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DIETARY PROTEIN REQUIREMENT OF MATURE, MODERATELY EXERCISED HORSES

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DIETARY PROTEIN REQUIREMENT OF MATURE, MODERATELY EXERCISED HORSES

By

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ABSTRACT

DIETARY PROTEIN REQUIREMENT OF MATURE, MODERATELY EXERCISED HORSES

By

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There is a dearth of empirical evidence demonstrating the degree to which exercise increases dietary protein requirement of the horse relative to maintenance. Knowledge of protein digestibility in diets fed to exercising horses is also essential to meet the protein need of the animal. The objectives of this research were first, to estimate dietary crude protein (CP) requirement using nitrogen (N) retention, serum urea-N, and serum amino acid (AA) concentration as response criteria for moderately exercised horses, and second, to determine apparent fecal N and AA digestibility of five hay:concentrate mix diets fed to horses performing moderate exercise. Five mature Arabian geldings were used in a 5 x 5 Latin Square design. Nitrogen retention, basal and post-feeding/exercise serum urea-N and serum AA results indicated that high (1016 g CP per d) and very high (1129 g CP per d) protein diets exceeded CP requirement and that very low (677 g CP per d) and low (790 g CP per d) protein diets provided sufficient amount of protein for a horse subjected to moderate exercise. Apparent fecal N and AA digestibility increased linearly ($P<0.05$) as dietary CP intake increased. Also, inclusion of soy bean meal to diet concentrates fed to exercising horses resulted in higher protein quality and contributed to an increase in apparent fecal N and AA digestibility.

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LIST OF ABBREVIATIONS

AA	amino acid
AA_i	amino acid intake
AA_f	amino acid losses in feces
ADF	acid detergent fiber
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BCS	body condition score
bpm	beats per minute
CP	crude protein
DAAO	direct amino acid oxidation
DE	digestible energy
FFA	free fatty acids
H^+	hydrogen ion
HR	heart rate
IAAO	indirect amino acid oxidation
IMP	inosine monophosphate
N	nitrogen
NADH	nicotinamide adenine dinucleotide, reduced form
NDF	neutral detergent fiber
NH_3	ammonia
NH_4^+	ammonium
N_i	nitrogen intake

N_f	nitrogen losses in feces
NPN	non-protein nitrogen
SBM	soy bean meal
TCA	tricarboxylic acid cycle

The Chapters in this thesis are written according to

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CHAPTER 1

INTRODUCTION

Introduction

The horse industry places great emphasis on health and performance of the exercising horse. Overall health and performance of the equine athlete depends primarily upon nutrition, with two key nutrients of interest being energy and protein. Research in the horse has shown that the requirement for digestible energy (DE) increases with exercise (Anderson et al., 1983; Glade, 1983; Pagan and Hintz, 1986). There is a dearth of empirical evidence demonstrating the degree to which exercise increases dietary protein requirement relative to maintenance. In the current NRC (1989), protein requirement for the moderately exercised horse is based on a crude protein (CP) to DE ratio estimated for the mature horse at maintenance, resulting in a protein requirement 1·5 fold that of maintenance. It is not known whether a CP to DE ratio of 40 g to 1 Mcal is a valid estimate of protein requirement for the exercising horse. We hypothesized that moderate exercise in horses increases protein requirement relative to maintenance below that recommended by NRC (1989). Interestingly, known methods used to estimate CP requirement in other species have not been tested in the horse. Thus, in testing our hypothesis, some of the common methods employed to estimate CP requirement were used and presented in this thesis.

In addition to the importance of providing accurate levels of dietary protein, the extent of protein availability in diets fed to horses is crucial to meeting the need of the animal. Thus, knowledge of protein digestibility is essential. Exercising horses are commonly fed diets consisting of increased concentrate relative to forage in order to meet the increased energy demands associated with exercise (Lawrence, 1990). Earlier studies provide estimates of protein digestibility of diets fed to horses and ponies at maintenance

ranging from 35 up to 79·8% (Slade et al., 1970; Hintz et al., 1971; Glade, 1984). Protein digestibility in diets fed to exercising horses has not been determined. Moreover, ingredients used in diets tested in previous studies (Slade et al., 1970; Glade, 1984) are not representative of those commonly used in formulating equine diets, especially in the Midwestern United States. The CP to DE ratio in NRC (1989) has been determined from horses consuming diets consisting solely of forage with a protein digestibility of 46%. However, total tract nitrogen (N) digestibility in ponies fed various types of forage ranged from 57 to 74% (Gibbs et al., 1988). We hypothesized that apparent fecal N digestibility would be higher than that reported in NRC (1989), and that apparent N and amino acid (AA) digestibility would increase when soy bean meal (SBM) is added to the concentrate portion of the diet.

The overall goal of this thesis is to provide further understanding of the protein requirement of the exercising horse. The specific objectives of this research were first, to estimate dietary protein requirement using N retention, serum urea-N, and serum AA concentration as response criteria in horses exposed to the same exercise regime and fed graded levels of CP, and second, to determine apparent fecal N and AA digestibility of five hay:concentrate mix diets fed to horses performing moderate exercise. The first and second objectives are addressed in chapters 2 and 3, respectively. The rationale for hypothesizing protein requirement for exercise to be less than that estimated from a ratio of 40 g CP to 1 Mcal DE is based on findings in the human literature. Thus, in the following section, the reader will gain greater understanding and appreciation behind the first research question. The rationale for the second hypothesis is based on evidence for increased utilization of concentrate diet in exercising horses (NRC, 1989; Lawrence,

1990) and the superior protein digestibility of the ingredients used to formulate such concentrates (Poultry NRC, 1994; Swine NRC, 1998). The study of nutrient digestibility in the horse is limited by the horse's extensive hind gut in itself and by the availability of methods that do not require invasive interventions. The following section of this introduction will allow the reader to understand some of the pitfalls associated with determining protein digestibility in feed ingredients fed to horses.

Thus, the objective of this introduction is to provide the reader with current knowledge on the impact of exercise on protein metabolism and with basic understanding regarding methods commonly used to estimate dietary protein requirement, as well as protein and AA digestibility.

1. **Exercise and nutrient metabolism**

1.1 Defining exercise intensity

For human athletes in particular, the level or intensity of exercise is usually divided into three categories; low intensity, submaximal, or high intensity. Because many other terms for these levels of exercise exist, defining them accurately and consistently can be challenging. This is especially true for the human and equine athlete since both species perform in many different disciplines, nonetheless some basic criteria for each category of intensity are provided herein. During low intensity exercise, subjects perform work at low speeds with low muscle force production. Lower work intensities are aerobic in nature and subjects usually perform at approximately 30-60% (Lawrence, 1990) of maximal oxygen uptake (VO_2 max). The current NRC (1989) refers to low intensity exercise in horses as light work which includes activities such as Western and English pleasure, bridle path hack, and equitation. In subjects performing

submaximal exercise, energy is supplied by both aerobic and anaerobic catabolism of available substrate (Miller-Graber et al., 1991a). Subjects performing submaximal exercise engage in activity of longer duration and/or at increased speeds and will reach approximately 75% of VO_2 max (Brooks, 1985). This type of exercise in horses is defined as moderate work and includes ranch work, roping, cutting, barrel racing, and jumping (NRC, 1989). During exercise trials designed to study electrolyte balance and serum and muscle AA profiles in endurance exercised horses (Danielsen et al., 1995; Trottier et al., 2002), horses were worked at medium speeds (2-8 m per sec), for longer duration. This type of work load correlates well with the definition of submaximal exercise given above, thus shorter competitive endurance rides could also be included in the moderate work category. Working heart rates in the study conducted by Danielsen et al. (1995) ranged from approximately 108 to 118 bpm. However, it should be recognized that many endurance horses are often required to perform for much longer duration and at greater speeds which would lead to further elevation in VO_2 max and heart rate. Endurance exercise of this nature may not correspond to a submaximal workload making it difficult to accurately classify endurance training or exercise in horses to one specific category of intensity.

Finally, high intensity exercise involves sprint work (Lawrence, 1990), thus higher speeds for shorter duration. This form of exercise is primarily anaerobic, and muscle glycogen serves as the major fuel source (Katz et al., 1986). Humans performing at high intensity have been reported to reach 95 to 100% of VO_2 max (Katz et al., 1986). In the current NRC (1989), high intensity exercise is defined as intense work and examples include race training and polo. In the study described in this thesis, protein

requirement and digestibility are estimated in horses subjected to submaximal exercise which will be referred to in the following chapters as moderate exercise.

1.2 *The role of nutrition in exercise performance*

During exercise, carbohydrate, fat, and protein can be used by working muscle as a source of fuel. The extent to which each substrate is utilized during exercise depends upon the intensity and duration of the exercise as well as the availability of each fuel source. Therefore, when formulating diets for exercising humans or horses, the level of exercise and the nutrient composition of the diet must be considered. The ultimate goal in formulating diets for athletes, human or animal, is to meet the individual's nutrient requirements in order to maximize performance and delay the onset of fatigue. Fatigue during exercise is brought on by substrate or fuel depletion and accumulation of end-products associated with nutrient and muscle metabolism such as lactate, H^+, and NH_3. Lactate and H^+ accumulation in muscle tissue causes a decline in muscle pH which diminishes ATP production and decreases force generation by muscle fibers (Lawrence, 1990). Ammonia production in muscle results primarily from the deamination of AMP to IMP in the purine nucleotide cycle (Lowenstein, 1972). Ammonia accumulation in muscle or blood may inhibit the TCA cycle enzyme isocitrate dehydrogenase, thus decreasing α-ketoglutarate synthesis and resulting in a reduction in ATP and NADH production (Miller and Lawrence, 1986). Fatigue in both submaximal and high intensity exercise is related to accumulation of end-products; however, accumulation of end-products and subsequent onset of fatigue occurs more rapidly at higher intensities (Parkhouse and McKenzie, 1984). Fatigue during both submaximal and high intensity exercise also results from depletion of carbohydrate (Lawrence, 1990), particularly

during work bouts or performances of longer duration. There is no evidence regarding the extent to which dietary protein intake influences the onset of fatigue. Thus, a dietary CP requirement to optimize performance is less than clear.

1.3 *Carbohydrate and fat*

Carbohydrate and fat are the primary energy sources during exercise (Mole and Johnson, 1971; Dohm et al., 1977). During high intensity exercise, carbohydrate serves as the primary fuel, while fat metabolism comprises a major source of energy in subjects performing low intensity exercise (Miller-Graber et al., 1991a). Carbohydrate is supplied to the working muscle primarily by the breakdown of muscle glycogen, but as muscle glycogen is depleted, blood glucose from hepatic glycogenolysis and gluconeogenesis becomes an important energy substrate (Winder, 1985; Bjorkman and Wahren, 1988). Glucose is converted to pyruvate via glycolysis for ATP generation needed for muscle contraction. At high intensities, oxidation of some glucose will be incomplete thus forming lactic acid (Hermansen, 1981).

Oxidation of free fatty acids (FFA) and circulating and intramuscular triglycerides can also supply energy to working muscle especially during submaximal exercise. Lipolysis in adipose tissue results in the release of FFA which are transported in the blood and taken up by the muscle for oxidation (Paul and Issekutz, 1967; Essen et al., 1977; Terjung et al., 1983). Fat utilization as an energy source can have a sparing effect on glycogen. Caloric intake composed of 55 to 65% carbohydrate, 20 to 30% fat, and 12 to 15% protein has been recommended in human athletes by Leaf and Frisa (1989). In studies conducted in humans and horses, it has been shown that exercise increases the demand for energy (Edwards et al., 1935; Pagan and Hintz, 1986; Grandjean, 1989).

However, much controversy exists regarding an increased demand for dietary protein in human and equine athletes.

1.4 *Protein*

The belief that protein intake could enhance athletic performance dates as far back as 450 BC (Leaf and Frisa, 1989). It was theorized then that consumption of meat would increase muscle strength. However, current researchers are still at odds regarding the increased need for protein as a result of physical activity, particularly in human athletes consuming adequate energy (Leaf and Frisa, 1989). Although selected AA, primarily alanine, are used in gluconeogenesis during prolonged exercise (Lawrence, 1990), Young (1986) suggested that in exercising humans, the oxidation of protein accounts for less than 5% of energy expenditure. Interestingly, in most species, it has been estimated that muscle protein turnover accounts for approximately 25 to 30% of whole body protein turnover (Young, 1986). In rats, endurance-type exercise results in decreased muscle protein synthesis and increased rates of AA oxidation, particularly leucine (Young, 1986). A study conducted in human endurance athletes (Meredith et al., 1989) provided evidence that protein requirement should be increased, as subjects attained N balance when ingesting protein at a level approximately 17% above World Health Organization recommendations (FAO/WHO/UNU, 1985).

Nitrogen retention increased in horses exposed to increasing workloads and fed increasing levels of dietary protein and energy (Freeman et al., 1988) indicating that protein requirement of the exercising horse may be increased relative to maintenance. Conversely, Gontzea et al. (1975) concluded from their N balance study, that habitual physical activity in humans did not increase protein requirement. In addition, 25 to 40

min of treadmill exercise at approximately 65% of VO2 max, did not affect nitrogen balance in young adults (Kido et al., 1997), and it was concluded from this study that exercise did not increase protein requirement relative to requirement of sedentary individuals. In exercising human (Millward et al., 1994) and equine (Lawrence, 1990) subjects, there is no known evidence that high protein intake relative to maintenance enhances athletic performance. Yet, Hallebeek et al. (2000) reported digestible protein intake in event horses to be 92% above protein requirement, thus suggesting critical problems behind the lack of knowledge on CP requirement.

A limitation to estimating dietary protein requirement is that known methods used to determine requirement in other species have not been tested in the horse. Methods employed to assess protein status and requirement in exercising humans include N balance, analysis of blood biochemistry, and isotopic tracer studies which measure AA oxidation in response to exercise. These methods will be discussed in the sections that follow.

2. **Methods for estimating protein requirement**

2.1 *Nitrogen retention*

Nitrogen retention has been used extensively in many species including humans and swine (Rose. 1957; Hegsted, 1976; Young and Scrimshaw, 1978; King et al., 1993; Otto et al., 2003) to estimate dietary protein requirement. Nitrogen retention is calculated by subtracting fecal and urinary N losses from N intake and is a measure of N retained by the animal for body protein synthesis and accretion. Figure 1 illustrates the relationship between N intake and N retention. Below dietary protein requirement, N retention increases linearly with increasing protein intake. When protein intake exceeds the

animal's potential for protein deposition, as estimated by N retention, N retention is maximized.

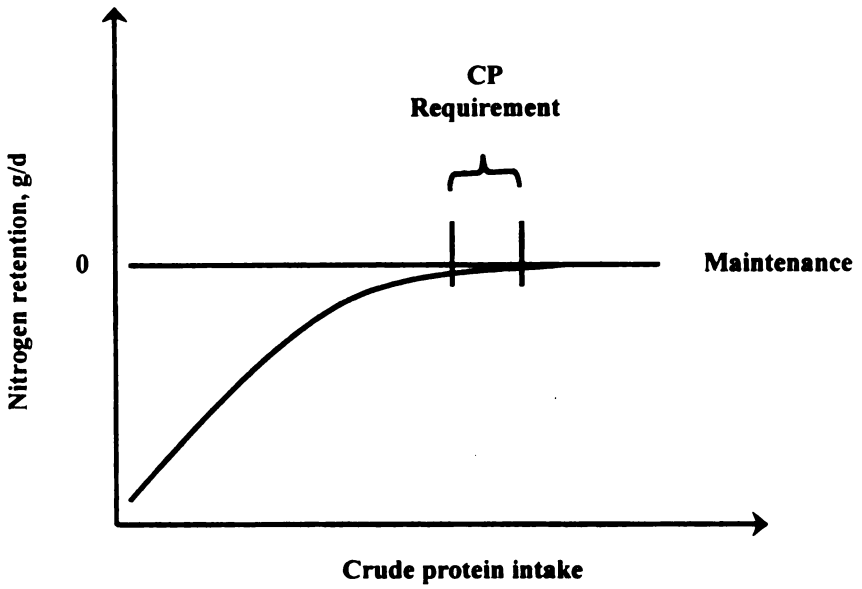

Figure 1. Relationship between crude protein intake and nitrogen retention.

