ANATOMICAL SITE AND MECHANISM OF LYSINE TRANSPORT IN THE EQUINE INTESTINE

By

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ABSTRACT

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The site and form of nitrogen (N) absorption across the gastrointestinal tract (GIT) in horses is unclear. While the role of the small intestine as a main site of amino acid (AA) absorption has been demonstrated using both *in vivo* and *in vitro* models of monogastric animals, contribution of the large intestine to N and AA homeostasis of the host is not known. Currently, there is very limited information on the anatomical site and mechanisms of AA uptake in the equine intestine. Understanding the functional and anatomical difference in AA absorption across the GIT of equids would provide critical information on the relative role of the large intestinal contribution to total GIT AA absorption and thus add to the overall understanding of protein utilization and AA requirements in equids.

The main hypothesis of this dissertation was that the capacity to transport lysine across the epithelium of the equine large intestine mucosa is similar to that of the small intestine. The overall objective was to characterize the molecular entities and kinetic processes of L-lysine transport in the equine small and large intestine. To test the hypothesis, three specific aims were addressed: (1) to determine the mRNA abundance of AA transporter proteins $b^{0,+}$AT (SLC7A9, of system $b^{0,+}$), CAT-1 (SLC7A1, of system y^{+}), and LAT-2 and LAT-3 (SLC7A8 and SLC43A1, respectively, of system L) in segments of the large intestine and the small intestine;

(2) to characterize total (sum of Na^+-dependent and Na^+-independent) L-lysine transport kinetics across equine and porcine small and large intestinal brush border membrane (BBM); and (3) to determine the distribution and epithelial cell membrane localization (apical, i.e., BBM vs. serosal, i.e., basolateral membrane) of the CAT-1 and LAT-2 protein in the small and large intestine mucosa. Results show that 1) transporters $b^{0,+}$AT and LAT-3 mRNA transcript abundance determined by qRT-PCR in the equine large intestine was similar to that of the small intestine; 2) L-lysine transport V_{max} and K_M across brush border membrane vesicles (BBMV) manufactured from equine intestinal epithelial cells did not differ between the large colon and the small intestine; 3) transporters CAT-1 and LAT-2 mRNA transcript abundance determined by qRT-PCR and protein abundance determined by immunohistochemistry and in the large intestine was lower than that in the small intestine; and 4) transporter LAT-2 appears to be localized to the equine intestinal basolateral epithelial membrane, while transporter CAT-1 is localized to the equine intestinal lamina propria and endothelial cells.

In conclusion, the equine large intestine is capable of L-lysine transport, most of which would originate from microbial proteins, across the intestinal epithelium. Thus, in horses, the large intestine may contribute to overall lysine homeostasis similar to that of the small intestine. This implies that feeding forages of lower protein quality which closer meets, rather than exceeds, requirements for the horse may reduce excretion levels of urinary N, leading to increased environmental sustainability. Overall, knowledge of the large intestinal role in lysine absorption as demonstrated *in vitro* can shed light on the actual protein utilization and availability of less apparently digestible forages in horses.

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KEY TO ABBREVIATIONS

AA = Amino acid

$b^{0,+}$ = Blastocyst amino acid transporter

BBM = Brush border membrane

BBMV = Brush border membrane vesicle

cDNA = Complementary DNA

CAT = Cationic amino acid transporter

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

GIT = Gastrointestinal tract

LAT = Large neutral amino acid transporter

Lys = Lysine

mRNA = Messenger RNA

N = Nitrogen

Na = Sodium

SLC = Solute carrier family

Q-RT-PCR = Quantitative reverse transcription polymerase chain reaction

VFA = Volatile fatty acid

INTRODUCTION

Feeding protein far in excess of requirement is a common practice in the horse industry (Glade, 1983; Lawrence et al., 2003; Ott, 2005). Nitrogen (N) intake in excess of utilization leads to higher energy demands for increased urea synthesis and excretion (Miller and Lawrence, 1988), and decreases performance measures such as growth (Yoakam et al., 1978) and racing speed (Glade, 1983). In addition, increased urinary N excretion contributes to surface and ground water contamination, and decreases housing and environmental air quality (Knowlton and Cobb, 2006). Strategies to mitigate the environmental and health impact of feeding excess protein to horses critically depend on better understanding of protein digestion and amino acid (AA) absorption processes in the horse gastrointestinal tract (GIT). The relative importance of anatomical sites of and capacity for AA digestion and absorption, i.e., pre- vs. post-cecal, remain poorly documented in the horse.

Nonruminant herbivores rely on the presence of microbes in the cecum and colon to help digest plant material (Hintz et al., 1978). In ruminants, these microbes reside proximal to the "true" stomach, allowing for the majority of microbial nitrogenous products to be absorbed by the small intestine (Wilson and Webb, 1990). As predominately hind-gut fermenters, major sites of microbial digestion in the horse occur distal to the small intestine, in the cecum and large colons (Reitnour et al., 1969; Reitnour and Salsbury, 1972; Gibbs et al., 1988). Currently, there is limited information on the overall contribution of the equine hindgut to nutrient homeostasis of the host, specifically that of nitrogenous products of dietary and microbial origin. Kern et al. (1973) compared the bacterial population profile in the pony cecum with that in steer rumen and found that ponies had more proteolytic bacteria, indicating that microbial-mediated protein

degradation in the cecum may be a mechanism increasing luminal AA availability. Dietary addition of starch from oats increased the total bacteria and viable bacteria, specifically *Blephacorys uncinata*, in the cecum of ponies (Kern et al., 1973). Cecal administration of starch from corn increased bacterial N digestion and apparent N digestibility (Reitnour, 1979; Reitnour, 1982), and, subsequently, increased N retention, presumably due to hindgut N absorption of microbial protein (Reitnour, 1982).

Though increase in bacterial protein synthesis and N retention is indicative of N uptake from the large intestine, the form in which N is absorbed, i.e., AA, urea or ammonia, remains unknown. Knowledge of whether or not AA in the large intestinal lumen are absorbed in the horse would improve the overall understanding of protein utilization and AA requirements in equids. In the long run, this understanding would contribute to the improvement of AA nutrition and environmental sustainability by allowing the design of diets based on both AA and N (crude protein (CP)) requirement rather than based on N requirement alone, as reported by Horse NRC (2007).

Currently, there is very limited information of the anatomical site and mechanisms of AA uptake in the equine intestine. *In vivo* studies measuring AA absorption from the small intestine and large intestine would require both fistulation of the cecum or ileum, and cecal and portal vein catheterization, procedures which are invasive to the horse and costly to investigators. These challenges have impeded progress in our understanding of the AA requirements by equids relative to other livestock species. Addressing the mechanisms of lysine absorption through the use of novel alternative approaches that combine relative quantitative real-time polymerase chain reaction with *in vitro* testing of lysine uptake by the equine GIT can improve knowledge of equine AA requirements in a less invasive fashion.

In the horse, neither lysine transport systems nor the molecular entities contributing to the uptake of AA by the intestinal epithelial cells are known. Knowledge of the relative role of the GI segments in AA utilization will allow a better understanding and definition of nutritional essentiality of lysine and thus, in part, the efficiency of dietary N utilization in horses.

Therefore, the overall objective of this dissertation was to characterize the molecular entities and the kinetic processes of L-lysine transport in the equine small and large intestine. The hypothesis was that the capacity to transport lysine across the epithelium of the equine large intestine mucosa is similar to that of the small intestine. To test the hypothesis, three specific aims were addressed: (1) to determine the mRNA abundance of AA transporter proteins $b^{0,+}$AT (SLC7A9, of system $b^{0,+}$), CAT-1 (SLC7A1, of system y^{+}), and LAT-2 and LAT-3 (SLC7A8 and SLC43A1, respectively, of system L) in segments of the large intestine and the small intestine; (2) to characterize total (sum of Na^{+}-dependent and Na^{+}-independent) L-lysine transport kinetics across equine and porcine small and large intestinal brush border membrane (BBM); and (3) to determine the distribution and epithelial cell membrane localization (apical, i.e., BBM vs. serosal, i.e., basolateral membrane) of the CAT-1 and LAT-2 protein in the small and large intestine mucosa.

This dissertation contains five chapters. The first chapter is a literature review, presenting the current knowledge of dietary protein utilization, including the functional anatomy of the GIT of the horse, the current knowledge of protein digestion of dietary feedstuffs, anatomical site of N absorption by the horse, and a brief overview of AA and peptide transport processes across epithelial cells of the intestine. The second chapter addresses the first objective, i.e., determination of the mRNA abundance of AA transporter proteins $b^{0,+}$AT (SLC7A9, of system

$b^{0,+}$), CAT-1 (SLC7A1, of system y^+), and LAT-2 and LAT-3 (SLC7A8 and SLC43A1, respectively, of system L) in segments of the large intestine and the small intestine. A preliminary study assessing *in vivo* metabolism and absorption of AA in the small intestine in horses fed timothy and alfalfa hay is presented in Appendix A. The third chapter addresses the second objective, i.e., characterization of total (sum of Na^+-dependent and Na^+-independent) L-lysine transport kinetics across equine and porcine small and large intestinal BBM. A concurrent study determining the functionality of brush border membrane vesicles (BBMV) and assessing glucose uptake in the porcine and equine small intestine and large colon, can be found in Appendix B. The fourth chapter addresses the third objective, i.e., distribution and epithelial cell membrane localization (apical, i.e., BBM vs. serosal, i.e., basolateral membrane) of the CAT-1 and LAT-2 protein in the small and large intestine mucosa. Finally, chapter five is a summary of the results and an overall conclusion.

CHAPTER ONE

LITERATURE REVIEW

Portions of this chapter are in review for the following manuscript:

Woodward, A. D.[*], M. L. Westendorf[†], C. A. Williams[†], K. L. Martinson[‡], A. O. Burk[§], and N. L. Trottier[*1]. Protein and nitrogen utilization in the horse: impact on environment. In preparation for submission to the *Equine Veterinary Journal*. Submitted to co-authors for review.

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CHAPTER ONE

LITERATURE REVIEW

1. Equine gastrointestinal anatomy and histology

1.1. Gross GI anatomy

The oral cavity of the horse contains incisors for cutting and molar teeth for grinding feed into small particles before swallowing (Dixon, 2002). Horses tend to chew feed particles into smaller sizes prior to swallowing compared to ruminants, although the rate of mastication (number of bites/minute) is not different between species (Meyer et al., 1975). Saliva, used to lubricate feedstuff, has a high concentration of bicarbonate to buffer stomach contents (Frape, 2004).

Once swallowed, peristaltic contractions push the ingesta down the esophagus and through the cardiac sphincter into the stomach. The extreme strength of the cardiac sphincter prevents reflux back into the esophagus (Moore et al., 2001). The equine stomach has a relatively small capacity (8 – 16 L) due in part to the adaptation of horses as foragers (Ralston, 1984). The stomach is divided into a glandular and non-glandular (squamous) region with the non-glandular region covering over one-half of the stomach. The glandular region is further subdivided into the fundic and the pyloric regions, where the pyloric region is responsible for secretion of gastrin, while the fundic region secretes hydrochloric acid and pepsin. The fundic region of the stomach also houses microorganisms capable of fermentation of soluble starch (Varloud et al., 2007). Volatile fatty acids (VFA) released through microbial fermentation of soluble carbohydrates in the stomach may serve as an energy source to the mucosa cells; VFA

escaping uptake and utilization in the stomach would be flushed into the small intestine (Varloud et al., 2007).

Following gastric digestion, digesta passes through the pyloric sphincter and enters the small intestine, composed of the duodenum, jejunum and ileum. The proximal duodenum (3 m) is attached to the dorsal body by a short mesentery, which stabilizes the section and keeps it from being involved in displacement (Moore et al., 2001). The duodenum passes over the dorsal aspect of the base of the cecum in the right paralumbar fossa region (Moore et al., 2001). The jejunum (20 m), the start of which is indicated by lengthening of the mesentery, rests on the dorsal cecum and large colon (Moore et al., 2001). The distal termination of the jejunum and the beginning of the ileum (45 cm) is signaled by thickening of the intestinal wall, narrowing of the intestinal lumen, and the beginning of the antimesenteric mesentery (Moore et al., 2001).

Upon passage through the ileocecal valve and into the cecum, the ingesta is subject to microbial degradation and fermentation by bacteria, protozoa, and other microorganisms which aid in the breakdown of plant fiber (Kern et al., 1973; Moore et al., 2001). The cecum, which extends from the paralumbar fossa region to the ventral midline of the horse (Moore et al., 2001), holds approximately 30 L of ingesta and is largely responsible for microbial synthesis of VFA and proteins (Argenzio et al., 1974; Reitnour, 1979; Reitnour, 1982). Cecal pH must be kept close to neutral in order for normal microbial fermentation to occur (Frape, 2004). Further mixing and microbial fermentation take place as the ingesta travels from the cecum through the cecacolic junction to the right ventral colon (Moore et al., 2001). The right ventral colon (1.2 m, 25 – 30 cm diameter) is sacculated by haustra, and muscular contractions move feed cranially towards the sternum (Moore et al., 2001).

As the digesta is further processed, it is pushed through the sternal flexure and into the left ventral colon (Moore et al., 2001). The left ventral colon (1.2 m) is also sacculated, and moves digesta caudally toward the flank on the left side (Moore et al., 2001). At the termination of the left ventral colon, the diameter begins to decrease from approximately 20 – 25 cm to approximately 10 cm at the junction of the pelvic flexure (Moore et al., 2001). After digesta moves through the pelvic flexure, it enters the left dorsal colon (1 m, 25 – 30 cm in diameter), which lies atop the left ventral colon. The left dorsal colon is unsacculated and carries digesta cranially from the flank to the diaphragm (Moore et al., 2001).

The digesta passes from the left dorsal colon into the right dorsal colon through the diaphragmatic flexure (Moore et al., 2001). The unsacculated right dorsal colon (1 m) is the largest colon in diameter, reaching approximately 30 – 35 cm, and serves as the last site for absorption of nutrients and one of the main sites of water and electrolyte uptake (Moore et al., 2001; Frape, 2004). The right dorsal colon is attached to the right ventral colon by a short, fibrous mesentery, again stabilizing the colon and preventing involvement in torsion (Moore et al., 2001). From the right dorsal colon, the digesta moves into the small colon (10 cm diameter), composed of the small transverse colon and the sacculated descending colon (4 – 5 m) (Moore et al., 2001).

1.2. Small and large intestinal histology

Both walls of the small and large intestine are composed of similar layers of cell arrangement: a mucosa, a sub-mucosa, a muscularis externa and a serosa, but the luminal mucosa of the small intestine has villi and microvilli to increase the surface area whereas the lumen of the large intestine has crypts. The mucosa contains 3 layers: the epithelium, the lamina propria and the muscularis mucosa. The cellular composition and morphology differ between the

duodenum and the distal small intestine and between the small and large intestine. The mucosa of the small intestine is characterized by villi and crypts, the latter referred to as crypts of Lieberkühn (Bacha, Jr. and Bacha, 2000), which secrete enzymes along with peptidases (Krause, 2000). Two layers of smooth muscle known as the muscularis mucosa separate the mucosal layer from the submucosal layer (Bacha, Jr. and Bacha, 2000). The duodenum and jejunum of the small intestine in the horse contain Brünners glands (Bacha, Jr. and Bacha, 2000), which secrete mucous to protect and lubricate the intestinal wall (Krause, 2000). In comparison to the small intestine, the large intestine has no villi; however, crypts are longer than in the small intestine (Bacha, Jr., and Bacha, 2000). The cecum and colon contain taenia ceci and taenia coli, respectively, which are longitudinal cells composed of elastic fibers (Bacha Jr. and Bacha, 2000; Klein, 1883).

2. Current knowledge on nitrogen product utilization by the GI of horse

2.1. Dietary constituents and their effect on nitrogen digestibility

Horses are hind-gut fermenters, thus they rely on microbial degradation of fibrous dietary components in order to synthesize proteins and VFA (Hintz et al., 1978). Unlike ruminants, where fermentation of structural polysaccharides occurs prior to passage through the stomach and small intestine, fermentation in equids mainly occurs distal to the small intestine in the cecum and proximal large intestine (Argenzio et al., 1974; Wootton and Argenzio, 1975). While there is no question the small intestine absorbs digested nutrients such as carbohydrates, proteins, and VFA (Reitnour et al., 1969; Reitnour and Salsbury, 1972), the site and extent of absorption of VFA and nitrogenous compounds released through fermentation in the large intestine remains unclear (Argenzio et al., 1974; Wootton and Argenzio, 1975). In addition, there is very limited information regarding the form in which N is absorbed, i.e., ammonia, urea, AA, or peptides.

Forage digestibility depends on the fractions of cell contents, i.e., lipids, sugars, pectin, starch, non-protein nitrogenous compounds, and soluble proteins, and cell wall constituents, i.e., fiber-bound protein, cellulose, hemicelluloses, lignin and lignified nitrogenous compounds (Van Soest, 1965; Van Soest, 1967). Of the cell contents, only sugars are completely digestible by non-ruminants and ruminants, and all other components are considered highly digestible (Van Soest, 1967). Of the cell wall constituents, cellulose, hemicelluloses and fiber-bound proteins are partially digestible upon fermentation (Van Soest, 1965; Van Soest, 1994), while lignin and lignified nitrogenous compounds are completely indigestible (Van Soest, 1967), in both hindgut fermenter and ruminant animals.

Soluble cell contents, insoluble cell wall constituents, and the nutritive value of forages are determined by the detergent system (Van Soest, 1994). Cell contents are first extracted and

removed from the fiber fraction of the forage with a neutral (pH = 7) solution, precipitating a fiber residue containing the cell wall constituents (Van Soest, 1965; Van Soest, 1994). The cell contents are considered the neutral detergent soluble fraction, and the cell wall constituents are the neutral detergent insoluble fraction, referred to as the neutral detergent fiber (NDF) (Van Soest, 1965). The NDF can be separated into an acid detergent soluble fraction and an acid detergent insoluble, or acid detergent fiber (ADF), fraction (Van Soest, 1965; Van Soest, 1994). The acid detergent soluble fraction of NDF contains the hemicelluloses and fiber-bound protein, also known as neutral detergent insoluble N (Van Soest, 1965; Van Soest, 1967; Van Soest, 1994), and the ADF contains cellulose, lignin, and lignified nitrogenous compounds (Van Soest, 1965; Van Soest, 1994). The lignified nitrogenous compounds and heat-damaged proteins in the ADF are indigestible and completely unavailable to non-ruminants or ruminants (Van Soest, 1967; Muscato et al., 1983; Van Soest, 1994; See Figure 1.1).

When comparing composition of grasses and legumes, hemicellulose content of grasses exceeds legumes by up to four fold (Van Soest, 1967). The high hemicellulose content slows the initial fermentation rate, thus increasing the availability of the neutral detergent-soluble fraction components and the neutral detergent insoluble N (Van Soest, 1965). Muscato et al. (1983) examined the AA profile of timothy grass hay fiber components and found over 65% of the total plant AA within the neutral detergent insoluble N fraction. Thus, over half of the amino-N in timothy hay is likely available to the large intestinal microflora of hindgut fermenters. However, *in vivo* utilization of this AA pool is unknown.

Differences in apparent N digestibility of grass hay are reported both between and within cultivars (Fonnesbeck et al., 1967; Darlington and Hershberger, 1968; Crozier et al., 1997; Takagi et al., 2002; Takagi et al., 2003). Between grass forages, lowest apparent N digestibility

is 32% in bermudagrass (Fonnesbeck et al., 1967), while highest apparent N digestibility is 68% in orchardgrass (Darlington and Hershberger, 1968). Estimates of whole tract apparent N digestibility range widely even within cultivars; timothy grass hay ranges from 36% (Cuddeford et al., 1992) to 65% (Darlington and Hershberger, 1968), tall fescue ranges from 38% (Fonnesbeck et al., 1967) to 67% (Crozier et al., 1997), canarygrass ranges from 48% to 62% (Fonnesbeck et al., 1967), and orchardgrass ranges from 40% (Takagi et al., 2003) to 68% (Darlignton and Hershberger, 1968).

Differences in apparent N digestibility among grass hay is likely related to differences in fiber composition, as reported in sheep, swine, rats (Keys et al., 1969) and horses (Glade, 1984). Digestibility of the cellulose component of fiber is directly related to lignification, which in turn decreases plant cell-wall protein digestibility (Van Soest, 1994). Further, hemicellulose is associated with lignin content (Van Soest, 1994) and is considered a limiting factor to fiber digestibility in horses (Glade, 1984). Cellulose, hemicelluloses, and lignin are all components of NDF; thus, low N digestibility of forage may be attributed to high NDF content, as reported in dairy cattle (Van Soest, 1994) and horses (Glade, 1984).

Cereal grains are commonly fed to horses as a source of dietary energy due to their high starch content, and the addition of grain can enhance whole tract apparent N digestibility of low protein forage, such as straw, from 50 to 66% (Kienzle et al., 2002). Whole oats is a common grain used in many mixed equine diets because they are readily digestible pre-cecally and very palatable (Hussein et al., 2004; Sarkijarvi and Saastamoinen, 2006). Palmgren Karlsson et al. (2000) reported a 9 and 18% increase in whole tract apparent N digestibility of the total diet when oats were provided in addition to grass hay at 0.3 and 0.6% BW, respectively. The increase in whole tract apparent N digestibility of timothy with the inclusion of oats may be due,

in part, to stimulation of the microbial protein synthesis and population by starch, in turn enhancing capacity for fiber digestion (Kern et al., 1973; Palmgren Karlsson et al., 2000). On the other hand, Hussein et al. (2004) supplemented alfalfa cubes with whole or naked oats and found no difference in whole tract apparent N digestibility. However, it should be noted that the forage source in the Hussein et al. (2004) study consisted of a processed alfalfa cube with a high CP content of 19%; therefore, addition of oats may increase whole tract apparent N digestibility only when low protein forage is used.

2.2. Evidence Supporting Nitrogen Absorption in the Large Intestine

Nitrogen utilization by the equine hindgut microflora and absorption was first reported by Slade et al. (1970), whereby dietary supplementation of urea improved N digestibility, presumably via cecal microbial breakdown of urea and ammonia utilization into free AA synthesis. Results of Slade et al. (1970) were substantiated by Nelson and Tyznik (1971), who demonstrated using cecally-cannulated ponies that urea, fed as the major dietary protein source, entered the cecum where it stimulated microbial protein synthesis. Similarly, Reitnour and Treece (1971) reported that although urea fed to ponies was absorbed largely from the small intestine, urea that reached the large intestine increased cellulose digestion of the basal diet. In a subsequent study, Reitnour and Salsbury (1972) infused cecally-fistulated ponies with three sources of protein, including fishmeal, soybean meal and linseed meal, and demonstrated increased plasma urea concentration and urinary N excretion following infusions. While those results indicate that N was absorbed by the large intestine, the form in which N is absorbed was not investigated.

In cecally fistulated ponies fed three different pelleted diets based on corn (89.8% corn, 8.7% purified cellulose), oat (59.8% oat, 33.1% corn starch, 5.5% purified cellulose), and barley (69.2% barley, 22.7% corn starch, 6.6% purified cellulose), only 11% of the whole GI tract (i.e., from mouth to the feces) protein digestion was attributed to the small intestine in contrast to 40% contribution from the large intestine alone based on a dietary indicator dilution approach (Reitnour et al., 1969). Poor protein digestibility rates (only 51%) were attributed to incomplete indicator recovery, as well as the contribution of metabolic N to apparent N digestibility (Reitnour et al., 1969). Similarly, in ponies solely offered Coastal Bermudagrass, low-protein alfalfa hay, or high-protein alfalfa hay, only 9.6%, 1.3%, and 21.0%, respectively, of N digestibility occurred pre-cecally, while 52.5%, 65.7%, and 66.9%, respectively, of N digestibility was post-ileal. Fitting the data to a linear regression indicated that 37% of true N digestibility occurred pre-cecally, while true post-ileal N digestibility calculated from total N presented to the large intestine was 96.3% (Gibbs et al., 1988). The high true post-ileal digestibility may also represent an artifact of metabolic N overestimation. Nonetheless, the relatively short time of passage through the small intestine (< 120 min) appears to contribute to the lower digestive efficiency in that segment of the equine GI tract, and thus digestive compensation of lower quality protein (i.e., less soluble) feeds may occur through post-cecal absorption (Reitnour et al., 1969; Gibbs et al., 1988).

Passage of digesta through the compartments of the equine large intestine, namely the cecum, ventral colon, and dorsal colon, may also play a role in post-ileal N absorption. Argenzio et al. (1974) and Wootton and Argenzio (1975) demonstrated that there was no retrograde flow between the hindgut compartments, and that each compartment independently absorbed VFA and N. This observation led Wootton and Argenzio (1975) to examine the equine large intestine

as a site of active protein appearance and disappearance. They noted that as much as 80 mg total protein N atoms $\cdot$ h^{-1} disappeared and appeared in different sections of the colon, yet less than 20 mg atoms N $\cdot$ h^{-1} was attributed to ammonia plus urea N, leaving 60 mg total protein N atoms$\cdot$ h^{-1} unaccounted (Wootton and Argenzio, 1975). The authors suggested AA may be generated and absorbed by the large intestine (Wootton and Argenzio, 1975), thusly accounting for the discrepancy in total protein N disappearance.

Compartmentalization of the equine large intestine was also observed by Glade (1983). Digesta from twelve mature horses fed a mixed diet composed of 30% corn, 30% oats, and 40% timothy grass hay was removed from 10 sections of the GI tract to determine N partitioning of neutral detergent soluble N, soluble N, and neutral detergent fiber (Glade, 1983). Only 20% of N disappearance was observed from the small intestine, with most absorption from the jejunum and ileum; the cecum and small colon accounted for the remaining N disappearance (Glade, 1983). In that study, Glade (1983) suggested that identifying colonic N transporters would allow separation of the true nature of N uptake by accounting for absorption of AA, peptides, and N-end products derived from microbial degradation.

Data suggests that the large intestine appears to be an important site of N absorption. This is in sharp contrast with NRC (2007), that reports "Proteins that are digested in the foregut are potentially available to the horse to contribute to the AA pool in the body, whereas those that pass to the hindgut are not." Contrary to this statement, early research has indicated (1) a significant portion of N absorption from the large intestine cannot be accounted for by ammonia and urea (Wootton and Argenzio, 1975), and (2) microbial protein-derived AA, specifically lysine and other essential AA, can be absorbed from the large intestine (Slade et al., 1971). If the

horse does have the ability to synthesize and absorb essential AA from the hindgut, then it may be reasonable to suggest that equids would have a relatively low dietary requirement for indispensable AA, or those which cannot be derived from the host and must be synthesized by microbes.

3. Amino acid and peptide uptake across epithelial cells

Utilization of dietary protein involves a series of steps, including hydrolyzing protein into small peptides and free AA and subsequent absorption of the N-end products by BBM of epithelial cells (Mello, 2003). Uptake of peptides and AA by intestinal epithelium is a complex process, involving several transport systems which facilitate the movement of specific AA either through passive diffusion or through the use of a facilitator, such as sodium (Na^+), hydrogen (H^+), potassium (K^+), and chloride (Cl^-) (Souba and Pacitti, 1992; Bröer, 2008). The general process behind peptide or AA transmembrane uptake includes (1) binding to a transporter domain on the apical membrane, (2) translocation through the apical membrane and into the cell, (3) release into the intracellular compartment (Souba and Pacitti, 1992). These steps must be repeated across the basolateral membrane to complete the transmembrane transport process across the intestinal epithelial cell and entry to the portal bloodstream for whole body utilization (Bröer, 2008). In the case of facilitated transport, both the substrate and the facilitator must bind to the transporter before translocation. Furthermore, the ability of a substrate to enter into a cell depends both on the affinity of that substrate to a transporter domain (i.e., as defined by the K_M) and the number of functional transporters on the cell membrane (i.e., as defined by the V_{max}) (Souba and Pachitti, 1992). Transport of peptides and subsequent hydrolysis into single AA molecules occurs through one of two mechanisms: (1) transport of the intact peptide through a specific membrane-bound peptide transporter followed by hydrolysis in the cell cytosol (Figure 1.2.A), or (2) hydrolysis of peptides by BBM peptidases prior to transport of individual AA into the epithelial cell (Figure 1.2.B) (Newey and Smyth, 1960; Newey and Smyth, 1962; Adibi, 1971; Adibi et al., 1975). Amino acid transport across the small intestinal epithelial cells occurs

via numerous transporter proteins, which define transport systems varying in capacity and overlapping in specificity (Souba and Pacitti, 1992; Bröer, 2008).

3.1. Transport of peptides across intestinal apical and basolateral membranes

The ability of the epithelial brush border membrane (BBM) to absorb intact peptides was first demonstrated in rats and humans using the dipeptide glycylglycine (Newey and Smyth, 1960; Adibi, 1971). In rats, addition of glycylglycine to the luminal (i.e., or mucosal), surface of everted gut sacs resulted in increased free glycine concentration on the serosal surface (Newey and Smyth, 1960). Further analysis of glycine concentration on the mucosal surface compared to the intestinal cell wall and serosal surface left little doubt to the authors that most hydrolysis of glycylglycine occurred intracellularly (Newey and Smyth, 1960), which was in turn verified in a subsequent experiment (Newey and Smyth, 1962). In humans, intact peptide absorption was demonstrated when intestinal infusion of glycylglycine led to increased glycine absorption, increased plasma-free glycine concentrations, and increased plasma glycylglycine concentrations compared to infusion with equimolar concentration of free glycine (Adibi, 1971). Subsequent research using the dipeptide glycylleucine supported the intact peptide absorption hypothesis, as intestinal infusion of glycylleucine increased the absorption rate of glycine and leucine compared to infusions with free glycine or free leucine (Adibi, 1971).

Because only 10% of peptidases reside on the BBM, compared to 80 to 90% localized intracellularly (Kim et al., 1972), tripeptides may also be absorbed intact prior to hydrolysis within the epithelial cell (Adibi et al., 1975). This is the case with triglycine, a small peptide which undergoes intracellular hydrolysis after intact absorption (Adibi et al., 1975). However, large, insoluble tripeptides, such as trileucine, may first require hydrolysis at the cell membrane

prior to absorption (Adibi et al., 1975). Regardless of whether peptides are hydrolyzed prior to absorption, PEPT1 is the only peptide transporter so far identified on the epithelial BBM (Gilbert et al., 2008), and the only one peptide transporter (PEPT1) reported on the epithelial basolateral membrane (Terada et al., 1999; Gilbert et al., 2008). Therefore, for optimal AA utilization, peptides are transported into the epithelial cells by PEPT1 (Gilbert et al., 2008), hydrolyzed into their individual AA components (Adibi, 1971; Adibi et al., 1975), and transported through the basolateral membrane and into circulation through individual AA transporters.

3.2. Transport of amino acids across intestinal apical and basolateral membranes

Amino acids enter and leave intestinal epithelial cells through the apical membrane via one of several AA transporters belonging to numerous systems. Passage of substrate specific AA from the intestinal lumen through the apical membrane can occur through AA transporters ASCT2 of system ASC; B^0AT1 of system B^0; $ATB^{0,+}$ of system $B^{0,+}$; $b^{0,+}AT$ of system $b^{0,+}$; IMINO of system IMINO; PAT1 of system PAT; and EAAT3 of system X^-_{AG} (Bröer, 2008) (Figure 1.3). Passage from the intestinal cell via the basolateral membrane to the blood involves AA transporters SNAT2 of system A; LAT2 and LAT3 of system L; TAT1 of system T; CAT-1 of system y^+; and y^+LAT1 and y^+LAT2 of system y^+L (Bröer, 2008) (Figure 1.4). Both AA transporters SNAT2 of system A and xCT of system x^-_c have ubiquitous expression; hence, transport of substrate specific AA across the apical and basolateral membrane can occur through either of these transporters (Bröer, 2008) (Figure 1.5).

All AA can be absorbed from the intestinal lumen through the apical membrane by AA transporters dependent on or independent of Na^+. Amino acid transporter SNAT2 is Na^+-dependent and responsible for translocation of neutral AA with high capacity and medium affinity (Sugawara et al., 2000; Bröer, 2008); it is likely the main transporter for absorption of glutamine for epithelial cell *in situ* utilization (Bröer, 2008). Sodium-dependent AA transporter ASCT2 has low capacity and high affinity and is responsible for the uptake of alanine, serine, cystine, threonine, and glutamine (Utsunomiya-Tate et al., 1996; Avissar et al., 2001). Amino acid transporter B^0AT1 is Na^+-dependent, has medium transport capacity and low transport affinity, and is believed to be the major transporter of neutral AA across the apical membrane of the intestine (Bröer et al., 2004; Bröer, 2008). Amino acid transporter $ATB^{0,+}$ has only been identified in the intestinal apical membrane (Munck, 1985; Nakanishi et al., 2001; Bröer, 2008), and it is both Na^+- and Cl^--dependent and transports neutral and cationic AA with medium capacity and high affinity (Munck, 1985; Sloan and Mager, 1999). Proline and other imino AA are absorbed largely through low-capacity, medium-affinity AA transporters IMINO, which is Na^+-dependent and Cl^--stimulated (Takanaga et al. 2005), and PAT1, which is H^+-dependent (Boll et al., 2002). Anionic AA are absorbed through medium-capacity, high-affinity transporter EAAT3, which requires K^+, three Na^+, and H^+ for movement of glutamate into the epithelial cell (Kanai et al., 1994; Zerangue and Kavanaugh, 1996). Finally, cystine and glutamate are exchanged across the apical membrane by low-capacity, high-affinity transporter xCT (Bröer, 2008).

