DIETARY AND DEVELOPMENTAL REGULATION OF NUTRIENT TRANSPORTER GENE EXPRESSION IN THE SMALL INTESTINE OF TWO LINES OF BROILERS

by

Elizabeth Ruth Gilbert

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Dr. Eric Wong, Chair
Dr. Kenneth Webb, Jr.
Dr. Julia Dibner
Dr. Audrey McElroy
Dr. Honglin Jiang

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(ABSTRACT)

To better understand the digestive and absorptive capacities of the chick intestine so that we may feed diets that better meet the nutritional needs of the chick, it is important to understand how expression of nutrient transporter genes changes in response to various factors. A series of feeding trials were conducted to evaluate the dietary and developmental regulation of nutrient transporter mRNA abundance in the small intestine of two lines of broilers selected on corn-based (Line A) or wheat-based (Line B) diets. Abundance of mRNA was quantified in all experiments using real time PCR and the absolute quantification method. The objective of the first study was to investigate intestinal nutrient transporter and enzyme mRNA in Line A and B broilers at embryo day 18 and 20, day of hatch, and d 1, 3, 7, and 14 posthatch. Genes evaluated included the peptide transporter, PepT1, 10 AA transporters (rBAT, $b^{o,+}$AT, $ATB^{o,+}$, CAT1, CAT2, LAT1, y^+LAT1, y^+LAT2, B^oAT and EAAT3), four sugar transporters (SGLT1, SGLT5, GLUT5, and GLUT2), and a digestive enzyme, APN. For PepT1, Line B had greater quantities of mRNA compared with Line A ($P = 0.001$), suggesting a greater capacity for absorption of AA as peptides. Levels of PepT1 mRNA were greatest in the duodenum ($P < 0.05$), whereas the abundances of SGLT1, GLUT5 and GLUT2 mRNA were greatest in the jejunum ($P < 0.05$). Abundances of EAAT3, $b^{o,+}$AT, rBAT, B^oAT, LAT1, CAT2, SGLT5 and APN mRNA were greatest in the ileum ($P < 0.05$). Quantities of PepT1,

EAAT3, B^oAT, SGLT1, GLUT5, and GLUT2 mRNA increased linearly ($P < 0.01$), while CAT1, CAT2, y^+LAT1, and LAT1 mRNA decreased linearly ($P < 0.05$) with age. The objective of the second study was to evaluate the effect of dietary protein quality on intestinal peptide, AA, and glucose transporter, and digestive enzyme mRNA abundance in Line A and B broilers. At day of hatch (doh), chicks from both lines were randomly assigned to corn-based diets containing 24% crude protein (CP) with either soybean meal (SBM) or corn gluten meal (CGM) as the supplemental protein source, ad libitum. Groups of chicks from both lines were also assigned to the SBM diet at a quantity restricted to that consumed by the CGM group (SBM-RT). Abundance of PepT1, EAAT3, and GLUT2 mRNA was greater in Line B ($P < 0.03$), while APN and SGLT1 were greater in Line A ($P < 0.04$). When feed intake was equal (CGM vs restricted SBM), a greater abundance of PepT1 and $b^{o,+}AT$ mRNA was associated with the higher quality SBM ($P < 0.04$), while a greater abundance of EAAT3 and GLUT2 mRNA was associated with the lower quality CGM ($P < 0.01$). When feed intake was restricted (SBM vs SBM-RT), a greater abundance of PepT1 mRNA was associated with the restricted intake ($P < 0.04$). The objective of the third study was to determine the effect of dietary protein composition on mRNA abundance of peptide and AA transporters, and a digestive enzyme. From day 8 to day 15 posthatch, Line A and B broilers were fed equal amounts of 1 of 3 diets (24% CP). Dietary protein sources included whey protein concentrate (whey), a partial whey hydrolysate (hydro), or a mixture of free amino acids (AA) similar to the composition of whey. Intestine was collected at days 8, 9, 11, 13, and 15. Expression of all genes except LAT1 was greater ($P < 0.05$) in Line B compared with A. Abundance of PepT1, EAAT3, y^+LAT2, CAT1, $b^{o,+}AT$, and APN mRNA varied little

across diets in Line A but for CAT1 mRNA was greatest ($P = 0.005$) in Line A birds that consumed the AA diet. Expression of these genes was greatest ($P < 0.006$) in Line B birds consuming the hydro diet. A greater ($P < 0.05$) age response of $b^{o,+}AT$, EAAT3, CAT1, and APN mRNA was observed in birds consuming the hydro or AA diets relative to the whey diet. Results from these studies collectively demonstrate that nutrient transporter gene expression is responsive to a variety of factors, including developmental stage, dietary manipulation, and genetic selection. Information from these studies can be used to improve dietary formulation so that nutrient utilization is enhanced, resulting in improved growth of the broiler.