At maintenance, N balance or N equilibrium is achieved when N losses are equal to N intake (Slade et al., 1970). Nitrogen requirements for horses and ponies at maintenance have been obtained using N retention (Slade et al., 1970; Hintz and Schryver, 1972; Prior et al., 1974), but there is a paucity of studies that have employed N retention as a response criterion to estimate protein requirement of the exercising horse fed graded levels of protein.

The N containing compounds in urine include urea, ammonium, and trace amounts of creatinine and AA including taurine and glycine. Most ammonium in urine originates from glutamine synthesized in the perivenous hepatocytes as a secondary route for ammonia detoxification (Boon et al., 1994). Glutamine is transported to the kidney

where it is deaminated and the ammonia formed excreted in the urine, a process known as renal ammoniagenesis (Boon et al., 1994). Creatinine in urine originates from the nonenzymatic dehydration of intracellular creatine, which, when phosphorylated, serves as the primary energy store in working muscle (Wang et al., 1996). Creatinine is proportional to skeletal muscle mass (Wang et al., 1996). In humans, muscle mass can be estimated from 24-h urinary creatinine excretion, where 1 g creatinine is proportional to 21·8 kg skeletal muscle (Wang et al., 1996). Creatinine has also been used as a clinical marker of renal function (Reyes et al., 1994).

2.2 *Blood urea nitrogen*

Urea is the major end product of N metabolism in mammals (Morris, 1985). Urea is excreted by the kidney (Reyes et al., 1994) and originates from the deamination of AA mobilized from body tissues or fed in excess of requirement. The urea cycle in the liver is vital in maintaining low plasma ammonia concentration (Reyes et al., 1994). The amino group generated as a result of AA catabolism is transferred to arginine by a series of enzymatic reactions. Arginine is then cleaved to form ornithine and urea by the enzyme arginase (Reyes et al., 1994). When an animal is in N balance, urea synthesis is a function of dietary protein level. In rats, pigs and humans, urea cycle activity has been shown to increase with increasing dietary protein intake (Edmonds et al., 1987; Matthews and Campbell, 1992; Bertolo et al., 2000). Blood urea-N has been used as a response criterion to determine protein and amino acid requirements of lactating sows (Coma et al., 1996). In exercising Quarter Horse mares, blood urea-N has been used to assess protein status when a high protein diet (18·5% CP) was fed (Miller and Lawrence, 1988). Figure 2 illustrates the hypothetical relationship between dietary protein intake and blood

urea-N. When balance between protein degradation and synthesis is achieved, blood urea-N should be minimized. Thus, at protein intake exceeding requirement, blood urea-N will increase exponentially.

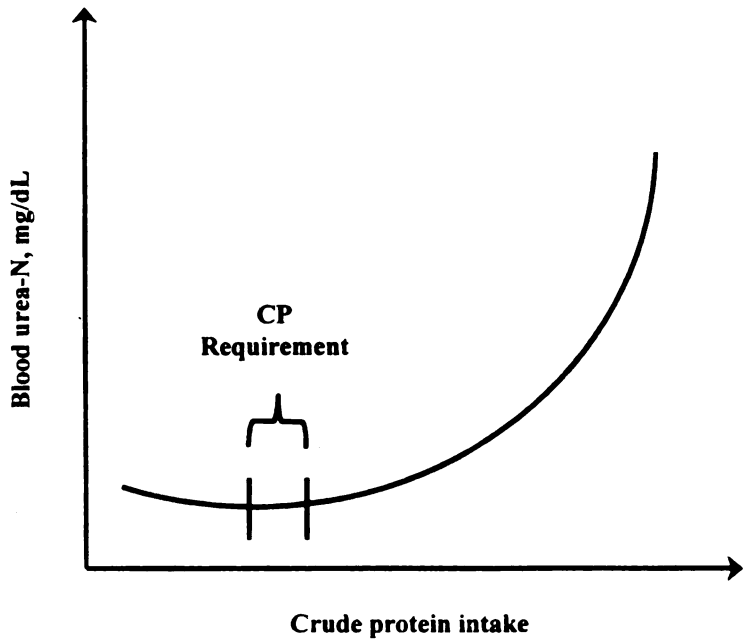

Figure 2. Relationship between crude protein intake and blood urea-nitrogen.

In addition to urea, concentrations of other metabolites involved in the urea cycle can be useful indicators of protein status. Figure 3 depicts the urea cycle beginning with incorporation of CO_2 and amino-N, resulting from deamination of AA, into carbamoyl phosphate inside the liver mitochondria. Ornithine and carbamoyl phosphate combine to form citrulline and the subsequent enzymatic reactions within the cytosol facilitate production of urea. Thus, ornithine and citrulline are key metabolites involved in the formation of urea and drive the enzymatic reactions of the urea cycle forward (Morris, 1985). Decreases in plasma or serum ornithine and citrulline concentration would

indicate impairment or saturation of urea cycle enzymatic activity (Reyes, 1994). Orotic acid is also a useful indicator of protein status. For instance, increased synthesis or decreased utilization of carbamoyl phosphate into the urea cycle leads to an increase in cytosolic orotic acid synthesis and subsequent urinary excretion (Fico et al., 1984).

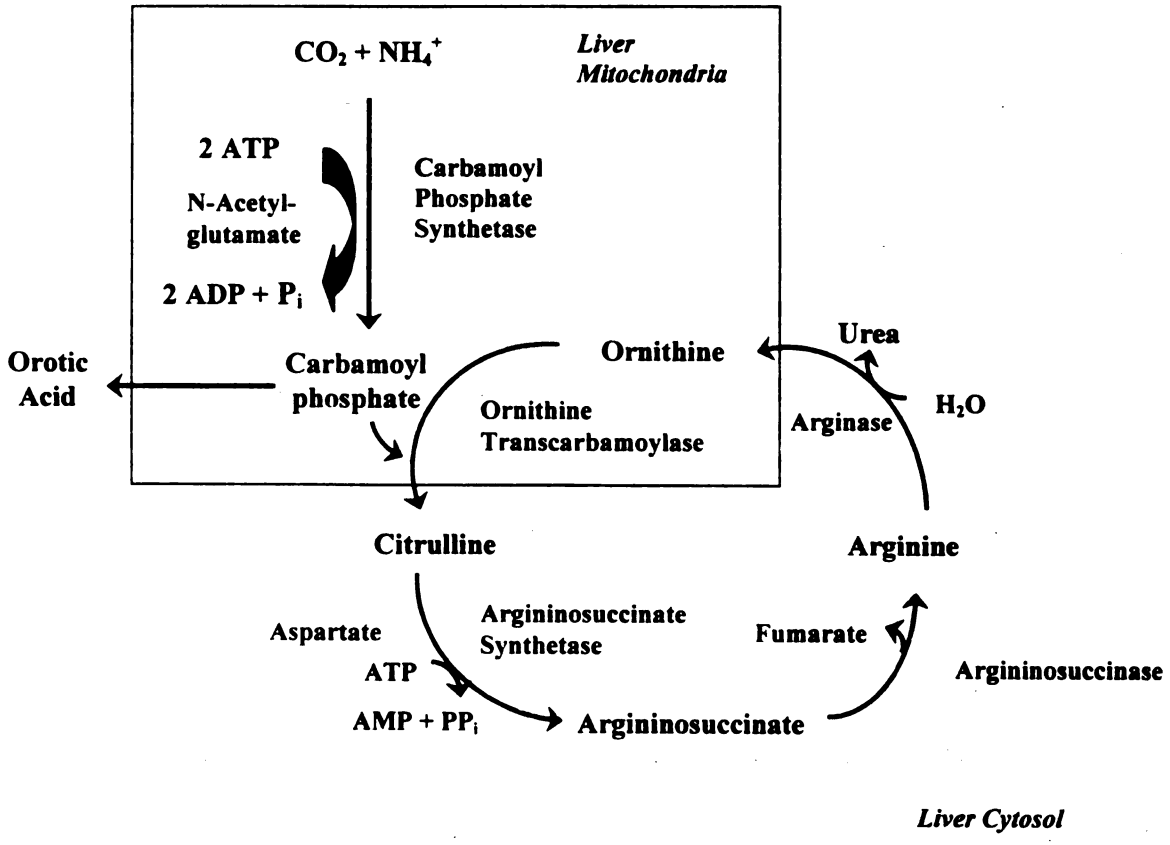

Figure 3. Urea cycle.

2.3 *Plasma free amino acid concentration*

Upon digestion of dietary protein, the majority of AA are transported in free form in the blood to the liver where they will be used for hepatic protein synthesis, catabolized, and/or sent to peripheral tissues for protein synthesis and utilization. Serum AA

concentrations represent a balance between dietary AA intake and protein turnover (Russell et al., 1986). Protein turnover refers to the continuous incorporation of AA into protein synthesis or degradation of tissue protein and subsequent release of AA into circulation. Concentration of AA in plasma can also be affected by the source and availability of AA from dietary protein (Richardson et al., 1965). While interpretations of changes in plasma AA concentrations can be difficult due to the multiple metabolic processes involved in AA entry into and removal from the plasma pool (Marchini et al., 1993), change in serum AA pattern can provide some information on the adequacy of dietary protein and AA intake. Plasma free AA measurement has been useful in swine and humans for evaluating dietary protein quality and protein status, and for estimating AA requirements (Puchal et al., 1962; Marchini et al., 1993).

2.4 *Indicator method: Direct and Indirect amino acid oxidation*

Other more recent techniques for determining protein status and AA requirements include direct and indirect AA oxidation (DAAO and IAAO, respectively). The DAAO method measures AA flux and oxidation in vivo by using amino acids labeled with stable or radioactive isotopes. When an AA is consumed at or below requirement, the labeled AA is conserved by the body and incorporated into protein synthesis, thus AA oxidation to CO_2 is minimized (Zello et al., 1990). These techniques have been used successfully in swine and humans to determine AA requirement (Kim et al., 1983; Zello et al., 1990; Zello et al., 1993), but have not been used in horses. For the IAAO method to be effective, the requirement of the labeled indicator AA must be known, as the indicator AA is most sensitive to intake of the test AA when it is fed at its required level (Wilson et

al., 2000). To date, indispensable AA requirements for the mature horse have not been determined, making it difficult to meet this criterion.

3. Methods for estimating nitrogen and amino acid digestibility

3.1 *Apparent fecal digestibility*

Digestion of protein and absorption of AA in the horse occurs in different segments of the gastrointestinal tract and is influenced by the type of diet consumed. Hintz et al. (1971) reported the major site of protein digestion in ponies was prececal. However, results of their study demonstrated significant disappearance of N from the hind gut as well. The extent to which products of protein digestion in the cecum and large intestine are absorbed and utilized by the horse remains unclear, and it is also not understood to what extent microbial protein and AA synthesis in the lower gut contributes to fecal N content in horses. Despite these uncertainties, several researchers have provided estimates of apparent fecal N digestibility in ponies and horses at maintenance (Slade and Robinson, 1970; Glade, 1984; Gibbs et al., 1988). However, apparent digestibility of AA in horse diets have not been investigated. Apparent fecal N and AA digestibility is defined as the difference between N or AA intake and N or AA losses in feces, expressed as a proportion of N or AA intake. While this method is noninvasive and provides some information on the availability of N and AA from the diet, it fails to differentiate between protein of dietary versus microbial protein origin, thus leading to inaccurate measurements of digestibility (Darragh and Hodgkinson, 2000).

3.2 *Apparent ileal digestibility*

An alternative method to apparent fecal digestibility is apparent ileal digestibility, which is believed to be a more sensitive and accurate technique (Darragh and Hodgkinson, 2000). Apparent ileal digestibility is determined by subtracting the N and AA measured in digesta collected at the distal ileum from the N and AA intake and dividing the difference by N and AA intake. Collection of digesta from the distal ileum at the ileal-cecal junction greatly reduces the confounding effect of microbial protein metabolism in the large intestine and results in a more accurate estimate of protein and AA digestibility. However, this method is more invasive as it requires surgical insertion of a cannula and can therefore be more expensive and labor intensive. This procedure has not been routinely used in the horse, and it is unlikely to be readily adopted in horses in the near future. In chapter 3 of this thesis, apparent fecal N and AA digestibility in diets fed to moderately exercised horses were determined.

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CHAPTER 2

NITROGEN BALANCE OVERESTIMATES DIETARY PROTEIN REQUIREMENT OF THE EXERCISING HORSE

ABSTRACT

NITROGEN BALANCE OVERESTIMATES DIETARY PROTEIN REQUIREMENT OF THE EXERCISING HORSE

By

Carissa Lee Wickens

Five mature Arabian geldings performing moderate exercise were randomly assigned to 1 of 5 dietary treatments in a 5x5 Latin Square design to estimate dietary crude protein (CP) requirement. Each period was 14 days in length and consisted of a 10-day diet adaptation followed by a 4-day total urine and fecal collection. All horses consumed mixed grass hay containing 10% CP at 1% of their body weight. Concentrate plus hay was fed to provide 677, 790, 903, 1016, and 1129 g CP daily corresponding to a very low (VL), low (L), Control, high (H) and very high (VH) protein diet, respectively. Nitrogen (N) retention, serum urea-N, and serum amino acid (AA) concentration were used as response criteria. Nitrogen retention, basal and post-feeding/exercise serum urea-N and serum AA concentration increased linearly ($P<0.05$) as daily CP intake increased. Nitrogen retention increased ($P<0.05$) in horses fed H compared to Control and was not different ($P=1.0$) compared to VH. Compared to Control, N retention was not different ($P>0.05$) and was positive in horses fed VL and L diets. Post-feeding/exercising serum urea-N increased in horses fed H compared to Control and was not different ($P=0.43$) compared to VH. For a majority of the indispensable AA, post-feeding/exercise serum concentration only differed ($P<0.05$) from Control in horses fed the VL protein diet. These results indicate that H and VH protein diets exceeded CP requirement and that the

VL and L protein diets provided sufficient amount of protein for a horse subjected to moderate exercise.

Key Words: Horse, Exercise, Protein, Nitrogen balance, Blood urea nitrogen

Introduction

There is a paucity of information on protein requirement of the exercising horse. In the current NRC (1989), protein requirement for a horse performing moderate work is based on a crude protein (CP) to digestible energy ratio estimated for the mature horse at maintenance, resulting in protein requirement being 1·5 times higher than that of maintenance. Protein catabolism comprises up to only 15% of energy production during exercise, and protein is a relatively inefficient source of energy compared to that of carbohydrate and fat (Lawrence, 1990). Moreover, feeding protein in excess of NRC (1989) recommendations is a common practice in the horse industry (Hiney and Potter, 1996), but positive benefits associated with dietary protein supplementation have not been documented in the horse (Lawrence, 1994). Thus, it is questionable whether the relationship between dietary requirement for protein and energy is similar between exercising and non-exercising horses. Excessive protein intakes are associated with increased water demands, increased plasma urea concentration, higher energy costs associated with nitrogen (N) excretion, and increased ammonia emission in barns (Meyer, 1987). In a study conducted by Freeman et al. (1988), N retention increased in horses exposed to increasing workloads and fed increasing levels of dietary protein and energy, indicating that exercise may increase the need for protein. However, to date, no empirical studies have been designed to estimate CP requirement in response to graded levels of dietary CP intake, thus the extent to which exercise increases dietary protein

requirement relative to maintenance remains unclear. We hypothesized that moderate exercise in horses increases protein requirement below that recommended by NRC (1989). The objective was to estimate dietary protein requirement using N retention, serum urea-N, and serum AA concentration as response criteria in horses exposed to the same workload and fed graded levels of CP.

