma amino acid concentrations in 6 yearlings, six 2-year-old horses, and 6 mature horses from which food was withheld for 18 hours and that then continued to have feed withheld (postabsorptive state) or were fed a high-protein pelleted diet (2 g/kg) at 0 and 30 minutes (postprandial state).

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However, the yearlings and 2-year-old horses did not differ significantly between the postprandial and postabsorptive states until 60 and 80 minutes, respectively, after a meal stimulus when the insulin concentration was greater for the postprandial state. During the postprandial state, insulin concentrations increased with time across all age groups. At both 0 and 30 minutes following the meal stimulus, there was no significant difference among age groups for plasma insulin concentrations in the postprandial state. However, at both 60 and 80 minutes after consumption of the high-protein pelleted meal, mature horses had significantly greater circulating insulin concentrations than did yearlings or 2-year-old horses. There was not a significant effect of age (P = 0.53) or age × time (P = 0.07) on plasma insulin concentration.

Plasma concentrations of amino acids—We detected a significant effect of treatment, time, and treatment × time on all plasma amino acid concentrations, except for glycine (Table 2). The glycine concentration at 30 minutes. However, the yearlings and 2-year-old horses did not differ significantly between the postprandial and postabsorptive states until 60 and 80 minutes, respectively, after a meal stimulus when the insulin concentration was greater for the postprandial state. During the postprandial state, insulin concentrations increased with time across all age groups. At both 0 and 30 minutes following the meal stimulus, there was no significant difference among age groups for plasma insulin concentrations in the postprandial state. However, at both 60 and 80 minutes after consumption of the high-protein pelleted meal, mature horses had significantly greater circulating insulin concentrations than did yearlings or 2-year-old horses. There was not a significant effect of age (P = 0.53) or age × time (P = 0.07) on plasma insulin concentration.

Plasma concentrations of amino acids—We detected a significant effect of treatment, time, and treatment × time on all plasma amino acid concentrations, except for glycine (Table 2). The glycine concentration
was significantly affected only by time. For the essential amino acids, plasma concentrations were increased at 80 minutes by a mean of 48% in the postprandial state, but the plasma amino acid concentrations in the postabsorptive state remained unchanged, compared with the baseline values. Except for glutamine and aspartate, there was no effect of age on any plasma amino acid concentration. There was no effect of treatment × time × age for any of the plasma amino acid concentrations examined.

Muscle translation initiation factors—The phosphorylation of Akt at Ser473 was significantly ($P < 0.001$) greater for the postprandial state in all ages of horses (Figure 3); however, there was not a significant effect of age ($P = 0.24$) or treatment × age ($P = 0.51$). There was not a significant effect of treatment ($P = 0.10$), age ($P = 0.90$), or treatment × age ($P = 0.48$) on Akt phosphorylation at Thr308 (Figure 4). There was significantly ($P < 0.001$) more phosphorylation of S6K1 at Thr389 between the postabsorptive and postprandial states (Figure 5). There was also a significant effect of age ($P = 0.008$) and treatment × age ($P = 0.003$) on phosphorylation of S6K1 at Thr389. Phosphorylation of S6K1 at Thr389 during the postprandial state was significantly greater in mature horses than in yearlings or 2-year-old horses, and the yearlings had a significantly ($P = 0.008$) greater abundance of phosphorylated S6K1 than did the 2-year-old horses.

The phosphorylation of rpS6 at Ser235/236 was significantly ($P < 0.001$) greater for the postprandial state, compared with that of the postabsorptive state (Figure 6). Age alone did not have a significant effects on the phosphorylation of rpS6 at Ser235/236 ($P = 0.07$). There was a significant ($P = 0.03$) treatment × age effect because yearlings (mean ± SE, 3.3 ± 0.3 arbitrary units; $P = 0.009$) and mature horses (mean ± SE, 3.6 ± 0.3 arbitrary units; $P = 0.002$) had significantly greater values for the postprandial state, compared with the value for the 2-year-old horses (mean ± SE, 2.0 ± 0.3 arbitrary units). There was significantly ($P < 0.001$) more phosphorylation of rpS6 at Ser235/236 for the postprandial state, compared with that for the postabsorptive state (Figure 7); however, there was not a significant effect of age ($P = 0.35$) or treatment × age ($P = 0.27$).