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Chapter I

Introduction

Intense selection for body weight (BW) and breast muscle yield has led to a much shorter time for broiler chickens to reach market age. The early posthatch period thus represents a greater proportion of the life of the bird. The transition from late embryogenesis to the early posthatch period is characterized by prompt development of the gastrointestinal tract allowing the bird to immediately consume nutrients after hatching. This transition is fundamental as the bird shifts from metabolism based on the lipid-rich yolk to a solid carbohydrate and protein-based diet at hatch. Although anatomically complete and functionally competent, the gastrointestinal tract is still relatively immature at hatch and undergoes striking morphological changes during the first few days posthatch. These morphological changes include enhancement in absorptive surface area through remodeling of the mucosal layer. During this period optimal nutrition is critical and sets the stage for overall lifetime performance.

The literature review in this dissertation will focus on the morphological and functional changes that occur during the late stages of embryogenesis and during the early posthatch period, as this represents the time during which the most dramatic changes are occurring. Particular attention will be paid to the effect of early posthatch nutrition on gastrointestinal development, and intestinal amino acid (AA), peptide and sugar transporters. This will establish the framework for the remainder of this dissertation that focuses on a series of studies aimed at evaluating the developmental and dietary regulation of sugar, peptide and AA transporter gene expression and intestinal morphology in two genetic lines of broilers that differ in growth characteristics. One of

the objectives of these studies is to determine if selection for growth on different diets has led to differences in expression of intestinal nutrient transporters. Dietary protein is the focus of the feeding trials, as it is a very costly nutrient and even minute improvements in its utilization could have substantial economic benefits for the poultry industry and reduce pollution by diminishing excess nitrogen excretion into the environment. Additionally, complexities in the mechanism of AA uptake vs. carbohydrate absorption make it an exciting target for studies aimed at improving nutrient utilization.

In recent years there has been an explosion of papers involving characterization and regulation of nutrient transporters, in particular the peptide transporter, PepT1, due to its pharmacological and clinical relevance, and the glucose transporter, SGLT1, due to its clinical relevance. Most of these studies are in a mammalian model and are undertaken with objectives related to improving the understanding of human nutrition and health. There has been much less done in livestock and poultry. Our lab was the first to characterize the peptide transporter in various livestock species and poultry. Understanding the regulation of nutrient transporters has tremendous implications for animal nutrition and health, as the intestine is a key organ that processes dietary nutrients for self-maintenance and growth, which then influences distribution to the rest of the body. Unless otherwise noted, broiler chickens are the focus of research in the papers discussed in the literature review.

Chapter II

Literature Review

Introduction

Morphology of the Intestinal Epithelium. Intestinal absorptive surface area is enhanced by finger-like projections called villi that extend out into the lumen (Mamajiwalla et al., 1992). Adjacent to villi are invaginations called crypts that contain stem cells, which give rise to daughter cells that differentiate into one of four cell types (enterocytes, goblet cells, entero-endocrine cells, and Paneth cells). Enterocytes, goblet cells, and entero-endocrine cells migrate out of the crypts and up the sides of the villi during which time they mature. Tight junctions between cells maintain a relatively impermeable epithelial layer. Paneth cells remain in the crypts and serve antimicrobial functions. Enterocytes serve digestive and absorptive functions and represent the greatest proportion of cells that line the villi.

Goblet cells represent approximately 13 %, 23 %, and 26 % of epithelial cells lining the villi of the duodenum, jejunum, and ileum, respectively (Uni et al., 2003b). Goblet cells secrete mucins, glycoproteins that mix with water to form the unstirred-water layer surrounding the epithelium. The mucus layer provides protective functions and is the primary barrier between luminal substances and the epithelial layer. The hydration radius, charge, hydrogen-bonding capability and molecular weight all influence the rate at which molecules traverse the unstirred water layer (Moran, 1985).

Microvilli, also known as brushborder membranes, are the point of contact between enterocytes and luminal nutrients. Microvilli constitute the apical membrane of

enterocytes facing the lumen and are anchored by bundles of actin filaments and associated binding proteins (Shibayama et al., 1987). Microvilli maximize absorptive surface area by further enhancing contact surface area with nutrients. A vast array of digestive enzymes and transporter proteins are inserted into the brushborder membrane.

Cells lining the villi eventually migrate to the tips where they are extruded into the lumen. In rodents, cell life span is approximately 2-3 d (Falk et al., 1998; Traber et al., 1991). Cell turnover in the chicken changes with age, but generally takes between 3 and 4 d (Uni et al., 2000). Paneth cells, which have anti-microbial functions, reside in the crypts and have a much longer life span (~ 28 d). This review will focus on the changes in intestinal morphology and function that take place during chick development and in response to diet.