Materials and Methods

Animals, experimental design, and diets

Five mature Arabian geldings with an initial body weight of 473·3 ± 16·4 kg were selected and kept on clover-grass mix pasture during a pre-experiment exercise and standardization period. Following conditioning, horses were randomly assigned to five dietary treatments in a 5 x 5 Latin square design. Horses were housed individually in box stalls (3·0 x 3·7 m) with free access to water. All horses consumed mixed grass hay containing 10% CP (as fed basis) at 1% of their BW. Five diet concentrates were formulated to achieve varying levels of crude protein intake. Ingredient and nutrient composition of the concentrate diets are provided in Table 1. A Control diet concentrate was first formulated to meet NRC (1989) daily protein requirement estimate for moderate exercise. A very low (VL) and a low (L) diet concentrate was formulated to provide 25% and 12·5% lower protein respectively, relative to Control, and a high (H) and very high (VH) diet concentrate was formulated to provide 12·5% and 25% higher protein respectively, relative to Control. Thus, total diet, i.e., hay plus concentrate, was fed to provide 677, 790, 903, 1016, and 1129 g CP daily corresponding to the very low (VL), low (L), Control, high (H), and very high (VH) protein diet, respectively. The level of protein intake in the VL, L, Control, H, and VH protein diets corresponded to a CP to

digestible energy (DE) ratio of 30, 35, 40, 45, and 50 g CP to 1 Mcal DE, respectively. To achieve a VL protein diet, corn was used as the primary feed ingredient. Protein concentration of the diet concentrates was increased by altering the corn to oat ratio and through the addition of soybean meal. Molasses was included in the concentrate mix as a binder. Diet concentrates varied slightly in DE content and were fed to achieve the desired protein intake levels. This resulted in minor energy deficiencies, thus corn oil was top-dressed in small amounts to assure NRC (1989) energy requirement for moderate exercise was met for each horse. Vitamin-mineral mix was top-dressed once daily to provide NRC (1989) recommended levels of Ca, P, Vitamin A, Vitamin D, Vitamin E, and Se. Meals were fed twice daily at 0700 and 1600. Acclimation of horses to treatment diets consisted of feeding mixed grass hay at 1·2-1·5 % of each horse's BW and gradually introducing the Control diet concentrate during the last week of conditioning.

Exercise Protocol

Prior to the start of the experiment, horses were subjected to a 6-week standardization period. During the initial week, horses were trotted daily for 6 consecutive d on a mechanical walker (Free Flow Equineciser, Centaur Horse Walkers, Mira Loma, CA) at a speed of 3·6 m per sec for 10 min. Thereafter, workload was increased each week by 10 min increments until horses were trotting 60 min per d. During the experimental period, horses were exercised bi-directionally on the walker at the trot at approximately 3·6 m per sec, 60 min per d, 6 d per week. Exercise was performed in the morning 2 h post-feeding. Heart rate (HR) monitors were used to assess work load and to verify that pre-experiment fitness levels, based on resting and working HR measured at the end of the standardization period, were maintained. Body weights

and body condition scores (BCS) were recorded every two weeks. Body condition score was assessed according to NRC (1989) guidelines.

This study was approved by Michigan State University All University Committee on Animal Use and Care.

Sample collection

The study consisted of five collection periods. Each period was 14 d in length and consisted of a 10-d diet adaptation followed by a 4-d total fecal and urine collection. Feces and urine were collected using gelding collection harnesses (Equisan Marketing, Melbourne, Australia) and emptied every 5 h or more frequently as needed. At each emptying, all feces were bagged and approximately 10% of the total urine volume was sampled. Feces and urine were immediately stored at -20°C. At the end of each collection period, daily fecal samples were thawed, pooled, weighed and homogenized using a 136-kg mechanical mixer. Sub samples were collected (~500 g) and frozen at -20°C. Daily urine samples were pooled, mixed, and a sample of pooled urine stored at -20°C. During exercise, harnesses were removed. Horses did not urinate during this time. If horses defecated, care was taken to collect and weigh the fecal matter. This fecal matter was then discarded.

Blood samples (20 mL) were drawn from each horse via jugular venipuncture on d 3 and 4 of the collection period. Blood samples were drawn prior to the morning meal (15 h post-feeding) to obtain basal metabolite values. Basal values were obtained to represent long-term metabolism. Horses were fed following blood collection and exercised 2 h later. An additional blood sample was obtained within 10-20 min of

completion of exercise. Blood samples were centrifuged (Beckman, GS-6KR Centrifuge, Fullerton, CA) at 3000 rpm for 15 min at 4°C and serum and plasma stored at -20°C.

Sample Analysis

For chemical analysis, fecal samples were freeze-dried (VirTis model 25-SRC, VirTis Co., Gardiner, NY). Fecal and feed samples (hay and concentrate) were finely ground using a cyclone mill (Foss Cyclotec sample mill 1093, Hoganas, Sweden) with a 1-mm mesh screen. Nitrogen concentration in feces, urine and feed was determined using an automated N analyzer (Leco FP-2000, Leco Co., St. Joseph, MI; AOAC No. 990·3). Dry matter of feed was determined following a 24-h drying period at 80°C using a drying oven (Fisher Isotemp, Fisher Scientific, Hanover Park, IL). Amino acid analysis was performed on feed samples using the Pico-Tag method (Waters Co., Milford, MA) following a 24-h acid hydrolysis in 6*N* HCl at 113°C and 121 mm Hg. Norleucine was used as an internal standard. Samples were derivatized with phenylisothiocyanate and analyzed by high pressure liquid chromatography (HPLC) (Alliance 2690, Waters Co., Milford, MA) fitted with a 30-cm Pico-Tag column (Waters Co., Milford, MA). Amino acid analysis was performed on basal and post-feeding/exercise serum samples using the Pico-Tag method (Waters Co., Milford, MA). Briefly, protein was first precipitated by mixing 200 μL serum with 1 mL Trifluoroacetic acid:methanol (1:10) and centrifuging (eppendorf centrifuge 5417R, Brinkmann Instruments, Westbury, NY) at 7000 rpm for 15 min at 4°C. The supernatant (155 μL) was removed and norleucine (1·25 mM) added as an internal standard. This was followed by evaporation to dryness at 37°C with a centrifuge evaporator (HETO Vacuum Concentration System, ATR, Laurel, MD). Samples were derivatized with phenylisothiocyanate and analyzed by HPLC (Waters Co.,

Milford, MA) fitted with a 30-cm Pico-Tag column (Waters Co., Milford, MA). Digestible lysine intake was calculated from the apparent fecal lysine digestibility coefficient determined by Wickens et al. (unpublished).

Urea-N analysis was performed on serum samples using a commercially available colorimetric assay (procedure no. 640-A, Sigma, St. Louis, MO). Absorbance was read at 570 nm using a spectrophotometer (Beckman, DU 7400, Schaumburg, IL). For urinary creatinine analysis, urine samples were diluted 1:10 and creatinine determined using a commercially available colorimetric assay (procedure no. 555, Sigma, St. Louis, MO). Absorbance was read at 500 nm using a spectrophotometer (Beckman, DU 7400, Schaumburg, IL). Urinary orotic acid was analyzed using a procedure adopted from Adachi et al. (1963). Briefly, urine was acidified to pH 2-3 and 1 mL urine was added to 2 mL citric acid-potassium citrate buffer (0·2 M) and 0·5 mL saturated bromine water. Samples stood for 1 min at room temperature followed by the addition of 1 mL ascorbic acid (5%). Samples were then placed in a warm water bath (40°C) for 5 min, followed by the addition of 2 mL p-dimethylaminobenzaldehyde (2·5%). Samples were then kept in the warm water bath for 10 additional min, cooled under running water and the absorbance read at 480 nm using a spectrophotometer (Beckman, DU 7400, Schaumburg, IL).

Statistical Analysis

Data were subjected to ANOVA using the PROC MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). Effects of horse, period, and diet were included in all statistical models. Horse was treated as a random effect. Differences between all pair-wise comparisons were evaluated using the Tukey-Kramer test (Younger, 1998).

Relationships between dietary CP intake and N balance parameters, serum amino acid concentration, serum urea-N concentration, urinary creatinine concentration, and urinary orotic acid concentration were determined using contrast (linear and quadratic) comparisons with orthogonal polynomials. Coefficients used for contrasts were based on the calculated CP intake of 677, 790, 903, 1016, and 1129 g per d. Statistical significance was based on an experiment-wise type-I error rate of 0·05. Regression analysis was performed using the GLM procedure of SAS (SAS Inst, Inc., Cary, NC) to determine the nature of the relationship between total and digestible lysine intake and N retention. Using the PROC UNIVARIATE procedure of SAS (SAS Inst., Inc., Cary, NC), total and digestible lysine intake versus N retention data were determined to be normally distributed with homogeneous variance.

Results

Horse performance data

Horse performance data including body weight, BCS, resting and working HR, as affected by period, are shown in Table 2. There was no effect of diet ($P>0·05$) on body weight, BCS, resting or working HR. Pre-experiment body weight was not different ($P>0·05$) compared to that in period 1. Body weight was higher ($P<0·05$) in period 5 compared to period 1, but was not different when compared to that in periods 2, 3, and 4. Pre-experiment BCS was not different ($P>0·05$) compared to that in period 1. Body condition score increased ($P<0·05$) from period 1 to 2, and there was no difference ($P>0·05$) in BCS between periods 2, 3, 4, and 5. There was no difference ($P>0·05$) in resting or working HR across periods, and HR throughout the trial did not change ($P>0·05$) with respect to HR taken at the end of the standardization period.

Nitrogen balance

Nitrogen intake, losses, and retention data, as affected by dietary CP level are presented in Table 3. Nitrogen intake, fecal N excretion, urinary volume, urinary N excretion, and N retention increased linearly ($P<0.05$) as daily CP intake increased. A quadratic relationship between these parameters and dietary CP intake was not found ($P>0.05$). Compared to Control, fecal N was not different ($P>0.05$) in horses fed VL, L, H and VH protein diets. Fecal N was higher ($P<0.05$) in horses fed the VH protein diet compared to that in horses fed the VL diet. Compared to Control, urinary volume was not different in horses fed VL, L, H, and VH protein diets. However, urinary volume was higher ($P<0.05$) in horses fed VH compared to that in horses fed VL. Compared to Control, there was no difference in urinary N excretion in horses fed L or H protein diets. Urinary N was higher ($P<0.05$) in horses fed VH and lower ($P<0.05$) in horses fed VL compared to that in horses fed Control. Compared to Control, there was no difference in N retention in horses fed the VL ($P=0.23$) and L ($P=0.80$) protein diets. Compared to Control, N retention increased ($P<0.05$) in horses fed H and was not different ($P=1.0$) compared to VH. There was no quadratic relationship ($P>0.05$) between N retention and increasing dietary CP intake. Relationship between N retention and daily total and digestible lysine intake is presented in figure 4 and 5, respectively. Nitrogen retention increased linearly ($P<0.05$) as daily total and digestible lysine intake increased. There was no quadratic relationship ($P>0.05$) between N retention and increasing total or digestible lysine intake.

Serum urea nitrogen, creatinine, and orotic acid

Basal and post-feeding/exercise serum urea-N, arginine, citrulline, ornithine, and glutamine concentration as affected by dietary CP level are presented in figures 6, 7, 8, 9, and 10, respectively. Basal and post-feeding/exercise serum urea-N concentration increased linearly ($P<0.001$) as dietary CP intake increased. Post-feeding/exercise serum urea-N in horses fed VL tended to differ ($P<0.06$) compared to that in horses fed the L protein diet but did not differ between horses fed L and Control ($P=0.23$). Post-feeding/exercise serum urea-N increased ($P<0.05$) in horses fed VH and H protein diets versus VL, L, or Control diets. Basal serum arginine, citrulline, and ornithine concentration were not different ($P>0.05$) among dietary treatments. A quadratic relationship ($P<0.05$) was found between dietary CP and post-feeding/exercise serum arginine concentration. Post-feeding/exercise serum arginine concentration increased ($P<0.05$) as dietary CP intake increased, and reached plateau in horses fed the Control diet. Post-feeding/exercise serum citrulline, ornithine, and glutamine concentration increased linearly ($P<0.05$) as dietary CP intake increased.

Urinary creatinine, urinary orotic acid, and orotic acid to creatinine ratio data are presented in Table 4. Urinary creatinine and orotic acid concentration were not affected ($P>0.05$) by dietary CP intake. Daily urinary orotic acid output increased linearly ($P<0.05$) with increasing daily CP intake. Orotic acid to creatinine ratio was not different ($P<0.05$) between dietary treatments. There was no quadratic relationship ($P>0.05$) between CP intake and the above parameters.

Serum amino acid concentration

Basal serum indispensable AA concentration data, as affected by dietary CP level are presented in Table 5. For a majority of the indispensable AA, basal serum concentration did not differ ($P>0{\cdot}05$) among dietary treatments. Serum isoleucine and valine concentration increased linearly ($P<0{\cdot}05$) as dietary CP intake increased. Post-exercise serum indispensable AA concentration data are presented in Table 6. Post-feeding/exercise serum concentration of all indispensable AA increased linearly ($P<0{\cdot}05$) as dietary CP intake increased. For a majority of the indispensable AA, post-feeding/exercise serum concentration only differed ($P<0{\cdot}05$) from Control in horses fed the VL protein diet. A quadratic relationship ($P<0{\cdot}05$) was found for serum total sulfur AA (TSAA) concentration. Total sulfur AA concentration increased ($P<0{\cdot}05$) as CP intake increased, and reached plateau in horses fed the Control diet.

Discussion

The current NRC (1989) crude protein (CP) requirement estimate for the moderately exercised horse is derived from a CP to digestible energy (DE) ratio of 40 g to 1 Mcal determined for the mature horse at maintenance. Consequently, dietary CP recommendation for the moderately exercised horse is 1·5 fold the recommended dietary CP requirement for maintenance. We raised the question whether this ratio can also be applied for the exercising horse. In this study, N retention, serum urea-N, and serum AA concentration were used as response criteria to estimate protein requirement of Arabian horses exposed to the same workload and fed varying levels of CP.

Freeman et al. (1988) showed that N retention increased in exercising horses exposed to increasing workloads and fed increasing level of dietary protein and energy.

In our study, in horses performing the same level of exercise, N retention increased as dietary CP increased. Nitrogen retention ranged from 21·0 g per d in horses fed the VL protein diet up to 43·0 g per d in horses fed VH. Gibbs et al. (1988) observed positive N retention of 31·1 g per d in mature ponies at maintenance consuming alfalfa hay (18·1% CP). Similarly, in a study conducted by Slade et al. (1970), positive N retention as great as 20 g per d was found in mature horses at maintenance consuming between 800 and 900 g CP per d. We had hypothesized protein requirement for moderate exercise to be lower than that of NRC (1989). Surprisingly, N retention increased and was seemingly maximized in horses fed 1016 g CP per d, corresponding to a protein level of 12·5% above that recommended by NRC (1989). Indeed, a linear rather than quadratic response between N intake and retention was obtained, which precluded estimating protein requirement using broken point analysis as performed by Dourmad and Etienne (2002). Considering the difference in protein quality between the lower and higher protein diets, the relationship between total or digestible lysine intake and N retention was examined and found also to be linear. Jackson (1999) has reported that at higher intakes of protein, measured N retention is consistently positive and suggested this to be an artifact inherent to N balance studies. Nitrogen retention at higher protein intake may be overestimated due to an overestimation of N intake and underestimation of N losses (Jackson, 1999). However, N retention has been used successfully to determine protein requirement in other species including humans and swine (Rose, 1957; Hegsted, 1976; Young and Scrimshaw, 1978; King et al., 1993; Dourmad and Etienne, 2002; Otto et al., 2003). In the current study, careful attention was given to collection and measurements of orts and waste materials consistently across treatments. Estimated N losses during the 60-min

work bout were 6, 5, 6, 6, and 7 g per day for the VL, L, Control, H, and VH protein diets, respectively. While this overestimated our N retention value, this overestimation was uniform across treatments. Other reasons for overestimation of protein requirement estimated from N balance studies include unmeasured N losses in sweat, skin desquamation, and moderate day to day changes in N metabolism (Hegsted, 1963; Young and Scrimshaw, 1978; Garlick et al., 1999). In our study, urine and fecal collection was performed over a 4-d period, thus accounting for possible daily fluctuations in N metabolism. The extent to which N loss in sweat in particular and skin desquamation contribute to the overall N losses in horses has not been addressed. In humans, N losses associated with sweat and skin desquamation represents a small contribution to the overall N losses (Jackson, 1999). Miller-Graber et al. (1991a) reported that in exercising Quarter Horse mares fed a high protein (18·5%) diet, urea-N excretion in sweat increased. Nitrogen losses in sweat were not accounted for in our study, and it is possible that sweat may have been an important route of N excretion at higher protein intake thus resulting in an overestimation of protein requirement.