Movement across the basolateral membrane of the intestinal epithelial cell occurs through fewer AA transporters, including SNAT2 and xCT, which are mentioned above. Amino acid transporter TAT1 is responsible for transport of the aromatic AA with high capacity and low affinity (Kim et al., 2001). Both AA transporters of the y^+L system, y^+LAT1 and y^+LAT2, are located on the basolateral membrane, display medium capacity and low affinity, and are Na^+-dependent for the transport of both neutral and cationic AA (Torrents et al., 1998; Pfeiffer et al., 1999).

Amino acid transporters of systems y^+, $b^{0,+}$, and L play a significant role in lysine absorption from the intestinal lumen across the apical membrane, through AA transporter $b^{0,+}$AT, and translocation into the blood through the basolateral membrane, through CAT1, LAT2, and LAT3. Accordingly, transporters belonging to these systems are discussed in more detail below.

3.2.1. Transport of lysine in intestinal epithelial cells by systems y^+, $b^{0,+}$, and L

The mechanisms and anatomical sites of intestinal lysine uptake have not previously been reported in horses. Several membrane systems have been shown to move lysine across both the apical and basolateral membranes of the small intestinal epithelium in the presence or absence of Na^+ in poultry, bovine, and eel (Angelo et al., 2002; Soriano-Garcia et al., 1999; Wilson and Webb, 1990, Vilella et al., 1990). Lysine must first be transported from the lumen into the epithelial cell through transporters located within the BBM of the microvilli, mainly through system $b^{0,+}$ (Bröer, 2008). Following apical transmembrane transport and intracellular travel, lysine efflux occurs via transporters and simple diffusion-mediated transport across the

basolateral membrane of the epithelial cell into the venous capillary bed, mainly via transporters of system y^+ and system L (Bröer, 2008).

Several systems of lysine uptake are likely utilized throughout the small intestine as evidenced by the presence of competitive inhibition among dietary AA in bovine (Wilson and Webb, 1990) and eel (Vilella et al., 1990). The affinity and capacity of Na^+-independent and Na^+-dependent transport systems and their transporters vary in different segments of the small intestinal tract (Wilson and Webb, 1990), and thus presumably compensates for possible inefficiencies in lysine uptake resulting from competitive inhibition. For example, both Na^+-dependent and Na^+-independent jejunal systems of lysine transport showed greater affinity but lower transport capacity for lysine, while the same ileal systems have lower affinity but higher capacity for lysine transport (Wilson and Webb, 1990). Whether these systems are under a coordinated regulation in response to dietary AA balance to ensure maximal lysine uptake is unknown. Collectively, transporter proteins CAT-1, $b^{0,+}$AT and LAT-2 are purported to significantly contribute to lysine uptake from the intestine in species studied to date.

Information pertaining specifically to AA uptake by the equine large intestine is limited to two studies (Slade et al., 1971; Bochroder et al., 1994). Slade et al. (1971) reported that cecal infusion of ^{15}N-labeled cecal bacteria in a pony resulted in label recovery in essential AA, including lysine, non-essential AA, urea, and ammonia appearing in the cecal vein. Recovery of the label was not measured; however, 4 hours after infusion, the label recovery in lysine was 5-fold that of in urea, with negligible recovery in ammonia (Slade et al., 1971). In contrast to Slade et al. (1971), Bochroder et al. (1994) suggested that the equine colon is incapable of AA

transport. In their study, whole tissue colonic discs were prepared from tissue obtained from 8 mature ponies and placed in buffer solution for 150 minutes prior to measurement of lysine, histidine and arginine appearance on to the serosal side. When AA concentration was raised 10 fold above physiological levels, there was only minimal measurable AA on the serosal side. However, apart from an unusually long incubation time of 150 minutes relative to other *in vitro* uptake studies using tissue explants or membrane vesicles (Wilson and Webb, 1990; Sepulveda and Smith, 1979; Ugawa et al., 2001), Bochroder et al. (1994) neglected to report or to measure AA disappearance from the mucosal side. Measurement of disappearance, or lack thereof, from the serosal side would have lended more confidence to their finding.

Assessment of AA uptake by the large intestine has also been limited in other species (Sepulveda and Smith, 1979; Ugawa et al., 2001). In the pig proximal colon, which lies immediately distal to the cecum and would be relative to the equine large colon, hydrophobic neutral AA were more readily absorbed by the epithelial cells than the hydrophilic neutral or basic AA from any transport system (Sepulveda and Smith, 1979).

3.2.1.1. System y^+

The Na^+-independent system y^+ is purported to be the primary AA transport system responsible for lysine uptake across intestinal tissue (Wilson and Webb, 1990; Angelo et al., 2002). The y^+ transport system displays high affinity and low transport capacity for cationic AA (Bröer, 2008), and is composed of CAT-1, CAT-2A, CAT-2B, CAT-3, and CAT-4, which is each uniquely responsible for uptake of cationic AA in different tissues. Amino acid transporter CAT-1 is the only transporter in system y^+ expressed in the intestine; therefore, further discussion will be focused to CAT-1.

The first transporter of system y^+ to be studied and cloned from mice was CAT-1 (Kim et al., 1991; Palacín et al., 1998). Since then, CAT-1 mRNA has been shown to have ubiquitous expression, including the small intestine of both broilers (Gilbert et al., 2007; Li et al., 2008) and steers (Liao et al., 2008; Liao et al., 2009). The CAT-1 transporter contains approximately 620 to 630 AA, has a molecular mass of 67 kDa, and is predicted to have 12 to 14 transmembrane domains (Deves and Boyd, 1998). Kinetic evaluation has shown the CAT-1 transporter is saturable for cationic AA substrates, with K_M values ranging from 70 to 100 μM (Deves and Boyd, 1998).

3.2.1.2. System $b^{0,+}$

Other Na^+-independent systems, including the $b^{0,+}$, also facilitate movement of lysine into epithelial cells (Angelo et al., 2002; Soriano-Garcia et al., 1999). System $b^{0,+}$ includes the heteromeric transporter rBAT/$b^{0,+}$AT and displays high affinity and medium transport capacity for cationic and neutral AA (Bröer, 2008). The heavy chain of the transporter complex, rBAT, was first identified in *Xenopus* oocytes (Bertran et al., 1993), and the light chain, $b^{0,+}$AT, was first discovered in mouse blastocysts (Van Winkle et al., 1988). Others have since reported $b^{0,+}$AT mRNA abundance throughout the small intestine of mice (Dave et al., 2004), broilers (Gilbert et al., 2007; Gilbert et al., 2008), pigs (Feng et al., 2008; Wang et al., 2009), and steers (Liao et al., 2009) and in the colon of mice (Dave et al., 2004) and pigs (Feng et al., 2008).

The rBAT heavy-chain has 685 AA, has a molecular mass of approximately 70 kDa, and contains a large, highly glycoslated extracellular domain (Bröer, 2008). The $b^{0,+}$AT light-chain

has 487 AA, a molecular mass of approximately 50 kDa, and 12 transmembrane domains (Bröer, 2008). The rBAT/$b^{0,+}$AT transporter has higher affinity for cationic than for neutral AA, with K_M values averaging 100 µm and 500 µm, respectively (Bröer, 2008).

3.2.1.3. System L

The Na^+-independent transporter system L is responsible for the uptake of neutral AA, including essential AA leucine, isoleucine, methionine, and phenylalanine, and has been reported in the small intestine of mice (Segawa et al., 1999) and in the small intestine and colon of rats (Fraga et al., 2005). Transporters within system L have been shown to possess medium affinity and high transport capacity for neutral AA (Bröer, 2008). System L is comprised of four individual transporters, the heteromeric amino acid transporters 4F2hc/LAT-1 and 4F2hc/LAT-2, and the uniport transporters LAT-3 and LAT-4 (Bröer, 2008).

The 4F2hc heavy-chain has between 520 and 530 AA, depending on species, has a molecular mass of approximately 85 kDa, and is heavily glycosylated (Bröer, 2008). Light-chain transporter LAT-1, first identified in rat using expression cloning in *Xenopus* oocytes by Kanai et al. (1998), has approximately 500 AA, a molecular mass of approximately 40 kDa, and 12 transmembrane domains (Verrey et al., 1999). Transporter LAT-1 is widely distributed in ovary, testis, placenta, and tumor cells (Verrey et al., 1999). Light-chain transporter LAT-2, localized to epithelial cells of intestine and kidney, has similar properties to LAT-1, with approximately 500 AA, a molecular mass of ~40 kDa, and 12 transmembrane domains (Verrey et al., 1999; Bröer, 2008). LAT-2 shares a 52% sequence identity with LAT-1, yet due to tissue location, it is believed to be involved in transepithelial movement of neutral AA (Verrey et al., 1999). While

kinetic analysis of LAT-1 is scarce, LAT-2 has been shown to have a micromolar affinity, with K_M values ranging from 40 to 200 μm (Segawa et al., 1999).

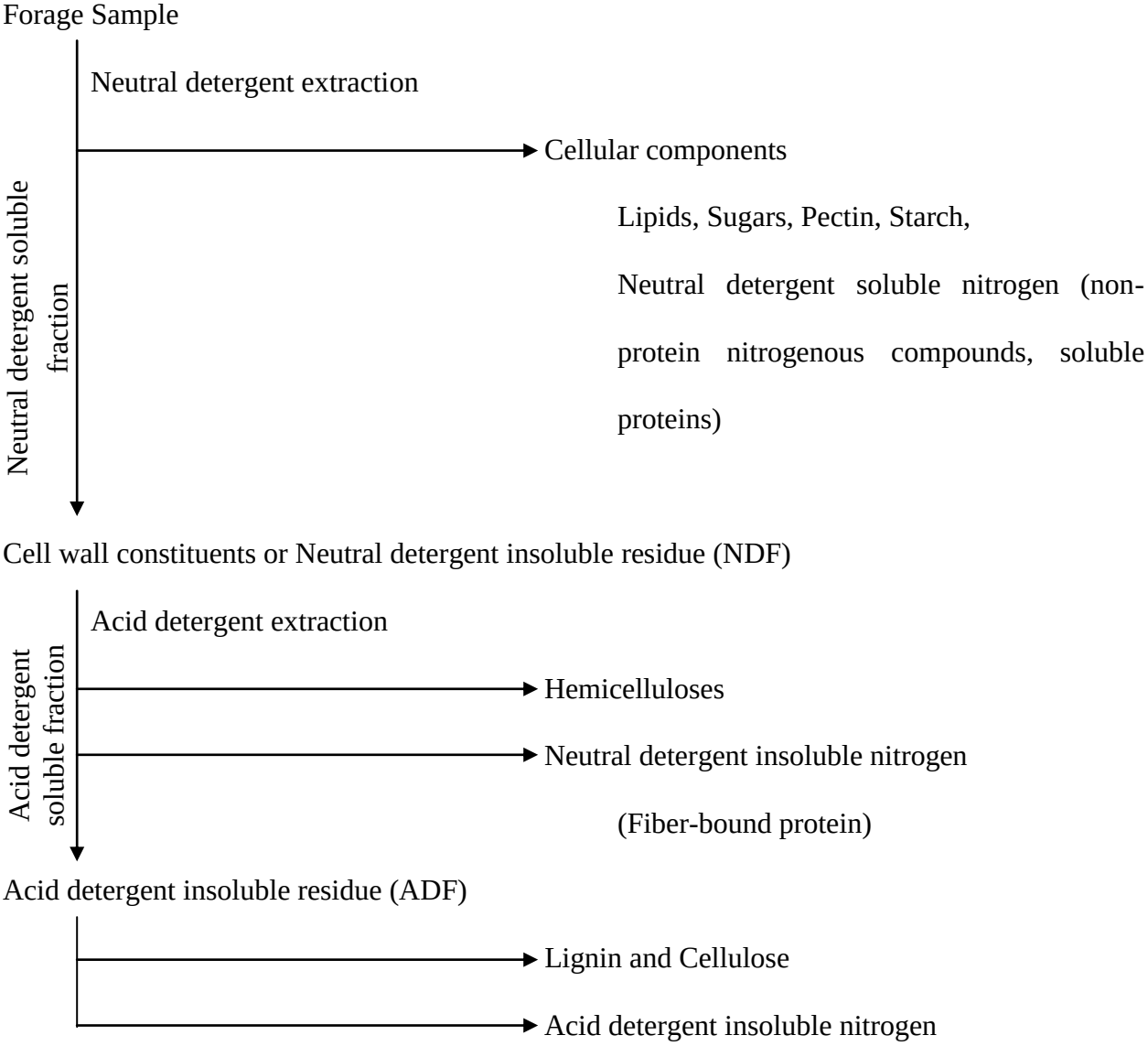
System L transporters LAT-3 and LAT-4 have similar properties to LAT-1 and LAT-2, yet they serve as uniporters of neutral AA, rather than having an antiporter mechanism (Bröer, 2008). Transporter LAT-3, first identified by expression cloning of human cancer cells in *Xenopus* oocytes (Babu et al., 2003), has limited published information available (Bröer, 2008). Babu et al. (2003) suggested it corresponds to a transporter designated system L2, yet further identification of AA sequence and molecular weight has not been performed (Bröer, 2008). Kinetic analysis of LAT-3 have shown a saturable AA transporter with two distinct affinity components, the low component having K_M values ranging from approximately 1.0 to 1.9 mM, and the high component having K_M values ranging from approximately 6.6 to 30.1 μM (Babu et al., 2003). Thus far, LAT-3 displays limited tissue distribution, with expression confined to pancreas, liver, and duodenum (Babu et al., 2003; Kim et al., 2007).

Transporter LAT-4, again first identified by expression cloning of human cells in Xenopus oocytes, has 57% identity to LAT-3 (Bodoy et al., 2005). Transporter LAT-4 has 569 AA, a molecular weight of 62.7 kDa, and 12 transmembrane domains (Bodoy et al., 2005). Similar to LAT-3, LAT-4 has two affinity components, the low component having K_M values ranging from approximately 3.7 to 4.7 mM, and the high component having K_M values ranging from approximately 100 to 180 μM (Bodoy et al., 2005). Transporter LAT-4 is widely distributed in the kidney, intestine, heart, lymphocytes, and placenta (Bodoy et al., 2005).

3.3. Equine dietary requirement of lysine

The ten AA, including lysine, presumed as essential for equids are based on the dietary needs of nonruminant species (NRC, 2007). Lysine is the only AA for which an estimated maintenance requirement exists in horses (NRC, 2007). However, the basis for this requirement estimate is not derived from empirical testing as "studies evaluating lysine requirements of sedentary adult horses have not been conducted" (NRC, 2007). Rather, lysine requirement for maintenance is deduced from N requirement at N equilibrium (NRC, 2007). A linear regression of N retention against N intake estimated N requirement at N equilibrium (N retention = 0) to be 126 mg/kg BW, while a broken line model fitted though the same data set estimated N requirement to be 202 mg/kg BW. Lysine intake corresponding to these crude protein (CP) (N × 6.25) requirement estimates were calculated and lysine requirement estimates were reported at 36 mg/kg BW and 54 mg/kg BW, respectively (NRC, 2007). The NRC recommends the 54 mg lysine/kg BW to be the optimum, which represents 18 mg/kg BW above the minimum lysine requirement for maintenance (zero N retention, 36 mg/kg BW). The basis for the 54 mg lysine/kg BW requirement for maintenance is unclear and is relatively high given that the requirement for the pregnant pig is 50 mg/kg BW (NRC, 1998). The current dietary requirement for lysine does not take into account the ability of the horse to synthesize AA from microbial fermentation (Slade et al., 1971), and potential contribution of microbial protein to large intestinal AA absorption (Wootton and Argenzio, 1975). Hence, the current lysine requirement may be overestimated because it is derived from CP intake and from a positive N retention. It is likely that equids have an obligate dietary requirement for N and may be able to derive a considerable proportion of lysine from the large intestine.

Figure 1.1. Forage composition as determined by detergent analysis.



The cellular components, neutral detergent soluble N, cellulose, hemicelluloses, and neutral detergent insoluble N are all available to non-ruminants through fermentation in the cecum. The lignin and acid detergent insoluble N is unavailable to non-ruminants (Van Soest, 1965; Van Soest, 1967; Van Soest, 1994).

Figure 1.2. Cellular transport of peptides and subsequent hydrolysis into single amino acid molecules. A. Transport of the intact peptide through PEPT1 followed by hydrolysis in the cell cytosol. B. Hydrolysis of peptides by brush border membrane peptidases prior to transport of individual AA into the cell cytosol.

A.

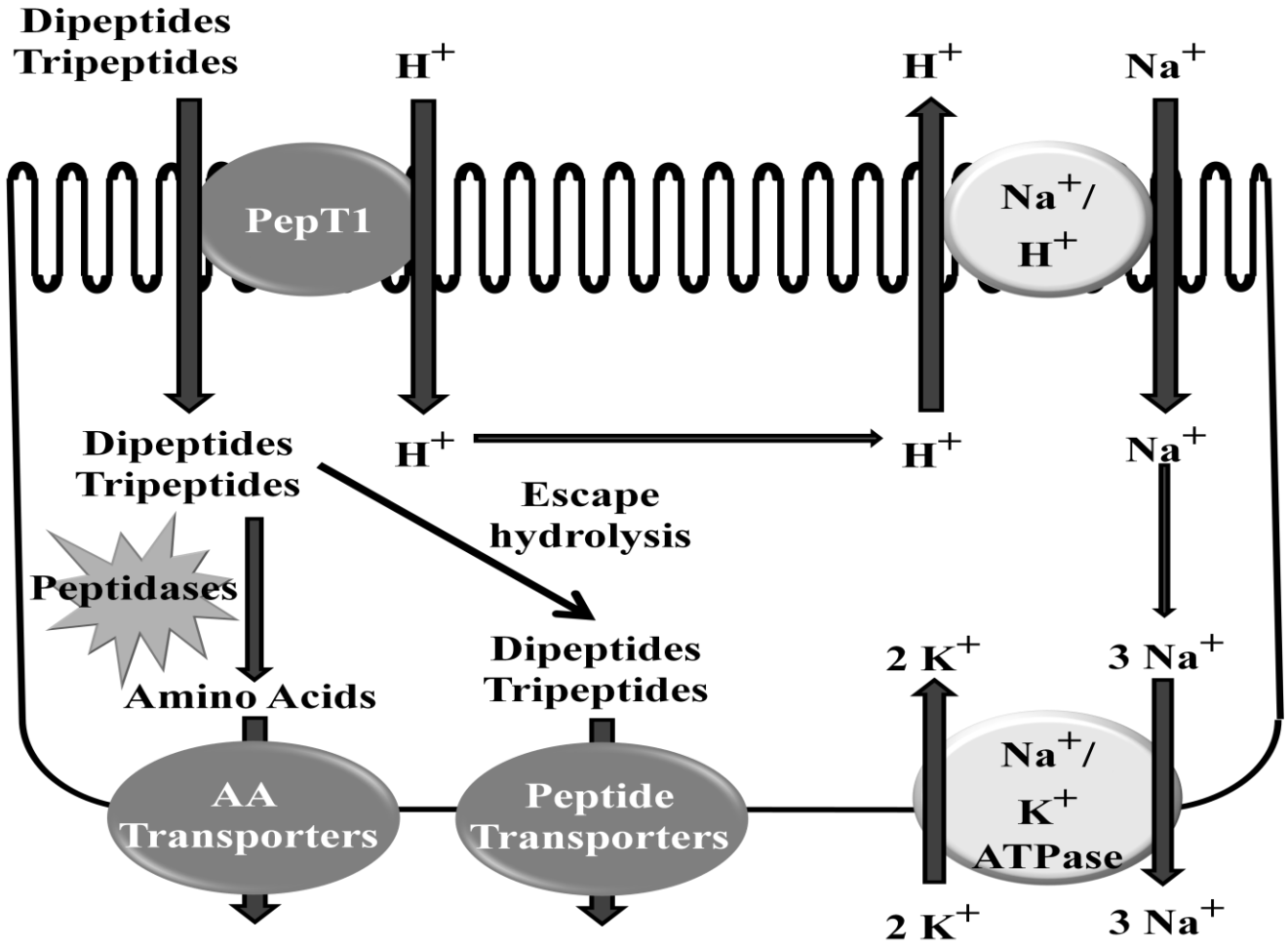


Figure 1.2. (Cont'd)

B.

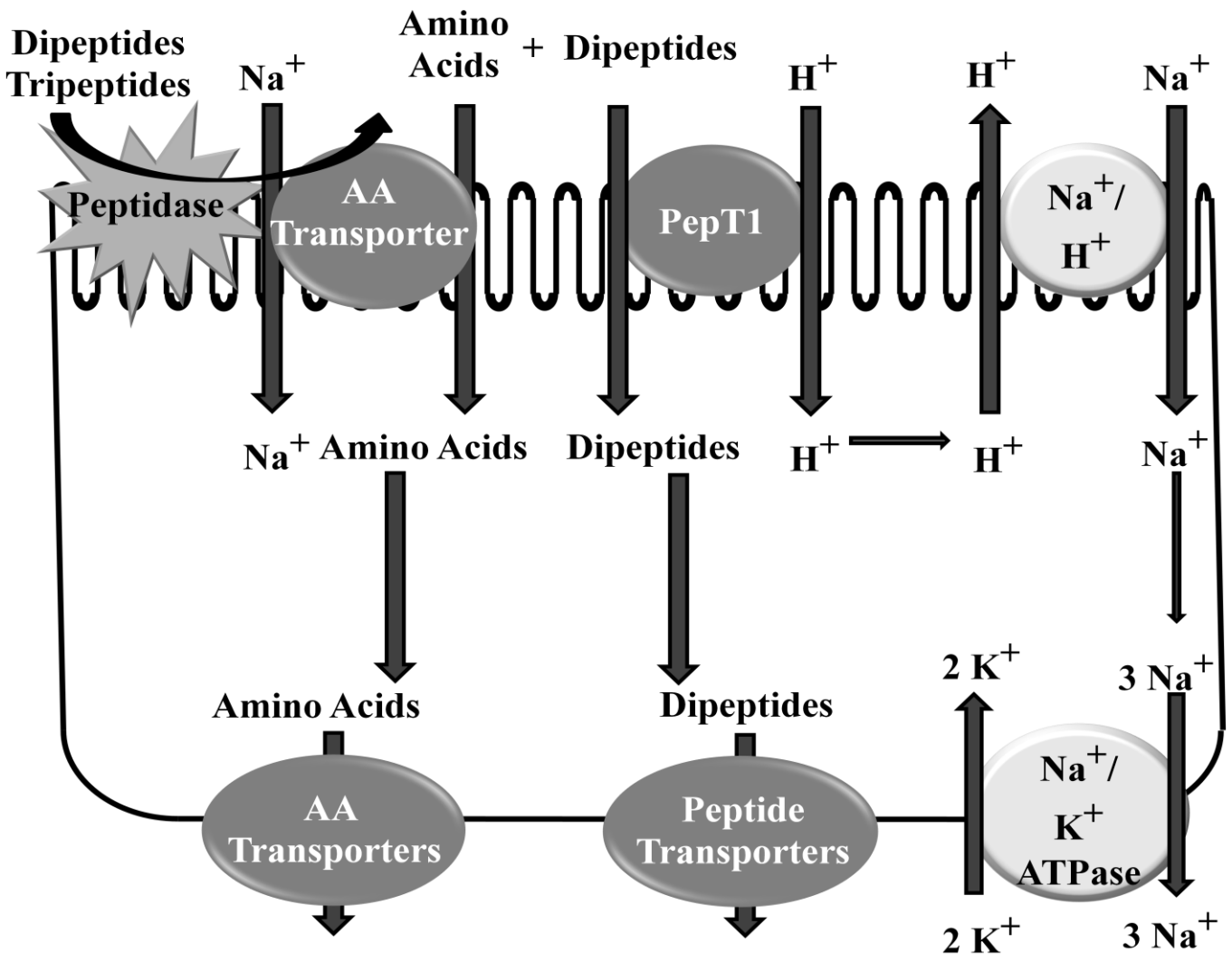


Figure 1.3. Passage of amino acid from the intestinal lumen through the apical membrane into the cell cytosol via brush border membrane specific amino acid transporters. A. Amino acid transporters ASCT2, B^0AT1, $ATB^{0,+}$, and $b^{0,+}AT$. B. Amino acid transporters IMINO, PAT1, and EAAT3.

A.

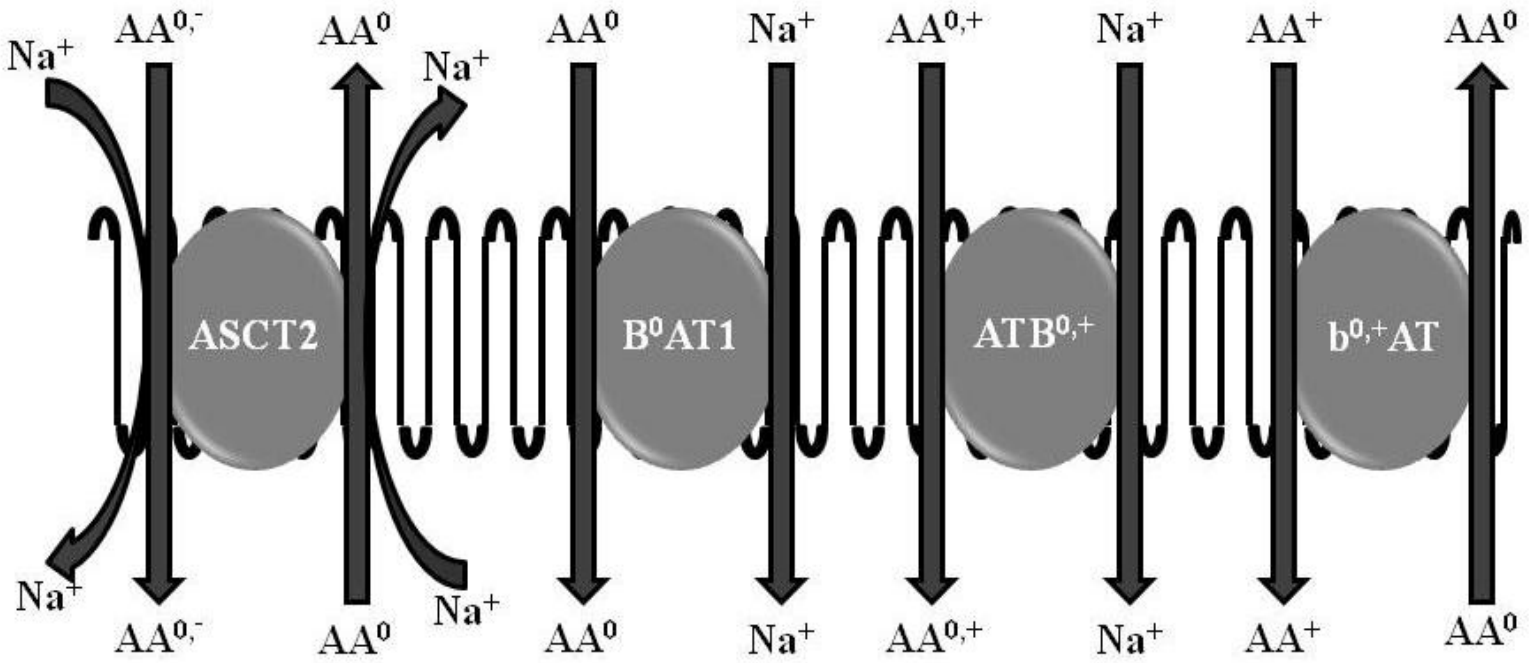


Figure 1.3. (Cont'd)

B.

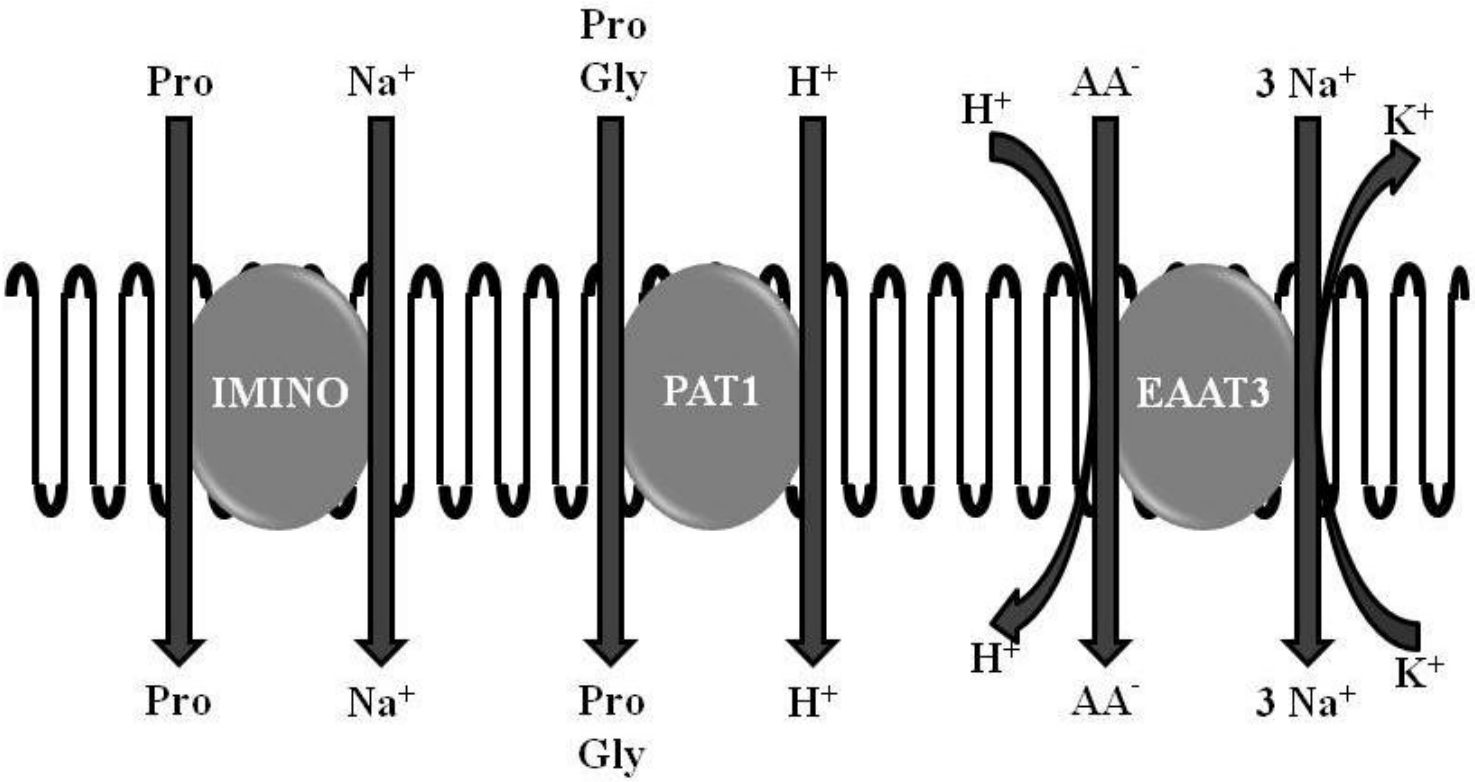


Figure 1.4. Passage of amino acid from the cell cytosol through the basolateral membrane into the blood via basolateral membrane specific amino acid transporters. A. Amino acid transporters LAT2, LAT3 and TAT1. B. Amino acid transporters CAT1, y^+LAT1, and y^+LAT2.

A.