Phosphorylation of 4EBP1 at Thr374/466 was significantly ($P = 0.003$) greater for the postprandial state, compared with that for the postabsorptive state (Figure 8); in addition, there was a significant ($P = 0.04$) effect of treatment × age. Yearlings had a significant ($P < 0.001$) increase in phosphorylation of 4EBP1 at Thr374/466 for the postprandial state, compared with that for the postabsorptive state, but there were no other significant effects of the feeding stimulus detected in the other age groups. There was not a significant effect of age ($P = 0.68$) on the phosphorylation of 4EBP1 at Thr374/466.

Discussion

To our knowledge, the present study is the first in which S6K1 has been examined in the skeletal muscle of horses of any age and the first in which the activation of translation initiation factors in response to anabolic stimuli has been examined in the skeletal muscle of adolescent animals of any species. There was a marked increase in the phosphorylation of both upstream and downstream mTOR signaling factors during the postprandial state, compared with that for the postabsorptive state, in gluteal muscle of yearlings, 2-year-old horses, and mature horses, with the highest degree of sensitivity to the anabolic stimulus in the yearlings.

After mTOR is activated, S6K1 and subsequently rpS6 are phosphorylated. Another study conducted by our laboratory group revealed that the protein abundance of the phosphorylated form of rpS6 was higher in the postprandial state than in the postabsorptive state for mature horses. This is in agreement with results of the present study, whereby protein abundance
of the phosphorylated forms of S6K1 and rpS6 was increased in the postprandial state. There is an occurrence of comparable literature on adolescent horses; therefore, studies18,30 of swine neonates were used to determine whether similar developmental trends continue during adolescence. The present study indicated that there was a decrease in the activation of translation initiation factors in response to a meal stimulus with adolescent development, which is a trend that appears to continue from neonatal development.18,30 There was a change of 158%, –7%, and 109% in the phosphorylation of S6K1 from the postabsorptive to postprandial state in yearlings, 2-year-old horses, and mature horses, respectively. In addition to the difference in the abundance of phosphorylated S6K1 at Thr389 in the postprandial state between the age groups, there were also differences in the postabsorptive state, whereby the yearlings had lower expression than did the mature or 2-year-old horses, which allowed for a more pronounced postprandial response in the younger horses. Developmental changes of the protein abundance of S6K1 at Thr389 in skeletal muscle during the postabsorptive state in neonatal pigs are inconclusive, with reports of no difference18,30 and increases31 from 7 to 26 days of age. Although adolescent horses were examined in the present study, we detected a phenomenon similar to that previously described in neonatal pigs,31 which indicated that horses are 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 Ser235/236 and Ser424/423. Phosphorylated rpS6 at Ser235/236 increased 147%, 73%, and 228% in yearlings, 2-year-old horses, and mature horses, respectively, from the postabsorptive to postprandial state. Increases of 144%, 76%, and 167% in the protein abundance of phosphorylated rpS6 at Ser424/423 from the postabsorptive to postprandial state were detected in yearlings, 2-year-old horses, and mature horses, respectively. Thus, the S6K1 portion of the signaling pathway after mTOR phosphorylation was activated in all age groups during the postprandial period, which indicated a potential increase in translation initiation. Results for the downstream effectors in the present study indicated that there was a decrease in the response to a meal-feeding stimulus in adolescent horses from 1 to 2 years of age, which may have been a continuation of the developmental decrease reported in neonates.18,19,30 However, further research is warranted to elucidate this phenomenon in equine neonates.

The other side of the downstream pathway after mTOR activation is the phosphorylation of 4EBP1 at Thr70S. In another study24 conducted by our laboratory group, we found that protein abundance of the phosphorylated form of 4EBP1 was higher in the postprandial state than in the postabsorptive state in mature horses; however, in the present study, the yearlings were the only age group that responded to the anabolic stimulus 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 aforementioned study,24 the 4EBP1 antibody was specific for all forms of 4EBP1 (α, β, and γ) and was expressed as the percentage of γ. However, in the present study, 2 antibodies were used: one was specific for phosphorylated Thr70S of 4EBP1, and the other recognized total 4EBP1. Activation of 4EBP1 during the postprandial state was 50%, 4%, and 15% greater in yearlings, 2-year-old horses, and mature horses, respectively, than during the postabsorptive state; however, activation for the postprandial state differed significantly from that for the postabsorptive state only in the yearlings. This may indicate that the yearlings were more sensitive to the anabolic stimulus of a meal than were the 2-year-old or mature horses, which is in alignment with findings of a decrease in the activation of translation initiation factors in response to a meal stimulus in swine neonates18,30 during development.