Blood urea-N has been used successfully to determine protein and amino acid requirements of the lactating sow (Coma et al., 1996). In this study, we have also measured blood urea-N as a response criterion to estimate protein requirement. Basal and post-feeding/exercise serum urea-N increased as daily CP intake increased indicating that N retention was overestimated at higher protein intake. Our serum urea-N results are in agreement with those obtained by Miller and Lawrence (1988). In their study, feeding a diet containing high protein concentration (18·5% CP) to exercising Quarter Horse mares increased plasma urea-N, indicating that protein intake was in excess of protein

requirement (Miller and Lawrence, 1988). In the current study, the fact that serum urea-N response to dietary CP intake was linear rather than exponential further indicates that an additional route of N excretion, possibly via sweat or salvage by the hind gut contributed to N losses at higher protein intake. In non-exercising humans consuming 74 g protein per d, 40% of urea produced was salvaged via colonic microflora activity (Danielsen and Jackson, 1992). Due to the high dependence on hind gut fermentation in the horse, it is possible that substantial amounts of urea are utilized by the microbial population.

Serum concentration of arginine, the direct precursor of urea, was maximized in horses fed the Control diet. Miller-Graber et al. (1991a) have suggested that in horses consuming a high protein diet, urea cycle capacity in the liver may be exceeded. However, in this study neither urinary orotic acid concentration nor orotic acid to creatinine ratio changed with CP intake, and both citrulline and ornithine concentration increased with increasing CP intake. Excretion of orotic acid and the orotic acid to creatine ratio have been used to evaluate urea cycle activity in exercising horses (Miller-Graber et al., 1991a; Miller-Graber et al., 1991b). Increased excretion of urinary orotic acid has been observed in humans and rats as indicative of impaired urea cycle activity (Fico et al., 1984; Reyes et al., 1994). In the current study, because urinary orotic acid concentration remained similar across dietary treatments, it is unlikely that the enzymatic capacity of the urea cycle was exceeded in horses consuming the high protein diets. Rather, the fact that urea increased despite a plateau in serum arginine concentration may have resulted from an increase in hepatic arginase activity in horses fed protein level

above requirement. Thus, arginine may be an important indicator of protein status in the horse.

Glutamine may be an important route for N excretion in mammals (Remesy et al., 1997), but has not been studied in horses. In this study, while basal serum glutamine concentration decreased linearly with increasing CP intake, post-feeding/exercise concentration increased linearly, indicating a reliance on glutamine for removal of N at higher protein intake.

Plasma free AA concentration has been used extensively in many species to evaluate dietary protein quality and indispensable AA requirement (Richardson et al., 1965; Young et al., 1971; Marchini et al., 1993). Although studies have been conducted in horses to investigate the effect of exercise on plasma free AA concentration (Russell et al., 1986; Poso et al., 1991; Trottier et al., 2002), no empirical studies have been conducted to address the response of circulating indispensable AA to graded levels of dietary CP intake in the exercised horse. In the current study, serum AA concentration was used as a response criterion to estimate CP requirement in horses fed varying levels of CP and exposed to moderate exercise. Except for histidine, post-feeding/exercise serum concentration for all indispensable AA increased as dietary protein intake increased. Mean serum concentration for the majority of indispensable AA, in particular lysine, was higher in horses fed the Control diet compared to that in horses fed the VL diet, indicating that these AA were in excess of requirement when fed in the Control diet.

The fact that response in N retention to increased CP intake was linear rather than quadratic could be the result of compensatory increase in N retention in horses fed from a low to higher protein diets as suggested by Slade et al. (1970). Increased lean body mass

or protein synthesis may explain in part the observed increase in N retention. Horse performance and level of condition remained consistent throughout the experiment. There were no differences ($P>0.05$) in body composition based on muscle mass calculated from 24-h urinary creatinine excretion (Appendix D) across dietary treatments or periods. However, from period 1 to period 5, there was a numerical increase in muscle mass of 36.8 kg. This response parallels the observed increase in protein accretion of 131 and 269 g per d in horses fed the VL and VH protein diets, respectively. This represents lean tissue accretion of 188 and 384 g per d in horses fed the VL and VH protein diets, respectively.

In conclusion, while horses fed protein levels below that of NRC (1989) maintained a positive N balance, N retention increased when horses were fed protein above that of NRC (1989). Although feeding protein at 12.5% above NRC (1989) level was necessary to achieve maximal N retention, this may not be indicative of requirement. The blood urea-N (BUN) results challenge the N retention response as both long and short term changes in BUN concentration indicated that protein intake was in excess of requirement, when fed above NRC (1989). In addition to the BUN results, serum AA concentrations were also elevated, thus casting doubt on the validity of the N retention response. Arginine concentration plateaued in horses fed NRC (1989) recommended protein level and may be an indicator of protein status in the horse. We had hypothesized that protein requirement for the moderately exercised horse to be less than that of NRC (1989). Our results indicate that protein requirement estimated from a ratio of 30 or 35 g CP to 1 Mcal DE is sufficient to meet the protein need for moderate exercise.

Table 1. Ingredient and nutrient composition of concentrate diets (as-fed basis)

	Diet†				
	VL	L	Control	H	VH
Ingredients					
Corn, yellow, (%)	94·0	50·0	40·0	34·0	28·0
Oats, (%)	0·0	44·0	48·0	46·0	44·0
Soybean meal, (%)	0·0	0·0	6·0	14·0	22·0
Molasses, (%)	6·0	6·0	6·0	6·0	6·0
Vitamin-mineral mix‡	+	+	+	+	+
Trace mineralized salt§	+	+	+	+	+
Corn oil‖	+	+	+	+	+
Nutrients					
Calculated					
Digestible energy, (kcal/kg)	3330·0	3100·0	3080·0	3090·0	3100·0
Crude protein, (%)	8·62	10·43	12·90	15·89	18·88
Lysine, (%)	0·24	0·30	0·47	0·70	0·92
Analyzed					
Dry matter, (%)	89·32	90·12	91·10	91·50	91·78
Gross energy, (kcal/kg)	3939·7	4069·3	4111·9	4161·0	4166·2
Crude protein, (%)	8·48	9·67	11·83	15·88	18·57
Acid detergent fibre, (%)	3·85	5·51	6·71	6·28	6·94
Neutral detergent fibre, (%)	7·48	15·05	17·55	15·97	16·41
Amino Acids					
Indispensable					
Arginine	0·41	0·58	0·78	1·12	1·37
Histidine	0·20	0·24	0·29	0·49	0·62
Isoleucine	0·30	0·36	0·47	0·69	0·83
Leucine	1·08	0·99	1·11	1·42	1·61
Lysine	0·17	0·25	0·40	0·71	0·93
Phenylalanine	0·36	0·43	0·53	0·77	0·92
Phenylalanine + Tyrosine	0·62	0·74	0·92	1·30	1·55
Threonine	0·24	0·28	0·35	0·48	0·59
Valine	0·39	0·48	0·59	0·82	0·97
Dispensable					
Alanine	0·64	0·62	0·68	0·85	0·95
Aspartate	0·66	0·84	1·13	1·69	2·08

Table 1. Continued

Glutamate	1·80	2·13	2·54	3·29	3·80
Glycine	0·26	0·37	0·45	0·66	0·80
Proline	0·81	0·74	0·86	1·07	1·20
Serine	0·39	0·47	0·57	0·78	0·92
Tyrosine	0·26	0·31	0·38	0·53	0·63

† VL, L, Control, H and VH diets correspond to 677, 790, 903, 1016 and 1129 g of crude protein intake per day, respectively.

‡ Vitamin-mineral mix was top-dressed at 28 g per day to provide 15000 IU Vitamin A, 3125 IU Vitamin D_3, 37·5 IU Vitamin E, 4480 mg Ca, 4480 mg P and 0·84 mg Se.

§ Trace mineralized salt was fed free choice in block form.

‖ Value of corn oil top dressed daily to meet NRC (1989) recommended digestible energy requirement for moderate exercise varied based on body weight of horse and averaged 609, 375, 259, 351, and 305 g for VL, L, Control, H, and VH protein diets respectively.

Table 2. Horse performance data

(Least squares mean values with standard errors)

Item	Mean	SEM
Body Weight, (kg)		
Pre-experimentl	473·3	16·4
Period 1	477·6[b]	17·5
Period 2	486·4[ab]	17·5
Period 3	488·4[ab]	17·5
Period 4	490·2[ab]	17·5
Period 5	491·4[a]	17·5
Body Condition Score		
Pre-experiment	6·3	0·4
Period 1	6·5[b]	0·4
Period 2	7·1[a]	0·4
Period 3	7·1[a]	0·4
Period 4	7·0[a]	0·4
Period 5	7·0[a]	0·4
Resting Heart Rate, (bpm)		
Pre-experiment	35·2	2·0
Period 1	33·0[a]	1·2
Period 2	32·4[a]	1·2
Period 3	32·9[a]	1·3
Period 4	31·8[a]	1·2
Period 5	32·8[a]	1·2
Working Heart Rate, (bpm)		
Pre-experiment	88·0	2·0
Period 1	87·2[a]	2·6
Period 2	90·4[a]	2·6
Period 3	90·7[a]	2·8
Period 4	86·6[a]	2·6
Period 5	89·8[a]	2·6

[a,b] Least squares mean values within a column with unlike superscript letters differ ($P<0·05$).

Table 3. Nitrogen balance in horses fed varying levels of protein

(Least squares mean values with their standard errors)

	Diet											
	VL (n=5)		L (n=4)		Control (n=5)		H (n=5)		VH (n=5)		P-Value	
Item	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Linear	Quadratic
N intake	107·8^{c}	3·0	123·9^{d}	3·3	145·8^{c}	3·0	165·5^{b}	3·0	185·3^{a}	3·0	*	NS†
Fecal DM output‡, (kg/d)	2·3^{c}	0·1	2·7^{b}	0·1	2·8^{ab}	0·1	2·8^{ab}	0·1	3·0^{a}	0·1	*	NS
Fecal DM‡, (%)	22·8^{a}	0·7	24·0^{a}	0·8	24·1^{a}	0·7	24·4^{a}	0·7	24·2^{a}	0·7	NS	NS
Fecal N, (g/d)	44·8^{b}	3·1	49·3^{ab}	3·3	50·8^{ab}	3·1	52·3^{ab}	3·1	57·6^{a}	3·1	*	NS
Urinary volume, (L/d)	6·3^{b}	1·0	7·8^{ab}	1·1	8·0^{ab}	1·0	8·0^{ab}	1·0	8·6^{a}	1·0	*	NS
Urinary N, (g/d)	42·0^{d}	4·5	49·0^{cd}	5·0	65·5^{bc}	4·5	70·6^{ab}	4·5	84·7^{a}	4·5	*	NS
N retention§, (g/d)	21·0^{b}	4·9	25·0^{b}	5·1	29·5^{b}	4·9	42·6^{a}	4·9	43·0^{a}	4·9	*	NS
N utilization$^{\|}$, (%)	32·7^{a}	4·8	34·1^{a}	5·2	30·8^{a}	4·8	37·5^{a}	4·8	34·3^{a}	4·8	NS	NS
N digestibility¶, (%)	58·8^{c}	2·0	61·0^{bc}	2·2	65·2^{ab}	2·0	68·2^{a}	2·0	68·9^{a}	2·0	*	NS

^{a,b,c,d,e} Least squares mean values within a row with unlike superscript letters differ (P<0·05).

* P<0·05.

† NS = not significant.

‡ Freeze dried basis.

§ N retention = N intake – Fecal N – Urinary N.

$^{\|}$N utilization = N retained / N absorbed, where N absorbed = N intake – Fecal N.

¶ N digestibility = N intake – Fecal N / N intake.

Table 4. Effect of diet on urinary creatinine, urinary orotic acid, and orotic acid to creatinine ratio

(Least squares mean values with standard errors)

	Diet											
	VL (n=5)		L (n=4)		Control (n=5)		H (n=5)		VH (n=5)		P-Value[§]	
Item	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	L	Q
Urinary creatinine[‡], (mg/dL)	125·6[a]	20·9	99·7[a]	21·6	122·3[a]	21·6	89·4[a]	20·9	103·6[a]	20·9	NS[†]	NS
Urinary creatinine[‡], (g/d)	7·2[a]	0·9	7·7[a]	1·0	9·2[a]	1·0	6·6[a]	0·9	7·7[a]	0·9	NS	NS
Urinary orotic acid, (ug/ml)	9·7[a]	0·8	8·9[a]	0·8	8·8[a]	0·8	9·7[a]	0·8	8·6[a]	0·8	NS	NS
Urinary orotic acid, (mg/d)	60·4[b]	7·2	68·9[ab]	7·5	69·4[ab]	7·2	76·8[a]	7·2	69·4[ab]	7·2	*	NS
Orotic acid/creatinine[‡], (ug/mg)	9·2[a]	2·8	10·2[a]	3·2	8·5[a]	3·2	14·8[a]	2·8	9·6[a]	2·8	NS	NS

[a,b] Least squares mean values within a row with unlike superscript letters differ (P<0·05).

[*] P<0·05.

[†] NS = not significant.

[‡] n = 4 for Control diet.

[§] L = linear response, Q = quadratic response.

Table 5. Effect of diet on basal serum indispensable amino acid concentration

(Least squares mean values with their standard errors)

	Diet											
	VL (n=5)		L (n=4)		Control (n=5)		H (n=5)		VH (n=5)		P-Value‡	
Item	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	L	Q
Serum amino acid, (umol/L)												
Basal												
Indispensable												
Histidine	$97{\cdot}4^{a}$	2·8	$93{\cdot}8^{ab}$	3·0	$90{\cdot}2^{ab}$	2·8	$81{\cdot}6^{c}$	2·8	$86{\cdot}5^{bc}$	2·8	*	NS†
Isoleucine	$45{\cdot}5^{c}$	3·1	$50{\cdot}1^{bc}$	3·2	$55{\cdot}6^{b}$	3·1	$54{\cdot}5^{b}$	3·1	$65{\cdot}5^{a}$	3·1	*	NS
Leucine	$125{\cdot}4^{ab}$	5·9	$116{\cdot}8^{ab}$	6·4	$122{\cdot}7^{ab}$	5·9	$117{\cdot}0^{b}$	5·9	$136{\cdot}0^{a}$	5·9	NS	*
Lysine	$50{\cdot}5^{a}$	4·9	$55{\cdot}1^{a}$	5·4	$56{\cdot}0^{a}$	4·9	$49{\cdot}8^{a}$	4·9	$59{\cdot}8^{a}$	4·9	NS	NS
Methionine	$26{\cdot}2^{a}$	0·7	$27{\cdot}1^{a}$	0·8	$27{\cdot}3^{a}$	0·7	$25{\cdot}5^{a}$	0·7	$26{\cdot}6^{a}$	0·7	NS	NS
Methionine + Cysteine	$30{\cdot}9^{a}$	1·1	$31{\cdot}5^{a}$	1·2	$31{\cdot}7^{a}$	1·1	$30{\cdot}4^{a}$	1·1	$31{\cdot}3^{a}$	1·1	NS	NS
Phenylalanine	$60{\cdot}4^{a}$	1·6	$58{\cdot}6^{a}$	1·7	$58{\cdot}9^{a}$	1·6	$56{\cdot}0^{a}$	1·6	$60{\cdot}0^{a}$	1·6	NS	NS
Phenylalanine + Tyrosine	$125{\cdot}9^{a}$	3·3	$122{\cdot}1^{a}$	3·7	$123{\cdot}9^{a}$	3·3	$115{\cdot}7^{a}$	3·3	$122{\cdot}3^{a}$	3·3	NS	NS
Threonine	$92{\cdot}0^{a}$	3·6	$81{\cdot}4^{ab}$	4·1	$86{\cdot}6^{ab}$	3·6	$74{\cdot}6^{b}$	3·6	$86{\cdot}7^{ab}$	3·6	NS	NS
Tryptophan	$108{\cdot}2^{a}$	5·7	$113{\cdot}6^{a}$	6·0	$114{\cdot}4^{a}$	5·7	$110{\cdot}8^{a}$	5·7	$112{\cdot}1^{a}$	5·7	NS	NS
Valine	$157{\cdot}2^{c}$	8·2	$174{\cdot}6^{bc}$	8·5	$185{\cdot}1^{b}$	8·2	$185{\cdot}9^{b}$	8·2	$213{\cdot}2^{a}$	8·2	*	NS

[a,b,c] Least squares mean values within a row with unlike superscript letters differ (P<0·05).