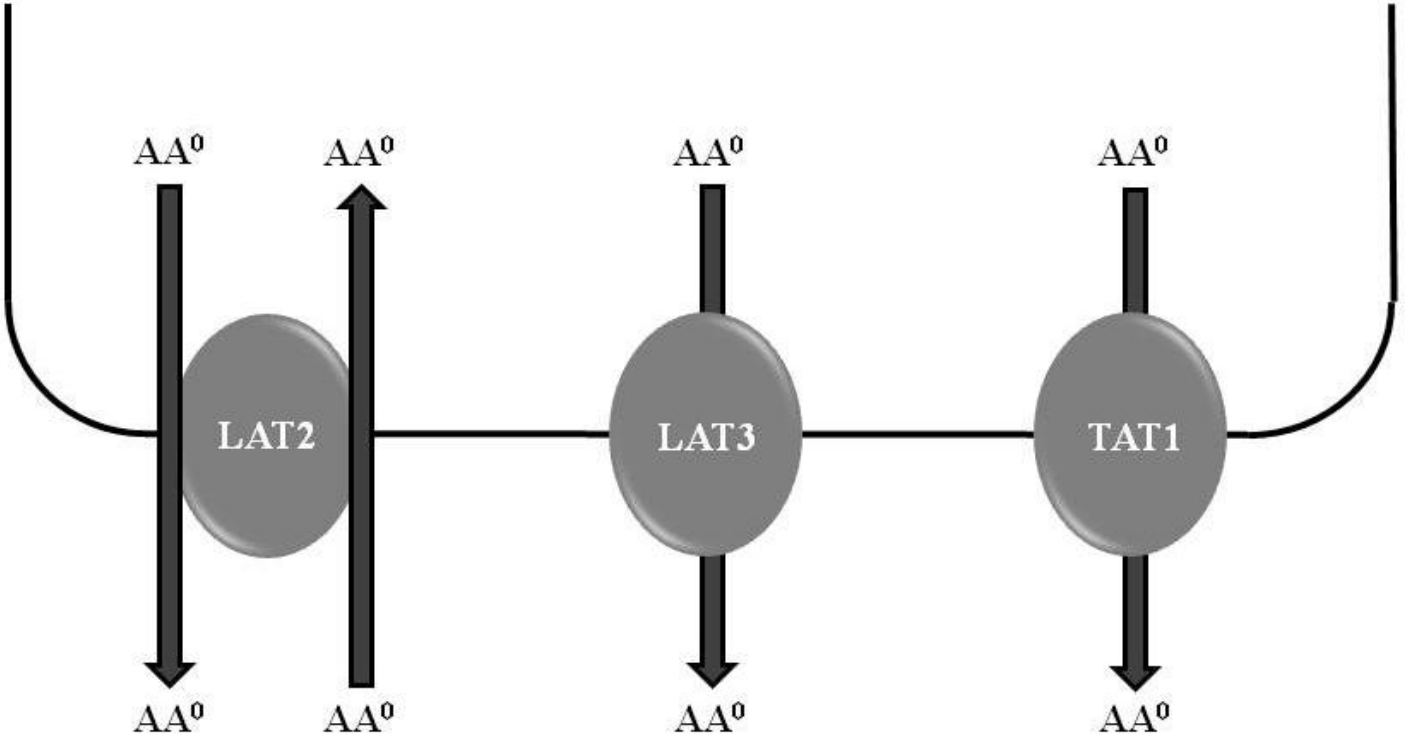


Figure 1.4. (Cont'd)

B.

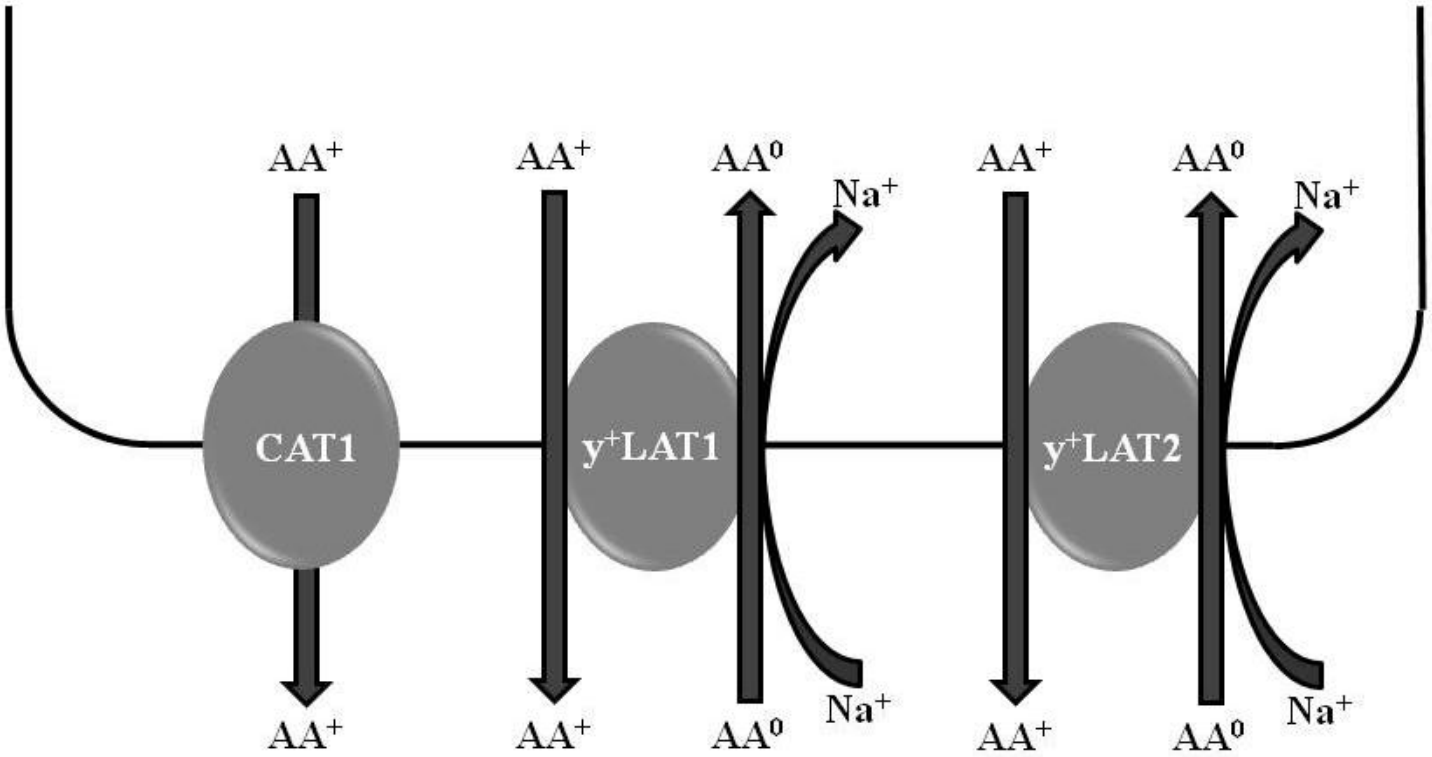
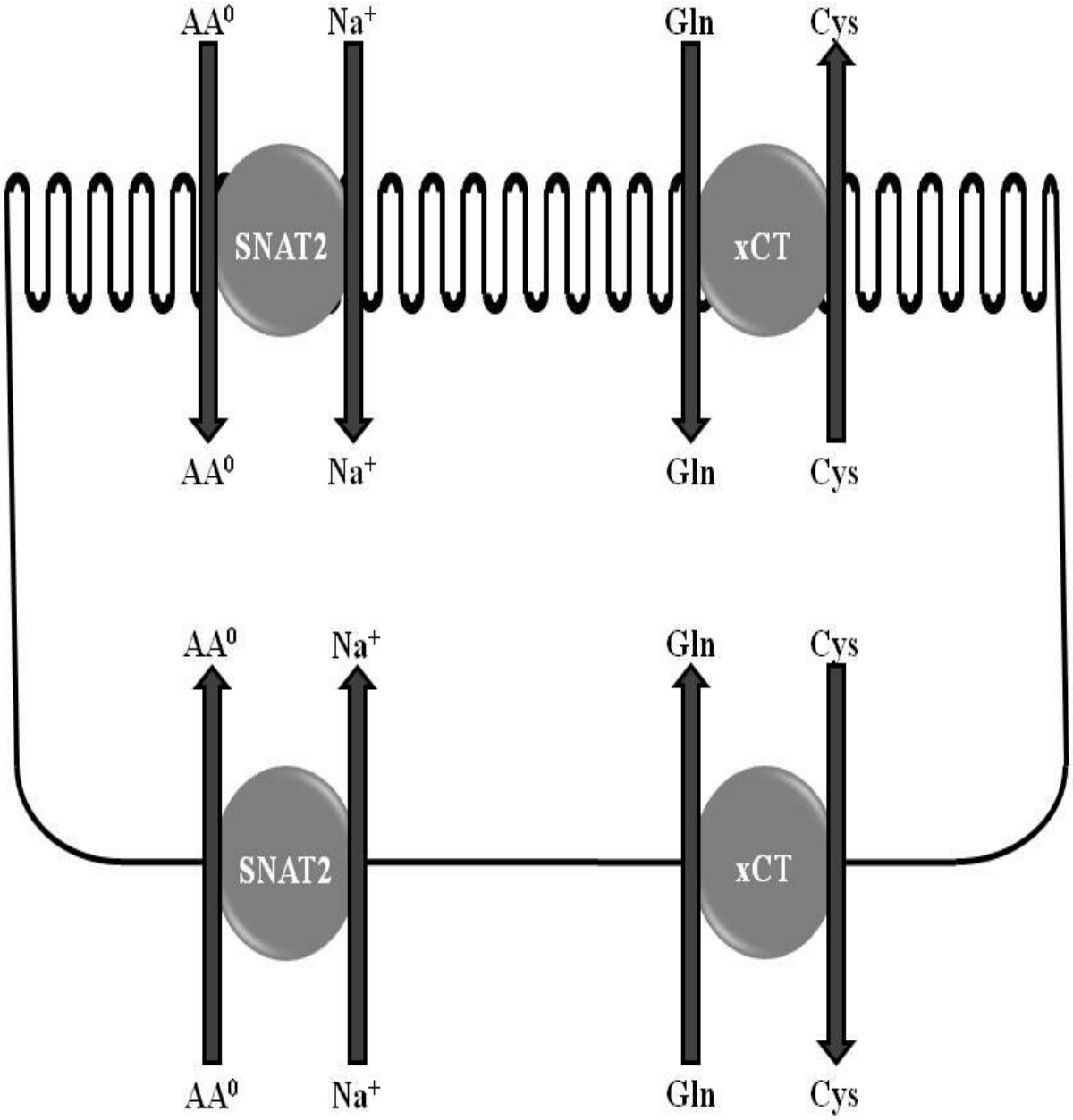


Figure 1.5. Passage of amino acids through both the apical and basolateral membrane via ubiquitously expressed amino acid transporters SNAT2 and xCT.



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CHAPTER TWO

Woodward, A. D.[*], S. J. Holcombe[†], J. P. Steibel[*,‡], W. B. Staniar[§], C. Colvin[*], and N. L. Trottier[*1]. 2009. Cationic and neutral amino acid transporter transcript abundances are differentially expressed in the equine intestinal tract. J. Anim. Sci. 88:1028-1033.

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CHAPTER TWO

CATIONIC AND NEUTRAL AMINO ACID TRANSPORTER TRANSCRIPT ABUNDANCES ARE DIFFERENTIALLY EXPRESSED IN THE EQUINE INTESTINAL TRACT

Abstract

To test the hypothesis that amino acid (AA) transporter transcripts are present in the large intestine and similarly expressed along the intestinal tract, messenger RNA (mRNA) abundance of candidate amino acid transporter genes *SLC7A9*, *SLC7A1*, *SLC7A8* and *SLC43A1*, respectively encoding AA transporters $b^{0,+}$AT, CAT-1, LAT-2 and LAT-3, was determined in small and large intestinal segments of the horse. Mucosa was collected from the equine small (jejunum and ileum) and large intestine (cecum, left ventral colon, and left dorsal colon), flash-frozen in liquid nitrogen, and stored at -80°C. Messenger RNA was isolated from tissue samples, followed by manufacture of complementary DNA. Relative quantitative reverse transcriptase polymerase chain reaction was conducted using the $2^{-\Delta\Delta C_T}$ method, with glyceraldehyde-3-phosphate dehydrogenase serving as the housekeeping gene. Compared to the jejunum, cationic and neutral AA transporter *SLC7A9* mRNA abundance was similar in the ileum, cecum and large intestinal segments. Compared to the jejunum, cationic AA transporter *SLC7A1* mRNA abundance was similar in the ileum, and lower in the cecum, left ventral colon and left dorsal colon ($P < 0.001$). Neutral AA transporter *SLC7A8* mRNA abundance decreased from the cranial to caudal end of the intestinal tract ($P < 0.001$). Neutral AA transporter *SLC43A1* mRNA abundance was similar in the ileum and left dorsal colon and higher in the cecum ($P < 0.01$) and left ventral colon ($P < 0.1$) compared to the jejunum. Cationic and neutral AA transporter *SLC7A9* mRNA abundance is similarly expressed in the large compared to small intestine, while

cationic AA transporter *SLC7A1* is of low abundance in the large intestine; neutral AA transporters *SLC7A8* and *SLC43A1* are differentially expressed with *SLC7A8* of lower abundance and *SLC43A1* of higher abundance, respectively, in the large intestine. Results indicate that the large intestine may contribute to both cationic and neutral AA uptake and absorption predominantly via transporters LAT-3 and $b^{0,+}AT$.

Key words: amino acids, equine, horse, mRNA abundance, transporters

Introduction

The gastrointestinal tract (GIT) absorbs nitrogen (N) of dietary and endogenous microbial origins (Fuller and Reeds, 1998). While there is no doubt as to the role of the small intestine in N absorption (Metges, 2000), the contribution of the large intestine to N homeostasis of the host is largely unknown. As predominately hindgut fermenters, major sites of microbial digestion in the horse occur distal to the small intestine, i.e., in the cecum and large intestine (Reitnour et al., 1969; Reitnour and Salsbury, 1972; Gibbs et al., 1988). While only 11 to 30 % of whole tract apparent N digestion is attributed to the small intestine, as much as 40 to 70 % appears to originate distal to the ileum (Reitnour et al., 1969; Gibbs et al., 1988). However, the form of N absorbed from the cecum and large intestine of the horse is unknown. Knowledge of whether or not AA are absorbed from the large intestine would further our understanding of AA utilization in horses.

Amino acid transport across the small intestinal epithelial cells occurs via numerous transporter proteins varying in capacity, and overlapping in specificity (Broer, 2008). Characterizing molecular entities of AA transport in the equine small and large intestine would provide both an initial step to understanding the functional difference of AA absorption at a

mechanistic level and an important clue on the relative role of intestinal segments in absorption of indispensable AA. To test the hypothesis that AA transporter transcripts are present in the large intestine and similarly expressed along the intestinal tract, mRNA abundance of candidate AA transporter genes *SLC7A9, SLC7A1, SLC7A8* and *SLC43A1*, respectively encoding for AA transporters $b^{0,+}$AT, CAT-1, LAT-2 and LAT-3, was determined in small and large intestinal segments of the horse.

Materials and Methods

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Animals and Collection of Tissue

Four mature horses (3 Standardbreds, 1 Thoroughbred; 2 geldings, 2 mares) fed a mixed alfalfa and timothy grass hay at 2% body wt, and 1.4 kg concentrate meal (10% CP, Purina Omelene) were used. Horses were between the age of 3 and 10 yr and were euthanized for reasons other than history of gastrointestinal problems. Following euthanasia, the horses were positioned in dorsal recumbency and a 2.5-m incision was made along the ventral midline in order to expose the entire GIT. Approximately 20-cm long sections were sampled from two segments of the small intestine, i.e., the distal jejunum and the ileum, the cecum, and two segments of the large intestine, i.e., the left ventral colon and left dorsal colon. Distal jejunal samples were obtained 12 m from the duodenal colic ligament. Ileal samples were collected within the segment of bowel located between the antimesenteric band and the linear artery. Cecal samples were obtained between the medial and ventral cecal bands, midway between the apex and the base. The left ventral colon was sampled between the sternal and pelvic flexures at

the lateral band. The left dorsal colon was sampled between the pelvic and diaphragmatic flexures in the antimesenteric area. After collection of each segment, the mucosal layer was gently pulled from the serosa, cut into small pieces (~1 g) and immediately flash frozen in liquid N. Samples were stored at -80°C for later quantification of messenger RNA (mRNA).

Isolation of RNA and preparation of cDNA

Ribonucleic acid was extracted from the intestinal mucosa tissue using the Manual PerfectPure RNA Cell & Tissue Kit according to the manufacturer's instructions (5 PRIME, Gaithersburg, MD). Briefly, approximately 80 mg of tissue was homogenized in 800 μL Lysis solution using a Polytron (PT10/35, Kinematica, Inc., Bohemia, NY). The homogenate was cleared of large particles by centrifugation at 400 × *g* for 1 min in a Preclear Column. Lysates were then transferred to a Purification Column and centrifuged at 16,000 × *g* for 1 min. Wash 1 Solution (400 μL) was added to each column and lysates were again centrifuged at 16,000 × *g* for 1 min. A DNase treatment was added, and the column was washed twice (200 μL) and centrifuged at 16,000 × *g* for 1 min after each wash. Wash 2 Solution (200 μL) was added to each column, the column was centrifuged at 16,000 × *g* for 1 min, and the wash was repeated. The resulting RNA was eluted from the Purification Column using 50 μL Elution Solution. Isolated RNA was tested for purity by spectophotometry (NanoDrop 1000, Thermo Scientific, Wilmington, DE) and for quality (2100 Bioanalyzer, Agilent Technologies, Foster City, CA).

Complementary DNA (cDNA) was manufactured using SuperScript II Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, 2 μg purified RNA was added to 1 μL dT_{18} primer and double deionized H_20 to provide a solution of 10 μL total reaction volume. Samples were placed in a thermocycler for 5 min at 70°C, 5 min at 20°C, 60

min at 50°C, 5 min at 70°C, 20 min at 37°C, followed by holding at 4°C. Master dNTP mix (10 μL) was added following the denaturation step, and 0.5 μL RNAse H (Invitrogen) was added after cDNA manufacturing. Once complete, 0.2 μL 0.5M EDTA was added to each sample and the contents were transferred to a new tube. Ethanol (125 μL) was added and the sample was stored at -20°C overnight to precipitate the cDNA pellet. After incubation, samples were centrifuged at 16,000 × *g* for 20 min at 4°C to obtain a cDNA pellet, which was washed in 1 mL 75% ethanol, dried, and quantified by spectrophotometry (NanoDrop 1000, Thermo Scientific). Complementary DNA was then diluted to a working stock of 10 ng/μL and stored at -20°C.

Measurement of SLC7A9, SLC7A1, SLC7A8 and SLC43A1 Relative mRNA Abundance by Quantitative Reverse Transcription PCR

Primers for quantifying mRNA abundance of the candidate AA transporter genes *SLC7A9, SLC7A1, SLC7A8,* and *SLC43A1* were designed based on the equine gene sequences deposited in the National Center for Biotechnology Information Entrez Gene (Table 2.1) using Primer Express software (v. 3.0, Applied Biosystems, Foster City, CA). Water and primers, optimized by matrix analysis as described by Mikeska and Dobrovic (2009), were added to a 96-well PCR plate, followed by the working cDNA stock and SYBR green (Applied Biosystems). Amplification of cDNA and fluorescence detection was carried out using an automated fluorometer (ABI 7000 using ABI Prism 7000 SDS software, Applied Biosystems) for quantitative reverse transcription (Q-RT)-PCR. Conditions for amplification and quantification included initial denaturing stages (50°C for 2 min and 95°C for 10 min) followed by 40 cycles of 2 amplification stages (95°C for 15 s and 60°C for 1 min) for primer annealing and elongation. A dissociation stage (95°C for 15 s, 60°C for 1 min and 95°C for 15 s) was added at the end of amplification to insure a single amplicon was produced and to validate the primer pairs.

Observed melting temperatures (T_m), a sensitive measure of length and sequence for DNA products, differed by less than 5°C from the predicted values (Table 2.1). A non-template control (RNase-free water) was used for every primer pair during each Q-RT-PCR run. Relative abundance of *SLC7A9, SLC7A1, SLC7A8,* and *SLC43A1* mRNA was quantified in duplicate using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH**)** as the normalizing gene. Fold change in AA transporter mRNA abundance in each intestinal segment was determined using the jejunum as a positive control, based on evidence that AA and peptide transporter expression is increased in the jejunum and ileum compared to the duodenum in other animal species (Chen et al., 1999; Segawa et al., 1999; Hatanaka et al., 2002; Fraga et al., 2005; Gilbert et al., 2007; Li et al., 2008; Liao et al., 2008; Wang et al., 2009).

Statistical Analysis

Data were analyzed using the PROC MIXED procedure (SAS version 9.0, SAS Institute Inc., Cary, NC). The model included the fixed effect of intestinal tract section and the random effect of horse. The student's *t*-test was used to compare the mean cycles to threshold (C_T) of each segment to the mean C_T of the jejunum for each transporter. Differences in C_T were log transformed to represent fold-change in mRNA abundance according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Results are reported as the fold-change in each segment relative to the jejunum. Data are expressed as mean fold-change ± SEM. Significant and marginally significant differences in expression are reported at $P < 0.05$ and $P < 0.1$, respectively.

Results

Messenger RNA abundance of GAPDH was not different between segments ($P = 0.98$); C_T in the jejunum, ileum, cecum, left ventral colon, and left dorsal colon were 23.3, 23.1, 22.7, 23.1, and 22.6, respectively, demonstrating the appropriateness of GAPDH as a normalizing gene in this study. Compared to the jejunum, mRNA abundance of transporter gene *SLC7A9* tended to decrease in the ileum ($P = 0.07$) and did not differ in the cecum ($P = 0.54$), left ventral ($P = 0.26$) and left dorsal colon ($P = 0.67$) (Figure 2.1.A.). Transporter genes *SLC7A1* (Figure 2.1.B.) and *SLC7A8* (Figure 2.1.C.) mRNA abundance in the ileum did not differ ($P = 0.46$ and 0.81, respectively) compared to the jejunum, and decreased ($P < 0.001$) in the cecum, left ventral colon, and left dorsal colon. For transporter gene *SLC43A1* (Figure 2.1.D.), compared to the jejunum, mRNA abundance in the ileum did not differ ($P = 0.57$), increased in the cecum ($P < 0.01$), tended to increase in the left ventral colon ($P = 0.07$), and did not differ in the left dorsal colon ($P = 0.77$).

Discussion

Equids are equipped with both significant hydrolytic and fermentative digestive capability in the proximal and distal GI respectively, making them an attractive model for the study of differential mechanisms of nutrient absorption. We hypothesized that the equine large intestinal mucosa expresses the transcript for AA transporters known in vertebrates to transfer indispensable AA across cell membranes. This hypothesis is based on the notion that horses meet both energy and protein demand through hindgut fermentation, and appear to assimilate a large proportion of dietary N from regions distal to the small intestine (Reitnour et al., 1969; Reitnour and Salsbury, 1972; Gibbs et al., 1988). Although the equine large intestine appears to

play a significant role in N absorption of both exogenous and microbial origin (Reitnour et al., 1969; Reitnour and Salsbury, 1972; Gibbs et al., 1988), very little is known regarding the form of N absorbed from the cecum and large intestine. Slade et al. (1971) demonstrated post-ileal uptake of ^{15}N-labeled lysine and other indispensable AA in ponies after placing uniformly ^{15}N-labeled bacteria in the cecum. Even in animals primarily relying on hydrolytic digestion, fermentation of dietary fiber leads to the synthesis of microbial proteins that could contribute to the N homeostasis in the host (Metges, 2000). For instance, in human subjects that have undergone ileostomy, digestibility of fiber components and AA is lower (Rowan et al., 1994), and circulating plasma AA concentrations are lesser compared to humans with intact tract digestive systems (Metges et al., 1999).

This study begins to address differential mechanisms of AA absorption between the orad and aborad regions of the GIT of the horse by profiling the mRNA abundance of target genes collectively responsible in part for transport of cationic and large neutral AA**.** Our candidate genes included *SLC7A9, SLC7A1*, *SLC7A8* and *SLC43A1*, respectively encoding for AA transporters $b^{0,+}$AT, CAT-1, LAT-2 and LAT-3. For the remainder of the manuscript, the common AA transporter protein names will be used in place of their gene names. All four AA transporters belong to Na^{+}-independent systems of AA transporters. Transporter CAT-1 belongs to the y^{+} transport system and transporter $b^{0,+}$ AT belongs to $b^{0,+}$ system of AA transport. Both systems share functional properties in that they display high affinity for cationic AA, and low and medium transport capacity (Broer, 2008). Transporters LAT subtype 2 (LAT-2) and LAT subtype 3 (LAT-3) belong to the L system of AA transport, and have medium and low affinity, respectively for neutral AA and high transport capacity (Broer, 2008).

In this study, abundance of $b^{0,+}$AT transcript was uniform across all segments of the equine intestinal tract. Others have reported $b^{0,+}$AT mRNA abundance throughout the duodenum, jejunum, and ileum of broilers (Gilbert et al., 2007; Gilbert et al., 2008), pigs (Wang et al., 2009), and steers (Liao et al., 2009), but did not determine $b^{0,+}$AT mRNA abundance in the large intestine. In contrast to our study, Dave et al. (2004) and Feng et al. (2008) reported a decrease in $b^{0,+}$AT mRNA abundance in mice and pig colon, respectively, compared to small intestine. Significant large intestinal $b^{0,+}$AT mRNA abundance has not been reported elsewhere.

Transporter CAT-1 mRNA has been reported throughout the small intestine of both broilers (Gilbert et al., 2007; Li et al., 2008) and steers (Liao et al., 2008; Liao et al., 2009). In our study, CAT-1 was predominately expressed in the jejunum and ileum, with little to no detectable mRNA in the cecum and large intestine, respectively. Gilbert et al. (2007) reported similar abundance of CAT-1 between the small intestinal segments of broilers, while greatest CAT-1 mRNA expression were found in either the jejunum or duodenum of growing steers (Liao et al., 2008; Liao et al., 2009). To the authors' knowledge, our study is the first to characterize and report on the mRNA expression profile of CAT-1 transporter pre- and post-ileally.

Similar to $b^{0,+}$AT, LAT-2 mRNA abundance was detected in all segments. However, level of abundance decreased from the proximal to distal segments, reaching an 18-fold reduction in the left dorsal colon. In mice, LAT-2 mRNA abundance was only found in the jejunum and ileum, with no transcript detected in the colon (Segawa et al., 1999). In rats, however, LAT-2 mRNA abundance was found in the jejunum, ileum, and also the colon, with no differential expression found between these segments (Fraga et al., 2005). In regards to the AA transporter LAT-3, limited information exists on its molecular and functional characteristics.

One study so far reported LAT-3 mRNA abundance in the duodenum of human, mice, and rat, (Kim et al., 2007). In that study, mRNA was isolated only from the duodenum, consequently differential abundance in other segments is unknown (Kim et al., 2007).

We found both $b^{0,+}$AT and LAT-3 to have similar or greater, respectively, mRNA abundance in all segments of the equine intestinal tract compared to the jejunum. Amino acid transporter $b^{0,+}$AT has a high affinity for cystine and cationic AA (Dave et al., 2004; Wang et al., 2009). The distribution of $b^{0,+}$AT in the cecum and colon appears to be unique to the horse, as others have not identified significant $b^{0,+}$AT expression in the large intestine of mice (Dave et al., 2004) or pigs (Feng et al., 2008). Expression of $b^{0,+}$AT is localized to the apical membrane of the small intestine in mice (Dave et al., 2004) and is considered the major cationic AA transporter in the intestine of mice (Wang et al., 2009). Feng et al. (2008) suggested the decreased expression in pig colon was due to low post-cecal AA availability; therefore, our findings may thus be relevant to the AA nutrition of horses and other mammals relying on microbial protein predominantly produced from dietary fiber fermentation occurring distal to the small intestine. In contrast to $b^{0,+}$AT, AA transporter LAT-3 is a low affinity transporter (Broer, 2008) with the greatest mRNA abundance in the large intestine. Fraga et al. (2005) found higher LAT-1 mRNA abundance in the rat colon compared to jejunum and suggested that may result from long-term adaptive response to decreased AA availability in the large intestine. Accordingly, increased presence of AA transporter LAT-3 transcript may be necessary to maximize absorption of neutral AA in the equine large intestine.

Of the 4 candidate genes characterized in this study and known to transport cationic and neutral AA across epithelial cells of other animal species, three were expressed in the equine large intestine. These transporters may facilitate the absorption of microbial and dietary-derived AA across the epithelium of the large intestine. Only CAT-1 was uniquely localized to segments of the small intestine. Together, the presence of both high affinity ($b^{0,+}AT$) and high capacity (LAT-2 and LAT-3) AA transporters indicates that the large intestine may contribute to both cationic and neutral AA uptake and absorption. Whether the observed differential mRNA abundances between segments and amongst the AA transporters studied translate into transporter protein levels, or functional and phenotypic expression, remain to be determined. Nonetheless, this study is novel and the first to report on the profile of AA transporter transcript abundances along the GIT of the horse. These results, along with future studies kinetically defining AA uptake, will be useful in estimating the contribution of the large intestine to N homeostasis of the horse.

Table 2.1. Primer sequences for quantitative reverse transcription PCR for target genes *SLC7A9*, *SLC7A1*, *SLC7A8* and *SLC43A1* and control gene glyceraldehyde-3-phosphate dehydrogenase.

Gene	Primer sequence (5' – 3')[1]	T_m	Accession No.[2]
GAPDH[3]	Forward: GCTGCCAAATACGATGAGATCA	84	XM001496020
	Reverse: GCCCAGGATGCCCTTGA		
SLC7A9	Forward: CAGCCTCATAGCGATGCTCAT	84	XM001489968
($b^{0,+}$AT)[4]	Reverse: CGGTCAGAGCCTCCTTTCC		
SLC7A1	Forward: GGCAGGCAGGCTCGTTT	82	XM001492839
(CAT-1)	Reverse: AATGTAAGAGAGCACTTTCATCATGTG		
SLC7A8	Forward: GGCCATGATCCACGTGAAG	84	XM001493818
(LAT-2)	Reverse: GGGTGGAGATGCATGTGAAGA		
SLC43A1	Forward: GCCTTCTTACCTGCCCCTTT	82	XM001498197
(LAT-3)	Reverse: GCTGTCCATGCAGTCCTTGA		

[1]Primers designed using Primer Express software (v. 3.0, Applied Biosystems, Foster City, CA).

[2]GenBank accession numbers of the gene sequences used for primer design and found on PubMed (www.pubmed.gov).

[3]GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

[4]Common amino acid transporter protein names appear in parenthesis.

1

Figure 2.1. Fold change in mRNA abundance of AA transporters in equine intestinal segments relative to mRNA abundance in the equine jejunum. Fold change in mRNA abundance was determined in each intestinal segment relative to the jejunum using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Each bar represents mean fold change ± SEM. Amino acid transporters *SLC7A9* ($b^{0,+}$AT, panel A); *SLC7A1* (CAT-1, panel B); *SLC7A8* (LAT-2, panel C); and *SLC43A1* (LAT-3, panel D). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{\dagger}P < 0.1$.

1

Figure 2.1.