On the basis of results of previous studies, we hypothesized that the anabolic stimulus of consuming a meal would cause greater activation of translation initiation factors in yearlings than in 2-year-old horses and that the 2-year-old horses would be more responsive than the mature horses; however, this was not observed. In the adolescent age groups examined in the present study, the downstream effectors were more responsive to a meal stimulus in the yearlings than in the 2-year-old horses. This is comparable with the results of studies performed in swine neonates, which indicate a decrease in the responsiveness of downstream effectors in 26-day-old pigs, compared with that in 7-day-old pigs, to a meal stimulus.18,30,31 Supplemental amino acids,7,10 or insulin.19 However, postprandial protein abundance of the phosphorylated forms of rpS6 and 4EBP1 was not different between the yearlings and mature horses, with the lowest abundance in the 2-year-old horses. This may have been partially attributed to the feeding protocol. In the present study, all age groups in the postprandial treatment were fed 4 g of feed/kg in an effort to standardize the protein intake among the age groups. This standardization was intended to meet the protein requirements of a mature sedentary horse with a mean body weight of 550 kg; as a result, this meal did not meet the requirements of the adolescent horses and supplied only 50% and 72% of the daily requirements of the yearlings and 2-year-old horses, respectively. The increased responsiveness in the yearlings, which were consuming only 50% of their daily protein intake, indicated that if the present study were repeated, standardizing the meal to meet 100% of the daily protein requirements in yearlings would likely provide a sufficient anabolic stimulus to elucidate a potential stepwise decrease in the activation of translation initiation factors.

Although increases in the downstream effectors were detected after a meal stimulus for all age groups, future studies that involve the use of isotopic techniques are needed to examine whether this indeed led to increased protein synthesis. It is also necessary to determine whether there are age-related differences among adolescent horses in whole-body and muscle protein synthesis rates in response to a meal stimulus. There is accretion of skeletal muscle when protein synthesis is greater than protein degradation. In mature sedentary animals, there should be a balance between protein syn-
thesis and degradation, which would result in maintenance of skeletal muscle but no accretion. Similar to the mTOR signaling pathway for protein synthesis, there are signaling pathways in skeletal muscle 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. Other investigators have detected an increase in the abundance of the factors associated with protein degradation with aging in other mammals and in the postabsorptive versus postprandial state. Although we detected lower activation of the downstream mTOR signaling effectors after a meal stimulus in the 2-year-old horses, compared with results for the mature horses, we expect that the mature horses had elevated rates of muscle protein breakdown, as reported in mature animals of other species; therefore, it is likely that the 2-year-old horses were accreting more muscle protein than were the mature horses, despite having a lower activation of the mTOR-related signaling factors.

Exercise and amino acid- and insulin-dependent mechanisms can all stimulate mTOR phosphorylation. In the present study, mTOR may have been phosphorylated in an insulin- and amino acid–dependent manner because both upstream and downstream effectors were activated. Although Akt phosphorylation at Thr308 did not change between the postabsorptive and postprandial states, Akt phosphorylation at Ser473 was increased approximately 86% for all age groups. Although Akt activation requires both sites to be phosphorylated, the contribution of each site is not equal. Phosphorylated Akt at Thr308 can sufficiently stimulate Akt activation without the phosphorylation of Ser473; however, the reverse is not true. Results of the present study may indicate that Akt was already sufficiently phosphorylated at Thr308 in the postabsorptive state, which resulted in a lack of a significant difference with the postprandial state. In agreement with results of the present study, phosphorylated Akt at Ser473 in human skeletal muscle is more responsive to postprandial insulin concentrations than to phosphorylation at the Thr308 site; however, further studies in horses that involve the use of a hyperinsulinemic-euglycemic isoaminoacidemic clamp technique are needed to confirm this outcome. The use of the hyperinsulinemic-euglycemic isoaminoacidemic clamp technique would allow for determination of the responsiveness of skeletal muscle Akt to administered insulin concentrations independent of other changes that occur in response to a meal. Results for the Akt phosphorylation at Ser473 in the mature horses during the postprandial state of the present study are in disagreement with results from another study conducted by our laboratory group, in which there was no difference in phosphorylation of Ser473 from the postabsorptive 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 the other study and the present study; however, the population of horses differed between the 2 studies. Despite the similar postprandial insulin concentrations at the time of biopsy in both groups, the horses of the previous study may have been more insulin resistant, which would explain the lack of change in Akt phosphorylation at Ser473 after feeding. In future studies, all horses should be screened for insulin sensitivity prior to inclusion in the study population.