* P<0·05.

† NS = not significant.

‡ L = linear response, Q = quadratic response.

Table 6. Effect of diet on post-feeding/exercise serum indispensable amino acid concentration

(Least squares mean values with their standard errors)

	Diet											
	VL (n=5)		L (n=4)		Control (n=5)		H (n=5)		VH (n=5)		P-Value‡	
Item	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	L	Q
Serum amino acid, (umol/L)												
Post-exercise												
Indispensable												
Histidine	$100{\cdot}0^{a}$	4·2	$95{\cdot}3^{a}$	5·0	$113{\cdot}1^{a}$	4·2	$110{\cdot}5^{a}$	4·2	$111{\cdot}9^{a}$	4·2	*	NS†
Isoleucine	$49{\cdot}6^{b}$	5·5	$48{\cdot}1^{b}$	6·6	$76{\cdot}7^{ab}$	5·5	$83{\cdot}1^{a}$	5·5	$92{\cdot}2^{a}$	5·5	*	NS
Leucine	$139{\cdot}7^{ab}$	10·0	$120{\cdot}2^{b}$	12·0	$172{\cdot}4^{ab}$	10·0	$176{\cdot}2^{a}$	10·0	$185{\cdot}4^{a}$	10·0	*	NS
Lysine	$53{\cdot}0^{c}$	6·3	$60{\cdot}9^{bc}$	7·2	$86{\cdot}9^{ab}$	6·3	$94{\cdot}4^{a}$	6·3	$102{\cdot}0^{a}$	6·3	*	NS
Methionine	$30{\cdot}0^{b}$	1·5	$33{\cdot}7^{ab}$	1·7	$38{\cdot}1^{a}$	1·5	$37{\cdot}1^{a}$	1·5	$39{\cdot}7^{a}$	1·5	*	NS
Methionine + Cysteine	$33{\cdot}7^{b}$	1·6	$38{\cdot}1^{ab}$	1·8	$42{\cdot}8^{a}$	1·6	$41{\cdot}8^{a}$	1·6	$43{\cdot}5^{a}$	1·6	*	*
Phenylalanine	$59{\cdot}4^{c}$	3·7	$59{\cdot}7^{bc}$	4·0	$71{\cdot}1^{ab}$	3·7	$72{\cdot}1^{a}$	3·7	$74{\cdot}0^{a}$	3·7	*	NS
Phenylalanine + Tyrosine	$137{\cdot}3^{c}$	7·4	$143{\cdot}1^{bc}$	8·2	$170{\cdot}6^{ab}$	7·4	$174{\cdot}0^{a}$	7·4	$184{\cdot}6^{a}$	7·4	*	NS
Threonine	$96{\cdot}1^{b}$	7·3	$94{\cdot}4^{b}$	8·6	$123{\cdot}5^{ab}$	7·3	$124{\cdot}4^{ab}$	7·3	$134{\cdot}0^{a}$	7·3	*	NS
Tryptophan	$110{\cdot}7^{b}$	8·0	$118{\cdot}8^{ab}$	8·6	$135{\cdot}0^{a}$	8·0	$134{\cdot}6^{a}$	8·0	$138{\cdot}7^{a}$	8·0	*	NS
Valine	$165{\cdot}1^{b}$	8·8	$173{\cdot}0^{b}$	10·5	$235{\cdot}0^{a}$	8·8	$251{\cdot}5^{a}$	8·8	$270{\cdot}0^{a}$	8·8	*	NS

[a,b,c] Least squares mean values within a row with unlike superscript letters differ (P<0·05).

* P<0·05.

† NS = not significant.

‡ L = linear response, Q = quadratic response.

Figure 4. Relationship between daily total lysine intake and nitrogen retention. Response between daily lysine intake and nitrogen retention was linear and described by $Y = 0{\cdot}734x + 4{\cdot}763$ ($r^2 = 0{\cdot}42$; $P<0{\cdot}05$).

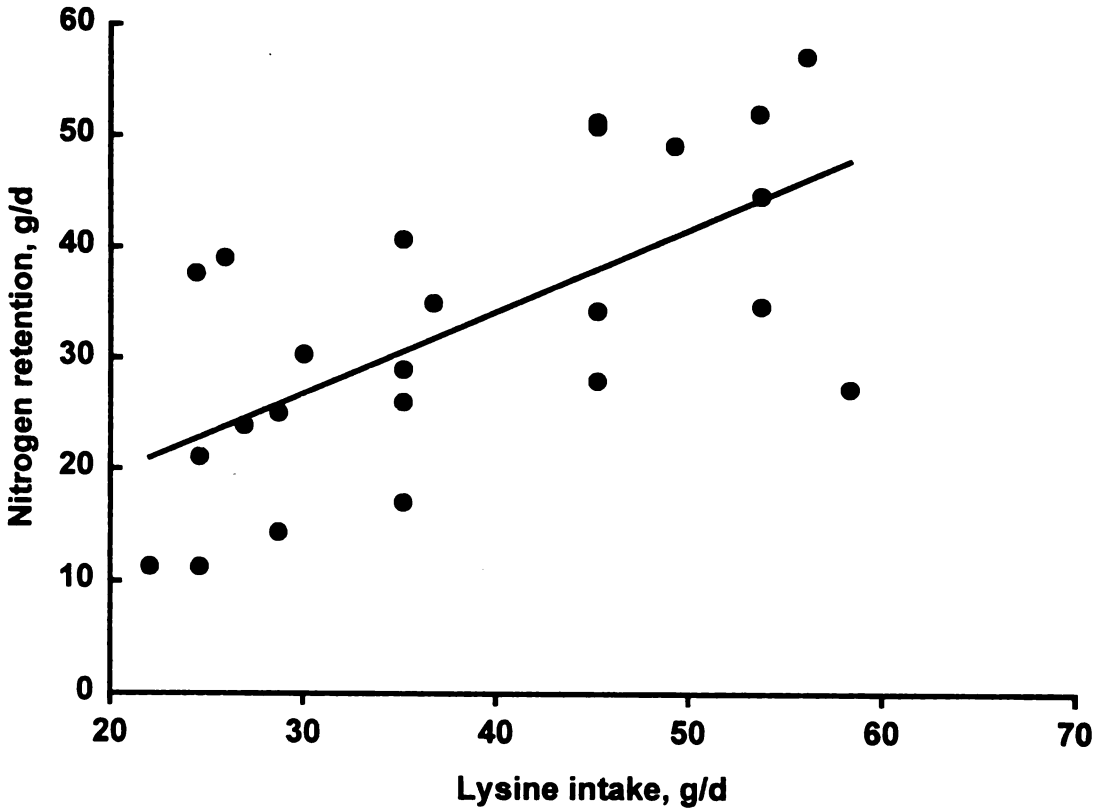

Nitrogen retention, g/d
60
50
40
30
20
10
0
20
30
40
50
60
70
Lysine intake, g/d

Figure 5. Relationship between daily digestible lysine intake and nitrogen retention. Response between daily digestible lysine intake and nitrogen retention was linear and described by $Y = 0{\cdot}823x + 11{\cdot}619$ ($r^2 = 0{\cdot}43$; $P<0{\cdot}05$).

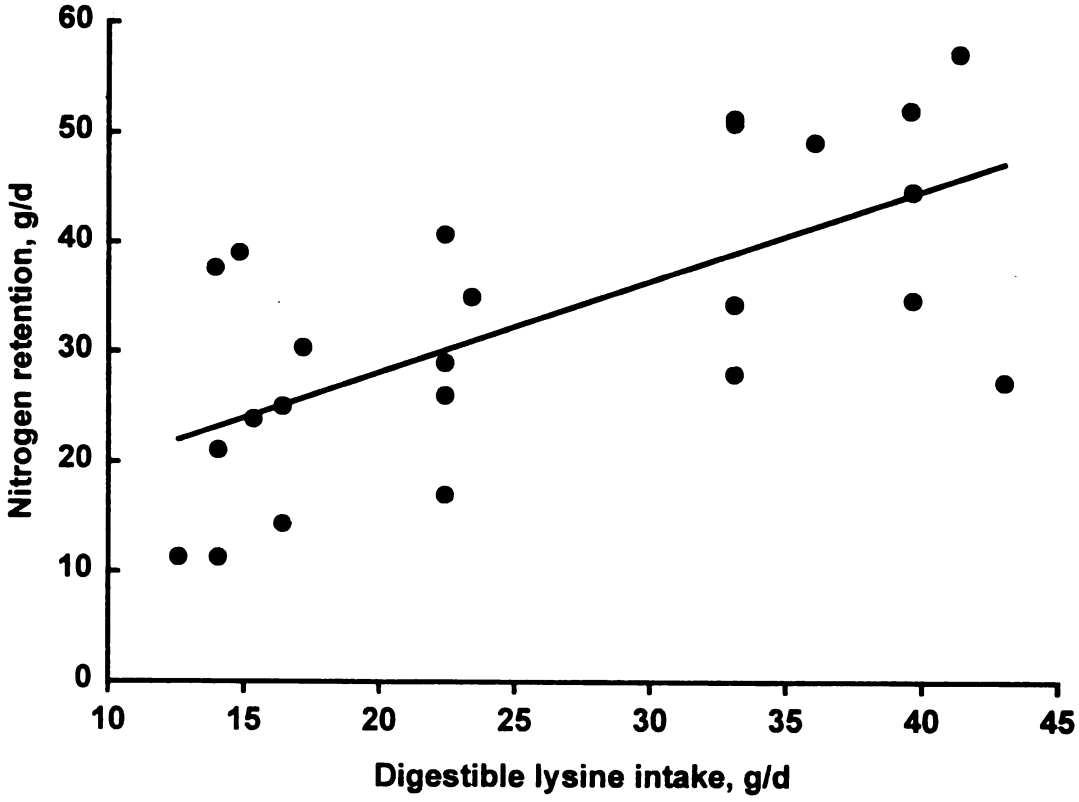

Nitrogen retention, g/d
60
50
40
30
20
10
0
10
15
20
25
30
35
40
45
Digestible lysine intake, g/d

Figure 6. Effect of dietary crude protein intake on basal and post-feeding/exercise serum urea-nitrogen concentration. Dietary crude protein level of 677, 790, 903, 1016, 1129 g CP per d corresponds to VL, L, Control, H, and VH protein diets, respectively. Closed (●) and opened (○) circle indicates basal and post-feeding/exercise serum urea-nitrogen concentration, respectively. Linear response between dietary crude protein intake and basal and post-feeding/exercise serum urea-nitrogen concentration was significant ($P<0.05$). Quadratic response between dietary crude protein intake and basal and post-feeding/exercise serum urea-nitrogen concentration was not significant ($P>0.05$).

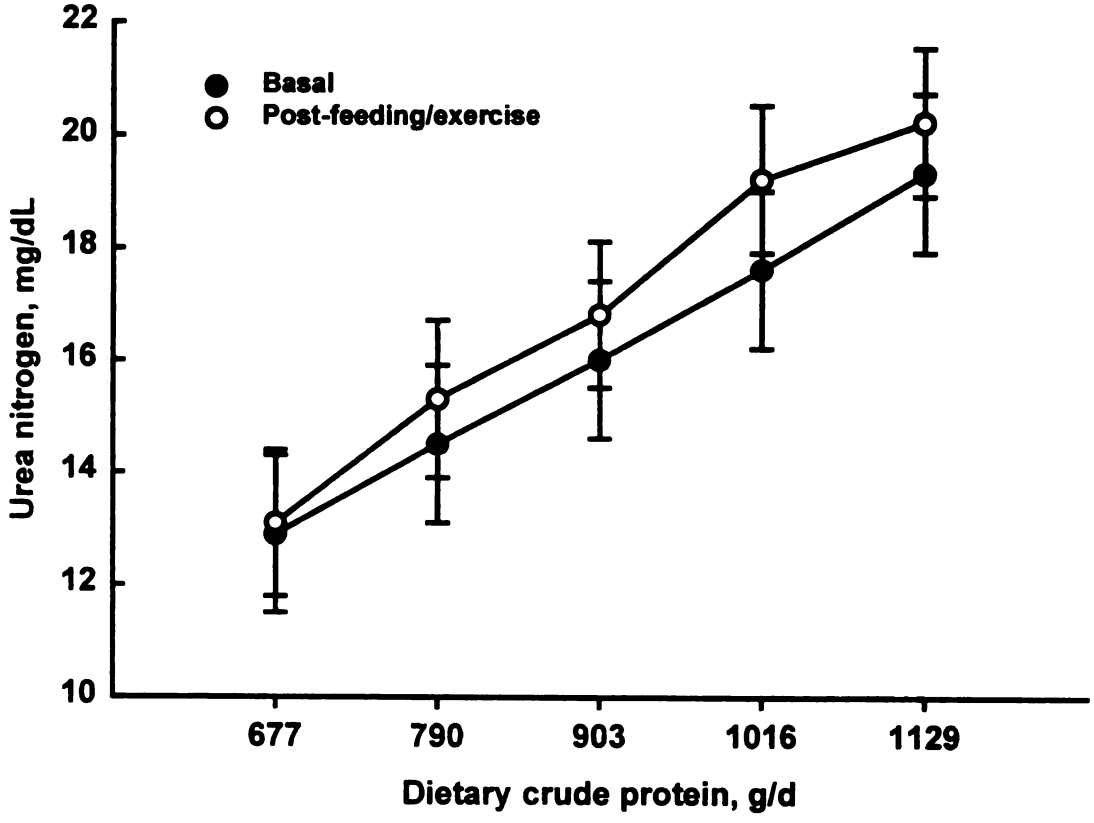
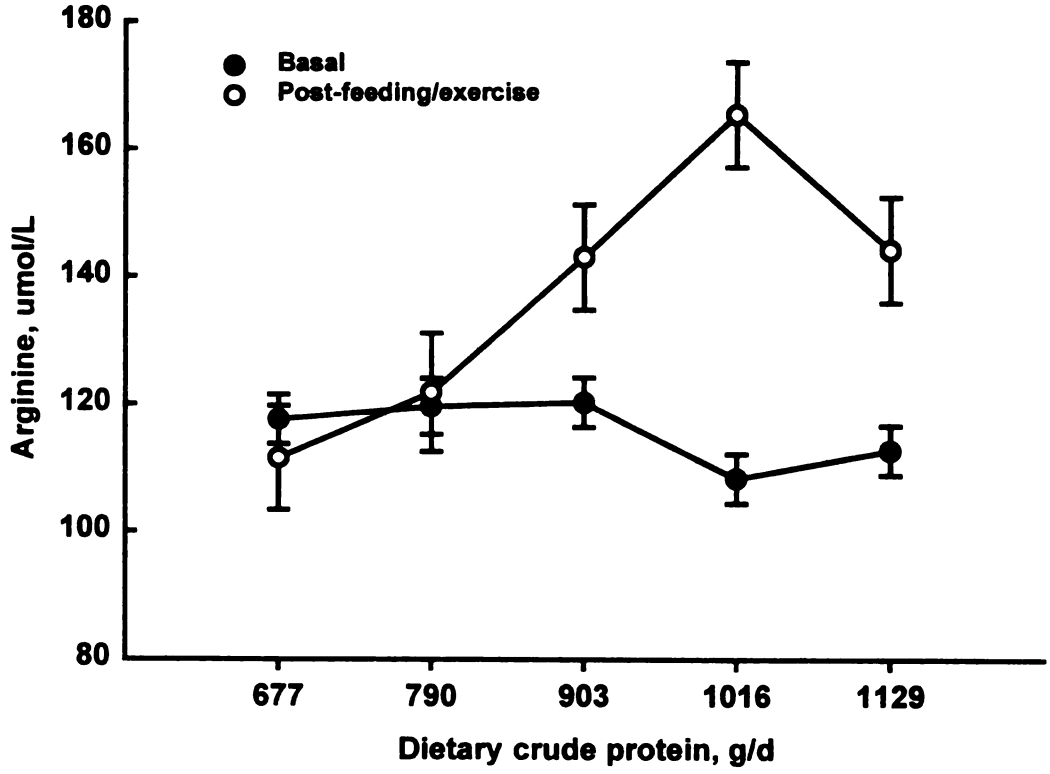
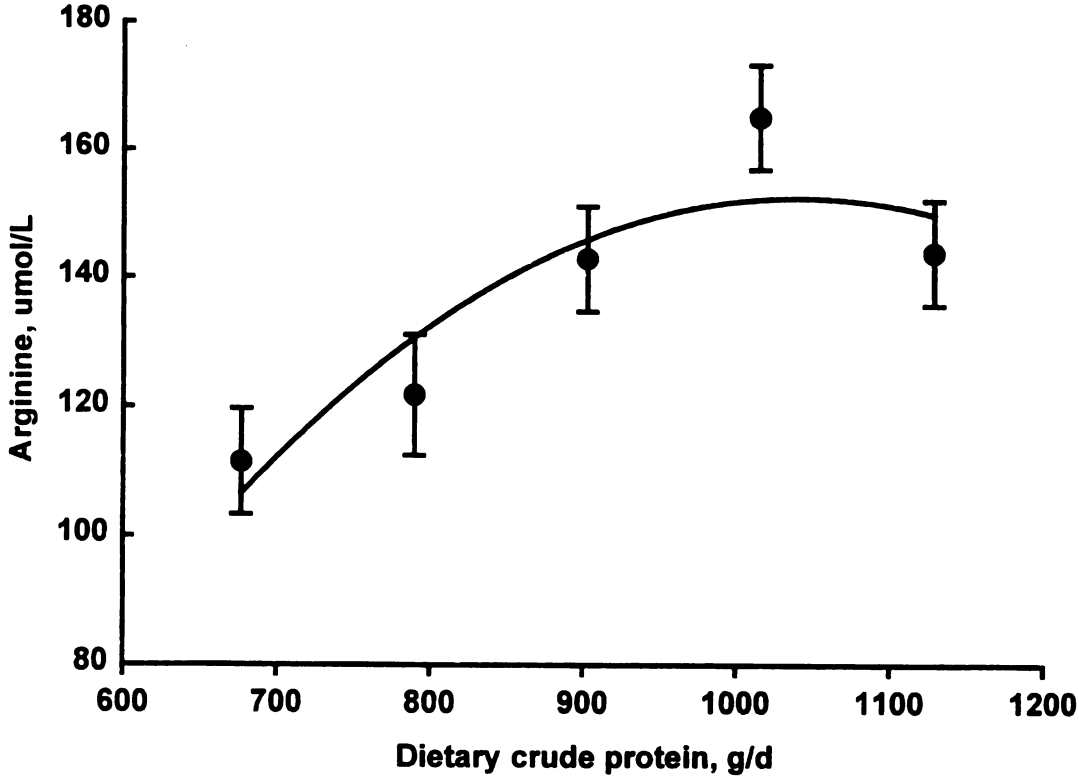