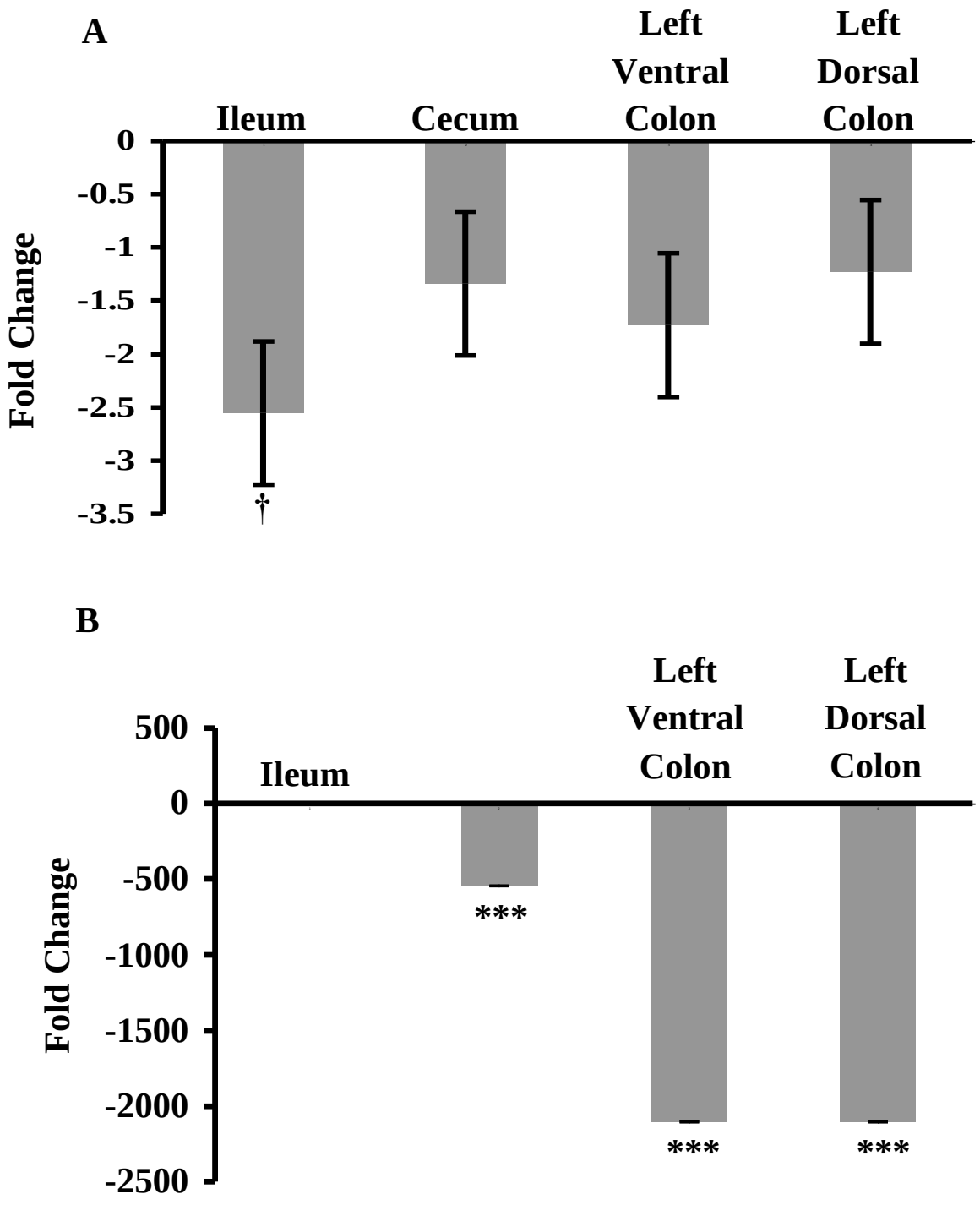
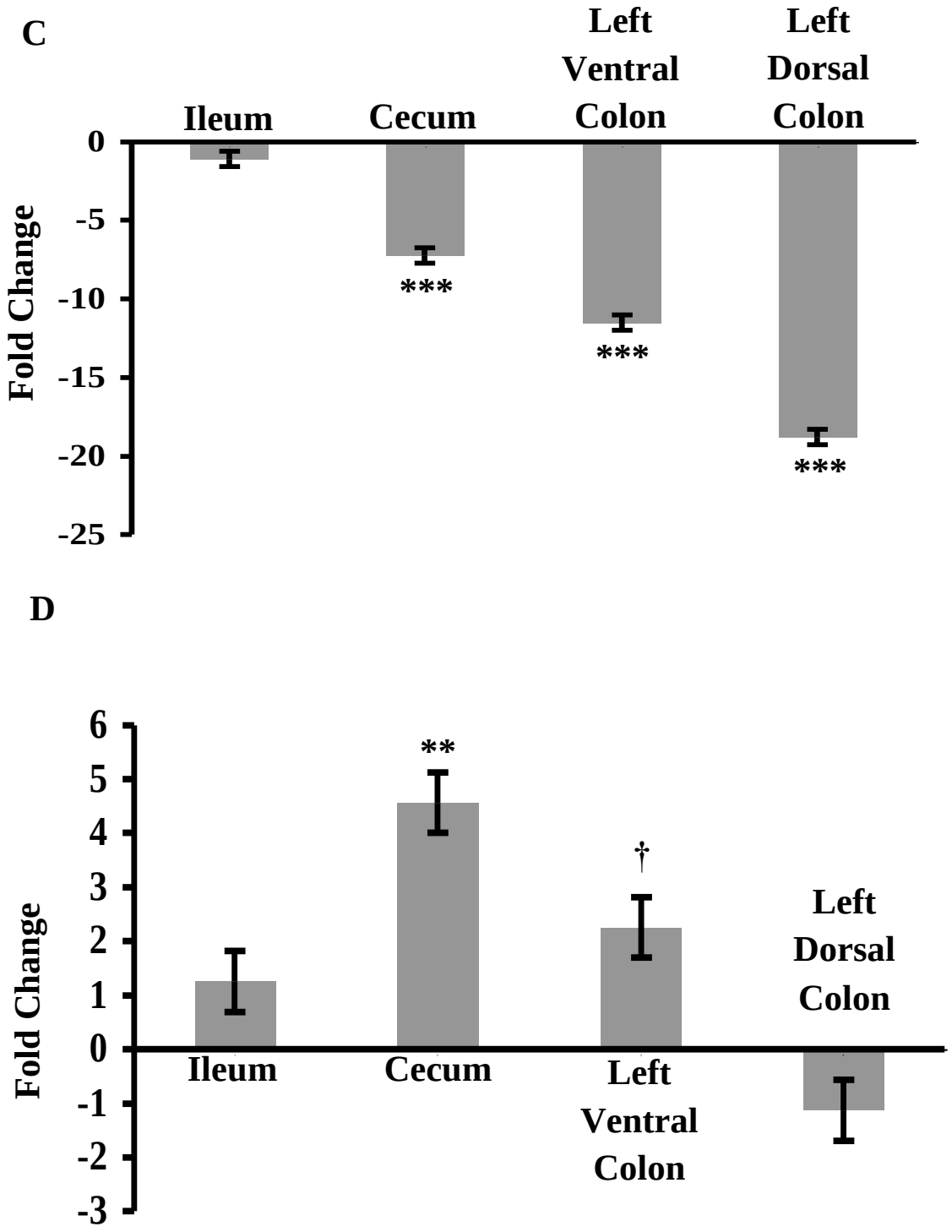

A
Ileum
Cecum
Left Ventral Colon
Left Dorsal Colon
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
Fold Change
†
B
Ileum
Left Ventral Colon
Left Dorsal Colon
500
0
-500
-1000
-1500
-2000
-2500
Fold Change
***
***
***

Figure 2.1. (Cont'd)



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CHAPTER THREE

Woodward, A. D.[*], M.Z. Fan[†], R. J. Goer[‡], N. P. Taylor[*], and N. L. Trottier[*1]. 2010. Kinetic analysis of L-lysine transport into equine and procine jejunal and colonic brush border membrane vesicles. In preparation for submission to the *Journal of Animal Science*. Submitted to co-authors for review.

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CHAPTER THREE

KINETIC ANALYSIS OF L-LYSINE TRANSPORT INTO EQUINE AND PORCINE JEJUNAL AND COLONIC BRUSH BORDER MEMBRANE VESICLES

Abstract

To test the hypothesis that Lys is transported across the large colon mucosa apical membrane with similar capacity to that of the small intestinal mucosa in the pony and pig, we examined transport *in vitro* using brush border membrane vesicles (BBMV). Mucosa was collected from the porcine (n = 3) and equine (n = 4) jejunum and large colon, flash-frozen in liquid nitrogen, and stored at -80°C. Jejunal and colonic BBMV were manufactured by Mg^{2+} precipitation and used to determine initial rates and kinetics of L-Lys transport into apical epithelia by rapid filtration technique in Na^{+}-gradient incubation buffer. Initial rates of total lysine uptake did not differ between segments in either pig or pony, nor in pony compared to pig, at each individual L-Lys concentration ($P > 0.1$). Further analysis of results indicated L-Lys uptake was mediated by a Na^{+}-dependent saturable component as well as by diffusion. In the pig, L-Lys capacity of 180 ± 26 ρmol · mg protein^{-1} · s^{-1} in the colon was not different than 121 ± 26 ρmol · mg protein^{-1} · s^{-1} in the jejunum (P = 0.14), while L-Lys affinity of 0.89 ± 0.22 mM in the colon tended to be higher than 0.23 ± 0.22 mM in the jejunum (P = 0.09). In the pony, L-Lys capacity of 149 ± 25 ρmol · mg protein^{-1} · s^{-1} and affinity of 1.05 ± 0.22 mM in the colon were higher than capacity of 62 ± 25 ρmol · mg protein^{-1} · s^{-1} and affinity of 0.08 ± 0.22 mM in the jejunum ($P < 0.05$). L-Lysine diffusion was not different across tissues; however, the pony

diffusion of 115 ± 10 ρmol · mg protein^{-1} · s^{-1} was higher than pig diffusion of 73 ± 10 ρmol · mg protein^{-1} · s^{-1} (P = 0.03). These results demonstrate that the large colon is capable of L-Lys transport across the apical epithelial membrane at higher capacity, but lower affinity, than the jejunum; thus, the large colon may play a significant role in Lys homeostasis in hind-gut fermenters.

Key words: L-lysine, transport kinetics, equine, porcine, intestine

Introduction

Both suids and equids are monogastric hind-gut fermenters; thus, they are equipped with both hydrolytic and fermentative digestive capability in the proximal and distal GI, respectively. The species differ in that equids are obligate hindgut fermenters; hence, they rely on dietary forages and are equipped with a much larger cecum and voluminous proximal colon. In monogastric animals, microbial fermentation of dietary fiber is essential to the synthesis of microbial proteins, which in turn contributes to the N homeostasis of the host (Metges, 2000). For instance, in human subjects that have undergone ileostomy, digestibility of fiber components and of AA is lower (Rowan et al., 1994), and circulating plasma AA concentrations are lesser compared to humans with intact tract digestive systems (Metges et al., 1999). Amino acids synthesized by the microbial population contribute to whole body AA homeostasis in the pig by meeting the equivalent of AA requirement estimates for maintenance (Torrallardona et al., 2003b). Few studies have been able to determine the anatomical site of microbial-derived AA uptake *in vivo*, but it appears that the ileum may be an important site for both synthesis and absorption of microbial AA (Torrallardona et al., 2003b).

In the horse, evidence points to the large intestine playing a significant role in N absorption of both exogenous and microbial origin (Reitnour et al., 1969; Gibbs et al., 1988). However, only two studies have described the capacity of the large intestine to absorb individual AA. Slade et al. (1971) demonstrated post-ileal uptake of ^{15}N-labeled Lys and other indispensable AA in ponies after placing uniformly ^{15}N-labeled bacteria in the cecum. Conversely, no uptake of Lys, Arg, or His was observed across the colon mucosa of the horse *in vitro* (Bochröder et al., 1994). However, in that study, mucosal buffer solutions did not contain Na^+, which acts as an activator of AA uptake, even in Na^+-independent transporters (Torras-Llort et al., 1998). Recently, we reported similar mRNA abundance of cationic AA transporter $b^{0,+}$AT in the proximal colon mucosa of the horse compared to the jejunum (Woodward et al., 2010), indicating the large intestine may play a role in AA absorption in equids.

Transport kinetics using brush border membrane vesicles (BBMV) manufactured from the intestinal mucosa apical membrane have been studied for Pro, Phe, Ala, Gly, and Lys in rabbit (Stevens et al., 1982), Arg and Lys in rat (Cassano et al., 1983; Wolffram et al., 1984), Leu, Thr and Gln in pig (Wolffram et al., 1986; Maenz and Patience, 1992; Fan et al., 1998), Leu in sheep (Wolffram et al., 1986), Lys and Met in steer (Wilson and Webb, 1990), and Lys in chicken (Torras-Llort, 1996). Despite the nutritional importance of Lys in the pig and presumably the horse, Lys transport kinetics across the small intestine using BBMV has not been characterized in these animal species. Furthermore, although colonic BBMV have been used to determine ion transport characteristics (Binder et al., 1986; Foster et al., 1986), there is no information on the use of colonic BBMV to address whether the apical membrane of the colon is

capable of AA transport. Knowledge of Lys contribution from the large intestine would allow for refinement of Lys requirement of the horse.

With these notions in mind, we hypothesized that Lys is transported across the large colon mucosa apical membrane with similar capacity to that of the small intestinal mucosa in the pony and pig. Furthermore, we hypothesized that Lys transport capacity across the large colon mucosa apical membrane of the pony is higher than that of the pig. The objective of this study was to quantify the uptake of Lys across the apical membrane of the jejunum and proximal large colon mucosa of the pony and the pig.

Materials and Methods

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Animals and Collection of Tissue

Three market pigs (Yorkshire crossbred, 122.5 ± 3.9 kg) and four mature ponies of mixed breeds were used. Pigs were grouped-housed at the MSU Swine Teaching and Research Center and were provided ad libitum access to a corn-soybean meal-diet containing 14 % CP. Pigs were transported to the Michigan State University Meat Laboratory, humanely killed for slaughter, and the entire intestinal tract was immediately removed. Approximately 20-cm long sections were obtained from the jejunum 2 m distal to the stomach and from the ascending colon 30 cm distal to the cecum. Ponies were of mature age and mixed breeds and had been maintained on low quality grass hay. Ponies were provided with their last forage meal at 1800 and euthanized between 1000 and 1400 on the following day. Ponies were euthanized for reasons other than history of gastrointestinal problems. Following euthanasia, a 2.5-m incision was made along the

ventral midline in order to expose the entire gastrointestinal tract. Approximately 20-cm long sections were sampled from the distal jejunum, located 12 m distal to the duodenal colic ligament, and from the left dorsal colon, located between the sternal and pelvic flexures at the lateral band.

For both species, intestinal segments were rinsed thoroughly in a 0.9% ice-cold NaCl solution and opened lengthwise to expose the mucosa. The mucosal layer was scraped from the serosal layer using a glass microscope slide, and scrapings were transferred to conical tubes until approximately 12 g were obtained. Samples were flash frozen in liquid nitrogen and stored at -80°C.

Preparation of Brush Border Membrane Vesicles

Brush border membrane vesicles were prepared using the approach of Fan et al. (1998, 2004) with the exception that mucosal scraping instead of whole mucosa was used. Briefly, mucosal scrapings (2.6 g) were homogenized over ice in homogenization buffer (52 mL; 50 mM D-mannitol, 10 mM HEPES, 0.2 mM PMSF; pH = 7.4) using a polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for three 1-min cycles. A sample of the resulting homogenate was collected for protein and enzyme analysis, and the remaining homogenate was centrifuged at 2000 × *g* for 15 min at 4°C. The supernatant was collected and the pellet discarded. A 200-mM $MgCl_2$ solution was added to the supernatant and mixed to a final concentration of 10 mM $MgCl_2$, gently shaken over ice for 15 min and centrifuged at 2400 × *g* for 15 min at 4°C. Again, the supernatant was collected and the pellet was discarded. The supernatant was divided into ultracentrifuge tubes and centrifuged at 19,000 × *g* for 30 min at 4°C. The resulting supernatant was discarded, and the remaining pellet representing the crude BBM pellet was suspended in vesicle pre-loading buffer (150 mM KSCN, 10 mM mannitol, 5

mM HEPES; pH = 7.4) using a Pasteur pipette. Samples were centrifuged for an additional 30 min at 39,000 × *g* and 4°C. The resulting supernatant was discarded and the final BBM pellet was suspended in 1 mL of vesicle pre-loading buffer. A sub-sample of the final BBMV suspension was collected for determination of protein concentration and enzyme activity. The remainder of the BBMV suspension was used for uptake experiments as described below.

Protein Concentration and Alkaline Phosphatase Enzyme Measurements

Protein concentration of the initial homogenate and the final BBMV suspension was determined with the Lowry assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions and using BSA as standard. Purity of BBMV was tested using alkaline phosphatase as a marker. Alkaline phosphatase was measured according to Fan et al. (1998) using p-nitrophenyl phosphate as substrate.

Measurement of Total (Na^+-dependent and independent) L-Lys Uptake into Brush Border Membrane Vesicles

Freshly prepared BBMV suspension was diluted with vesicle pre-loading buffer to contain 6 to 10 mg protein · mL^{-1}. The final BBMV suspension remained on ice until uptake experiments were performed (not more than 6 hr after final suspension). Uptake experiments were carried out using the rapid filtration procedure as outlined by Fan et al. (1998, 2004). Uptake buffer (50 µL; 150 mM NaSCN, 10 mM mannitol, 5 mM HEPES; pH = 7.4) containing [^{3}H]-Lys was first loaded into polystyrene tubes and allowed to warm to room temperature (22 to 24°C). Two separate 5-µL droplets of BBMV suspension were added along the side of the tube using a Microman pipette (Gibson S.A.S., Villiers-le-Bel, France). After warming to room temperature for 10 s, uptake was initiated by a foot-switch activated vibromixer attached to an

electronic timer (GraLab model 545). Uptake was terminated by immediate addition of ice-cold stop and wash buffer (1.2 mL; 150 mM KSCN, 10 mM mannitol, 10 mM HEPES, 0.1 mM $HgCl_2$; pH = 7.4) at the end of the timing cycle. Immediately after termination, 1 mL of the uptake media was collected and pipetted onto 0.22-μm nitrocellulose membrane filters (Millipore, Billerica, MA) mounted on a Manifold filtration unit (Millipore, Billerica, MA) connected to a vacuum source. Filters were immediately washed three times with 5 mL stop and wash buffer and transferred to scintillation vials pre-filled with 10 mL scintillation fluid. All filters were allowed to dissolve in scintillation fluid for 30 min before radioactivity was determined with a liquid scintillation counter (Beckman, Brea, CA). Remaining uptake media in the polystyrene tubes was pooled per substrate concentration, and 10 μL counted to determine the average initial radioactivity.

Functionality of BBMV was assessed by uptake of glucose in the presence of an inwardly directed Na^+-gradient in freshly manufactured BBMV immediately prior to each Lys kinetic experiment (submitted). Time course experiments were performed under a Na^+-gradient using the pig jejunum and the resulting incubation time used for all kinetic uptake experiments. Non-specific binding of Lys to BBMV and filters was corrected for time course experiments by subtracting time-zero radioactivity counting. To eliminate non-specific binding of substrate to filter during kinetic analysis, uptake buffer without the addition of BBMV was treated with stop and wash buffer, filtered, and washed as mentioned previously. Uptake buffers for kinetic experiments consisted of replacing mannitol with Lys at the following concentrations: 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mM.

Each uptake experiment was conducted in triplicate. Fourteen different batches of BBMV prepared from the mucosal scrapings of the small and large intestine of three pigs and four ponies were used to conduct 14 separate uptake experiments, with small and large intestinal Lys transport from a single animal measure on the same day. Composition of incubation buffer is described in detail in figure legends.

Calculations to Determine Lys Kinetics

To determine uptake at various time points and under Na^+-dependent conditions, the following equation was used:

$$J = [((R_F - R_B) \times S)/R_I]/(W \times T)$$

where, J = the initial rate of total Lys uptake into BBMV (ρmol · mg protein^{-1} · s^{-1}); R_F = the radioactivity in disintegration per min of filters (DPM/filter); R_B = the radioactivity for non-specific binding to filters (DPM/filter); S = the extravesicular Lys concentrations (mM); R_I = the radioactivity in the uptake media (DPM/μL); W = the amount of membrane protein provided for the incubations (mg protein); and T = the time of incubation for initial uptake (s).

Kinetic parameters, namely V_{max} and K_M, were analyzed according to the method of Wolffram et al. (1986) using a computer program for multiparameter curve fitting based on the following two-component equation:

$$J = \frac{V_{max}[S]}{K_M + [S]} + D[S]$$

where, J = the initial rate of Lys uptake into BBMV (ρmol · mg protein^{-1} · s^{-1}); V_{max} = the maximal transport rate (ρmol · mg protein^{-1} · s^{-1}); S = the extravesicular Lys concentrations (mM); K_M is the Michaelis constant (50% saturation; mM); and D = the diffusion component.

Statistical Analysis

The initial rate of Lys uptake into BBMV (J, ρmol · mg protein^{-1} · s^{-1}) at each Lys concentration was analyzed using the PROC MIXED procedure (SAS version 9.0, SAS Institute, Inc., Cary, NC). The model included the fixed effects of animal species, intestinal segment, and Lys concentration, the interaction between animal species and intestinal segment, and the random effect of individual animal nested within animal species. Kinetic parameters, i.e., V_{max} and K_M, were analyzed using the PROC MIXED procedure (SAS version 9.0, SAS Institute Inc., Cary, NC). The model included the fixed effects of animal species and intestinal segment, the interaction between animal species and intestinal segment, and the random effect of individual animal nested within animal species. Contrasts were used to determine differences between intestinal segments and between animal species within intestinal segment. Differences in variance between tissues were accounted for through grouping of the interaction. Results are reported as LSMEANS ± SEM. Significant and marginally significant differences in V_{max} and K_M were determined at $P < 0.05$ and $P < 0.1$, respectively.

Results

Membrane Purity

Comparison of alkaline phosphatase enzyme activity in the BBMV relative to the mucosal scraping tissue homogenate yielded an average enrichment factor of 4.55 ± 0.43 fold, indicating the BBMV had little contamination by the basolateral membrane. This falls in the reported range of alkaline phosphatase activity enrichment in BBMV compared to crude homogenate, from 2.3-fold in Aedes aegypti larvae mid-gut (Abdul-Rauf and Ellar, 1999) to 31.1-fold in rat jejunum (Hopfer et al., 1973); it closely resembles the 5.1-fold enrichment in bovine jejunum and ileum (Wilson and Webb, 1990). Experiments determining uptake of D-glucose in the presence of a Na^+-gradient further verified the functional properties of the isolated BBMV (Woodward et al., submitted), a technique which has been used by Torras-Llort et al. (1996, 1998).

Initial Time Course of L-Lys Uptake

Time course experiments of L-Lys uptake into porcine jejunal BBMV indicated Lys uptake was linear up to 30 s at L-Lys concentrations of 0.001 and 5 mM and up to 60 s at L-Lys concentration of 1 mM in Na^+-gradient uptake buffer (Figure 3.1.A., 3.1.C., and 3.1.B., respectively). Therefore, 30 s was used to measure the initial uptake of L-Lys. Our time course results are in agreement with Wolfram et al. (1984) and Torras-Llort et al. (1996) who reported that Lys required a 20 to 30-s incubation time for maximum uptake above control conditions in rat and chicken jejunal BBMV, respectively.

Initial Rate of L-Lys Uptake into Brush Border Membrane Vesicles

Initial rates of total L-Lys (*J*) uptake in porcine and equine colon compared to small intestine BBMV under various Lys concentrations were calculated from Eq. (1) and shown in

Figures 3.2. and 3.3., respectively. At each individual concentration, uptake of Lys into BBMV did not differ in pig colon compared to pig jejunum, pony colon compared to pony jejunum, pony jejunum compared to pig jejunum, or pony colon compared to pig colon ($P > 0.1$).

Kinetics of L-Lys Uptake in Porcine and Equine Small versus Large Intestine

In order to determine Lys kinetic parameters of the pig and pony small intestine and colon, total Lys uptake was divided into a saturable and a diffusion component as determined in Eq. (2) (Wolffram et al., 1986). Calculated kinetic parameters V_{max}, K_M, and diffusion are presented in Table 3.1. Across animal species, compared to the jejunum, the colon had increased V_{max} ($P = 0.03$) and K_M ($P = 0.01$). In the pig, V_{max} did not differ between the colon and jejunum and K_M of Lys uptake tended to be higher ($P = 0.09$). In the pony, V_{max} and K_M were higher ($P < 0.05$) in the colon compared to jejunum. Horses had a higher diffusion rate than pigs ($P < 0.05$) across segments.

Discussion

In vitro techniques, such as BBMV and everted gut sacs, have been an effective tool for determining transport kinetic parameters of different nutrients in mice (Robinson et al., 1973; Ugawa et al., 2001), rat (Evered and Nunn, 1968; Cassano et al., 1983), eel (Vilella et al., 1990), chicken (Lind et al., 1980; Torras-Llort et al., 1996), dog (Robinson et al., 1973), pig (Fan et al., 1998), steer (Wilson and Webb, 1990), and horse (Salloum et al., 1993). This study is the first to characterize the kinetics of Lys transport across the porcine and equine small intestine using BBMV, and to manufacture BBMV from porcine and equine colon to further characterize kinetics of Lys transport in the distal GI tract.

Lysine transport through the apical epithelial membrane occurs through Na^+-dependent symport transport systems which rely on a Na^+ gradient for transfer into the cell, and through Na^+-independent antiport transport systems which exchange Lys in the lumen for another intracellular neutral AA (Broer, 2008). Previous reports of Lys transport in rat (Cassano et al., 1983; Wolfram et al., 1984), rabbit (Stevens et al., 1982), and chicken (Torras-Llort et al., 1996) jejunal BBMV indicated no difference in uptake using either a Na^+-enriched or Na^+-free gradient; however, Na^+ is believed to have a stimulating role on L-Lys influx without being transported into the vesicles itself (Torras-Llort et al., 1998). Therefore, in order to measure total Lys uptake into apical BBMV, all uptake experiments were conducted in the presence of a Na^+ gradient.

Lysine uptake was divided into a saturable and a diffusive component, according to the methods of Wolffram et al. (1986), in both the jejunum and colon of the pig and pony. Others have reported a significant contribution of apparent simple diffusion to total nutrient uptake when utilizing BBMV (Stevens et al., 1982; Cassano et al., 1983; Wolffram et al., 1986; Wilson and Webb, 1990; Fan et al., 2001). Lysine diffusion into jejunal BBMV in this study was significantly higher than reported values of 0.5 ρmol · mg protein^{-1} · s^{-1} in rabbits (Stevens et al., 1982). However, pony Lys diffusion in both the jejunum and colon was similar to jejunal Lys diffusion of 130 ρmol · mg protein^{-1} · s^{-1} reported in steers (Wilson and Webb, 1990). Diffusion of L-Leu into jejunal BBMV manufactured from sheep and pigs was higher and lower, respectively, compared to our Lys diffusion rate (Wolffram et al., 1986). Significant L-Gln diffusion into jejunal BBMV has also been reported in both pigs (Fan et al., 1998) and horses

(Salloum et al., 1993). The significant contribution of diffusion to total uptake is most likely due to disruption of the BBM extracellular matrix during preparation, increasing membrane permeability (Fan et al., 1998; Fan et al., 2001) and therefore would not occur *in vivo*.

In the pig, there was no difference in Lys transport capacity between the jejunum and colon; however, as indicated by the higher K_M value, Lys transport affinity tended to be three-fold lower in the pig colon compared to that of the pig jejunum. Wolffram et al. (1986) manufactured BBMV from pig jejunum and reported V_{max} and K_M for L-leucine transport to be 2-fold higher compared to our Lys values. On the other hand, kinetic parameters of L-Thr in a Na^+-gradient condition were significantly lower than those of Lys in pig jejunum, with a V_{max} of 14 ρmol · mg protein^{-1} · s^{-1} and a K_M of 0.03 mM (Maenz and Patience, 1992). Kinetic parameters of L-Gln uptake in a Na^+-gradient condition were intermediate to those reported for L-Leu and L-Thr and similar to Lys results reported here, with a V_{max} of 70.7 ρmol mg · protein^{-1} · s^{-1} and a K_M of 0.77 mM (Fan et al., 1998). Collectively, *in vitro* determination of kinetic parameters of AA uptake in pig jejunal BBMV demonstrates that transport capacity is highest for L-Leu, followed by L-Lys, L-Gln, and L-Thr; transporter affinity is greatest for L-Thr, followed by L-Lys, L-Leu, and L-Gln. Transport kinetics does not necessarily reflect *in vivo* AA disappearance, however, as measurements of uptake utilizing BBMV are performed in an environment free of inhibition or transporter competition (Wilson and Webb, 1990). Physiological uptake of Lys may be hindered by L-Arg, L-Ala, or L-Phe, which display a 96%, 78%, and 90%, respectively, inhibition of L-Lys uptake in chicken jejunum (Torras-Llort et al., 1996); this indicates a shared transporter for both cationic and neutral AA. It is believed that

systems $b^{0,+}$ and y^+ act as the main transport systems for Lys uptake across the apical epithelium in chicken intestine (Torral-Llort et al., 1996). Both transport systems can absorb cationic and neutral AA across the apical epithelium, and system y^+ has a high affinity for neutral AA in the presence of Na^+ (White, 1985; Torral-Llort et al., 1996); therefore, the presence of inhibitory AA in the intestinal lumen can prevent Lys absorption *in vivo*.

Thus far, pig colonic BBMV have not been used to characterize AA transport kinetics; therefore, this study is the first to report similar Lys transport capacity in the jejunum and colon of the pig. Both *in vitro* and *in vivo* studies indicate that AA uptake can occur from the cecum and large colon (Holmes et al., 1974; Sepulveda and Smith, 1979; Torrallardona et al., 2003a). Sepulveda and Smith (1979) demonstrated on isolated colonic mucosa from new-born pigs that all AA tested, including Lys were absorbed. It has been estimated that approximately 25% of intestinal Lys absorption in the growing pig occurs in the large intestine *in vivo* (Torrallardona et al., 2003a). Others have determined that *in vivo* AA disappearance and absorption can occur in the large intestine of pigs fed either a soybean meal or rapeseed meal-based diet (Holmes et al., 1974). These studies, in combination with results presented here, suggest that the large intestine may play a role in Lys absorption in pigs.

Unlike the pig, the pony colon had a 10-fold decrease in transport affinity coupled with a 2-fold increase in transport capacity compared to the jejunum. Very little information using *in vitro* approaches to assess intestinal AA uptake in the horse is available. Compared to Lys kinetic parameters reported herein, significantly higher V_{max} of 308 ρmol · mg protein^{-1} · s^{-1} and K_M of 0.52 mM were estimated for L-Gln in a Na^+-gradient condition in horse jejunal BBMV (Salloum et al., 1993). This may be explained based on the large abundance of Gln in

feeds, and the high Gln extraction and utilization by intestinal tissue. Glutamine plays a central role in mucosal growth and function, and thus serves as a primary fuel for small intestinal epithelial cells (Windmueller, 1982; Souba et al., 1990; Salloum et al., 1994).

Studies of L-Lys uptake by the large intestine using both *in vivo* and *in vitro* methods have yielded contradictory results (Slade et al., 1971; Bochröder et al., 1994). Our results support those of Slade et al. (1971) whom demonstrated appearance of labelled Lys and other essential AA in the cecal vein after ^{15}N-labelled microbes were cecally infused in a pony, indicating significant absorption of AA by the large intestine *in vivo*. We have also recently reported that mRNA transcript abundance of AA transporter $b^{0,+}$AT was similarly expressed in jejunum, cecum, and proximal colon of the horse (Woodward et al., 2010). Amino acid transporter $b^{0,+}$AT is considered the primary transporter of cationic AA across the BBM of the intestine (Broer, 2008). Therefore, it is possible that $b^{0,+}$AT is responsible for Lys uptake from the intestinal lumen both in the jejunum and colon of the horse. On the other hand, transmucosal potential difference of Lys, His, and Arg measured across proximal colonic discs was low, indicating minimal transport (Bochröder et al., 1994). Also, Freeman et al. (1989) found no transport of L-Ala determined with transmucosal potential difference across cecal mucosa harvested from horses. Bochröder et al. (1994) and Freeman et al. (1989) used mucosal sheets; hence, they characterized transport across both the BBM and the basolateral membrane. In our study, transport was characterized across the BBM only. It is conceivable that the pony and pig colon are equipped for transport of AA only across the BBM for intracellular epithelial cell utilization. On the other hand, it is recognized that, at least in the proximal GIT regions, intestinal cells use a significant proportion of arterially-derived AA (Wu, 1998) and are equipped

with AA transporters at the basolateral aspect to ensure AA absorption (Broer, 2008). Additionally, Bochröder et al. (1994) used incubation buffers with equal Na^+ molarity on both the mucosal and serosal membranes, eliminating the Na^+-gradient essential for movement of Lys across the BBM. Further, Na^+ may act as an activator of Lys uptake through the BB (Torras-Llort et al., 1998), and rat colon has been shown to contain a Na^+-H^+ exchange in colonic BBMV (Binder et al., 1986; Foster et al., 1986). Therefore, it may be suggested that transport of Lys by the colon may not occur in the absence of a Na^+ gradient.

In other species, kinetic transport properties of Lys uptake have been measured using BBMV isolated from rat jejunum (Cassano et al., 1983), bovine jejunum and ileum (Wilson and Webb, 1990), and chicken jejunum (Torras-Llort et al., 1996). Our study yielded a higher V_{max} for jejunal Lys transport in pigs and ponies compared to rats, which had a transport capacity of only 27.2 ρmol · mg protein^{-1} · s^{-1} (Cassano et al., 1983). Lysine jejunal V_{max} in the pig and pony was more closely related to bovine and chicken jejunum, with transport capacities of 120 ρmol · mg protein^{-1} · s^{-1} and 97 ρmol mg protein^{-1} · s^{-1}, respectively (Wilson and Webb, 1990; Torras-Llort et al., 1996). Transport capacity across the colon of the pig and pony most resembled that of the bovine ileum, with a reported V_{max} of 150 ρmol · mg protein^{-1} · s^{-1} (Wilson and Webb, 1990). Lysine jejunal transport affinity in the pig and pony closely resembles that of the rat, with a reported K_M of 0.26 mM (Cassano et al., 1986). Lysine

transport affinity in chicken jejunum, with a K_M of 0.16 mM, is lower than in pig and pony, but transport affinity in bovine jejunum, with a K_M of 0.36 mM, is higher than in pig and pony.