The lack of an age effect on Akt may have been attributable to differences in insulin sensitivity among the 3 age groups of horses in the present study. Postprandial insulin concentrations were higher in the mature horses than in the yearlings or 2-year-old horses at the time of biopsy; therefore, we expected the phosphorylation of Akt to be greater in the mature horses than in the yearlings or 2-year-old horses; however, this was not the case. This may indicate that the younger age groups had higher insulin sensitivity than did the mature horses, which corresponds with findings of an epidemiological study on the prevalence of obesity and hyperinsulinemia in horses. Furthermore, most obese and hyperinsulinemic horses are between 5 and 15 years of age, which is substantially older than yearlings or 2-year-old horses and similar to the age of mature horses used in the present study, the mean ± SD age of which was 13.8 ± 2.9 years. The response of Akt phosphorylation may have also been maximized at the lower insulin concentrations in the yearlings and 2-year-old horses; however, confirmation of this requires further investigation.

Exogenous insulin alone does not appear to maximally stimulate protein synthesis in adult humans and rats. Thus, it is more likely that the increase in translation initiation in the horses of the present study was the result of a combination of increased concentrations of amino acids and insulin following provision of the meal that contained a high amount of crude protein. There was no difference among age groups for most of the plasma concentrations of essential amino acids at the time of biopsy; however, the mature horses had an increase in the S6K1 response, compared with that of the yearlings or 2-year-old horses. This may indicate an increase in amino acid sensitivity with age. However, amino acid sensitivity decreases with development in neonatal pigs and decreases from maturity to old age in humans. Therefore, studies that involve the use of amino acid clamp techniques are needed, which would allow for administration of a controlled dose of amino acids in horses to determine whether mature horses truly have an increase in amino acid sensitivity.

The present study revealed that feeding a high-protein diet after an 18-hour period of feed withholding resulted in an increase in the activation of translation initiation factors Akt, S6K1, rpS6, and 4EBP1 in mature horses, 2-year-old horses, and yearlings. 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 with that of the 2-year-old horses, 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 were the yearlings or 2-year-old horses.
horses with regard to the activation of translation initiation factors. Although this finding warrants further investigation, it may have implications for designing feeding and management strategies specific for young adolescent and mature horses.

c. Dairy One Forage Laboratory, Ithaca, NY.
d. 14-gauge × 1.40-cm, Abbacath, Abbott Laboratories, North Chicago, Ill.
e. Vacutainer, Becton-Dickinson, Franklin Lakes, NJ.
f. HS equine stocks, Priefert Rodeo & Ranch Equipment, Mount Pleasant, Tex.
g. YSI 2300 STAT Plus glucose and lactate analyzer, YSI Inc Life Sciences, Yellow Springs, Ohio.
i. 3.9 X 300-mm PICO-TAG reverse-phase column, Waters Corp, Millford, Mass.
j. Sigma P8340, Sigma-Aldrich Corp, St Louis, Mo.
k. Thermo Scientific, Rockford, Ill.
l. Bio-Rad Laboratories Inc, Hercules, Calif.
m. Cell Signaling Technology Inc, Boston, Mass.
n. Amersham ECL Plus western blotting detection reagents, GE Healthcare, Piscataway, NJ.
o. Kodak X-OMAT film processor, Kodak Health Imaging Divi-
sion, Rochester, NY.
p. Adobe Photoshop elements, version 8.0, Alpha Innotech Corp,
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