22
20
18
16
14
12
10
Urea nitrogen, mg/dL
Basal
Post-feeding/exercise
677
790
903
1016
1129
Dietary crude protein, g/d

Figure 7a. Effect of dietary crude protein intake on basal and post-feeding/exercise serum arginine concentration. Dietary crude protein level of 677, 790, 903, 1016, 1129 g CP per d corresponds to VL, L, Control, H, and VH protein diets, respectively. Closed (●) and opened (○) circle indicates basal and post-feeding/exercise serum arginine concentration, respectively. Linear and quadratic response between dietary crude protein intake and basal serum arginine concentration was not significant ($P>0{\cdot}05$). Linear and quadratic response between dietary crude protein intake and post-feeding/exercise serum arginine concentration was significant ($P<0{\cdot}05$).

Figure 7b. Effect of dietary crude protein intake on post-feeding/exercise serum arginine concentration. Solid (–) line represents predicted post-feeding/exercise serum arginine concentration in response to dietary CP intake. Response was quadratic ($P<0{\cdot}05$).


Basal
Post-feeding/exercise
Arginine, umol/L
180
160
140
120
100
80
677
790
903
1016
1129
Dietary crude protein, g/d


Arginine, umol/L
180
160
140
120
100
80
600
700
800
900
1000
1100
1200
Dietary crude protein, g/d

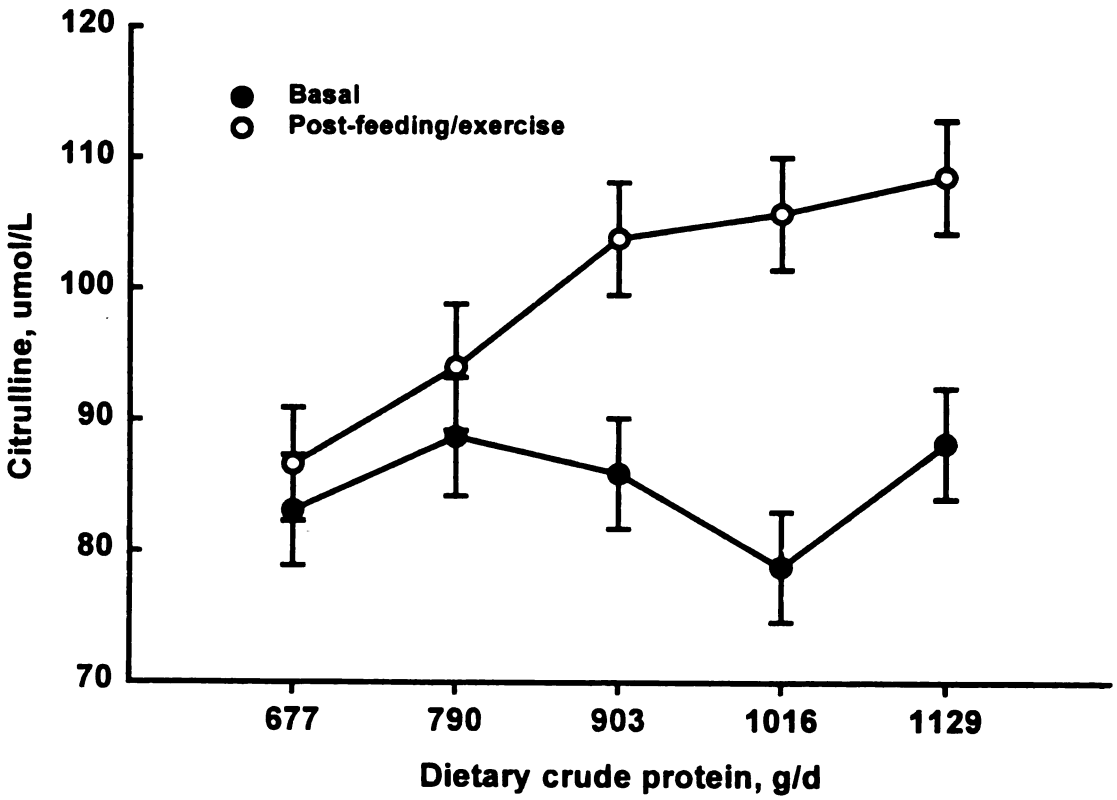

120
110
100
90
80
70
Citrulline, umol/L
Basal
Post-feeding/exercise
677
790
903
1016
1129
Dietary crude protein, g/d

Figure 8. Effect of dietary crude protein intake on basal and post-feeding/exercise serum citrulline concentration. Dietary crude protein level of 677, 790, 903, 1016, 1129 g CP per d corresponds to VL, L, Control, H, and VH protein diets, respectively. Closed (●) and opened (○) circle indicates basal and post-feeding/exercise serum citrulline concentration, respectively. Linear and quadratic response between dietary crude protein intake and basal serum citrulline concentration was not significant ($P>0{\cdot}05$). Linear response between dietary crude protein and post-feeding/exercise serum citrulline concentration was significant ($P<0{\cdot}05$). Quadratic response for post-feeding/exercise serum citrulline concentration was not significant ($P>0{\cdot}05$).

Figure 9. Effect of dietary crude protein intake on basal and post-feeding/exercise serum ornithine concentration. Dietary crude protein level of 677, 790, 903, 1016, 1129 g CP per d corresponds to VL, L, Control, H, and VH protein diets, respectively. Closed (●) and opened (○) circle indicates basal and post-feeding/exercise serum ornithine concentration, respectively. Linear and quadratic response between dietary crude protein intake and basal serum ornithine concentration was not significant ($P>0{\cdot}05$). Linear response between dietary crude protein and post-feeding/exercise serum ornithine concentration was significant ($P<0{\cdot}05$). Quadratic response for post-feeding/exercise serum ornithine concentration was not significant ($P>0{\cdot}05$).

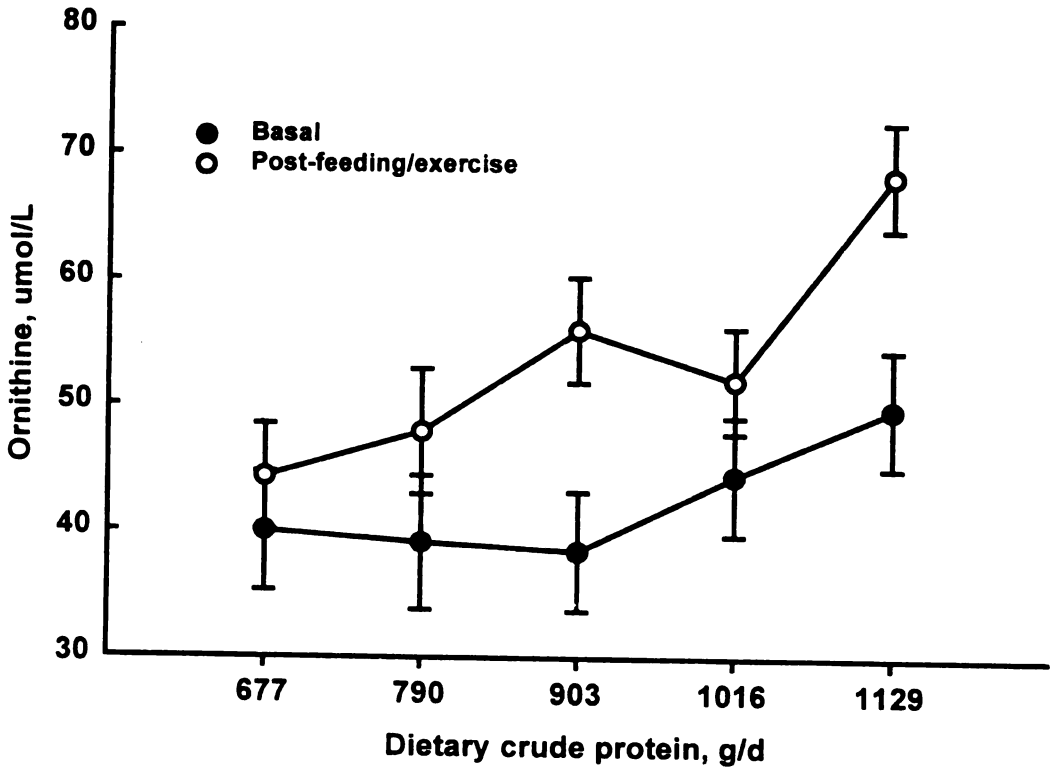
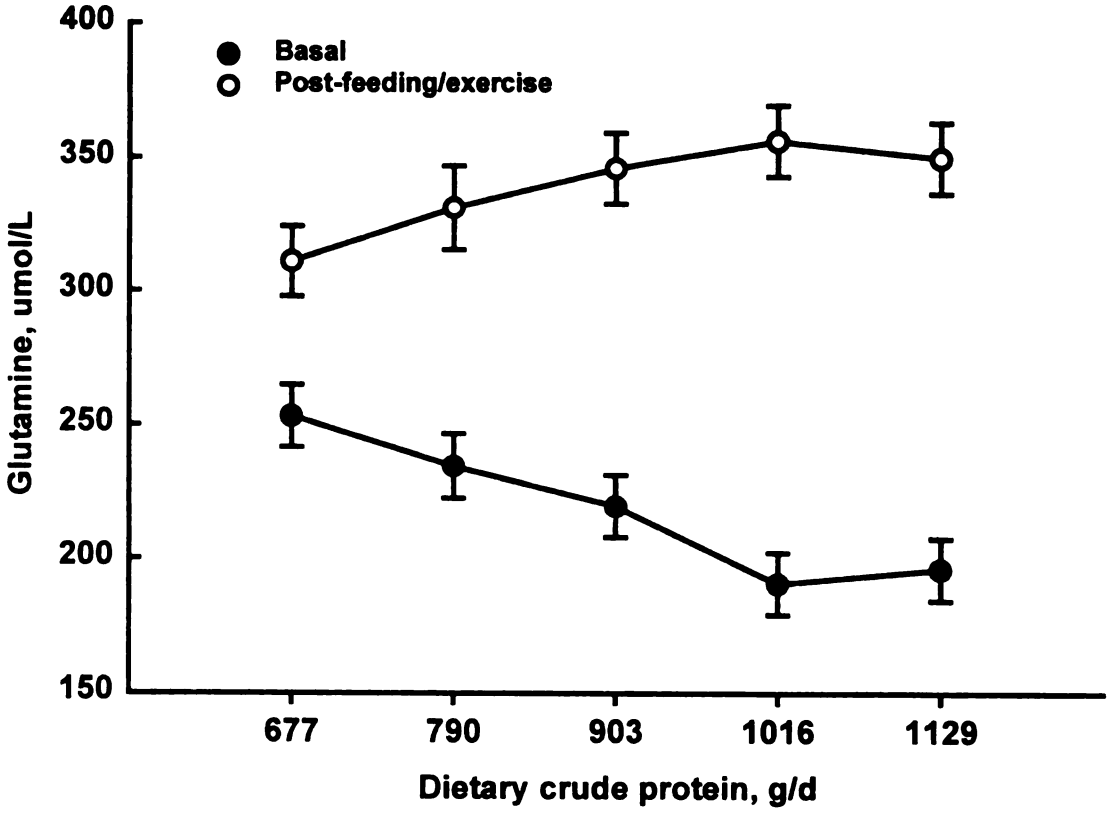

80
70
60
50
40
30
Ornithine, umol/L
Basal
Post-feeding/exercise
677
790
903
1016
1129
Dietary crude protein, g/d

Figure 10. Effect of dietary crude protein intake on basal and post-feeding/exercise serum glutamine concentration. Dietary crude protein level of 677, 790, 903, 1016, 1129 g CP per d corresponds to VL, L, Control, H, and VH protein diets, respectively. Closed (●) and opened (○) circle indicates basal and post-feeding/exercise serum glutamine concentration, respectively. Linear response between dietary crude protein intake and basal and post-feeding/exercise serum glutamine was significant ($P<0.05$). Quadratic response between dietary crude protein intake and basal and post-feeding/exercise serum glutamine was not significant ($P>0.05$).


400
350
300
250
200
150
Glutamine, umol/L
Basal
Post-feeding/exercise
677
790
903
1016
1129
Dietary crude protein, g/d

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CHAPTER 3

APPARENT FECAL NITROGEN AND AMINO ACID DIGESTIBILITY OF DIETS FED TO EXERCISING HORSES

ABSTRACT

APPARENT FECAL NITROGEN AND AMINO ACID DIGESTIBILITY OF DIETS FED TO EXERCISING HORSES

By

Carissa Lee Wickens

Five mature Arabian geldings were used in a 5x5 Latin square design to determine apparent nitrogen (N) and amino acid (AA) digestibility of five hay:concentrate mix diets. Horses were randomly assigned to 1 of 5 dietary treatments. Each period consisted of a 10-day diet adaptation followed by a 4-day total urine and fecal collection. All horses consumed mixed grass hay containing 10% crude protein (CP) at 1% of their body weight. Total diet (hay plus concentrate) was formulated to provide 677, 790, 903, 1016, and 1129 g CP daily corresponding to a very low (VL), low (L), Control, high (H) and very high (VH) protein diet respectively. Diets were composed of corn, oat, and SBM in varying proportions. Apparent fecal N and AA digestibility increased linearly ($P<0.05$) as dietary CP intake increased. Compared to Control, N digestibility was not different ($P>0.05$) in horses fed L, H, and VH. Apparent fecal lysine digestibility was not different ($P>0.05$) in horses fed VL and L compared to that in horses fed Control and was higher ($P<0.05$) in horses fed H and VH. Inclusion of SBM to the Control, H, and VH protein diet concentrates resulted in higher protein quality and contributed to the increase in apparent fecal N and AA digestibility.