Across species, transport capacity increased and transport affinity decreased in the colon. In fact, Lys transport affinity in the colon of both pig and pony was noticeably lower than other reported affinity values of Lys in the small intestine. It is recognized that AA transport across the small intestinal mucosa increases in the presence of increasing levels of AA. Transport of Lys, Leu, and Met increased with increasing dietary levels above maintenance in everted small intestine of mice (Diamond and Karasov, 1987). Increased capacity and decreased affinity for L-Leu transport were observed in rats fed a high protein diet compared to rats fed a high carbohydrate diet (Wolffram and Scharrer, 1984). In BBMV isolated from bovine jejunum and ileum, higher AA in the incubation medium increased uptake of Met and Lys (Wilson and Webb, 1990). Both pigs and ponies are hind-gut fermenters, and thus have a prominent microbial population in the cecum distal to the small intestine; whether Lys exists at higher luminal concentration in the colon compared to the small intestine is unknown. Alternatively, the colon may compensate for a lower protein level per unit of mucosa. Indeed, when expressed per mg of tissue, average Lys uptake by the pig and pony jejunum was calculated to be approximately 1.14 and 1.16 ρmol · mg tissue^{-1}, respectively, while uptake by the pig and pony colon was approximately 0.84 and 0.91 ρmol · mg tissue^{-1}, respectively.

In our study, there was no difference in total Lys uptake or kinetic parameters of Lys transport in the pony jejunum compared to the pig jejunum, nor was there a difference in pony colon compared to pig colon, exemplifying the relatively close physiological similarity between the two species.

Herein, we have demonstrated using BBMV that the large colon apical membrane is remarkably capable of Lys transport in both the pig and the pony via a high capacity and lower affinity transport than that of the small intestine. We have also reported novel Lys transport characteristics in the jejunum of the pig and the pony that are indicative of a medium affinity transport relative to other identified AA transport kinetics. Though the large colon has a lower affinity for Lys compared to the small intestine, the increased capacity of Lys transport in the large colon is indicative of the potential role it plays in the uptake of essential AA. Further characterization of Lys transport into Na^+-dependent and Na^+-independent components will allow identification of potential AA transport systems responsible for Lys uptake in the large colon. These results will be useful in estimating the contribution of the large colon to Lys absorption and N homeostasis.

Table 3.1. Kinetic parameters of L-Lys uptake in porcine and equine small (jejunum) and large (colon) intestinal brush border membrane vesicles.

Species	Segment	V_{max}[1,3] (ρmol mg protein^{-1} s^{-1})	K_M[1,4] mM	Diffusion[2,5] (ρmol mg protein^{-1} s^{-1})
Pig	Jejunum	121.37 ± 25.55	0.23 ± 0.22[y]	65.74 ± 12.03
	Colon	180.40 ± 25.55	0.89 ± 0.22[x]	80.65 ± 12.03
Pony	Jejunum	61.82 ± 25.39[b]	0.08 ± 0.22[b]	111.95 ± 11.75
	Colon	149.43 ± 25.39[a]	1.05 ± 0.22[a]	117.97 ± 11.75

[1]Colon is higher than jejunum across species ($P < 0.05$).

[2]Pony is higher than pig across segments ($P < 0.01$).

[3]Species × intestinal segment not significant ($P = 0.56$).

[4]Species × intestinal segment not significant ($P = 0.51$).

[5]Species × intestinal segment not significant ($P = 0.32$).

[ab]Within column and species, means with uncommon superscripts differ at $P < 0.05$.

[xy]Within column and species, means with uncommon superscripts tend to differ ($P = 0.09$).

Figure 3.1. Time courses of 0.001 (A), 1 (B), and 5 (C) mM L-Lys uptake using Na^+-mediated uptake. Brush border membrane vesicles were pre-loaded with a buffer containing 150 mM KSCN, 10 mM mannitol, 5 mM HEPES; pH 7.4. Incubation buffer contained 150 mM NaSCN, 5 mM HEPES, pH 7.4, and mannitol:Lys in the following combination: (A) 9.99 mM D-mannitol:0.001 mM L-Lys, including 0.8 µM L-[^{3}H]Lys, (B) 8.99 mM D-mannitol:1.001 mM L-Lys, including 0.8 µM L-[^{3}H]Lys, or (C) 4.99 mM D-mannitol:5.001 mM L-Lys, including 0.8 µM L-[^{3}H]Lys. Each point represents the average of a triplicate observation in brush border membrane vesicles manufactured from porcine jejunal mucosal scrapings.

Figure 3.1.

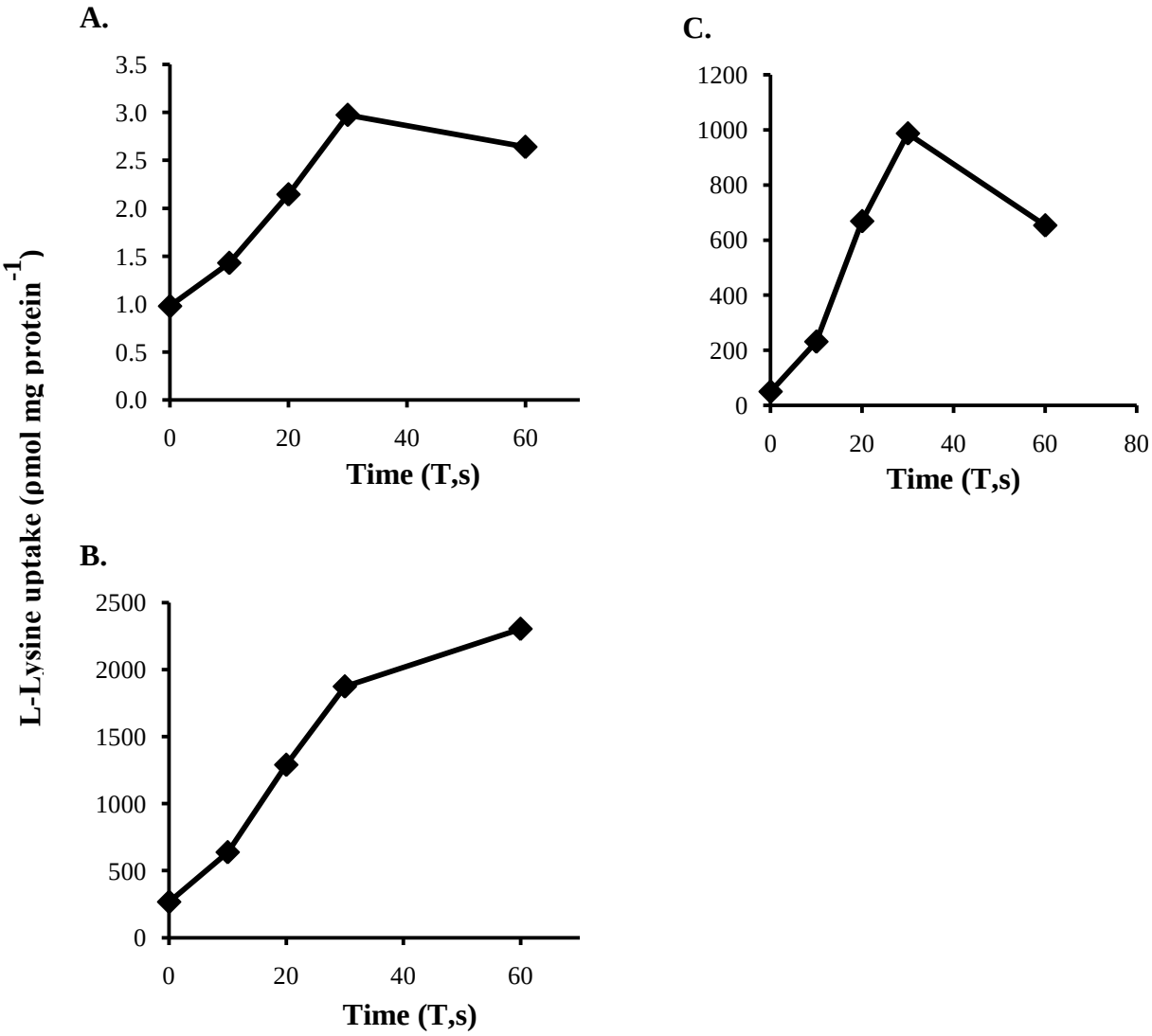
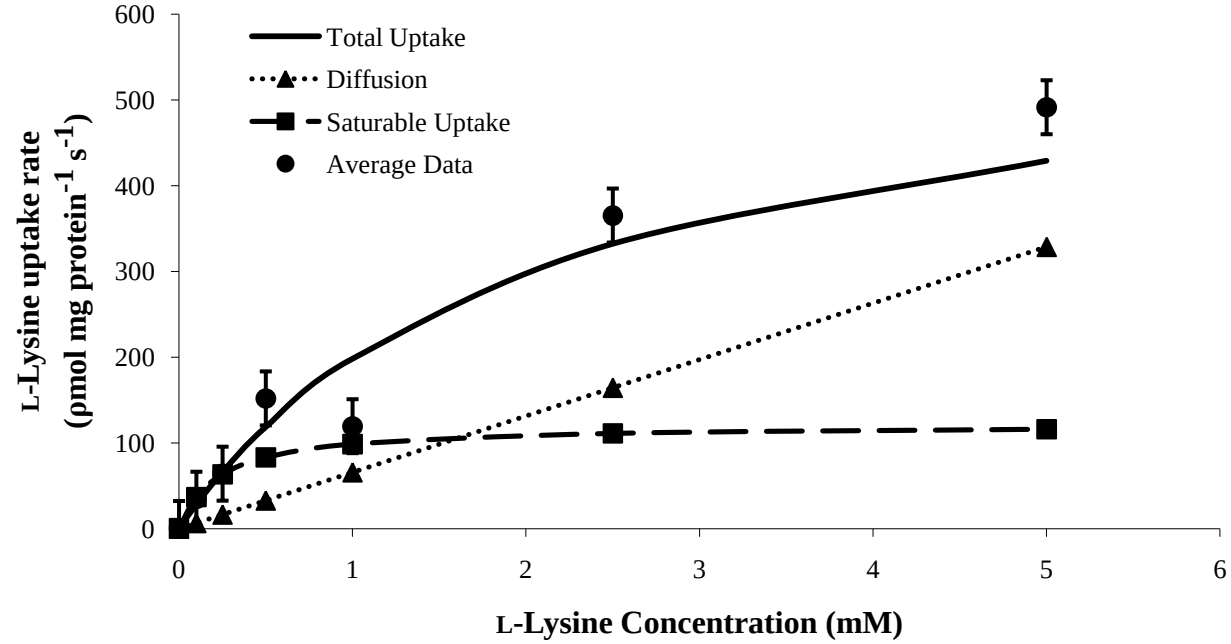

A.
B.
C.
L-Lysine uptake (ρmol mg protein-1)
Time (T,s)
Time (T,s)
Time (T,s)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
500
1000
1500
2000
2500
0
200
400
600
800
1000
1200
0
20
40
60
80

Figure 3.2. Kinetics of L-Lys uptake into porcine jejunal (A) and colonic (B) brush border membrane vesicles. Brush border membrane vesicles were pre-loaded with a buffer containing 150 mM KSCN, 10 mM mannitol, 5 mM HEPES; pH 7.4. Incubation buffer contained 150 mM NaSCN, 5 mM HEPES, pH 7.4, 0.8 µM L-[^{3}H]Lys, and non-labelled L-Lys at 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mM. D-mannitol was added to maintain osmolarity. Each point represents the mean ± SEM of uptake experiments from three pigs.

Figure 3.2.

A.


Total Uptake
Diffusion
Saturable Uptake
Average Data
L-Lysine uptake rate (ρmol mg protein-1 s-1)
L-Lysine Concentration (mM)
0
100
200
300
400
500
600
0
1
2
3
4
5
6

B.

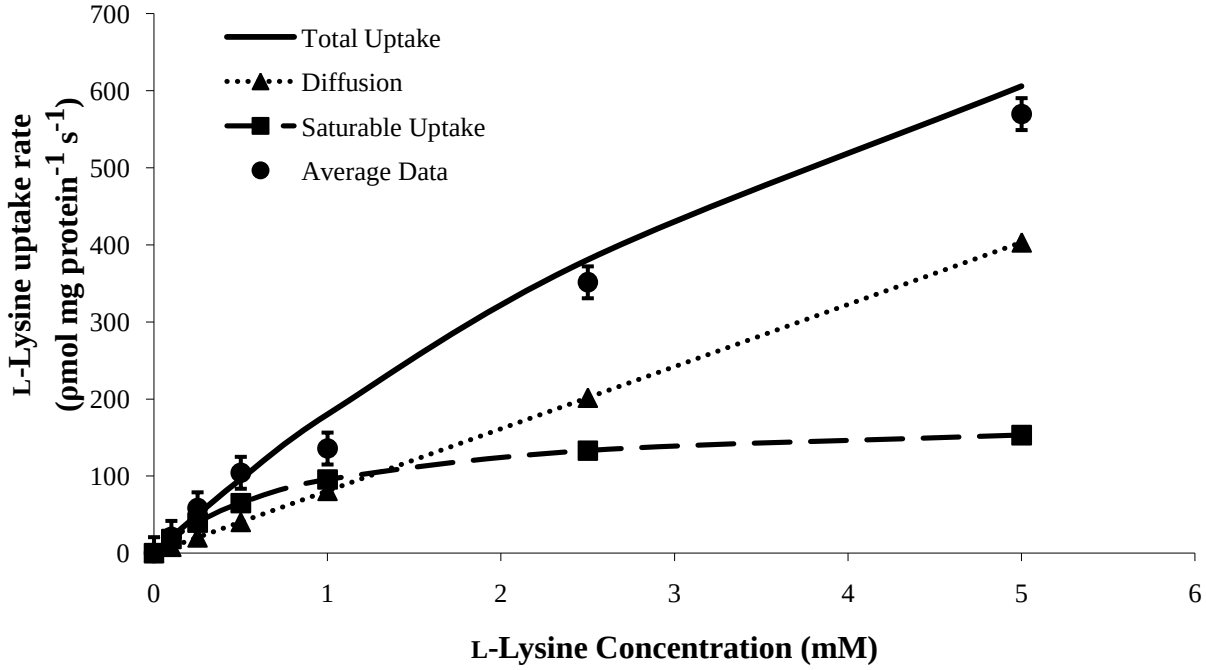
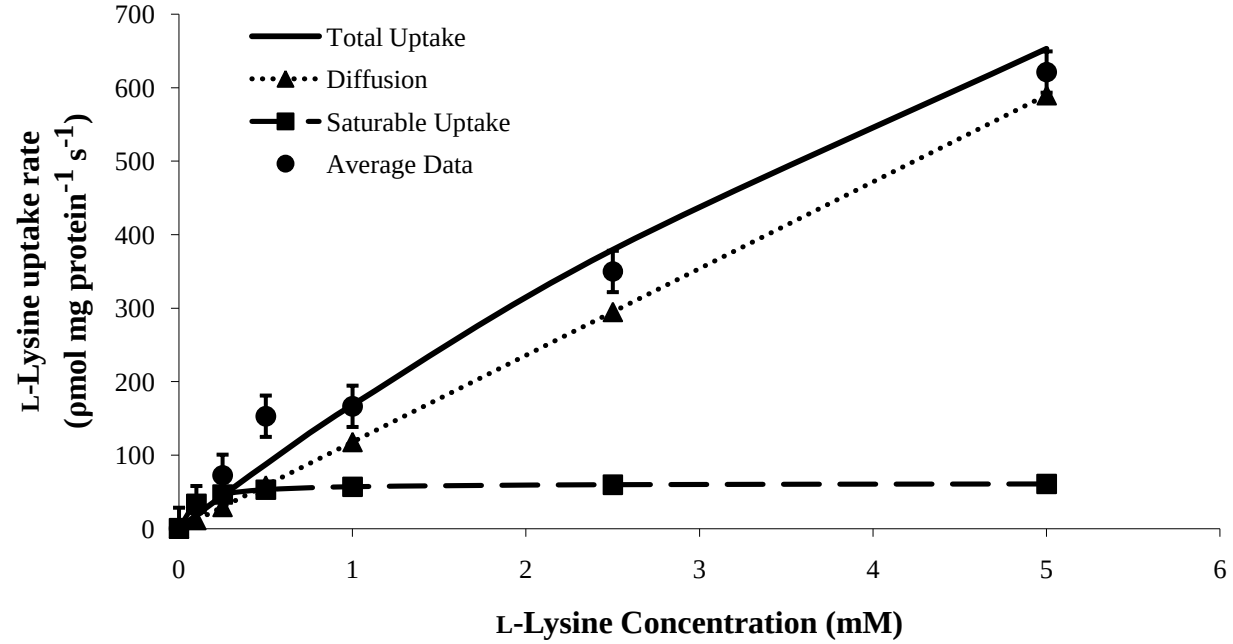

Total Uptake
Diffusion
Saturable Uptake
Average Data
L-Lysine uptake rate (ρmol mg protein-1 s-1)
L-Lysine Concentration (mM)
0
100
200
300
400
500
600
700
0
1
2
3
4
5
6

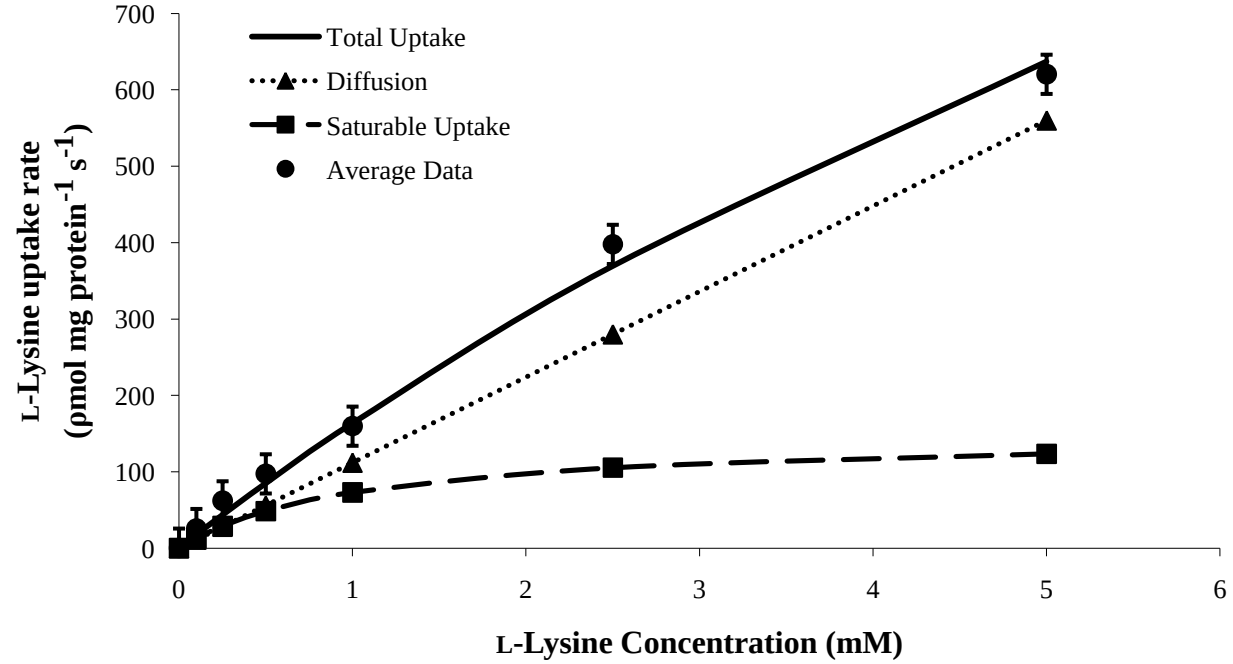
Figure 3.3. Kinetics of L-Lys uptake into equine jejunal (A) and colonic (B) brush border membrane vesicles. Brush border membrane vesicles were pre-loaded with a buffer containing 150 mM KSCN, 10 mM mannitol, 5 mM HEPES; pH 7.4. Incubation buffer contained 150 mM NaSCN, 5 mM HEPES, pH 7.4, 0.8 µM L-[^{3}H]Lys, and non-labelled L-Lys at 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mM. D-mannitol was added to maintain osmolarity. Each point represents the mean ± SEM of uptake experiments from four ponies.

Figure 3.3.

A.


Total Uptake
Diffusion
Saturable Uptake
Average Data
L-Lysine uptake rate (ρmol mg protein-1 s-1)
L-Lysine Concentration (mM)
0
100
200
300
400
500
600
700
0
1
2
3
4
5
6

B.


Total Uptake
Diffusion
Saturable Uptake
Average Data
L-Lysine uptake rate (ρmol mg protein-1 s-1)
L-Lysine Concentration (mM)
0
100
200
300
400
500
600
700
0
1
2
3
4
5
6

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Literature Cited

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CHAPTER FOUR

Woodward, A. D.[*], M. Kiupel[†], S. J. Holcombe[‡], and N. L. Trottier[*1]. 2010. Localization and distribution of amino acid transporters CAT-1 and LAT-2 in the small intestine and large colon mucosa of horses. In preparation for submission to the *Equine Veterinary Journal*. Submitted to co-authors for review.

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CHAPTER FOUR

LOCALIZATION AND DISTRIBUTION OF AMINO ACID TRANSPORTERS CAT-1 AND LAT-2 IN SMALL INTESTINE AND LARGE COLON MUCOSA OF HORSES

Abstract

To test the hypothesis that expression of CAT-1 and LAT-2 proteins is localized to the epithelial cells with relatively less distribution in the large colon compared to the small intestine in horses, the relative distribution of CAT-1 and LAT-2 proteins in the equine small intestine and large colon was assessed using immunohistochemical staining. Jejunal and large colonic whole intestinal tissue was collected from three mature horses. Tissues were paraffin-embedded, cut with a microtome, fixed to a microscope slide, and stained with either rabbit polyclonal anti-human CAT-1 or goat polyclonal anti-human LAT-2 to assess sub-cellular localization along either the apical or basolateral membrane of the intestinal epithelium. Visual analysis showed CAT-1 staining was weaker in the colon compared to the jejunum, with most signal occurring in the lamina propria of the small intestinal villi and in endothelial cells. For LAT-2, staining was detected along the basolateral membrane of intestinal epithelial cells, with weaker signal in the large colon compared to the jejunum. The results indicate that CAT-1 is most likely not responsible for AA transport in the intestinal epithelium, and that LAT-2 may be involved in neutral AA transport across the basolateral membrane of the equine intestine.

Key words: equine, intestinal localization, amino acid, transport

Introduction

Amino acid (AA) absorption through the intestinal epithelial cells is a complex process, involving AA transport across the brush border membrane (BBM) and intracellular travelling to the opposing basolateral membrane for transport into the portal circulatory system. Transmembrane movement not only entails many transporters of varying capacity and overlapping specificity, but transporters also differ between the apical and basolateral membrane (Bröer, 2008). For example, in rodents, cationic AA move through the small intestinal apical membrane via systems $b^{0,+}$ and $B^{0,+}$ and across the basolateral membrane using systems y^+ and y^+L (Bröer, 2008). It is well recognized that the small intestine is the major site of AA absorption, but much less is known on the large intestinal contribution to post-gut AA homeostasis in the horse. In fact, the available information is conflicting. Slade et al. (1971) demonstrated that the equine large intestine is capable of microbially-derived AA absorption; in contrast, Bochroder et al. (1994) found no transmembrane movement of lysine, histidine or arginine across whole tissue colonic discs prepared from mature ponies. *In vivo* studies measuring AA absorption from the equine small and large intestine would require procedures which are invasive to the horse and costly to investigators. These challenges have impeded progress in our understanding of the AA requirements by equids relative to other livestock species. Addressing the mechanisms of AA absorption through the use of novel and alternative *in vitro* approaches may be very useful in determining large intestinal AA contribution in the horse.

Recently, we have found the transcripts encoding cationic AA transporter CAT-1 (SLC7A1) and neutral AA transporter LAT-2 (SLC7A8) are present in both the small and large

intestinal tissue of the horse (Woodward et al., 2010). Transporter CAT-1 belongs to the y^+ transport system and displays high affinity and low capacity for cationic AA, while LAT-2 belongs to the L system of AA transport and has medium affinity and high capacity for neutral AA (Bröer, 2008). In the horse, CAT-1 is nearly exclusively expressed at the transcription level in the small intestinal mucosa, and LAT-2 mRNA abundance is 18-fold lower in the large colonic mucosa (Woodward et al., 2010); however, distribution of CAT-1 in intestinal epithelial cells is not known, and information on LAT-2 localization in the intestine is limited to very few studies (Rossier et al., 1999; Dave et al., 2004; Kobayashi, 2007). Nevertheless, the presence of LAT-2 transcript in the equine large colonic mucosa indicates that some form of neutral AA transport exists post-ilealy. To further elucidate whether CAT-1 and LAT-2 play a role in movement of AA across the epithelium of the small intestine and large colon, the relative distribution of CAT-1 and LAT-2 proteins in the small intestine and large colon was assessed using immunohistochemical staining. Based on the relative mRNA abundance of CAT-1 and LAT-2 in the small and large intestinal mucosa of the horse, we hypothesized that expression of CAT-1 and LAT-2 proteins is localized to the epithelial cells with relatively less distribution in the large colon compared to the small intestine.

Materials and Methods

Animals and Collection of Tissue

Three mature horses fed timothy grass hay at 2% BW were used. Horses were between the age of 7 and 10 yr and were clinically diagnosed with Cushing's syndrome; however, horses were euthanized for reasons other than observation of gastrointestinal problems. Following euthanasia, the horses were positioned in dorsal recumbency and a 2.5-m incision was made

along the ventral midline in order to expose the entire gastrointestinal tract. A 20-cm long section was obtained 12 m from the duodenal colic ligament for the distal jejunum, and a second sample was collected between the sternal and pelvic flexures at the lateral band for the left ventral colon. After collection of each segment, tissue was thoroughly washed in two separate ice-cold saline bath solutions. Tissue was then placed in 10% neutral buffered formalin for 24 h to allow formalin fixation of the cellular components.

Paraffin-Embedding and Slide Preparation of Tissue

After 24 h in formalin, tissue was removed, trimmed, and placed in a biopsy cassette before paraffin-embedding. For paraffin-embedding, tissues were dehydrated by 2-hr incubations in a series of graded ethanol washes (60%, 70%, 80%, 95%, 100%) and xylene. The tissue was then embedded with infiltrating paraffin for four 1-h time periods. The paraffin-embedded tissue was then dried, cut with a microtome into 5 μm sections, and placed in a water bath (55°C) for less than 20 seconds. A microscope slide coated with 3-aminopropyl-triethoxysilane was floated underneath the tissue, the tissue was allowed to adhere to the slide, and the finished slide for immunohistological staining was set in a drying rack for 24 h. Finished slides were heated to 56°C for 1 to 2 h and stained with hematoxylin for 90 sec and eosin for 120 sec.

Immunohistochemistry

Immunostaining was performed using a Dako Autostainer (Dako, Carpenteria, CA) at the Michigan State University Diagnostic Center for Population and Animal Health. Briefly, slides were incubated overnight at 60°C followed by two 10-min xylene incubations for paraffin removal. Tissue was rehydrated through a series of graded ethanol washes as described above two times for 5 min per wash, followed by rinsing with distilled water. Slides were then placed

into sodium citrate solution and heated for 20 min to further remove formalin cross-links and allow for antigen retrieval. To block endogenous peroxidase background staining, slides were placed in a 3% hydrogen peroxide:methanol solution for 15 min, followed by two 3-min washes in PBS wash solution. A protein-blocking agent (Dako) was then added to the slide for 10 min in order to reduce non-specific binding. Slides were then incubated with rabbit polyclonal anti-human CAT-1 (Abcam Inc., Cambridge MA, 95% homology) or goat polyclonal anti-human LAT-2 (Santa Cruz Biotechnology, Santa Cruz, CA, 97% homology). Slides were rinsed with PBS and incubated with secondary antibody (Dako). The binding of the antibodies was followed by a streptavidin-biotin wash (Dako), which binds the biotin in the secondary antibody, and a 3'3' diaminobenzidine wash (Dako), which reacts with the streptavidin to produce a brown precipitate for visualization. Slides were counter stained with Mayer's hematoxylin. Tissue was then dehydrated again through the graded ethanol washes, and finally a xylene wash was performed to remove pap pen circles. Coverslips were mounted over the tissue and slide to protect the stain, and the slides were stored in a darkened box until image analysis was performed.

Results and Discussion

Previously, we have profiled cationic and neutral AA transporter mRNA abundance along the equine small and large intestine mucosa (Woodward et al., 2010); however, relative cellular and subcellular distribution of AA transporter proteins along the intestinal epithelium has not been defined. This study is the first to address the localization of AA transporters to the apical or basolateral membrane of equine intestinal epithelial cells in both the small intestine and large colon.

In all three horses, there was weaker mucosal staining of CAT-1 in the colon than in the small intestine (Figure 4.1), which is consistent with little to no detectable CAT-1 mRNA abundance in both the left ventral and left dorsal colon of the horse (Woodward et al., 2010). There was little epithelial CAT-1 signal in both the colon and small intestine, but strong staining in the small intestinal lamina propria of villi and in endothelial cells (Figure 4.1). No other studies have documented the localization of CAT-1 to either the brush border or basolateral membrane of the intestine. Even so, several studies purport that CAT-1 plays a central role in lysine uptake by intestinal epithelial cells; for instance, Thwaites et al. (1996) suggested CAT-1 was partially responsible for lysine transport based on demonstrated lysine uptake across both apical and basolateral membrane of human intestine-derived Caco-2 cells, and Gilbert et al. (2007) and Liao et al. (2008) made similar claims based on CAT-1 mRNA abundance in the small intestine of broilers and steers, respectively. On the other hand, CAT-1 is localized to the basolateral membrane of polarized Madin-Darby canine kidney epithelial cells (Cariappa et al., 2002; Kizhatil and Albritton, 2002) and renal medulla epithelial cells (Kakoki et al., 2004).

Prominent CAT-1 staining in endothelial cells in the small intestinal villi in this study is consistent with the notion that the colon has less vasculature than the small intestine and is not equipped with villi (Bacha, Jr., and Bacha, 2000). In porcine pulmonary artery endothelial cells, CAT-1 protein is highly expressed and is responsible for arginine uptake, the primary substrate for generation of nitric oxide (Woodard et al., 1994; McDonald et al., 1997; Krotova et al., 2003). Labeling of CAT-1 in the intestinal lamina propria and endothelial cells indicates that CAT-1 is likely not involved in movement of cationic AA across intestinal epithelial cells in horses. It is noteworthy to mention, however, that additional studies, including Western blotting, would be valuable in supporting the sub-cellular distribution of CAT-1 reported herein.

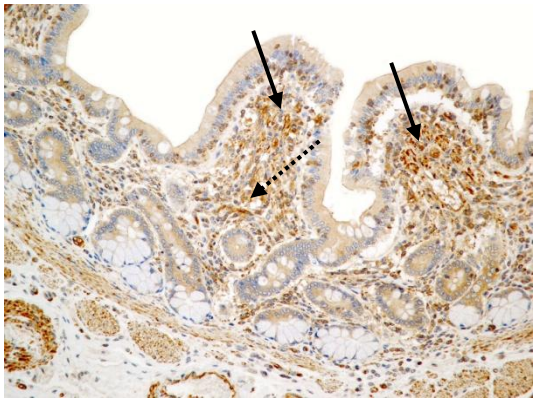
In all three horses, LAT-2 showed intense staining along the basolateral membrane of epithelial cells, with weaker signal in the colon relative to the small intestine (Figure 4.2). Conversely, Fraga et al. (2005) found LAT-2 protein expression in the rat colon was similar to expression in the jejunum and ileum. As for CAT-1, LAT-2 protein expression between the large and the small intestine of the horse is consistent with the previously reported (Woodward et al., 2010) relatively lower mRNA abundance of LAT-2 in the colon compared to the small intestine. Others have also shown that LAT-2 is localized to the basolateral membrane of the mouse small intestine (Rossier et al., 1999; Dave et al., 2004) and rat duodenum (Kobayashi, 2007). Thus, results indicate that in the horse, movement of large neutral AA across the basolateral membrane of small intestinal and large colonic epithelial cells may occur via LAT-2.

Of the two candidate proteins, only LAT-2 was detected in the epithelial cells and confined to the basolateral membrane. The remarkable labeling of CAT-1 within the lamina propria rather than epithelium suggests that CAT-1 is not involved in the absorption of lysine across the intestine of the horse. Other AA transporter transcripts known to transport lysine in other mammalian models are expressed along the equine intestinal tract (Woodward et al., 2010); however, the localization of their respective transport protein to epithelial cells remains to be determined. Future studies addressing the distribution and sub-cellular localization of other AA transport proteins along the small and large intestinal epithelium of the horse would provide valuable information on AA transport from the intestinal lumen to the blood supply, and thus on the potential relative contribution to the animal's AA requirement.