Key words: Horse, protein, amino acid, digestibility

Introduction

The current NRC (1989) protein requirement estimate for the exercising horse is derived from a crude protein (CP) to digestible energy (DE) ratio of 40 g to 1 Mcal estimated for the horse at maintenance. This ratio was determined from horses consuming a forage diet with a protein digestibility of 46%. Several studies have reported protein digestibility to be higher than 46%. In ponies fed Coastal Bermuda grass, low-protein alfalfa, or high-protein alfalfa hay, Gibbs et al. (1988) reported total tract N digestibility of 57, 66, and 74%, respectively. Similarly, Slade et al. (1970), Hintz et al. (1971), and Glade (1984) have provided estimates of protein digestibility of diets fed to horses and ponies at maintenance ranging from 35 up to 79·8%. However, ingredients used in diets tested in earlier studies (Slade et al., 1970; Reitnour and Treece, 1971; Glade, 1984) are not representative of those routinely fed to horses.

Exercising horses are commonly fed a mixed ration consisting of increased level of concentrate relative to forage in order to meet the increased energy demands associated with physical activity. The concentrate portion is typically composed of ingredients of higher protein quality than that found in most forages. Thus, it is expected that protein digestion of a diet formulated for the exercising horse will be higher than that reported by the current NRC (1989). Moreover, protein digestibility of diets fed to exercising horses has not been determined. The objective of this study was to determine apparent fecal N and AA digestibility of five hay:concentrate mix diets fed to horses performing moderate exercise. We hypothesized that apparent N digestibility would be higher than values reported in NRC (1989) and that inclusion of soy bean meal (SBM) increases apparent N and AA digestibility.

Materials and Methods

Animals, experimental design, and diets

As part of a N balance study conducted to estimate the protein requirement of the moderately exercised horse (Wickens et al., unpublished), five mature Arabian geldings with an initial body weight of 473·3 ± 16·4 kg were randomly assigned to five dietary treatments in a 5 x 5 Latin square design. Horses were housed individually in box stalls (3·0 x 3·7 m) with free access to water. All horses consumed mixed grass hay containing 10% CP (as fed basis) at 1% of their BW. Five diet concentrates were formulated to achieve varying levels of crude protein intake. Ingredient and nutrient composition of the concentrate diets are provided in Table 7. A Control diet concentrate was first formulated to meet NRC (1989) daily protein requirement estimate for moderate exercise. A very low (VL) and a low (L) diet concentrate was formulated to provide 25% and 12·5% lower protein respectively, relative to Control, and a high (H) and very high (VH) diet concentrate was formulated to provide 12·5% and 25% higher protein respectively, relative to Control. Thus, total diet (hay plus concentrate) was formulated and fed to provide 677, 790, 903, 1016, and 1129 g CP daily corresponding to the very low (VL), low (L), Control, high (H), and very high (VH) protein diet, respectively. To achieve a very low protein diet, corn was used as the primary ingredient. Protein concentrations of the diet concentrates were increased by altering the corn to oat ratio and through the addition of soybean meal to the Control, H, and VH protein concentrates. Molasses was included in the concentrate mix as a binder. Diet concentrates varied slightly in DE content and were fed to achieve the desired protein levels. This resulted in minor energy deficiencies, thus corn oil was top-dressed in small amounts to assure NRC (1989) energy

requirement for moderate exercise was met for each horse. Vitamin-mineral mix was top-dressed once daily to provide NRC (1989) recommended levels of Ca, P, Vitamin A, Vitamin D, Vitamin E, and Se. Meals were fed twice daily at 0700 and 1600. Forage to concentrate ratios of the total diet (hay plus concentrate) are shown in Table 8.

This study was approved by Michigan State University All University Committee on Animal Use and Care.

Sample collection

The study consisted of five collection periods. Each period was 14 d in length and consisted of a 10-d diet adaptation followed by a 4-d total fecal and urine collection. Feces and urine were collected using gelding collection harnesses (Equisan Marketing, Melbourne, Australia) and emptied every 5 h or more frequently as needed. At each emptying, all feces were bagged, and immediately stored at -20°C. At the end of each collection period, daily fecal samples were thawed, pooled, weighed, and homogenized using a 136-kg mechanical mixer. Sub samples were collected (~500 g) and frozen at -20°C for N and AA analysis.

Sample Analysis

For chemical analysis, fecal samples were freeze-dried (VirTis model 25-SRC, VirTis Co., Gardiner, NY). Feed and fecal samples were finely ground using a cyclone mill (Foss Cyclotec sample mill 1093, Hoganas, Sweden) with a 1-mm mesh screen. Nitrogen content in feed and feces was determined using an automated N analyzer (Leco FP-2000, Leco Co., St. Joseph, MI; AOAC No. 990·3). Dry matter of feed was determined following a 24-h drying period at 80°C using a drying oven (Fisher Isotemp, Fisher Scientific, Hanover Park, IL). Amino acid analysis was performed on feed and

fecal samples using the Pico-Tag method (Waters Co., Milford, MA) following a 24-h acid hydrolysis in 6*N* HCl at 113°C and 121 mm Hg. Norleucine was used as an internal standard. Samples were derivatized with phenylisothiocyanate and analyzed by high pressure liquid chromatography (Alliance 2690, Waters Co., Milford, MA) fitted with a 30-cm Pico-Tag column (Waters Co., Milford, MA).

Calculations

Apparent fecal N digestibility (AND) and AA digestibility (AAD) was calculated in the following manner:

$$AND = (N_i - N_f)/ N_i$$

$$AAD = (AA_i - AA_f)/ AA_i$$

where N_i and AA_i are the total N and AA intake, and N_f and AA_f are the total N and AA losses in feces.

Statistical Analysis

Data were subjected to ANOVA using the PROC MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). Effects of horse, period, and diet were included in the statistical model. Horse was treated as a random effect. Differences between all pair-wise comparisons were evaluated using the Tukey-Kramer test (Younger, 1998). Relationships between dietary CP intake and N and AA digestibility were determined using contrast (linear and quadratic) comparisons with orthogonal polynomials. Coefficients used for contrasts were based on the calculated CP intake of 677, 790, 903, 1016, and 1129 g per d. Statistical significance was based on an experiment-wise type-I error rate of 0·05. Regression equations for apparent fecal CP, N, and AA digestibility were calculated using the PROC REG procedure of SAS (SAS Inst, Inc., Cary, NC).

Results and Discussion

The forage to concentrate ratio for each diet is shown in Table 8. Ratios ranged from 53:47 forage to concentrate to 63:37 forage to concentrate. These ratios correspond to those currently recommended by the NRC (1989) for horses performing moderate work. Apparent fecal N digestibility data, as affected by dietary CP intake are presented in Table 9. Nitrogen digestibility increased linearly ($P<0.001$) as daily CP intake increased. Slade et al. (1970) found similar results with increases in dietary CP in horses fed at maintenance. Compared to Control, N digestibility was not different ($P>0.05$) in horses fed L, H and VH protein diets. Nitrogen digestibility was lower ($P<0.05$) in horses fed the VL protein diet compared to that in horses fed Control. Slade et al. (1970) suggested that the increase in apparent N digestibility on higher protein intakes is due to a reduction in the endogenous contribution to fecal N and an increased N availability for microbial activity in the hind gut. The presence of many types of microflora in the large intestine of the horse with proteolytic activity has been shown (Kern et al., 1973; Frape, 1975); however, the contribution of microbial protein and AA synthesis and absorption across the wall of the large intestine to post-gut AA availability in the horse is unknown. Hintz et al. (1971) reported significant disappearance of N from the large intestine of the horse. In humans and pigs, it is well recognized that some protein digestion and amino acid absorption occurs in the large intestine (Metges, 2000). Thus it is possible that the increased apparent digestibility of N in horses fed higher protein intakes is a result of increased utilization by hind gut microflora. According to Frape (1975) this may also partially explain increases in N retention in horses fed diets containing higher protein concentration due to an expanded urea pool and a consequential utilization of NPN by the

microbial population in the large intestine. Indeed, we have found that N retention increased as dietary CP increased above predicted requirement (Wickens et al., unpublished).

Protein digestibility is also affected by dietary fiber content. Glade (1984) reported lower apparent N digestibility in horses consuming a diet containing straw and a higher percent NDF. In our study, percent NDF of the total diet (hay plus concentrate) was similar across all dietary treatments, thus it is unlikely that fiber content affected apparent N digestibility.

In mature geldings fed various mixtures of feed ingredients, such as corn, SBM, ground alfalfa, straw, and urea, apparent N digestibility ranged from 64·0 to 79·6% (Glade, 1984). In our study, apparent N digestibility ranged from 58·8% to 68·9% when diets containing feed ingredients such as mixed grass hay, corn, oat, and SBM where fed to mature, moderately exercised horses. The current NRC (1989) provides equations for estimating apparent protein digestibility of selected feed ingredients. These equations were derived using horses fed diets consisting primarily of almond hulls, fishmeal, and oat hay (Slade and Robinson, 1970). However, these feed ingredients are limited and may not be representative of others commonly fed to horses such as corn, oat, and SBM. Regression coefficients for apparent CP and N digestibility were calculated using our feed ingredients including mixed grass hay, corn, oat, and SBM and are presented in Table 10.

Individual AA requirements for the mature horse have not been determined, and information on AA digestibility in feeds commonly fed to horses is not available. Thus, in this study apparent fecal AA digestibility of the five hay:concentrate mix diets was

measured. Apparent fecal AA digestibility data as affected by dietary CP intake are presented in Table 9. For all indispensable AA, apparent digestibility increased linearly ($P<0.05$) as dietary CP intake increased. With the exception of alanine, apparent digestibility of dispensable AA also increased linearly ($P<0.05$) with increasing dietary CP intake. Lysine has been shown to be the first limiting AA in the diet of young growing horses (Breuer et al., 1971; Ott et al., 1979, 1981) and is most likely the first limiting AA in diets fed to mature horses as well. In our study, apparent fecal digestibility of lysine increased linearly ($P<0.05$) and was higher ($P<0.05$) in horses fed the H and VH protein diets compared to that in horses fed Control, L, and VL. Apparent fecal lysine digestibility was 57.0 and 57.2% in the VL and L protein diets, respectively. The concentrate portion of these lower protein diets consisted of primarily corn or a corn:oat mixture. Thus, apparent lysine digestibility of a diet containing mixed grass hay and corn is 57.0%, and the similar digestibility between the VL and L protein diets indicates comparable digestibility between corn and oats. With soybean meal added to the Control, H and VH protein diet concentrates, apparent lysine digestibility increased ($P<0.05$) to 63.7, 73.2, and 73.8%, respectively. Lysine to protein ratio in SBM is 6.4 to 1 compared to 2.7 to 1 in corn, and lysine digestibility in SBM is higher when fed to poultry (NRC, 1994) and swine (NRC, 1998) compared to that of corn. Thus, the inclusion of SBM to the Control, H, and VH protein diet concentrates resulted in a concentrate feed with higher protein quality. It may be favorable to use lysine digestibility in formulating equine diets because it is likely to be the first limiting amino acid in diets fed to mature horses and contributes to the improved N digestibility observed in horses fed the Control, H and VH protein diets. Using CP digestibility in

diets containing SBM would lead to overestimation of protein need. Regression coefficients have also been calculated for apparent AA digestibility in our study and are presented in Table 10.

So far, the apparent fecal N and AA digestibility method has been used for determining the availability of dietary N and AA in horses. In other species, in particular swine (Donkoh and Moughan, 1994; Stein et al., 1999; Otto et al., 2003), AA recovered at the distal ileum have been used to estimate AA digestibility and availability. The major problem associated with apparent fecal digestibility is that it fails to account for the confounding effect of microbial metabolism of dietary and endogenous protein (Darragh and Hodgkinson, 2000). Microbial protein degradation can result in fecal N digestibility coefficients which overestimate N and AA availability, and any net synthesis of protein and AA by hind gut microbes could lead to underestimation of protein digestibility (Darragh and Hodgkinson, 2000). Interestingly, for some indispensable AA including lysine, histidine, isoleucine, leucine, and valine, Darragh and Hodgkinson (2000) found no difference between apparent fecal and apparent ileal digestibility coefficients in humans fed a meat, vegetable, cereal, or dairy product diet. It is not known whether this is similar in the horse as microbial fermentation is of greater significance compared to that in humans, and the extent to which microbial protein and AA synthesis in the large intestine contributes to fecal N content remains unclear.

Collection of ileal content in horses has not been routinely performed. Recovery of ileal content is more invasive and has only been reported on a small number of ponies (Gibbs et al., 1988). It is unlikely that ileal collection to estimate pre-cecal N and AA digestibility would be a readily acceptable and commonly used method in horses.

Although apparent fecal N and AA digestibility provide some indication of post-gut N and AA availability, it is important to recognize the limitation associated with this technique. New methods will need to be developed to assess pre-cecal N and AA digestibility in horses, and to study hind-gut N and AA metabolism and the contribution of microbial protein and AA synthesis to fecal N output.

In conclusion, results of this study provide apparent fecal N and AA digestibility of diets containing varying ratios of feed ingredients typically used in the horse industry for exercising horses including mixed grass hay, corn, oat, and SBM. Apparent fecal N and AA digestibility increased as dietary CP increased, and apparent N digestibility in our study was higher than values reported in the current NRC (1989). Apparent fecal lysine digestibility was higher in diets containing SBM and thus contributed to the improved apparent fecal N digestibility. Because lysine is likely to be the first limiting amino acid in horse diets, it may be preferable to formulate equine diets using apparent lysine digestibility. Using CP digestibility would overestimate total protein requirement of the exercising horse in diets containing ingredients of high protein quality, such as SBM.

Table 7. Ingredient and nutrient composition of concentrate diets (as-fed basis)

	Diet†				
	VL	L	Control	H	VH
Ingredients					
Corn, yellow, (%)	94·0	50·0	40·0	34·0	28·0
Oats, (%)	0·0	44·0	48·0	46·0	44·0
Soybean meal, (%)	0·0	0·0	6·0	14·0	22·0
Molasses, (%)	6·0	6·0	6·0	6·0	6·0
Vitamin-mineral mix‡	+	+	+	+	+
Trace mineralized salt§	+	+	+	+	+
Corn oil‖	+	+	+	+	+
Nutrients					
Calculated					
Digestible energy, (kcal/kg)	3330·0	3100·0	3080·0	3090·0	3100·0
Crude protein, (%)	8·62	10·43	12·90	15·89	18·88
Lysine, (%)	0·24	0·30	0·47	0·70	0·92
Analyzed					
Dry matter, (%)	89·32	90·12	91·10	91·50	91·78
Gross energy, (kcal/kg)	3939·7	4069·3	4111·9	4161·0	4166·2
Crude protein, (%)	8·48	9·67	11·83	15·88	18·57
Acid detergent fibre, (%)	3·85	5·51	6·71	6·28	6·94
Neutral detergent fibre, (%)	7·48	15·05	17·55	15·97	16·41
Amino Acids					
Indispensable					
Arginine	0·41	0·58	0·78	1·12	1·37
Histidine	0·20	0·24	0·29	0·49	0·62
Isoleucine	0·30	0·36	0·47	0·69	0·83
Leucine	1·08	0·99	1·11	1·42	1·61
Lysine	0·17	0·25	0·40	0·71	0·93
Phenylalanine	0·36	0·43	0·53	0·77	0·92
Phenylalanine + Tyrosine	0·62	0·74	0·92	1·30	1·55
Threonine	0·24	0·28	0·35	0·48	0·59
Valine	0·39	0·48	0·59	0·82	0·97
Dispensable					
Alanine	0·64	0·62	0·68	0·85	0·95
Aspartate	0·66	0·84	1·13	1·69	2·08

Table 7. Continued

Glutamate	1·80	2·13	2·54	3·29	3·80
Glycine	0·26	0·37	0·45	0·66	0·80
Proline	0·81	0·74	0·86	1·07	1·20
Serine	0·39	0·47	0·57	0·78	0·92
Tyrosine	0·26	0·31	0·38	0·53	0·63

† VL, L, Control, H and VH diets correspond to 677, 790, 903, 1016 and 1129 g of crude protein intake per day, respectively.

‡ Vitamin-mineral mix was top-dressed at 28 g per day to provide 15000 IU Vitamin A, 3125 IU Vitamin D_3, 37·5 IU Vitamin E, 4480 mg Ca, 4480 mg P and 0·84 mg Se.

§ Trace mineralized salt was fed free choice in block form.

‖ Value of corn oil top dressed daily to meet NRC (1989) recommended digestible energy requirement for moderate exercise varied based on body weight of horse and averaged 609, 375, 259, 351, and 305 g for VL, L, Control, H, and VH protein diets respectively.

Table 8. Forage to concentrate ratio (percent of total diet)

	Diet				
	VL	L	Control	H	VH
Forage	62·8	54·7	53·0	54·6	53·9
Concentrate	37·2	45·3	47·0	45·4	46·1

Table 9. Apparent nitrogen and amino acid digestibility of experimental diets, percent

(Least squares mean values with their standard errors)

	Diet											
	VL (n=5)		L (n=4)		Control (n=5)		H (n=5)		VH (n=5)		P-Value	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Linear	Quadratic
Nitrogen	58·8^{c}	2·0	61·0^{bc}	2·2	65·2^{ab}	2·0	68·2^{a}	2·0	68·9^{a}	2·0	*	NS†
Amino acid												
Indispensable												
Arginine	76·4^{d}	1·4	79·3^{cd}	1·5	81·6^{bc}	1·4	83·7^{ab}	1·4	85·5^{a}	1·4	*	NS
Histidine	71·6^{c}	1·9	73·7^{bc}	2·1	76·2^{bc}	1·9	79·9^{ab}	1·9	82·6^{a}	1·9	*	NS
Isoleucine	60·5^{c}	2·4	63·4^{bc}	2·6	67·5^{abc}	2·4	71·1^{ab}	2·4	73·7^{a}	2·4	*	NS
Leucine	73·1^{b}	1·8	74·0^{ab}	1·9	76·2^{ab}	1·8	77·3^{ab}	1·8	79·0^{a}	1·8	*	NS
Lysine	57·0^{b}	2·5	57·2^{b}	2·7	63·7^{b}	2·5	73·2^{a}	2·5	73·8^{a}	2·5	*	NS
Phenylalanine	65·0^{c}	2·1	68·1^{bc}	2·3	71·4^{ab}	2·1	74·5^{ab}	2·1	77·0^{a}	2·1	*	NS
Threonine	66·9^{b}	2·2	70·4^{ab}	2·4	72·4^{ab}	2·2	75·3^{a}	2·2	77·6^{a}	2·2	*	NS
Valine	63·5^{c}	2·2	67·3^{bc}	2·4	70·1^{ab}	2·2	72·3^{ab}	2·2	74·8^{a}	2·2	*	NS
Dispensable												
Alanine	68·0^{a}	2·3	68·3^{a}	2·6	68·3^{a}	2·3	70·0^{a}	2·3	72·4^{a}	2·3	NS	NS
Aspartate	68·2^{d}	2·0	76·7^{bc}	2·1	74·2^{b}	2·0	78·6^{ac}	2·0	81·9^{a}	2·0	*	NS
Glutamate	75·4^{c}	1·5	79·6^{b}	1·6	80·7^{ab}	1·5	82·5^{ab}	1·5	84·2^{a}	1·5	*	NS
Glycine	63·0^{b}	2·1	68·2^{ab}	2·3	68·9^{ab}	2·1	72·3^{a}	2·1	74·6^{a}	2·1	*	NS
Proline	75·7^{b}	1·6	76·3^{b}	1·8	79·5^{ab}	1·6	79·7^{ab}	1·6	82·2^{a}	1·6	*	NS
Serine	73·4^{c}	1·5	77·4^{bc}	1·7	78·8^{ab}	1·5	80·7^{ab}	1·5	82·7^{a}	1·5	*	NS
Tyrosine	64·9^{c}	2·4	70·6^{bc}	2·6	73·6^{ab}	2·4	75·4^{ab}	2·4	78·8^{a}	2·4	*	NS

Table 9. Continued

[a,b,c,d] Least squares mean values within a row with unlike superscript letters differ ($P<0.05$).

[*] $P<0.05$.

[†] NS = not significant.

Table 10. Parameter estimates for crude protein, nitrogen and indispensable amino acid digestibilities

	Parameter				
	B_0	P Value	B_1	P Value	R^2
CP, (%)†	38·5	<0·001	2·015	0·012	0·26
N, (g/d)	46·6	<0·001	0·123	0·017	0·23
Indispensable AA, (g/d)					
Arginine	69·5	<0·001	0·199	0·001	0·41
Histidine	62·9	<0·001	0·541	0·002	0·35
Isoleucine	47·5	<0·001	0·486	0·003	0·34
Leucine	65·5	<0·001	0·125	0·099	0·12
Lysine	43·3	<0·001	0·577	0·001	0·40
Phenylalanine	53·5	<0·001	0·409	0·002	0·36
Threonine	55·6	<0·001	0·537	0·009	0·27
Valine	52·3	<0·001	0·345	0·009	0·27

† CP %, dry matter basis.

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SUMMARY AND CONCLUSIONS

Summary and Conclusion

The horse industry prioritizes health and performance of the equine athlete, thus great emphasis is placed on the nutritional needs of the exercising horse. Controversy exists regarding the degree to which exercise increases the demand for dietary protein. There is no known evidence that higher protein intake relative to maintenance enhances athletic performance, yet surveys of feeding practices in the industry report horses are often fed protein in excess of currently recommended levels. In addition, there is limited information on the digestibility of protein in diets fed to exercising horses and digestibility of individual amino acids is unavailable. Research designed to estimate protein requirement for exercise and to determine the availability of protein and AA in diets typically fed to exercising horses is critical. The overall goal of this study was to provide further understanding of the protein requirement of the exercising horse. The major findings of this experiment were as follows. First, N retention increased when horses were fed protein 12·5% above NRC (1989). Although this level of dietary protein was necessary to achieve maximal N retention, this response was paralleled by an increase in serum urea-N. Nitrogen retention thus overestimated protein requirement. This overestimation may have resulted from unmeasured nitrogen losses in sweat and utilization of N by hind gut microflora at higher protein intake. In addition to serum urea-N, serum AA concentration increased in horses fed Control relative to the low protein diets indicating that protein intake was in excess when fed above NRC (1989). The linear response in serum urea-N to graded level of protein intake for both long and short-term metabolism suggests that protein level fed at a level 25% below NRC (1989) may still have been in excess of protein requirement. However, it is important to

recognize that blood urea-N as a response criterion may be limited in the horse as serum urea-nitrogen concentration could also be affected by alternative routes of nitrogen excretion such as sweat or salvage by hind gut microflora. Concentration of serum arginine, the direct precursor of urea, plateaued in horses fed NRC (1989) recommended protein level and thus may be a useful indicator of protein status in the horse.

It was hypothesized that protein requirement reported in NRC (1989) overestimated the need for the moderately exercised horse. Response between nitrogen retention, serum urea-N, the majority of serum AA and dietary protein intake was linear rather than quadratic, thus precluding precise estimation of protein requirement using break point analysis. Nonetheless, our results suggest that AA utilization for protein synthesis and retention was not superior in horses fed NRC (1989) level compared to that of horses fed 12·5 and 25% below NRC (1989). Thus a protein to energy ratio as low as 30 g to 1 Mcal may be appropriate to meet the demand for protein for the moderately exercised horse.

Second, apparent fecal N and AA digestibility of diets containing varying ratios of feed ingredients typically used in the horse industry for exercising horses including mixed grass hay, corn, oat, and SBM were determined. Apparent fecal N and AA digestibility increased as dietary crude protein increased, and apparent N digestibility was higher than values reported in the current NRC (1989) ranging from 59 up to 69%. Apparent fecal lysine digestibility was higher in diets containing SBM and ranged from 61 up to 76%, thus contributing to the improved apparent fecal N digestibility. These results suggest protein digestibility is higher than currently reported in NRC (1989) in diets formulated according to a ratio of 40 g CP to 1 Mcal DE and to meet the DE

demand for exercise. Underestimation of protein digestibility contributes to feeding protein in excess of requirement. In addition, using CP digestibility in diet formulation rather than apparent lysine digestibility would overestimate total protein requirement of the exercising horse in diets containing ingredients higher in protein quality, such as SBM.

APPENDICES

Appendix A

Individual horse performance data

(Mean values with standard errors)

	Horse						
	1	2	3	4	5	Mean	SEM
Body Weight, (kg)							
Pre-experiment	498	524	452	455	436	473	16·4
Period 1	505	536	456	455	436	478	17·5
Period 2	503	542	465	486	436	486	17·5
Period 3	500	539	472	486	445	488	17·5
Period 4	507	546	467	486	445	490	17·5
Period 5	503	552	471	486	445	491	17·5
Body Condition Score							
Pre-experiment	6·5	7	7	6	5	6·3	0·4
Period 1	7	7	7	6	5·5	6·5	0·4
Period 2	8	8	7	6·5	6	7·1	0·4
Period 3	8	8	7	6·5	6	7·1	0·4
Period 4	7·5	8	7	6·5	6	7	0·4
Period 5	7·5	8	7	6·5	6	7	0·4
Resting Heart Rate, (bpm)							
Pre-experiment	36	40	28	35	37	35·2	2·0
Period 1	32	34	34	30	35	33	1·2
Period 2	32	36	32	27	35	32·4	1·2
Period 3	35	36	-	28	33	32·9	1·3
Period 4	29	35	31	31	33	31·8	1·2
Period 5	31	35	32	32	34	32·8	1·2
Working Heart Rate, (bpm)							
Pre-experiment	87	93	88	90	82	88	2·0
Period 1	80	94	96	83	83	87·2	2·6
Period 2	90	98	89	87	88	90·4	2·6
Period 3	87	99	-	89	86	90·7	2·8
Period 4	80	94	90	82	87	86·6	2·6
Period 5	82	99	89	88	91	89·8	2·6

Appendix B

Effect of diet on basal serum dispensable amino acid concentration

(Least squares mean values with their standard errors)

	Diet											
	VL (n=5)		L (n=4)		Control (n=5)		H (n=5)		VH (n=5)		P-Value‡	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	L	Q
Serum amino acid, (umol/L)												
Basal												
Dispensable												
Alanine	209·1[a]	12·5	163·1[ab]	13·8	145·4[b]	12·5	135·9[b]	12·5	141·2[b]	12·5	*	*
Asparagine	100·3[a]	4·8	89·3[ab]	5·4	90·5[ab]	4·8	79·4[b]	4·8	86·9[ab]	4·8	*	NS†
Aspartate	7·0[a]	0·6	5·7[a]	0·7	6·4[a]	0·6	5·7[a]	0·6	6·4[a]	0·6	NS	NS
Citrulline	83·1[a]	4·2	88·7[a]	4·5	85·9[a]	4·2	78·8[a]	4·2	88·2[a]	4·2	NS	NS
Cysteine	4·7[a]	0·6	4·5[a]	0·7	4·4[a]	0·6	4·8[a]	0·6	4·7[a]	0·6	NS	NS
Glutamine	253·2[a]	11·6	234·5[ab]	12·1	219·5[bd]	11·6	190·5[c]	11·6	195·8[cd]	11·6	*	NS
Glutamate	67·4[a]	5·3	53·5[a]	6·1	60·2[a]	5·3	63·7[a]	5·3	61·9[a]	5·3	NS	NS
Glycine	368·2[a]	17·0	400·7[a]	18·9	405·7[a]	17·0	356·6[a]	17·0	409·3[a]	17·0	NS	NS
Ornithine	40·0[a]	4·7	39·1[a]	5·3	38·4[a]	4·7	44·4[a]	4·7	50·0[a]	4·7	NS	NS
Proline	120·0[a]	3·2	92·7[b]	3·7	88·6[b]	3·2	80·6[b]	3·2	84·2[b]	3·2	*	*
Serine	285·6[a]	17·6	240·8[ab]	18·5	213·7[bc]	17·6	186·5[c]	17·6	182·1[c]	17·6	*	*
Taurine	40·5[b]	2·3	47·6[ab]	2·5	47·7[ab]	2·3	45·8[ab]	2·3	51·5[a]	2·3	*	NS
Tyrosine	65·5[a]	2·3	63·6[a]	2·5	65·0[a]	2·3	59·6[a]	2·3	62·3[a]	2·3	*	NS

Appendix B Continued

[a,b,c,d] Least squares mean values within a row with unlike superscript letters differ ($P<0.05$).

[*] $P<0.05$.

[†] NS = not significant.

[‡] L = linear response, Q = quadratic response.

Appendix C

Effect of diet on post-feeding/exercise serum dispensable amino acid concentration

(Least squares mean values with their standard errors)

	Diet											
	VL (n=5)		L (n=4)		Control (n=5)		H (n=5)		VH (n=5)		P-Value‡	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	L	Q
Serum amino acid, (umol/L)												
Post exercise												
Dispensable												
Alanine	216·7[a]	16·3	191·1[a]	17·0	214·5[a]	16·3	222·9[a]	16·3	218·9[a]	16·3	NS†	NS
Asparagine	162·3[a]	9·6	152·2[a]	11·1	194·2[a]	9·6	195·0[a]	9·6	194·8[a]	9·6	*	NS
Aspartate	5·1[a]	0·5	5·6[a]	0·6	5·7[a]	0·5	6·5[a]	0·5	5·4[a]	0·5	NS	NS
Citrulline	86·6[b]	4·3	94·0[ab]	4·8	103·8[a]	4·3	105·7[a]	4·3	108·5[a]	4·3	*	NS
Cysteine	3·7[bc]	0·3	4·4[ac]	0·3	4·7[a]	0·3	4·6[a]	0·3	3·8[c]	0·3	NS	*
Glutamine	310·9[a]	13·2	330·9[a]	15·7	345·7[a]	13·2	355·9[a]	13·2	349·4[a]	13·2	*	NS
Glutamate	57·7[a]	3·4	51·7[a]	3·9	55·2[a]	3·4	54·4[a]	3·4	53·3[a]	3·4	NS	NS
Glycine	361·8[a]	23·8	358·7[a]	27·8	421·5[a]	23·8	400·0[a]	23·8	405·8[a]	23·8	NS	NS
Ornithine	44·3[b]	4·2	47·9[ab]	5·0	56·0[ab]	4·2	52·0[ab]	4·2	68·2[a]	4·2	*	NS
Proline	156·8[a]	6·3	150·9[a]	7·3	172·9[a]	6·3	173·0[a]	6·3	175·1[a]	6·3	*	NS
Serine	298·2[a]	14·3	248·1[b]	15·4	280·2[ab]	14·2	265·4[ab]	14·2	246·3[b]	14·2	*	NS
Taurine	33·3[b]	1·8	37·3[ab]	2·1	42·3[a]	1·8	40·1[ab]	1·8	45·6[a]	1·8	*	NS
Tyrosine	77·8[c]	4·3	83·3[bc]	4·9	99·3[ab]	4·3	101·9[a]	4·3	110·7[a]	4·3	*	NS

Appendix C Continued

[a,b,c] Least squares mean values within a row with unlike superscript letters differ ($P<0.05$).

[*] $P<0.05$.

[†] NS = not significant.

[‡] L = linear response, Q = quadratic response.

Appendix D

Effect of diet and period on calculated muscle mass, kg[†]

(Least squares mean values with their standard errors)

Item	Mean	SEM
Diet		
VL	155·9[a]	19·3
L	167·4[a]	21·1
Control	199·7[a]	21·1
H	144·6[a]	19·3
VH	167·4[a]	19·3
Period		
1	146·4[a]	19·3
2	161·0[a]	21·1
3	171·7[a]	21·1
4	172·8[a]	19·3
5	183·2[a]	19·3

[a] Least squares mean values within a column with unlike superscripts differ ($P<0·05$).

[†] Muscle mass calculated from 24-h urinary creatinine excretion, where 1 g creatinine = 21·8 kg skeletal muscle (Wang et al., 1996).