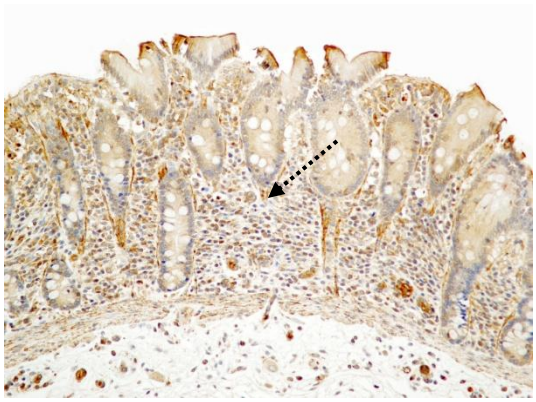
Figure 4.1. Immunohistochemical staining of AA transporter CAT-1 in the small intestine (panels A1, B1, and C1) and large colon (A2, B2, and C2) of three horses. Immunoreactivity was detected in the villi of small intestine (solid black arrows, panels A, C, and E) and lamina propria of both the small intestine and large colon (dotted black arrows). Each row of images is grouped by horse, i.e., the A panels are the small intestine (A1) and large colon (A2) of horse 1. In all panels, original magnification equals 400×. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 4.1.

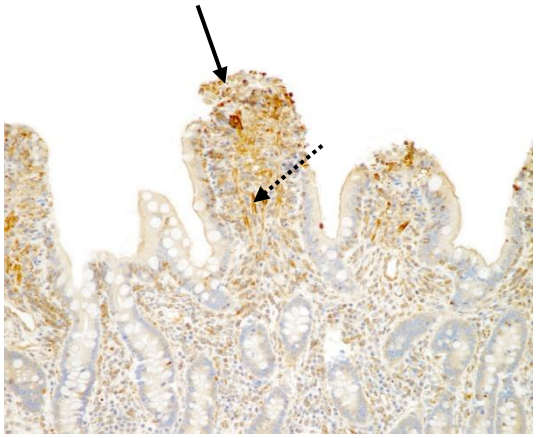
A1.



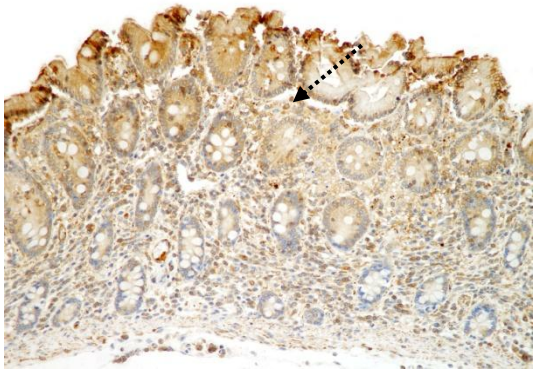
A2.



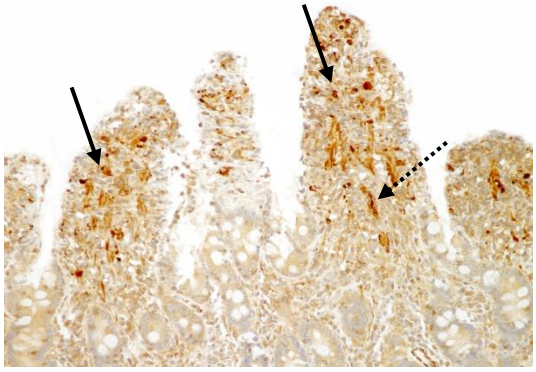
B1.



B2.



C1.



C2.

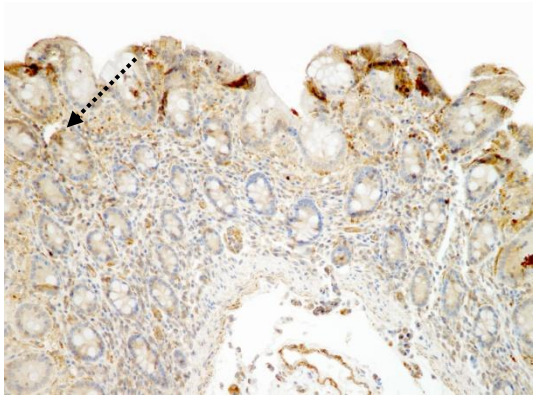
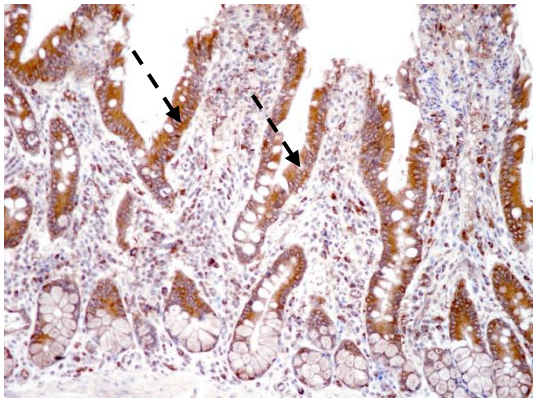


Figure 4.2. Immunohistochemical staining of AA transporter LAT-2 in the small intestine (panels A1, B1, and C1) and large colon (A2, B2, and C2) of three horses. Immunoreactivity was detected along the basolateral membrane of the epithelial cells in both the small intestine and large colon (dashed black arrows). Each row of images is grouped by horse, i.e., the A panels are the small intestine (A1) and large colon (A2) of horse 1. In all panels, original magnification equals 400×.

Figure 4.2.

A1.



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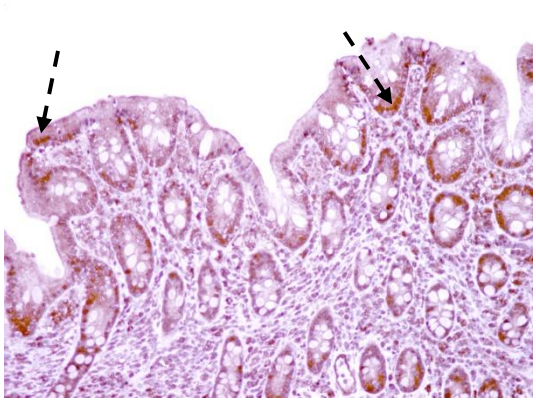

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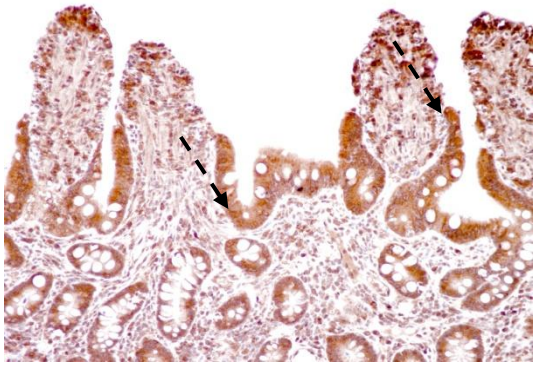

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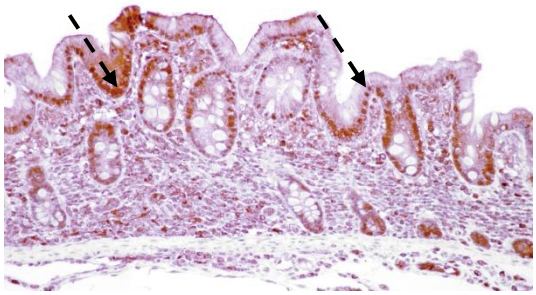
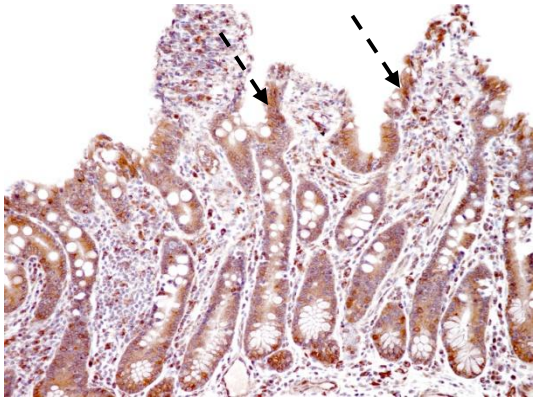
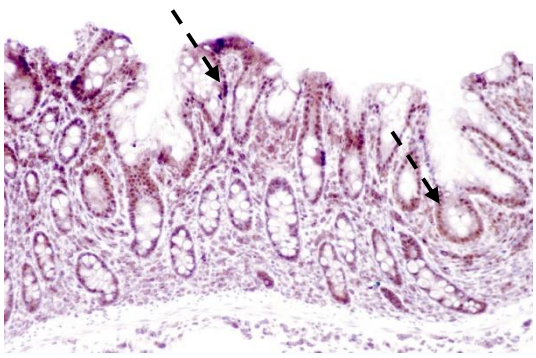


C1.



C2.



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CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Contribution of the equine large intestine to N and AA homeostasis of the host is unclear. Several studies have demonstrated that the equine large intestine significantly contributes to whole intestinal apparent N digestibility; however, there is limited research demonstrating large intestinal absorption of AA derived from microbial fermentation. Knowledge of large intestinal contribution to total gastrointestinal AA absorption in the horse would contribute to the overall understanding of protein utilization and AA requirements in equids. The overall goal of this dissertation research was to characterize the molecular entities and transport kinetics of lysine in the equine small and large intestine. The main hypothesis was that the capacity to transport lysine across the epithelium of the equine large intestine is similar to that of the small intestine. To test this hypothesis, the following three specific aims were addressed: (1) to determine the mRNA abundance of AA transporter proteins $b^{0,+}$AT (SLC7A9 of system $b^{0,+}$), CAT-1 (SLC7A1 of system y^{+}), and LAT-2 and LAT-3 (SLC7A8 and SLC43A1, respectively, of system L) in the colon relative to the small intestine; (2) to characterize lysine transport kinetics across the brush border membrane (BBM) of equine and porcine small and large intestinal BBM; and (3) to determine the localization and distribution (of the CAT-1 and LAT-2 protein in the small and large intestinal mucosa.

Both $b^{0,+}$AT and CAT-1 are purported to be responsible for transport of cationic AA across intestinal epithelial cells. Because transporter $b^{0,+}$AT is a symport transporter and moves neutral AA out of the cell in exchange for cationic AA, the level of transcript expression of

neutral AA transporters SLC7A8 (LAT-2) and SLC43A1 (LAT-3) was also profiled along the equine GIT. Although SLC7A9 ($b^{0,+}AT$) and SLC43A1 (LAT-3) mRNA abundance did not differ in the large compared to that of the small intestine, SLC7A1 (CAT-1) and SLC7A8 (LAT-2) had lower abundance in the large intestine. Many studies purport CAT-1 to be an important transporter responsible for lysine absorption from the small intestine. The research presented herein showed that there was a remarkable and significant decrease of SLC7A1 (CAT-1) mRNA abundance in all segments of the large intestine, i.e., the cecum, left ventral colon, and left dorsal colon compared to that in the jejunum and ileum, strongly indicating that lysine transport from the large intestine, if any, would not be facilitated by CAT-1. Amino acid transporter $b^{0,+}AT$ has been increasingly recognized as playing a significant role in lysine transport across the apical membrane. Hence, the mRNA abundance of this transporter in the large intestine of the horse represents a potential facilitated lysine transport process from the large intestine. If both of these cationic AA transporters move lysine across the intestinal epithelium, then the presence of both CAT-1 and $b^{0,+}AT$ in the small intestine may allow increased opportunity for lysine absorption through the small intestine of the horse, with the large intestine playing a lesser role in lysine absorption via AA transporter $b^{0,+}AT$.

In order to demonstrate whether the presence of targeted AA transporter transcript in the equine large intestine was related to functional lysine uptake across the mucosal epithelium, lysine transport uptake across the equine large colon apical membrane was compared to that of the small intestine using brush border membrane vesicles (BBMV). Because of the lack of published material pertaining to lysine uptake into equine BBMV, the pig was included as an additional animal model, along with glucose as test substrate for all lysine uptake experiments.

In both the pig and the pony, kinetic parameters V_{max} and K_M of L-lysine transport across the large colon were higher than across the jejunum when expressed on mg protein basis, indicating higher capacity and lower affinity per mg protein for L-lysine transport in the large colon. These results agree well with the transcript abundance of SLC7A9 ($b^{0,+}$AT) and SLC43A1 (LAT-3) in the equine large intestine, whereby mRNA is indicative of transport capacity. On the other hand, because the large intestine is not equipped with villi and thus has less protein per unit of mucosa weight, parameters of transport kinetics were expressed on a per mg of mucosal tissue. On such basis, L-lysine uptake by the pony was similar in the small intestine compared to the large colon, with total L-lysine uptake of 1.16 ρmol per mg of small intestinal tissue, and of 0.91 ρmol per mg of large colonic tissue. Knowledge of total mucosal tissue weight per intestinal segment would allow a clearer assessment of lysine uptake capacity in the different segments of the equine intestine. Nonetheless, these results demonstrate that the large intestinal tissue apical membrane is capable of L-lysine transport, suggesting a likely role for the large intestine contribution to whole body lysine homeostasis. Based on the finding that transporter SLC7A9 ($b^{0,+}$AT) showed similar transcript abundance in the small and large intestine, and that functional L-lysine uptake was also similar in both the small and large intestine, it is suggested that $b^{0,+}$AT plays a significant role in lysine absorption in equids.

To further confirm the above findings, AA transporter sub-cellular localization to the apical and basolateral membrane, and distribution along the small intestine and large colon, was determined. Antibody against $b^{0,+}$AT and LAT-3 proteins were not specific, thus these proteins could not be assessed. Immunohistochemical staining showed that LAT-2 was located on the basolateral membrane in both the small and large intestinal epithelium of horses, indicating that

the intracellular neutral AA pool in the intestinal epithelial cell may be used either as exchangers for $b^{0,+}$AT or for neutral AA transport into the portal circulation. Surprisingly, AA transporter CAT-1 was localized to the lamina propria, with very little labeling found in the intestinal epithelium of either the small or large intestine, suggesting that the CAT-1 is not a major pathway for lysine transport across the intestinal epithelium of the horse.

While breakdown of forage proteins can occur in the stomach and small intestine, research presented herein demonstrates that the large intestine is capable of L-lysine transport, most of which would originate from microbial proteins. Future studies which quantify protein abundance and subcellular location of AA transporters, such as Western blots or immunohistochemistry, would further confirm the large intestinal role of L-lysine transport. Feeding horses forages that contain high protein concentration leads to high excretion levels of urinary N, and reduced dietary protein utilization efficiency, and thus is not environmentally sustainable. Adaptation of both small and large intestinal AA transporters to different diets, i.e. high concentrate versus high forage, also needs to be examined in order to determine feeding regimens which benefit the horse while decreasing environmental contamination. In all, knowledge of the large intestinal role in lysine absorption as demonstrated *in vitro* sheds light on the actual protein utilization and availability of less apparently digestible forages in horses.

APPENDICES

APPENDIX A

Woodward, A. D.[*], B. D. Nielsen[*], J. Liesman[*], T. Lavin[†], and N. L. Trottier[*1]. 2010. Protein quality and utilization of alfalfa, timothy, and oat-supplemented timothy hay in exercised Arabian horses. In preparation for submission to the *Journal of Animal Science*. Submitted to co-authors for review.

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APPENDIX A

PROTEIN QUALITY AND UTILIZATION OF ALFALFA, TIMOTHY, AND OAT-SUPPLEMENTED TIMOTHY HAY IN EXERCISED ARABIAN HORSES

Abstract

In order to evaluate the protein quality of alfalfa hay harvested at different maturities, of first-harvest timothy hay, and of oat-supplemented timothy-hay diets, apparent whole tract N digestibility and serum AA profiles were determined in light to moderately exercised Arabian horses. Six Arabian geldings (16.0 ± 0.3 yr, 467 ± 11 kg) were randomly allocated to a 6 × 6 Latin square design. Diets included first cutting timothy grass hay (G), G + 0.2% BW oat (G1), G + 0.4% BW oat (G2), first cutting alfalfa (A1), second cutting alfalfa (A2), and third cutting alfalfa hay (A3). Forages were fed at 1.6% of body weight (as fed). Each period consisted of 14 d with an11-d adaptation period followed by a 3-d total collection period. Blood samples were collected on d 11 for analysis of serum AA profile. During the 3-d collection period, urine and feces were collected every 8 h, measured and weighed, and samples representing 10% of the total were retained and stored at -20°C. Fecal and urine subsamples from each collection time and period were thawed, pooled, and a representative sample per period was retained. Nitrogen intake, urine volume, urinary N excretion, N absorbed, and apparent whole tract N digestibility were higher in A1, A2, and A3 compared to G ($P < 0.05$). Fecal DM output, urine volume, and urine N output did not differ between G, G1, and G2. Nitrogen intake, N absorbed, and apparent whole tract N digestibility were higher in G1 and G2 compared to G ($P < 0.05$). Serum AA profiles were higher in horses fed A1 and A3 compared to G ($P < 0.05$); horses fed A2 has

similar serum AA profiles compared to G. Most AA showed a curvilinear response (quadratic, $P < 0.05$), with peak AA concentrations met at 2-h post feeding. The results show efficient utilization of N is decreased in horses fed alfalfa hay of increasing CP concentration, indicating CP intake was in excess of requirement. Thus, feeding grass hay may be more desirable than feeding legume hay in order to decrease environmental N contamination.

Key words: nitrogen, horse, digestibility, protein utilization

Introduction

Feeding protein far in excess of requirement is common in the horse industry (Lawrence et al., 2003; Ott, 2005). Protein intake in excess of utilization by the animal leads to increased urinary N excretion, in turn contributing to ground water contamination and decreasing environmental air quality (Knowlton and Cobb, 2006). Strategies to mitigate the impact of equine feeding practices on the environment critically depend on knowledge of the protein quality of feeds and diets fed to horses. Such information in horses remains limited for common feedstuffs such as alfalfa and timothy hay, and oats. Alfalfa hay is used extensively due to its higher protein concentration (Ralston et al., 1989) and superior nutrient digestibility (Cymbaluk and Christensen, 1986) compared to grass hay. However, fiber composition, protein concentration and protein digestibility of alfalfa are greatly affected by the plant maturity at harvest (Darlington and Hershberger, 1968). The extent to which plant maturity impacts alfalfa protein composition and utilization in horses and on N losses to the environment is poorly documented. Timothy hay protein concentration and apparent protein digestibility are typically lower than alfalfa (Fonnesbeck et al., 1967; Darlington and Hershberger, 1968; Cuddeford et al., 1992); but timothy hay fed alone or in conjunction with oats when energy is limiting (Kienzle et

al., 2002) may be more environmentally sustainable than feeding alfalfa, in particular for horses at maintenance or under light exercise. Thus the complementary effect of oat grains and timothy hay on the protein quality and utilization, and N losses to the environment remains to be assessed. The objective of this study was to evaluate the protein quality of alfalfa (*Medicago sativa*) hay harvested at different maturities, of first-harvest timothy hay (*Phelum pretense*), and of oat-supplemented timothy-hay diets. The specific aims were to 1) determine apparent whole tract N digestibility of timothy and alfalfa hay of different maturities at harvest; 2) evaluate the AA profile and availability of timothy grass hay and of alfalfa hay harvested at different maturities; and 3) determine apparent whole tract N digestibility and utilization of grass hay with two levels of oats inclusion.

Materials and Methods

All animal use and handling were approved by the Michigan State University Institutional Animal Care and Use Committee.

Animal Management

Six mature Arabian geldings (16.0 ± 0.3 yr, 467 ± 11 kg) were used. Prior to the start of the experiment, horses were housed together on a large mixed-grass pasture with free access to water. For the duration of each study, horses were individually housed in 3 × 3 m box stalls bedded with wood shavings. Body weight and body condition score (BCS; Henneke et al., 1983) were recorded one day prior to the start of each adaptation period. The BCS of each horse represented the averaged BCS determined by three independent skilled observers. In order to minimize overfeeding and orts, the BW was adjusted using one BCS unit per 20-kg gain or loss relative to an average 500-kg horse (NRC, 2007) as follows:

Adjusted BW = BW- [(BCS-5) × 0.04 × BW]

If a horse had a BCS of 5, no adjustment was made. No horses had a BCS below 5.

During the entire study, horses were used in horsemanship classes and equestrian team practices three to five days per week. The weekly workload on average consisted of 35% walk, 55% trot and 10% canter, corresponding to a light to moderate exercise workload, as described by NRC (2007).

Experimental Design and Diets

Horses were allocated to 6 diets over 6 time-periods in a 6 × 6 Latin square design balanced for residual effects. Diets included first cutting timothy grass hay (G), G + 0.2% BW oat (G1), G + 0.4% BW oat (G2), first cutting alfalfa (A1), second cutting alfalfa (A2), and third cutting alfalfa hay (A3). Timothy hay was harvested from seven fields from June 11 through June 22, 2007. The three alfalfa harvests originated from the same field on June 12, 2007 (A1), July 8 (A2) and August 13 (A3). Forages were fed daily at 1.6% BW (as fed), with one-half at 700 and 1600, and free access to water was provided during the entire study. Orts were collected and weighed prior to the morning feeding to determine 24-h feed refusal, if any. Each period was 14 d, consisting of 11 d of adaptation followed 3 d of total feces and urine collection.

Sample Collection

Forages and oats. Sufficient bales of each hay type (G, A1, A2, and A3) were allocated to 6 periods. Each bale was core-sampled and the samples pooled for each period for nutrient analysis per period. Oats were sampled and samples pooled for each period for nutrient analysis.

Feces and urine. On d 12 of each adaptation period, total collection devices (Equisan Marketing, Melbourne, Australia) were fitted to each horse for collection of feces and urine over a 3-d period (i.e., d 12, 13, and 14). Feces and urine were removed every 8 h. Eight-hour urine

volume was measured and 10% of total volume sampled and stored in tightly capped Nalgene bottles at -20°C. Eight-hour fecal mass was weighed and 10% of total mass was sampled and stored in a plastic bag at -20°C. After completion of each 3-d collection periods, fecal and urine samples from each horse were thawed. Urine was strained through cheesecloth to remove debris, pooled and thoroughly homogenized. Fecal samples were pooled and thoroughly mixed. Representative fecal and urine samples were retained and stored at -20°C until analysis.

Blood. Blood samples (10 mL) were collected on the morning of the last day of each adaptation period, i.e. d 11, via jugular venipuncture using a 20-G needle and serum Vacutainer tubes containing no additives (Becton Dickenson) following feeding of hay at 0.8% BW and oats at 0.1 and 0.2% of BW for diets G1 and G2. Oats were offered simultaneously with hay in G1 and G2. Samples were collected 30 min prior to feeding and at 60, 120, 180, and 240 min post-feeding, allowed to coagulate on ice for 20 min, and centrifuged at 1,340 × *g* for 10 min. Serum was harvested and stored at -20°C for later analysis of AA concentrations.

Laboratory Analyses

Hay and oat samples were air dried at 105°C and ground through a 1-mm screen (Cyclotec 1093 sample mill, Foss, Eden Prairie, MN) for DM, fiber composition, and AA analyses.

Fiber composition. For each period, ADF, NDF, and ADL analyses were carried out on timothy and alfalfa hay samples according to the methods of Goering and Van Soest (1970). Hemicellulose was determined by subtraction of ADF from NDF and cellulose by subtraction of ADF from ADL.

Dietary, fecal, and urinary N. Fecal samples were thawed, freeze-dried rather than oven dried to prevent N evaporative losses, and ground to pass through a 1-mm screen (Cyclotec 1093

sample mill, Foss, Eden Prairie, MN). Feed, fecal, and urine samples were analyzed for N by thermal combustion (LECO, St. Joseph, MI).

Amino acids. Samples of timothy and alfalfa hay from each period were pooled and AA concentrations determined by HPLC (Waters, Milford, MA) as described by Guay et al. (2006) following hydrolysis in 6*N* HCL for 24 h at 110°C. Serum AA concentrations were determined as described by Guay and Trottier (2006). All AA analyses were run in duplicate.

Statistical Analysis

Forage composition, DM, and CP were analyzed using the GLM model analysis of variance in SAS (Version 9.1, SAS Inst., Inc., Cary, NC). The model included the fixed effects of forage and period. Dietary, fecal, and urinary N, N digestibility and serum AA concentrations were analyzed using the MIXED model procedure in SAS (Version 9.1, SAS Inst., Inc., Cary, NC). The model included the fixed effects of diet and period, and the random effect of horse. Orthogonal contrasts were performed to compare oat-supplemented timothy grass (G1 and G2) versus timothy grass alone (G), alfalfa (A1, A2, and A3) versus G, as well as A1 and A2 versus A3. For serum AA concentrations, the model included the fixed effects of diet, time, period, the interaction of diet × time and the random effect of horse, and pre-feeding (i.e. -30 min) AA concentrations as a covariable. Differences between diets were tested using least squares means and multiple comparisons were accounted for by correcting with Bonferroni adjustment. Serum AA concentration response to feeding over a 240-min period was evaluated using linear and quadratic polynomial functions. Significance and tendencies toward significance are reported at $P < 0.05$ and $P < 0.1$, respectively.

Results

Forage Nutrient Profile

Crude protein and fiber composition of timothy and alfalfa hay for each period (1 to 6) is presented in Table A.1 and summarized across periods presented in Table A.2. Dry matter did not differ in any alfalfa hay cuttings compared to timothy. Crude protein concentration was highest in A3, followed by A2, A1, and G ($P < 0.001$). Fiber components NDF, ADF, and cellulose were lowest in A3, followed by A2, A1, and G ($P < 0.001$). Hemicellulose was lowest in A3, and across A1, A2 and A3, was lower than G ($P < 0.001$). Lignin content was highest in A1 followed by A2, A3, and G ($P < 0.001$); lignin did not differ between A3 and G.

Amino acid composition of timothy and alfalfa hay is presented in Tables A.3 (g AA/100 g hay) and A.4 (g AA/100 g N × 6.25). Amino acid concentration was numerically higher for A1, A2, and A3 compared to G (Table 3), in particular for Lys, which was 3.1- to 3.7-fold lower in G compared to alfalfa. Crude protein AA composition, i.e., g AA/100 g CP, in G was similar to that of A1, A2, and A3 (Table 4). Protein AA composition of alfalfa remained similar across the different plant maturities (i.e., A1, A2, and A3).

Nitrogen Balance and Utilization

Contrasts between alfalfa (A1, A2, and A3) and timothy hay (G) are presented in Table A.5. Nitrogen intake was higher in A1, A2, and A3 compared to G ($P < 0.001$) and was higher in A3 compared to A1 and A2 ($P < 0.001$). Fecal DM output was lower in A2 and A3 ($P < 0.05$ and $P < 0.01$, respectively), and tended to be lower in A1 ($P = 0.06$), compared to G, and did not differ between A3 and either A1 or A2. Urine volume was higher in A1, A2, and A3 compared to G ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively), and not different in A1 and A2 compared to A3. Compared to G, fecal N output tended to be higher in A3 ($P = 0.07$), and urinary N

excretion was higher in A1, A2, and A3 ($P < 0.001$). Nitrogen absorbed and apparent whole tract N digestibility were higher in A1, A2 and A3 compared to G ($P < 0.001$). Nitrogen absorbed was higher in A3 compared to A1 and A2 ($P < 0.001$). Nitrogen retained tended to be higher in A3 compared to G ($P = 0.05$).

Contrasts between oat-supplemented timothy hay diets (G1 and G2) and timothy hay alone (G) are presented in Table A.6. Fecal DM output, urine volume, and urine N output did not differ between G, G1, and G2. Nitrogen intake was higher in G1 and G2 compared to G ($P < 0.001$) and it was higher in G2 compared to G1 ($P < 0.01$). Fecal N output was higher in G2 compared to G ($P < 0.05$). Absorbed N was higher in G1 and G2 compared to G ($P < 0.001$) and higher in G2 compared to G1 ($P < 0.01$). Compared to G, apparent whole tract N digestibility was higher for G1 ($P < 0.01$) and G2 ($P < 0.001$).

Serum AA Concentration

Serum AA concentrations in response to feeding G, A1, A2, and A3 are presented in Figure A.1. Overall, compared to horses fed G, horses fed A1 and A3 had higher serum concentrations of arginine ($P = 0.04$ and $P < 0.001$, respectively), isoleucine ($P = 0.02$ and $P < 0.001$, respectively), leucine ($P = 0.03$ and $P < 0.001$, respectively), lysine ($P < 0.01$ and $P < 0.01$, respectively), threonine ($P = 0.02$ and $P < 0.01$, respectively), tryptophan ($P < 0.01$ and $P < 0.01$, respectively) and valine ($P < 0.01$ and $P < 0.001$, respectively). Compared to horses fed G, serum methionine concentrations were higher ($P < 0.01$) and tended to be higher ($P = 0.08$) in horses fed A3 and A1, respectively. In horses fed A2, serum arginine concentrations were higher ($P = 0.02$) and serum tryptophan concentrations tended to be higher ($P = 0.08$) compared to horses fed G.

Over the 240 min post-feeding period, there was a curvilinear response in horses fed G for threonine (quadratic, $P = 0.02$), methionine (quadratic, $P = 0.04$), leucine (quadratic, $P = 0.01$), phenylalanine (quadratic, $P < 0.01$), and lysine (quadratic, $P < 0.01$), with serum AA concentration appearing to peak 120 min post-feeding. In horses fed A1, threonine increased linearly ($P < 0.01$), while other AA increased curvilinearly: methionine (quadratic, $P < 0.01$), leucine (quadratic, $P = 0.02$), phenylalanine (quadratic, $P = 0.02$), tryptophan (quadratic, $P < 0.01$), and lysine (quadratic, $P < 0.001$). In horses fed A2, the majority of essential AA increased curvilinearly over time (quadratic, *P*-values range from < 0.001 to 0.02); histidine and valine did not change over the 240-min post-feeding period. In horses fed A3, the majority of essential AA increased curvilinearly (quadratic, $P < 0.001$), threonine and valine increased linearly ($P < 0.001$ and 0.01, respectively), and histidine tended to increase linearly ($P = 0.07$).

Discussion

Nitrogen requirement for the 500-kg horse ranges from 67.5 to 90 mg/kg BW/d for maintenance and from 73 to 101 mg/kg BW/d for light to moderate work (NRC, 2007). Sustainable feeding strategies aim to ensure that dietary N requirements are met and minimize N excess. Optimizing efficiency of N utilization is dependent upon knowledge of feed ingredient protein quality, which is defined by its digestibility, AA composition, and post-gut utilization. Our objective was to assess the apparent protein digestibility and utilization of commonly used feeds, i.e., timothy and alfalfa hay of varying maturity, and of oat-supplemented timothy-hay diets.

Estimates of whole tract apparent N digestibility of forages range widely both between and within cultivars. Whole tract apparent N digestibility of timothy hay ranges from 36%

(Cuddeford et al., 1992) to 66% (Darlington and Hershberger, 1968). Timothy hay whole tract apparent N digestibility was 54% for first cutting fed to Standardbred geldings (Fonnesbeck et al., 1967) and 65, 62, and 55% for immature, mid-mature, and mature cutting, respectively fed to ponies (Darlington and Hershberger, 1969). Ordakowski-Burk et al. (2006) reported as low as 48% for third cutting fed to Thoroughbred geldings, while Cuddeford et al. (1992) and Hintz et al. (1971) reported values of 36% in horses and 42% in ponies, respectively. Darlington and Hershberger (1968) suggested that forage whole tract apparent N digestibility in equids was directly proportional to its CP concentration. However, Darlington and Hershberger (1968) reported timothy hay CP concentration of 6.5% with an apparent N digestibility of 55%, in sharp contrast to Ordakowski-Burk et al. (2006) who reported timothy hay CP as high as 14% with an apparent N digestibility of 48%.

Estimates of whole tract apparent N digestibility of alfalfa hay fed to equids range from 55 (Darlington and Hershberger, 1968) to 81% (Potts et al., 2009). Whole tract apparent N digestibility of first cutting alfalfa (A1) in this study was 73%, which is higher than others; for instance, Fonnesbeck et al., (1967) reported whole tract apparent N digestibility of 65% in first cutting alfalfa, while Darlington and Hershberger, (1969) reported a value of 55% in their most mature alfalfa. The apparent N digestibility values for second and third cutting alfalfa hay (73 and 75%, respectively) in this study are in agreement with others; Darlington and Hershberger (1968) reported 72 and 75% whole tract apparent N digestibility in mid-mature and immature alfalfa, respectively. Others have reported comparable whole tract apparent N digestibility values of 73% in ponies (Gibbs et al., 1988), 73% in Arabian geldings (Crozier et al., 1997), 74% in Thoroughbred geldings (Cuddeford et al., 1992), and 76% in stock-type geldings (Sturgeon et al., 1999). The relationship between CP concentration and apparent N digestibility

in horses appears to be more consistent for alfalfa than for timothy hay; alfalfa hay with as low as 9% CP was 55% digestible (Darlington and Hershberger, 1969) compared to alfalfa hay in this study containing as high as 25% CP with 78% N digestibility. However, Fonnesbeck et al. (1967) reported first and second cutting alfalfa with CP of 11% and 16%, respectively, and apparent N digestibility of 65% and 59% apparent N digestibility, respectively. The discrepancy in CP content and whole tract apparent N digestibility in the study of Fonnesbeck et al. (1967) is unclear.

Lower apparent N digestibility of timothy compared to that of alfalfa hay in our study is consistent with others (Gibbs et al., 1988; Cuddeford et al., 1992; Crozier et al., 1997). Darlington and Hershberger (1969) reported higher whole tract apparent N digestibility of immature and mid-mature alfalfa compared to timothy and orchardgrass hay of similar maturity to that of alfalfa; however, mature alfalfa had similar digestibility values compared to mature timothy and mature orchardgrass.

Fiber composition may play a significant role in whole tract apparent N digestibility differences between grass and legume forages, and between maturity stages at harvest. Differences in fiber composition have been shown to affect apparent N digestibility in sheep, swine, rats (Keys et al., 1969), and horses (Glade, 1984). Digestibility of the cellulose component of fiber is directly related to lignification, which in turn decreases plant cell-wall protein digestibility (Van Soest, 1994). In our study, as alfalfa maturity decreased, lignification decreased and apparent N digestibility increased. In timothy hay, lignin content was similar to third cut alfalfa (A3), yet N digestibility was dramatically lower. However, timothy NDF content was 30 to 100% higher than alfalfa hay (first to third cut, respectively); in dairy cattle (Van Soest, 1994) and horses (Glade, 1984), NDF is inversely related to N digestibility,

suggesting that the low N digestibility of timothy hay is mainly attributed to high NDF content. In addition, hemicellulose content of timothy was 3 to 4-fold higher than that of alfalfa hay. Hemicellulose is associated with lignin content (Van Soest, 1994) and is considered a limiting factor to fiber digestibility in horses (Glade, 1984). Consequently, the low hemicellulose of alfalfa compared to high hemicellulose of timothy hay is likely to be an important contributing factor to the higher whole tract apparent N digestibility of alfalfa compared to timothy hay. It is critical however to recognize that apparent N digestibility underestimates the true digestibility of lower-protein containing feeds relative to that of higher protein-containing feeds. Endogenous losses associated with feeding timothy hay are likely to be of significance compared to those associated with alfalfa. Correcting for endogenous losses would allow for a better assessment of timothy hay protein digestibility. However, such information is currently not available in the literature; endogenous losses reported so far (NRC, 2007) pertain to losses associated with mixed feeds rather than feed specific and as such cannot be used to estimate true protein digestibility of feeds fed to horses.

Cereal grains are commonly fed to horses as a source of dietary energy due to their high starch content. Addition of grain can enhance whole tract apparent N digestibility of low-protein forages, such as straw, from 50 to 66% (Kienzle et al., 2002). Whole oats is a common grain used in many mixed equine diets because they are readily digestible pre-cecally and very palatable (Hussein et al., 2004; Sarkijarvi and Saastamoinen, 2006). In this study, addition of oats as low as 0.2 and 0.4 % of the horse's BW to the low-protein timothy hay improved the total diet apparent N digestibility by as much as 9.1 and 14.6%, respectively. Similarly, Palmgren Karlsson et al. (2000) reported a 9 and 18% increase in whole tract apparent N digestibility of the total diet when oats were provided in addition to grass hay at 0.3 and 0.6% of the horses BW.

The increase in whole tract apparent N digestibility of timothy with the inclusion of oats may be due, in part, to stimulation of the microbial protein synthesis and population by starch, in turn enhancing capacity for fiber digestion (Kern et al., 1973; Palmgren Karlsson et al., 2000). On the other hand, Hussein et al. (2004) supplemented alfalfa cubes with whole or naked oats and found no difference in whole tract apparent N digestibility. However, it should be noted that the forage source in the Hussein et al. (2004) study consisted of a processed alfalfa cube containing 19% CP; therefore, addition of oats may increase whole tract apparent N digestibility only when low protein forage is used.

All horses consumed forages at 1.6% BW (as fed basis). Horses fed timothy hay diets consumed N within NRC (2007) requirement estimates for horses under light to moderate level of exercise, ranging from 163 mg/kg for timothy hay alone to 244 mg/kg for timothy hay with oats fed at 0.4% BW. Horses consuming the alfalfa diets exceeded N intake by 2 to 2.5-fold that of NRC (2007) estimates. Despite the wide differences in N intake, N retention only differed in horses fed A3. Gibbs et al. (1998) reported increased N retention of ponies fed high- compared to low-protein alfalfa or coastal bermudagrass and suggested that the increase in N retention was a result of replenishing N stores depleted upon surgical ileal cannulation. Nitrogen retention in horses fed coastal bermudagrass with a corn-oat concentrate also increased in parallel to an incremental workload (Freeman et al., 1988). In our study, N retention was expected to be near equilibrium as all horses were mature, performed light to moderate exercise, and exhibited no change in body weight. It is unclear as to why horses fed the third cutting alfalfa were in positive balance. It is possible that intake of large quantities of proteins from forage feeds require a much longer adaptation period to reach N equilibrium. In contrast to N retention, urinary volume and urinary N excretion differed markedly between the alfalfa diets and timothy,

with a 3- to 4-fold higher urinary volume and 4- to 5-fold higher urinary N excretion, indicating a decrease in utilization efficiency of protein when feeding alfalfa at 1.6% of the horse's BW. In contrast, feeding timothy with oats included at either 0.2% of 0.4% of the horse's BW had minimal effect on urine volume; in fact, oats inclusion markedly enhanced N digestibility without increasing N excretion, suggesting improvement in post-gut N utilization.

As expected, when expressed as g AA/100 g hay, AA concentration of timothy hay was lower than any of the alfalfa hays. Total AA composition of timothy and alfalfa hay was similar to previously reported values of AA composition of timothy hay expressed as percent of DM (Muscato et al., 1983) and to timothy and alfalfa hay expressed as percent of CP (Teceschi et al., 2001). Amino acid composition of timothy and alfalfa hay CP (i.e., g AA/g N × 6.25) was remarkably similar; however, when expressed as g AA/100 g hay, lysine was 3.1- to 3.7-fold lower in G compared to alfalfa. Thus, timothy fed alone at 1.6% of the horse's BW met requirement for CP (NRC, 2007), yet provided only 50% of the lysine requirement estimate for exercised horses. Addition of oats at 0.2% BW to timothy hay increased daily ration lysine content to 24.5 g, which met the lysine requirement for horses at maintenance NRC (2007); addition at 0.4% BW increased daily lysine content to 30.0 g, meeting requirement for light exercise, but not moderate, exercise. Alfalfa AA composition of CP was similar across all maturities, indicating that the protein quality of alfalfa is a function of protein digestibility rather than AA composition and post-gut N utilization; thus, intake of alfalfa hay alone at any maturity exceeded lysine requirements for light to moderate exercising horses by as much as 50%; hence, low protein timothy hay fed alone may be provided at 2.5% BW in order to meet the horse's requirement for lysine rather than for protein over a prolonged time period or fed in conjunction with oats if hay supply is limited.

Profiling serum AA concentrations post-feeding allowed additional qualitative assessment of N utilization of hays differing in apparent N digestibility. Serum AA concentrations in horses have previously been related to dietary AA composition (Reitnour et al., 1970; Wilson and Graham-Thiers, 2009; Graham-Thiers and Bowen, 2009), but very little information is available on serum AA response to forage feeding alone in horses (Graham-Thiers and Bowen, 2009). Consistent with the higher AA concentration and N digestibility of alfalfa compared to timothy hay, serum AA concentrations increased postprandially in horses fed alfalfa, reaching a peak at approximately 2-h post-feeding. Johnson and Hart (1974) also reported AA concentrations to increase within two hours and to return to basal within four hours following feeding of a pelleted diet consisting of alfalfa, orchardgrass hay, wheat straw, barley, oats, and soybean. This response was also seen for timothy hay, with apparent peak in AA serum concentrations at 2-h post feeding. Because passage rate through the small intestine ranges from 2 to 4 hours for grains and forages, respectively, our results and those of Reitnour et al. (1970) indicate the small intestine plays a significant role in dietary AA absorption of forage proteins in the horse. However, DePew et al. (1994) reported a secondary spike in plasma indispensable AA concentrations 6 h after feeding a diet consisting of bermudagrass hay plus concentrate, indicating contribution of the hindgut to AA absorption. In the current study, serum AA concentration was profiled over a 4-h window, which may have been insufficient to detect blood AA appearance, if any, from the hindgut.

In conclusion, we found that whole tract apparent N digestibility of alfalfa hay is higher than timothy hay, digestibility decreases with increasing maturity, and that high lignin and hemicelluloses content may lead to low N digestibility. The AA composition of timothy hay was low compared to alfalfa, and AA did not change with increasing maturity in alfalfa. Low lysine

content of timothy hay could indicate that timothy alone is not a sufficient source of protein when fed at 2% BW; hence, the addition of oats to low protein feeds may be necessary to insure AA requirements are met. Most serum AA concentrations in horses fed the forage diets exhibited a curvilinear response to feeding, with peak absorption occurring approximately 2 hours post-feeding; this response signifies the small intestine plays an important role in forage AA absorption. In all, increasing urinary N excretion in horses fed alfalfa hay of increasing CP concentration (decreasing maturity) indicates that CP intake was in excess of CP requirement. When grass hay availability is not limiting, feeding grass hay may be more environmentally desirable than feeding legume hay, in particular for the pleasure horse.

Table A.1. Nutrient composition of forages fed corresponding to each balance period (DM basis)[1]

Item, %	Balance Period						Standard Deviation
	1	2	3	4	5	6	
Timothy (G)							
CP	7.3	7.2	9.1	7.5	8.1	8.2	0.7
NDF	63.7	63.5	61.5	63.0	62.0	61.8	0.9
ADF	38.7	39.7	39.2	37.1	37.2	38.2	1.1
Cellulose	34.9	36.0	35.0	32.5	32.3	32.8	1.6
Hemicellulose	25.0	23.8	22.3	26.0	24.9	23.6	1.3
ADL[2]	3.8	3.7	4.3	4.5	4.9	5.4	0.7
Alfalfa, 1st cutting (A1)							
CP	18.4	18.3	19.7	17.7	19.9	18.8	0.8
NDF	41.3	44.9	46.5	44.3	40.8	41.0	2.4
ADF	35.8	37.5	38.9	37.3	33.7	33.7	2.1
Cellulose	28.9	29.8	31.3	28.8	25.4	25.5	2.4
Hemicellulose	5.5	7.4	7.6	7.0	7.1	7.3	0.8
ADL	6.9	7.8	7.7	8.5	8.3	8.2	0.6
Alfalfa, 2nd cutting (A2)							
CP	20.6	20.8	20.8	19.5	19.9	19.9	0.5
NDF	39.8	41.7	40.9	38.1	39.8	37.7	1.4
ADF	35.1	34.1	32.8	29.5	32.0	29.6	2.3
Cellulose	29.3	27.5	25.7	23.2	24.2	22.5	2.6
Hemicellulose	4.8	7.6	8.1	9.6	7.8	8.1	1.6
ADL	5.7	6.6	7.0	6.3	7.7	7.1	0.7
Alfalfa, 3rd cutting (A3)							
CP	24.4	25.7	25.5	25.3	24.0	24.4	0.7
NDF	33.4	29.2	29.9	29.7	29.2	33.7	2.1
ADF	27.1	24.5	24.7	24.5	24.4	26.8	1.3
Cellulose	22.1	20.1	20.2	18.9	18.8	20.6	1.2
Hemicellulose	6.3	4.6	5.2	5.3	4.8	6.9	0.9
ADL	5.0	4.5	4.5	5.5	5.5	6.2	0.7

[1]Values are means of duplicate sample analyses. Samples were collected by coring each hay bale fed during each collection period and pooled per period for analyses.

[2]Acid detergent lignin.

Table A.2. Analysis and comparison of crude protein and fiber composition of forages[1]

Item, %	Timothy (G)[2]	Alfalfa (A)[3]			SEM
		A1	A2	A3	
DM	85.4	86.3	85.7	86.8	-
CP	7.9[d]	18.8[c]	20.2[b]	24.9[a]	0.7
NDF	62.6[a]	43.1[b]	39.8[c]	30.9[d]	1.8
ADF	38.3[a]	36.2[b]	32.2[c]	25.3[d]	2.0
Cellulose	33.9[a]	28.3[b]	25.4[c]	20.1[d]	2.0
Hemicellulose	24.2[a]	7.0[bc]	7.7[b]	5.5[c]	1.2
ADL[4]	4.4[c]	7.9[a]	6.7[b]	5.2[c]	0.7

[abcd]Means with uncommon superscript letters differ at $P < 0.05$.

[1]Values are LSMEANS of duplicate samples collected over each period. Samples were collected by coring each hay bale fed during each collection period and pooled per period for analyses.

[2]First cut of the season. Harvested from seven fields between June 11 and June 22, 2007.

[3]The three alfalfa harvests originated from the same field. A1 is the first cutting harvested on June 12, 2007. A2 is the second cutting harvested on July 8, 2007. A3 is the third cutting harvested on August 13, 2007.

[4]Acid detergent lignin

Table A.3. Amino acid composition of forages (g AA/100 g hay), %[1]

Item	Timothy (G)	Alfalfa (A)		
		A1	A2	A3
Alanine	0.39	0.69	0.77	0.89
Arginine	0.36	0.72	0.91	0.96
Aspartate	0.21	0.57	0.70	0.73
Cystine	0.03	0.04	0.04	0.04
Glutamate	0.45	0.84	0.97	1.05
Glycine	0.26	0.52	0.56	0.67
Histidine	0.12	0.36	0.40	0.46
Isoleucine	0.24	0.53	0.60	0.68
Leucine	0.41	0.86	0.97	1.18
Lysine	0.24	0.73	0.76	0.89
Methionine	0.07	0.11	0.12	0.15
Phenylalanine	0.27	0.55	0.63	0.76
Proline	0.36	0.78	0.95	1.36
Serine	0.22	0.48	0.50	0.51
Threonine	0.28	0.62	0.69	0.77
Tyrosine	0.14	0.28	0.31	0.37
Valine	0.31	0.66	0.73	0.89

[1]Values are means of duplicate sample analyses. Samples were collected by coring each hay bale fed during each collection period and pooled per period for analyses.

Table A.4. Amino acid composition of forages (g AA/g N × 6.25), %[1]

Item	Timothy (G)	Alfalfa (A)		
		A1	A2	A3
Alanine	4.9	3.6	3.8	3.6
Arginine	4.5	3.8	4.5	3.9
Aspartate	2.7	3.0	3.4	2.9
Cystine	0.4	0.2	0.2	0.2
Glutamate	5.7	4.5	4.8	4.2
Glycine	3.3	2.7	2.8	2.7
Histidine	1.5	1.9	2.0	1.9
Isoleucine	3.0	2.8	3.0	2.7
Leucine	5.2	4.6	4.8	4.8
Lysine	3.0	3.9	3.7	3.6
Methionine	0.9	0.6	0.6	0.6
Phenylalanine	3.4	2.9	3.1	3.1
Proline	4.5	4.2	4.7	5.5
Serine	2.7	2.5	2.5	2.0
Threonine	3.6	3.3	3.4	3.1
Tyrosine	1.7	1.5	1.5	1.5
Valine	3.9	3.5	3.6	3.5

[1]Values are means of duplicate sample analyses. Samples were collected by coring each hay bale fed during each collection period and pooled per period for analyses.

Table A.5. Nitrogen balance and utilization of horses fed timothy grass hay (G) and alfalfa hay harvested at different times in the summer season (A1, A2, A3)

Item	Diets				SEM
	G	A1	A2	A3	
DM intake, kg/d	6.1	6.1	5.9	6.0	0.1
Fecal DM output, kg/d	3.4[a]	3.0[b]	2.9[b]	2.7[b]	0.2
Urine volume, L/d	3.2[c]	8.3[ab]	7.8[b]	10.8[a]	1.1
N intake, mg/kg BW	163.2[d]	393.2[c]	409.5[b]	518.8[a]	7.7
N fecal, mg/kg BW	101.0	104.1	104.3	114.0[†]	5.2
N urine, mg/kg BW	83.3[c]	251.4[b]	264.8[ab]	326.6[a]	30.2
N absorbed, mg/kg BW	62.1[d]	289.1[c]	305.2[b]	404.9[a]	9.3
N retained, mg/kg BW[1]	-21.2	37.8	40.4	78.3	32.6
N digestibility, %	38.0[c]	73.4[b]	74.5[ab]	78.1[a]	2.2

[abcd]Means with uncommon superscript letters differ at $P < 0.05$.

[†]Tendency toward significantly different from G at $P < 0.1$.

[1]N retention differed from zero for diet A3 at $P < 0.05$.

Table A.6. Nitrogen balance and utilization of horses fed timothy grass hay alone (G) and timothy grass hay supplemented with oats at 0.2% BW (G1) and 0.4% BW (G2)

Item	Diets			SEM
	G	G1	G2	
DM intake, kg/d	6.1[c]	7.0[b]	7.7[a]	0.1
Fecal DM output, kg/d	3.4	3.6	3.8	0.2
Urine volume, L/d	3.2	3.5	4.6	1.1
N intake, mg/kg BW	163.2[c]	205.9[b]	244.2[a]	7.7
N fecal, mg/kg BW	101.0[b]	107.8[ab]	115.3[a]	5.2
N urine, mg/kg BW	83.3	92.9	131.0	30.2
N absorbed, mg/kg BW	62.1[c]	98.1[b]	128.9[a]	9.3
N retained, mg/kg BW[1]	-21.2	5.2	-2.1	32.6
N digestibility, %	38.0[b]	47.1[a]	52.6[a]	2.17

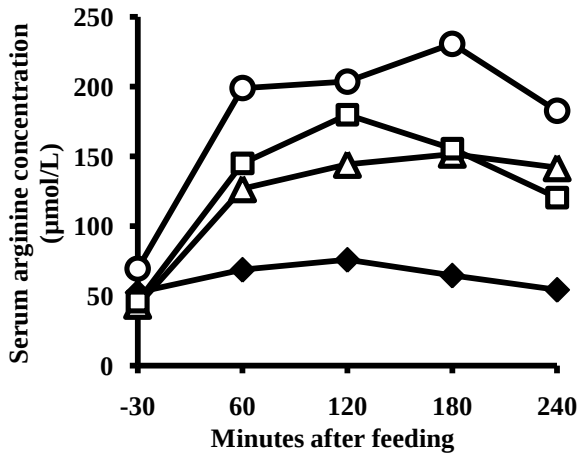
[abc]Means with uncommon superscript letters differ at $P < 0.05$.

[1]N retention did not differ from zero.

Figure A.1. Serum essential amino acid concentrations pre- and post-feeding from horses fed diets consisting of timothy grass hay (G, ◆), first cutting alfalfa (A1, △), second cutting alfalfa (A2, □), or third cutting alfalfa (A3, ○). Time -30 indicates basal serum amino acid concentrations. In each accompanying table, at each time point, ** and * indicate different from G at $P < 0.01$ and $P < 0.05$, respectively. Tendencies towards significantly different from G ($P < 0.1$) are indicated by †. Panels are as follows: arginine (Panel A, SEM = 27.41), histidine (Panel B, SEM = 5.83), isoleucine (Panel C, SEM = 18.05), leucine (Panel D, SEM = 28.60), lysine (Panel E, SEM = 6.22), methionine (Panel F, SEM = 3.80), phenylalanine (Panel G, SEM = 5.25), threonine (Panel H, SEM = 18.16), tryptophan (Panel I, SEM = 4.60), and valine (Panel J, SEM = 43.74).

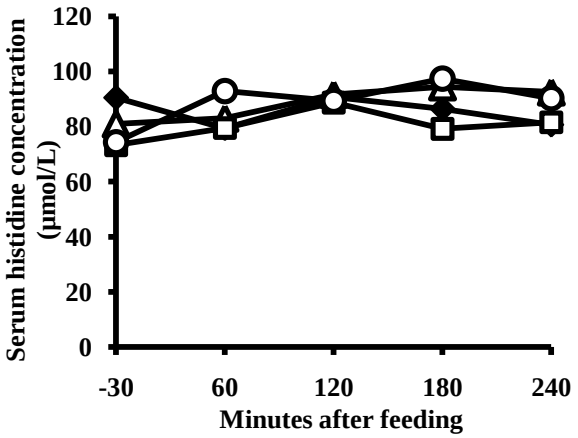
Figure A.1.

A.



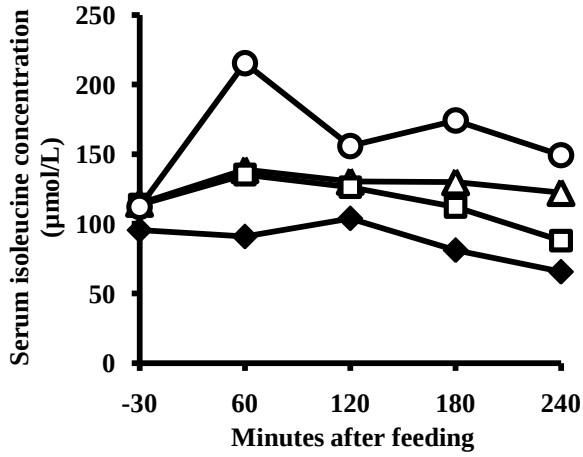
Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.93	0.53
A1 △	ns	†	*	*	0.10	0.23
A2 □	*	**	*	†	0.22	0.20
A3 ○	**	**	**	**	<.01	<.01

B.



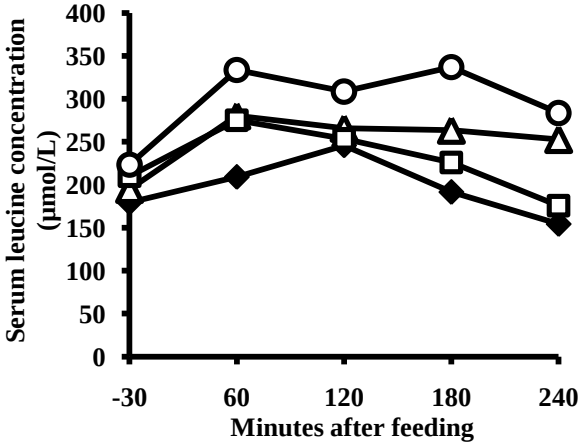
Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.70	0.60
A1 △	ns	ns	ns	ns	0.05	0.53
A2 □	ns	ns	ns	ns	0.55	0.32
A3 ○	ns	ns	ns	ns	0.07	0.10

C.



Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.11	0.29
A1 △	†	ns	†	*	0.48	0.13
A2 □	†	ns	ns	ns	0.25	0.02
A3 ○	**	*	**	**	0.22	<.01

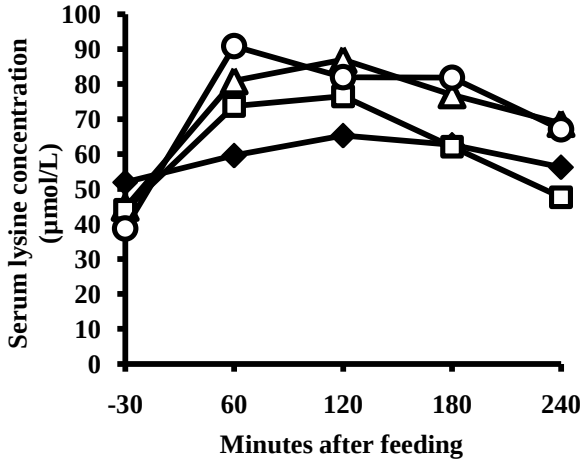
D.



Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.32	0.01
A1 △	†	ns	†	*	0.12	0.02
A2 □	ns	ns	ns	ns	0.22	<.01
A3 ○	**	ns	**	**	0.02	<.01

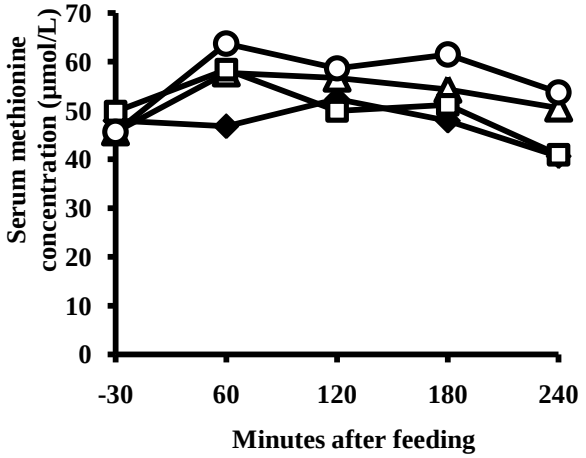
Figure A.1. (Cont'd)

E.



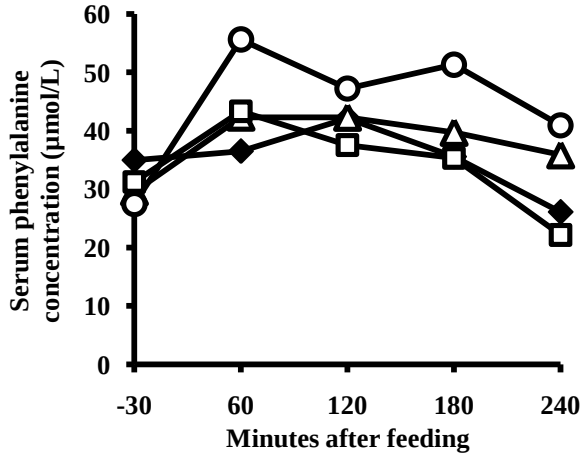
Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.08	<.01
A1 △	*	*	†	ns	<.01	<.01
A2 □	†	ns	ns	ns	0.57	<.01
A3 ○	**	†	*	ns	0.05	<.01

F.



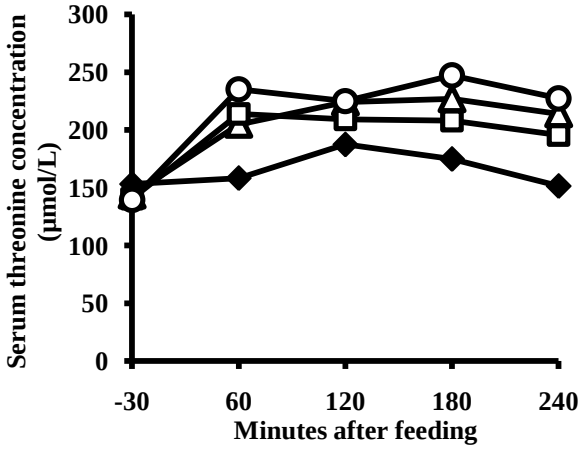
Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.22	0.04
A1 △	*	ns	ns	†	0.64	<.01
A2 □	*	ns	ns	ns	0.03	<.01
A3 ○	**	ns	*	*	0.18	<.01

G.



Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.30	<.01
A1 △	ns	ns	ns	ns	0.50	0.02
A2 □	ns	ns	ns	ns	0.05	<.01
A3 ○	*	ns	*	*	0.15	<.01

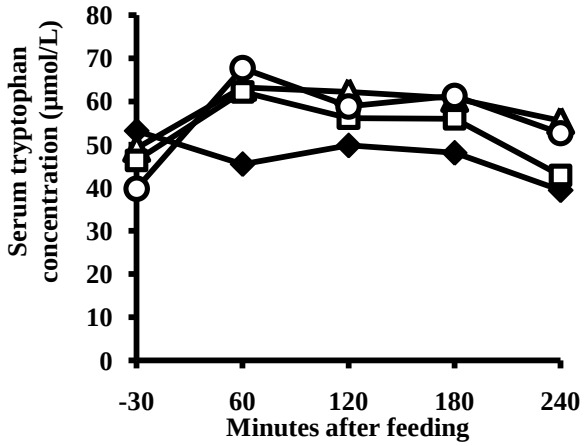
H.



Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.37	0.02
A1 △	†	ns	*	*	<.01	<.01
A2 □	†	ns	ns	ns	0.55	<.01
A3 ○	**	ns	**	**	<.01	<.01

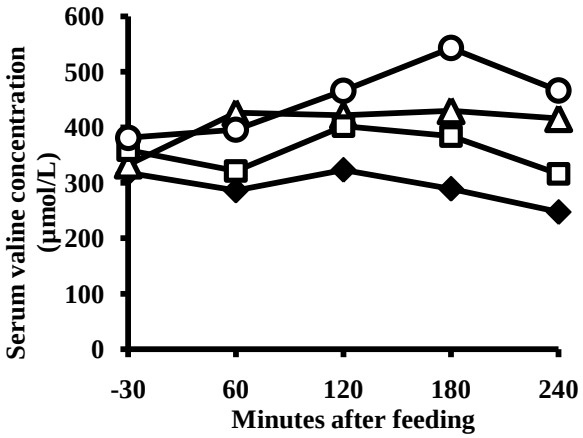
Figure A.1. (Cont'd)

I.



Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.20	0.19
A1 △	**	†	†	*	0.25	<.01
A2 □	*	ns	ns	ns	0.19	<.01
A3 ○	**	ns	*	*	0.62	<.01

J.



Item	Minutes after Feeding				*P*-value	
	60	120	180	240	L	Q
G ◆					0.23	0.51
A1 △	*	ns	*	**	0.11	0.11
A2 □	ns	ns	ns	ns	0.87	0.14
A3 ○	†	*	**	**	<.01	0.08

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APPENDIX B

Woodward, A. D.[*], M.Z. Fan[†], R. J. Goer[‡], N. P. Taylor[*], and N. L. Trottier[*1]. 2010. Kinetic analysis and comparison of D-glucose transport into procine and equine jejunal and colonic brush border membrane vesicles. In preparation for submission to the *Journal of Animal Science*. Submitted to co-authors for review.

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APPENDIX B

KINETIC ANALYSIS AND COMPARISON OF D-GLUCOSE TRANSPORT INTO PORCINE AND EQUINE JEJUNAL AND COLONIC BRUSH BORDER MEMBRANE VESICLES

Abstract

To test the hypotheses that glucose is transported across the large colon mucosa apical membrane at a lower capacity to that of the small intestinal mucosa in both the pig and pony and that glucose transport capacity across the intestine of the pony is lower than that of the pig, we examined transport *in vitro* using brush border membrane vesicles (BBMV). Mucosa was collected from the porcine (n = 3) and equine (n = 4) jejunum and large colon, flash-frozen in liquid nitrogen, and stored at -80°C. Jejunal and colonic BBMV were manufactured by Mg^{2+} precipitation and used to determine initial rates and kinetics of D-glucose transport into apical epithelia by rapid filtration technique in Na^{+}-gradient incubation buffer. No active uptake of glucose was noted in the equine colon. Initial rates of D-glucose uptake were higher in pig jejunum compared to pig colon ($P < 0.001$) and to pony jejunum ($P < 0.001$). Further analysis of results indicated D-glucose uptake was mediated by both a Na^{+}-dependent saturable component and diffusion. In the pig, D-glucose capacity of 571 ± 331 ρmol · mg protein^{-1} · s^{-1} and affinity of 0.54 ± 0.07 mM in the colon was lower than 2595 ± 331 ρmol · mg protein^{-1} · s^{-1} capacity and 0.09 ± 0.07 mM affinity in the jejunum ($P < 0.05$). In the pony jejunum, D-glucose capacity of 655 ± 286 ρmol · mg protein^{-1} · s^{-1} was lower than pig jejunum ($P < 0.01$), while pony jejunal affinity of 0.27 ± 0.06 mM was not different than pig jejunum ($P = 0.11$). Diffusion was lower

in both the pig colon and pony jejunum compared to the pig jejunum ($P < 0.01$). These results demonstrate that the pig jejunum is capable of glucose transport at a higher capacity and lower affinity than either pig colon or pony jejunum. Further, the equine large colon is incapable of D-glucose transport across the apical epithelial membrane, thus indicating the equine small intestine importance in glucose clearing.

Key words: D-glucose, transport kinetics, equine, porcine, intestine

Introduction

Equids are obligate hindgut fermenters; hence, they rely on dietary forages and are equipped with a large cecum and voluminous proximal colon for microbial fermentation of cellular fiber components. When fed a diet high in soluble carbohydrates, glucose absorption in the small intestine must be efficient in order to minimize the flow of glucose into the cecum and colon. The highly specific Na^{+}-dependent SGLT1 transporter, the main vehicle for movement of monosaccharides glucose and galactose from the intestinal lumen to the epithelial cell cytosol, is located in the brush border membrane (BBM; apical membrane) of the equine small intestine and capable of increased expression in horses presented with increased soluble carbohydrate-based diets (Dyer et al., 2000; Dyer et al., 2009). However, SGLT1 is a small capacity glucose transporter relative to GLUT-2, a high capacity Na-dependent transporter, which is inserted in the BBM of rats immediately following a meal (Kellett and Helliwell, 2000), but to this date has not been found along the apical membrane of equine intestine.

Excess soluble carbohydrate ingestion in equids frequently results in the flow of surplus soluble carbohydrates to the cecum and large colon, often leading to rapid fermentation of these soluble carbohydrates and high levels of lactic acid and bacterial-derived endotoxin production,

and laminitis. While the microbial events leading to equine carbohydrate-induced laminitis have been chronicled (Milinovich et al., 2010), mechanisms of glucose absorption in the large colon have not been documented. In other hind-gut fermenters, such as suids consumption of soluble carbohydrate-rich diets are not associated with distal GIT disturbances. Compared to equids, suids have a relatively slower passage rate through the stomach and small intestine and have evolved on the consumption of highly diverse diets, including tubers and roots in addition to forages. Hence, suids likely have a higher capacity for small intestinal soluble carbohydrate absorption than their equine counterpart, which have solely evolved on grass and low-quality forages (Ralston, 1984). However, the possibility for glucose absorption and portal clearance from the large colon has not been explored in suids. Although the majority of dietary glucose should be absorbed from the small intestine in both species, there are no studies reporting on the ability of the large colon to transport luminal glucose in either suids or equids. We have recently demonstrated that the equine large colon has very limited transcript abundance of glucose transporters compared to the jejunum (Taylor et al., submitted), but it is still unknown whether these transcripts are functionally expressed at the BBM.

With these notions in mind, we hypothesized that glucose is transported across the large colon mucosa apical membrane at a lower capacity to that of the small intestinal mucosa in both the pig and pony. Furthermore, we hypothesized that glucose transport capacity across the small intestinal and colonic mucosa apical membrane of the pony is lower than that of the pig. The objective of this study was to quantify the uptake of glucose across the BBM membrane of the jejunum and proximal large colon mucosa of the pony and the pig.

Materials and Methods

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Animals and Collection of Tissue

Three market pigs (Yorkshire crossbred, 122.5 ± 3.9 kg) and four mature ponies of mixed breeds were used. Pigs were grouped-housed at the MSU Swine Teaching and Research Center and were provided ad libitum access to a corn-soybean meal-diet containing 14 % CP. Pigs were transported to the Michigan State University Meat Laboratory, humanely killed for slaughter, and the entire intestinal tract was immediately removed. Approximately 20-cm long sections were obtained from the jejunum 2 m distal to the stomach and from the descending colon 30 cm distal to the cecum. Ponies were of mature age and mixed breeds and had been maintained on low quality grass hay. Ponies were provided with their last forage meal at 1800 and euthanized between 1000 and 1400 on the following day. Ponies were euthanized for reasons other than history of gastrointestinal problems. Following euthanasia, a 2.5-m incision was made along the ventral midline in order to expose the entire gastrointestinal tract. Approximately 20-cm long sections were sampled from the distal jejunum, located 12 m distal to the duodenal colic ligament, and from the left dorsal colon, located between the sternal and pelvic flexures at the lateral band.

For both species, intestinal segments were rinsed thoroughly in a 0.9% ice-cold NaCl solution and opened lengthwise to expose the mucosa. The mucosal layer was scraped from the serosal layer using a glass microscope slide, and scrapings were transferred to conical tubes until approximately 12 g were obtained. Samples were flash frozen in liquid nitrogen and stored at -80°C.

Preparation of Brush Border Membrane Vesicles

Brush border membrane vesicles were prepared using the approach of Fan et al. (1998, 2004) with the exception that mucosal scraping instead of whole mucosa was used. Briefly, mucosal scrapings (2.6 g) were homogenized over ice in homogenization buffer (52 mL; 50 mM D-mannitol, 10 mM HEPES, 0.2 mM PMSF; pH = 7.4) using a polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for three 1-min cycles. A sample of the resulting homogenate was collected for protein and enzyme analysis, and the remaining homogenate was centrifuged at 2000 × *g* for 15 min at 4°C. The supernatant was collected and the pellet discarded. A 200-mM $MgCl_2$ solution was added to the supernatant and mixed to a final concentration of 10 mM $MgCl_2$, gently shaken over ice for 15 min and centrifuged at 2400 × *g* for 15 min at 4°C. Again, the supernatant was collected and the pellet was discarded. The supernatant was divided into ultracentrifuge tubes and centrifuged at 19,000 × *g* for 30 min at 4°C. The resulting supernatant was discarded, and the remaining pellet representing the crude BBM pellet was suspended in vesicle pre-loading buffer (150 mM KSCN, 10 mM mannitol, 5 mM HEPES; pH = 7.4) using a Pasteur pipette. Samples were centrifuged for an additional 30 min at 39,000 × *g* and 4°C. The resulting supernatant was discarded and the final BBM pellet was suspended in 1 mL of vesicle pre-loading buffer. A sub-sample of the final BBMV suspension was collected for determination of protein concentration and enzyme activity. The remainder of the BBMV suspension was used for uptake experiments as described below.

Protein Concentration and Alkaline Phosphatase Enzyme Measurements

Protein concentration of the initial homogenate and the final BBMV suspension was determined with the Lowry assay (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions and using BSA as standard. Purity of BBMV was tested using

alkaline phosphatase as a marker. Alkaline phosphatase was measured according to Fan et al. (1998) using p-nitrophenyl phosphate as substrate.

Measurement of Total (Na^+-dependent and independent) D-Glucose Uptake into Brush Border Membrane Vesicles

Freshly prepared BBMV suspension was diluted with vesicle pre-loading buffer to contain 6 to 10 mg protein mL^{-1}. The final BBMV suspension remained on ice until uptake experiments were performed (not more than 6 hr after final suspension). Uptake experiments were carried out using the rapid filtration procedure as outlined by Fan et al. (1998, 2004). Uptake buffer (50 µL; 150 mM NaSCN, 10 mM mannitol, 5 mM HEPES; pH = 7.4) containing [^{3}H]-D-glucose was first loaded into polystyrene tubes and allowed to warm to room temperature (22 to 24°C). Two separate 5-µL droplets of BBMV suspension were added along the side of the tube using a Microman pipette (Gibson S.A.S., Villiers-le-Bel, France). After warming to room temperature for 10 s, uptake was initiated by a foot-switch activated vibromixer attached to an electronic timer (GraLab model 545). Uptake was terminated by immediate addition of ice-cold stop and wash buffer (1.2 mL; 150 mM KSCN, 10 mM mannitol, 10 mM HEPES, 0.1 mM $HgCl_2$; pH = 7.4) at the end of the timing cycle. Immediately after termination, 1 mL of the uptake media was collected and pipetted onto 0.22-µm nitrocellulose membrane filters (Millipore, Billerica, MA) mounted on a Manifold filtration unit (Millipore, Billerica, MA) connected to a vacuum source. Filters were immediately washed three times with 5 mL stop and wash buffer and transferred to scintillation vials pre-filled with 10 mL scintillation fluid. All filters were allowed to dissolve in scintillation fluid for 30 min before radioactivity was determined with a liquid scintillation counter (Beckman, Brea, CA). Remaining uptake media in

the polystyrene tubes was pooled per substrate concentration, and 10 μL counted to determine the average initial radioactivity.

Time course experiments were performed under a Na^+-gradient using pig and horse jejunum and pig colon and the resulting incubation time used for all kinetic uptake experiments. Non-specific binding of glucose to BBMV and filters was corrected for time course experiments by subtracting time-zero radioactivity counting. To eliminate non-specific binding of substrate to filter during kinetic analysis, uptake buffer without the addition of BBMV was treated with stop and wash buffer, filtered, and washed as mentioned previously. Uptake buffers for kinetic experiments consisted of replacing mannitol with glucose at the following concentrations: 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mM for pig and 0.1, 0.25, 0.5, 1.0, 2.5 for pony.

Each uptake experiment was conducted in triplicate. Fourteen different batches of BBMV prepared from the mucosal scrapings of the small and large intestine of three pigs and four ponies were used to conduct 14 separate uptake experiments, with small and large intestinal glucose transport from a single animal measured on the same day. Composition of incubation buffer is described in detail in figure legends.

Calculations to Determine Glucose Kinetics

To determine uptake at various time points and under Na^+-dependent conditions, the following equation was used:

$$J = [((R_F - R_B) \times S)/R_I]/(W \times T)$$

where, J = the initial rate of total glucose uptake into BBMV (ρmol mg protein^{-1} s^{-1}); R_F = the radioactivity in disintegration per min of filters (DPM/filter); R_B = the radioactivity for non-specific binding to filters (DPM/filter); S = the extravesicular glucose concentrations (mM);

R_I = the radioactivity in the uptake media (DPM/μL); W = the amount of membrane protein provided for the incubations (mg protein); and T = the time of incubation for initial uptake (s).

Kinetic parameters, namely V_{max} and K_M, were analyzed according to the method of Wolffram et al. (1986) using a computer program for multiparameter curve fitting based on the following two-component equation:

$$J = \frac{V_{max}[S]}{K_M + [S]} + D[S]$$

where, J = the initial rate of glucose uptake into BBMV (ρmol mg protein^{-1} s^{-1}); V_{max} = the maximal transport rate (ρmol mg protein^{-1} s^{-1}); S = the extravesicular glucose concentrations (mM); K_M is the Michaelis constant (50% saturation; mM); and D = the diffusion component.

Statistical Analysis

The initial rate of glucose uptake into BBMV (J, ρmol mg protein^{-1} s^{-1}) at each glucose concentration was analyzed using the PROC MIXED procedure (SAS version 9.0, SAS Institute, Inc., Cary, NC). Equine colonic data was omitted from statistical analysis because data did not conform to the multiparameter analysis software used. The model included the fixed effects of animal species, intestinal segment, and D-glucose concentration, the interaction between species and intestinal segment, and the random effect of individual animal nested within species. Kinetic parameters, i.e., V_{max} and K_M, were analyzed using the PROC MIXED procedure (SAS version 9.0, SAS Institute Inc., Cary, NC). The model included the interaction between species and intestinal segment, and the random effect of individual animal nested within species. Results are

reported as LSMEANS ± SEM. Significant and marginally significant differences in V_{max} and K_M were determined at $P < 0.05$ and $P < 0.1$, respectively.

Results

Membrane Purity

Comparison of alkaline phosphatase enzyme activity in the BBMV relative to the mucosal scraping tissue homogenate yielded an average enrichment factor of 4.55 ± 0.43 fold, indicating the BBMV had little contamination by the basolateral membrane. This value falls within the reported range of alkaline phosphatase activity enrichment in BBMV compared to crude homogenate, from 2.3-fold in Aedes aegypti larvae mid-gut (Abdul-Rauf and Ellar, 1999) to 31.1-fold in rat jejunum (Hopfer et al., 1973); it is close to the 5.1-fold enrichment in bovine jejunum and ileum (Wilson and Webb, 1990).

Initial Time Course of D-Glucose Uptake into Brush Border Membrane Vesicles

For statistical analysis, time course experiments of D-glucose uptake into porcine jejunal ($n = 3$) and colonic ($n = 1$) and equine jejunal ($n = 2$) BBMV were combined to achieve a total of six animal observations. Equine colonic data was omitted because data did not conform to the multiparameter analysis software used. Time course experiments indicated D-glucose uptake was linear up to 20 s at D-glucose concentrations of 0.001 (Figure B.1). Because D-glucose is transported rapidly into BBMV (Hopfer et al., 1973; Lücke et al., 1978; Wolffram et al., 1986; Wood et al., 2000; Dyer et al., 2002), an incubation time of 3 s was used to measure the initial uptake of D-glucose. Our time course results are in agreement with Wolfram et al. (1986) and Dyer et al. (2002) who used a incubation time of 3 s or less to measure glucose kinetics in jejunum of pig and horse, respectively.

Initial Rate of D-Glucose Uptake into Brush Border Membrane Vesicles

Initial rates of total D-glucose (*J*) uptake in BBMV of porcine and equine small intestine and large colon under various glucose concentrations were calculated from Eq. (1) and shown in Figures B.2 and B.3, respectively. No active uptake of glucose was noted in the equine colon. At D-glucose concentrations of 0.001 mM, total uptake was not different between pig jejunum and pig colon or between pig jejunum and pony jejunum. At all other concentrations, total D-glucose uptake was higher in pig jejunum compared to pig colon ($P < 0.001$) and to pony jejunum ($P < 0.001$).

Kinetics of D-Glucose Uptake into Brush Border Membrane Vesicles

To determine glucose kinetic parameters, total glucose uptake was divided into a saturable and a diffusion component as determined in Eq. (2) (Wolffram et al., 1986). Calculated kinetic parameters V_{max}, K_M, and diffusion are presented in Table B.1. In the pig, V_{max} and K_M of glucose uptake was higher in the jejunum compared to the colon ($P < 0.05$). Transport capacity, V_{max}, was lower ($P < 0.01$) in the pony compared to the pig jejunum. Transport affinity, K_M, was not different between pony and pig jejunum ($P = 0.11$). Diffusion was lower in both the pig colon and pony jejunum compared to that of the pig jejunum ($P < 0.01$).

Discussion

The main objective of this work was to determine the uptake of glucose across the apical membrane of the jejunum and proximal large colon mucosa of the pony and the pig using BBMV. Brush border membrane vesicles have been an effective tool for characterizing glucose

transport kinetics in a host of animals species, including humans (Lücke et al., 1978; Harig et al., 1989; Malo and Berteloot, 1991), rabbits (Kaunitz and Wright, 1984; Ikeda et al., 1989), pig (Wolffram et al., 1986), sheep (Wolffram et al., 1986; Wood et al., 2000), cattle (Kaunitz and Wright, 1984; Wood et al., 2000), chicken (Garriga et al., 1999; Gal-Barber et al., 2000), and horses (Dyer et al., 2002; Dyer et al., 2009). This study is the first to compare kinetics of glucose transport across jejunal BBMV between suids and equids and to determine kinetics of glucose uptake across the apical colonic BBM. We recently demonstrated the equine colon has very low mRNA abundance of glucose transporters compared to the jejunum (Taylor et al., submitted); hence, the absence of active uptake in the pony colon was not surprising.

Glucose is transported into the intestinal cell through the apical membrane through the Na^+-dependent SGLT1 or by diffusion (Kaunitz and Wright, 1984; Stevens et al., 1984; Storelli et al., 1986; Wolffram et al., 1986). Glucose uptake via diffusion component was noticeably higher in pig jejunum in our study compared to Wolffram et al. (1986). In pig jejunum, only 78 ρmol glucose · mg protein^{-1} · s^{-1} was explained by diffusion (Wolffram et al., 1986), which is closer to the diffusion parameters of pig colon and pony jejunum reported here. Diffusion of glucose into BBMV ranges from a low of 18 ρmol · mg protein^{-1} · s^{-1} in bovine jejunum (Kaunitz and Wright, 1984), to 24 and 27 ρmol · mg protein^{-1} · s^{-1} in eel (Storelli et al., 1986) and sheep jejunum (Wolffram et al., 1986), respectively, to a high of 63 ρmol · mg protein^{-1} · s^{-1} in rabbit jejunum (Kaunitz and Wright, 1984). The reason for the high glucose diffusion in this study is unknown and could most likely be due to disruption of the BBM extracellular matrix during preparation, increasing membrane permeability (Fan et al., 1998; Fan et al., 2001); therefore, it is unlikely such diffusion rates would occur *in vivo*.

Transport capacity across the pony jejunum BBMV was 4-fold lower and the affinity 3-fold higher than that of the pig jejunum. Wolffram et al. (1986) reported Na^+-gradient glucose transport capacity of 398 ρmol · mg protein^{-1} · s^{-1} and affinity of 0.69 mM in pig jejunum, which is 6-fold lower and higher, respectively, than our values in pig jejunum. Our pony V_{max} value of 655 is in close agreement with that of Dyer et al. (2002) for the horse ileum, who reported V_{max} of 698 ρmol · mg protein^{-1} · s^{-1}. The K_M for glucose transport across the pony jejunum is lower than the previously reported value of 0.48 mM in the horse (Dyer et al., 2002; Dyer et al., 2009). However, in those studies (Dyer et al., 2002; Dyer et al., 2009), the diffusion component was not estimated. When including the diffusion component, transport capacity increases to 1250 ρmol · mg protein^{-1} · s^{-1}, which is similar to that of BBMV manufactured from duodenum (1312 ± 142 ρmol · mg protein^{-1} · s^{-1}), jejunum (918 ± 73 ρmol · mg protein^{-1} · s^{-1}), and ileum (698 ± 61 ρmol · mg protein^{-1} · s^{-1}) of horses maintained on a grass-hay only diet (Dyer et al., 2002). The lower K_M value, indicating higher transport affinity, could also be an artifact of the diffusion component, as K_M increased to 0.74 ± 0.46 mM and is similar to reported values (Dyer et al., 2002; Dyer et al., 2009) when diffusion is included in the kinetic values.

Nonetheless, variation in glucose transport kinetics depends on several factors, including diet, age, and BBMV preparation and incubation (Ferraris et al., 1993; Dyer et al., 2002; Fan et al., 2001). Glucose transporter SGLT1 protein abundance is increased 2- and 5-fold in the jejunum and ileum, respectively, of horses fed a grain-based diet compared to those fed solely grass-hay (Dyer et al., 2009). Furthermore, given the recognized higher incidence of diet-

induced colic and laminitis in ponies, it is not unlikely that the V_{max} for glucose transport across BBMV could be lower in ponies compared to horses. The difference in transport capacity of the pig jejunum in our study compared to previous reports is not as easily explained, as animal diet and age was not reported (Wolffram et al., 1986). In our study, pigs were maintained on a highly digestible corn-soybean meal-based diet; hence, a higher V_{max} in pig jejunum compared to pony jejunum would be expected. However, pigs in our study were deprived of any access to feed for at least 14 h prior to euthanasia. On the other hand, when comparing our pig values to those of horses maintained on a corn and oat-based diet, the reported V_{max} of 2883 ρmol · mg protein^{-1} · s^{-1} in horse jejunum (Dyer et al., 2009) is similar to our pig jejunum value. However, pig jejunal total V_{max} value increases to 8,877 ρmol · mg protein^{-1} · s^{-1} when the diffusion component is included. Therefore, the high transport capacity found in pig jejunum in this study may be related to the highly digestible corn-soybean meal-based diet. Furthermore, glucose transporter expression both at the transcript and protein level appears to be regulated on a short-term basis (Dyer et al., 2009); thus, short-term feed restriction would allow for basal comparison of glucose uptake between pigs and ponies.

Our values of glucose transport capacity and affinity fall in the range of those reported for other monogastric animals (Table B.2), being most similar to human jejunum (Harig et al., 1989; Malo and Berteloot, 1991). Transport kinetics in the pig jejunum were slightly higher than what others have reported; however, the pig is able to adapt to a wide variety of dietary ingredients. Thus, the high values for glucose transport capacity and affinity are most likely reflective of the consumption of a high soluble carbohydrate diet.

As expected, our glucose transport capacity estimates for the pig and pony are remarkably higher than those for ruminant animals, with bovine jejunum V_{max} ranging from 0.52 to 146 ρmol · mg protein^{-1} · s^{-1} (Wood et al., 2000; Kaunitz and Wright, 1984) and ovine jejunum V_{max} ranging from 2.7 to 31.7 ρmol · mg protein^{-1} · s^{-1} (Wood et al., 2000; Wolffram et al., 1986). This is most likely reflective of the fact that little glucose enters the small intestine of ruminants due to fermentation of carbohydrates into volatile fatty acids (Van Soest, 1994). One surprising finding of this study is the similarity of glucose transport kinetics between the pig colon and pony jejunum. The pig jejunum appears extremely efficient in clearing glucose from the intestinal lumen, leaving little absorption of glucose in the colon. Nonetheless, the pig colon capacity for glucose uptake is remarkably high. Furthermore, the near absence of glucose transporters (Taylor et al., submitted) in the pony colon coupled with only diffusional glucose uptake further confirms the critical role of the small intestine in glucose clearance in the horse in particular.

This study is the first to characterize the kinetics of glucose transport across the apical membrane of the large colon. Herein, we have determined the maximal uptake of glucose across the apical membrane of the jejunum and proximal large colon mucosa of the pig and pony. We have shown that the pony jejunum has a remarkably lower transport capacity and affinity for glucose uptake compared to the pig. We also found that the pig colon is capable of significant glucose transport, in contrast to the pony, whose large colon is completely devoid of facilitated glucose transport. Thus our results further confirm the importance of glucose clearing by the small intestine in the pony. This information contributes to the understanding of the underlying mechanisms that increase the susceptibility and risk to carbohydrate-induced laminitis in equids.

Table B.1. Kinetic parameters of D-glucose uptake in porcine and equine small (jejunum) and large (colon) intestinal brush border membrane vesicles.

Species	Segment	V_{max}[1] (ρmol mg protein^{-1} s^{-1})	K_M[2] mM	Diffusion[3] (ρmol mg protein^{-1} s^{-1})
Pig	Jejunum	2595.21 ± 330.56[a]	0.09 ± 0.07[a]	974.17 ± 86.90[a]
	Colon	571.04 ± 330.56[b]	0.54 ± 0.07[b]	171.05 ± 86.90[b]
Pony	Jejunum	655.37 ± 286.28[b]	0.27 ± 0.06[a]	201.00 ± 86.90[b]

[1]Animal species * intestinal segment differs $P = 0.01$.

[2]Animal species * intestinal segment differs $P = 0.02$.

[3]Animal species * intestinal segment differs $P < 0.01$.

[ab]Within a column, means with uncommon superscripts differ at $P < 0.05$.

Table B.2. Kinetic parameters of D-glucose uptake in monogastric small intestinal brush border membrane vesicles.

Species	Segment	V_{max} (ρmol mg protein^{-1} s^{-1})	K_M mM	Author
Human	Jejunum	931	0.86	Harig et al., 1989
Human	Jejunum	805	0.75	Malo and Berteloot, 1991
Rabbit	Jejunum	470	0.57	Ikeda et al., 1989
Calf	Jejunum	360	0.1	Wood et al., 2000
Lamb	Jejunum	602	0.04	Wood et al., 2000

Figure B.1. Time course of 0.001 mM D-glucose uptake under Na^+ gradient. Brush border membrane vesicles were pre-loaded with a buffer containing 150 mM KSCN, 10 mM mannitol, 5 mM HEPES; pH 7.4. Incubation buffer contained 150 mM NaSCN, 5 mM HEPES, pH 7.4, and mannitol:glucose at 9.99 mM D-mannitol:0.001 mM D-glucose, including 0.8 µM D-[^{3}H]glucose. Each point represents the average of triplicate observations of six animals in brush border membrane vesicles manufactured from porcine jejunal (n = 3), colonic (n = 1), and pony jejunal (n = 2) mucosal scrapings.

Figure B.1.

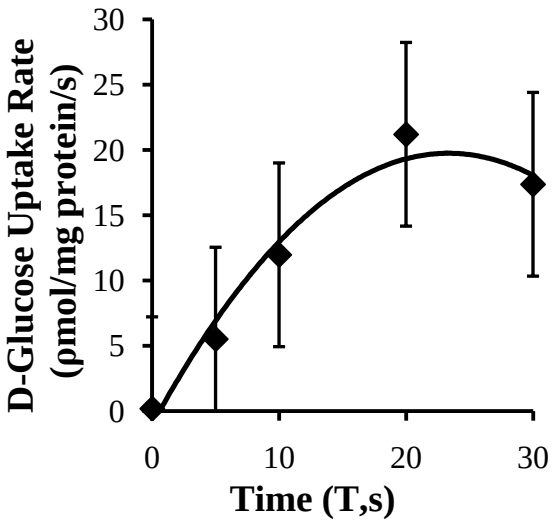
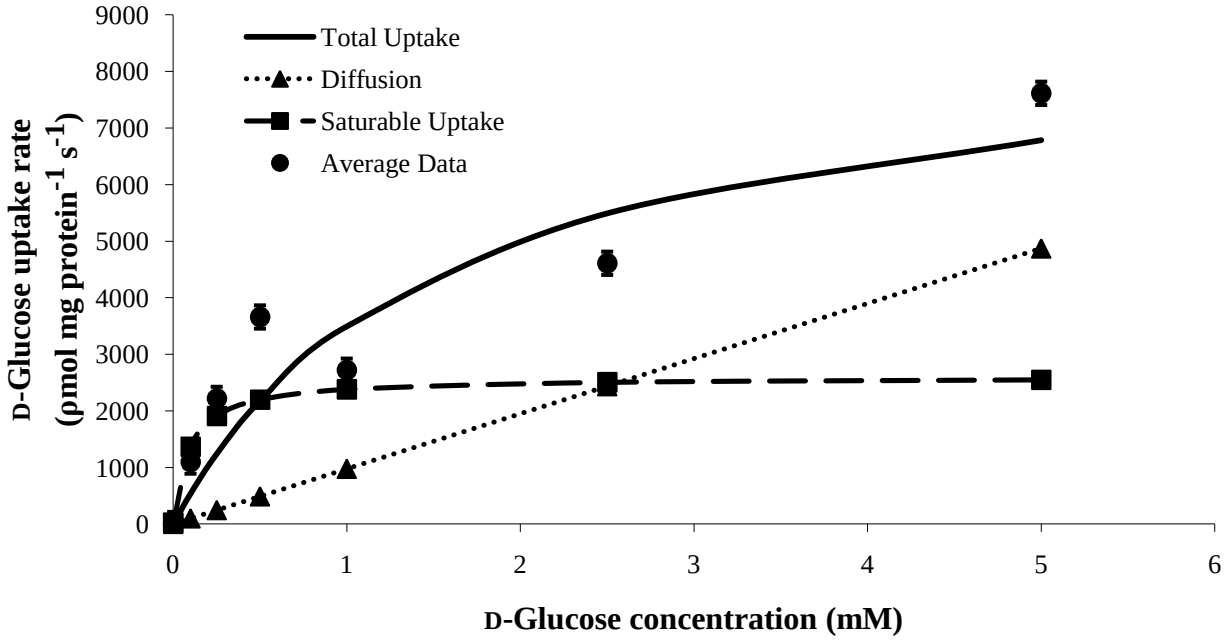

D-Glucose Uptake Rate
(ρmol/mg protein/s)
30
25
20
15
10
5
0
0
10
20
30
Time (T,s)

Figure B.2. Kinetics of D-glucose uptake into porcine jejunal (A) and colonic (B) brush border membrane vesicles. Brush border membrane vesicles were pre-loaded with a buffer containing 150 mM KSCN, 10 mM mannitol, 5 mM HEPES; pH 7.4. Incubation buffer contained 150 mM NaSCN, 5 mM HEPES, pH 7.4, 0.8 μM D-[^{3}H]glucose, and non-labelled D-glucose at 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 mM. D-Mannitol was added to maintain osmolarity. Each point represents the mean ± SEM of uptake experiments using brush border membrane vesicles manufactured from jejunum and colon mucosal scrapings of three pigs.

Figure B.2.

A.


Total Uptake
Diffusion
Saturable Uptake
Average Data
D-Glucose uptake rate (ρmol mg protein-1 s-1)
D-Glucose concentration (mM)
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
0
1
2
3
4
5
6

B.

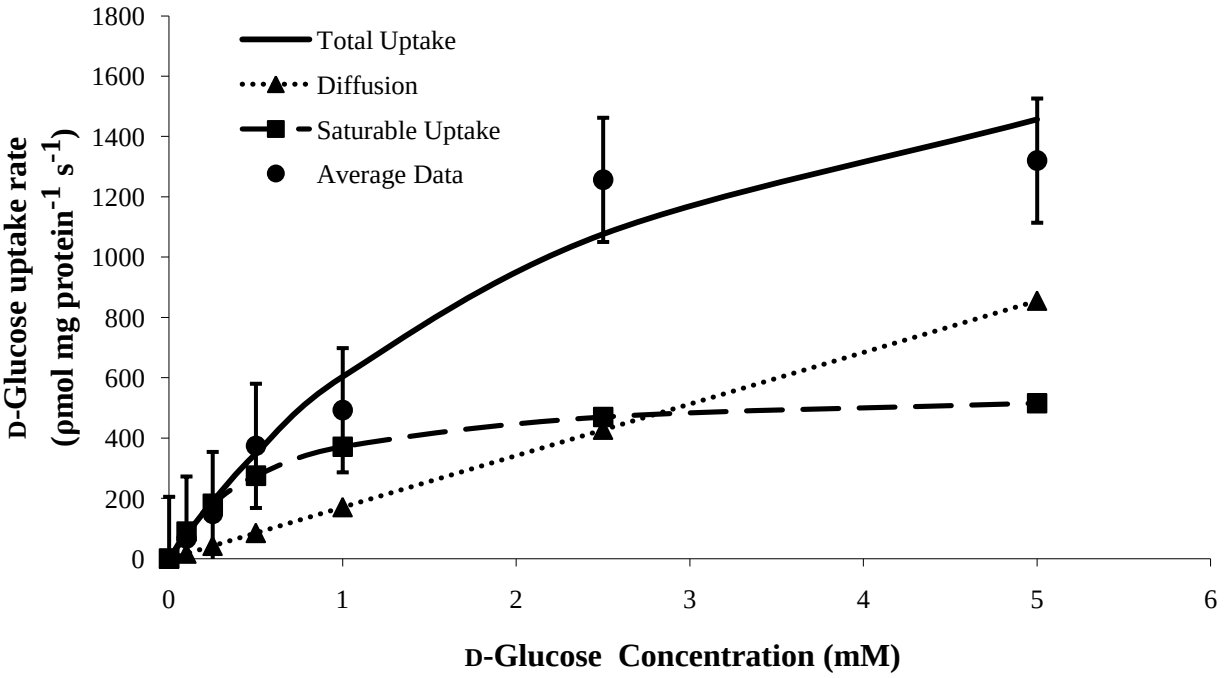
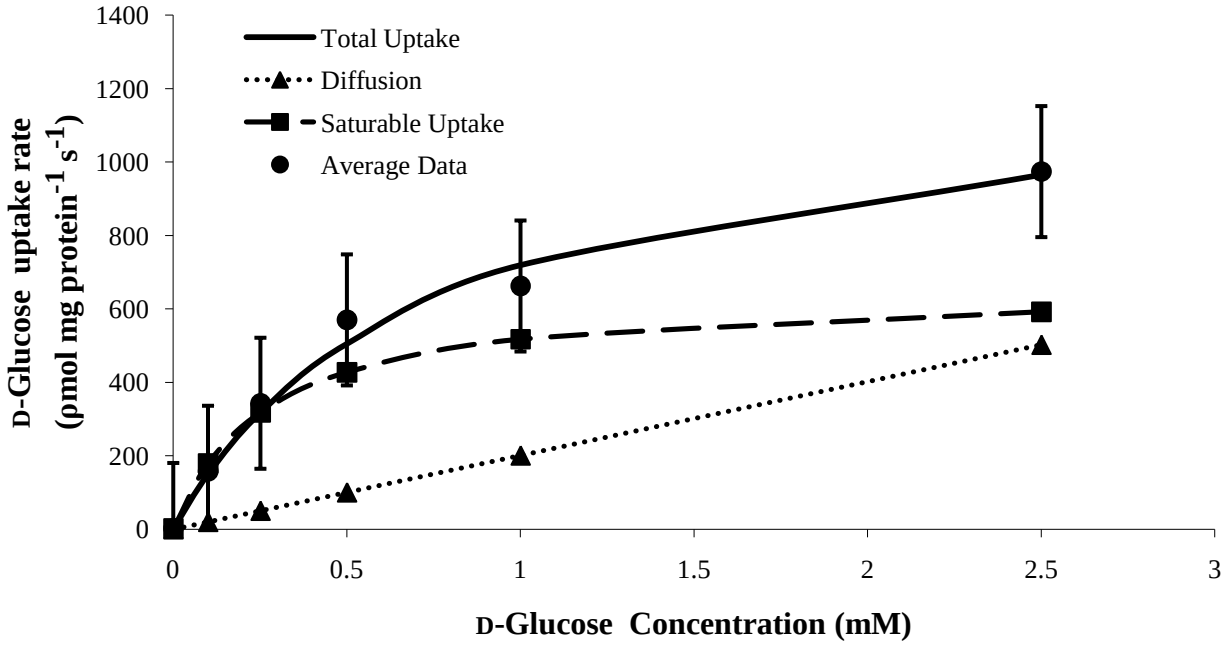

Total Uptake
Diffusion
Saturable Uptake
Average Data
D-Glucose uptake rate (ρmol mg protein-1 s-1)
D-Glucose Concentration (mM)
0
200
400
600
800
1000
1200
1400
1600
1800
0
1
2
3
4
5
6

Figure B.3. Kinetics of D-glucose uptake into equine jejunal brush border membrane vesicles. Brush border membrane vesicles were pre-loaded with a buffer containing 150 mM KSCN, 10 mM mannitol, 5 mM HEPES; pH 7.4. Incubation buffer contained 150 mM NaSCN, 5 mM HEPES, pH 7.4, 0.8 µM D-[^{3}H]glucose, and non-labelled D-glucose at 0.1, 0.25, 0.5, 1.0, and 2.5 mM. D-mannitol was added to maintain osmolarity. Each point represents the mean ± SEM of uptake experiments using brush border membrane vesicles manufactured from jejunum of four ponies.

Figure B.3.

A.


1400
1200
1000
800
600
400
200
0
0
0.5
1
1.5
2
2.5
3
Total Uptake
Diffusion
Saturable Uptake
Average Data
D-Glucose uptake rate (ρmol mg protein-1 s-1)
D-Glucose Concentration (mM)

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