Nutrition during Embryogenesis

During incubation the chick embryo obtains nutrients primarily from the yolk sac and also by swallowing the amniotic fluid. The yolk sac supplies nutrients directly to the circulation through the yolk sac membrane throughout embryogenesis, but also directly to the small intestine through the yolk stalk towards the end of incubation. At the end of the second week of embryo growth, the seroamniotic connection ruptures and albumen enters the amniotic sac from which fluids are swallowed by the embryo (Moran, 2007; Vieira and Moran, 1999).

The Yolk Sac Membrane. The yolk sac membrane (YSM) swathes the surface of the yolk and the advancing edge contains a nonvascular region of endodermal cells referred to as the area vitelline (Moran, 2007; Vieira and Moran, 1999). Mesodermal tissue spreads over the endodermal layer and forms blood vessels and connective tissue.

Between embryo d 5 and 21 the yolk becomes completely enveloped by the yolk sac membrane and the endodermal cells maintain contact with the yolk surface and are responsible for nutrient uptake (Speake et al., 1998). The yolk sac membrane is permeable and relatively non-selective in both directions (Noy et al., 1996). Transfer of nutrients occurs from circulation to the yolk, from yolk to circulation, and between the yolk and small intestine.

Yolk Sac Composition. The yolk sac is a source of nutrients for the growing embryo. Its composition is approximately 50 % water with lipids and proteins comprising approximately 60 and 30 % of its dry matter, respectively (Noy and Sklan, 2001; Noy et al., 1996; Speake et al., 1998). The yolk sac protein contains maternal antibodies that represent an important source of passive immunity for the chick (Dibner et al., 1998). Most of the yolk lipids are in the form of very low density lipoproteins (VLDL). The lipid distribution of the chick yolk is 72.5 % triglycerides, 24.4 % phospholipids, and 3.9 % cholesterol (Vieira and Moran, 1999). The poult has a similar lipid yolk content consisting of 62 % triglycerides, 28 % phospholipids, and 8 % cholesterol esters (Noy and Sklan, 1998b). Lipid metabolism is an important source of energy in the developing chick embryo as reflected by elevated levels of ketone bodies in the blood that persist until consumption of a carbohydrate-based diet at hatch (Best, 1966). In addition to serving as a source of energy through beta oxidation, fatty acids as well as other lipid components serve other functions in the body, including synthesis of cell membranes, steroidogenesis, and signal transduction, to name a few (Speake et al., 1998).

Distribution of Yolk Nutrients to the Circulation. There has been some controversy as to the exact mechanism of VLDL transfer from yolk to the circulation

(Powell et al., 2004). An older theory was that yolk VLDL were transcytosed intact across the endodermal cell and delivered intact to the circulation of the embryo. More recent evidence supports the hypothesis that yolk-derived VLDL use receptor-mediated endocytosis to enter the endodermal cell (Moran, 2007). Once inside the cell, VLDL are hydrolyzed by lysosomal lipase and subsequent products are delivered to the endoplasmic reticulum for re-assembly of VLDL, which are then secreted and delivered to the circulation (Powell et al., 2004). Powell et al. (2004) observed that label from radio-labeled palmitic acid that was incubated with YSM pieces were recovered in triglycerides (84 %), phospholipids (12 %), and cholesterol esters (1 %), supporting the idea that YSM endodermal cells possess the enzymatic equipment for lipid esterification.

The VLDL particles that are exocytosed from the basal surface of the YSM to the circulation are at least twofold greater in size than those detected within the yolk (Kanai et al., 1996), also supporting the idea that there is substantial lipid remodeling within the endodermal cells. Activities of acyltransferases involved in re-esterification were detected at levels in the YSM that were greater than the liver or any other tissue of the chick embryo throughout incubation (Murray et al., 1999). Activities of protein hydrolases were also detected in the YSM. These hydrolases increased after embryo d 5 and became maximal from embryo d 14 to 20, demonstrating the potential for digestion of proteinaceous yolk material (Sugimoto and Yamada, 1986).

Delivery of Yolk Nutrients to the Intestine. Close to hatching, there is a rise in active transport systems for sugars and AA in the YSM (Holdsworth and Wilson, 1967; Moran and Reinhart, 1980). Movement of yolk to the small intestine is also stimulated during the last few days before hatch and yolk sac contents enter the small intestine

through the yolk stalk at Meckel's diverticulum. Its contents enter the proximal small intestine by antiperistalsis and are subjected to some hydrolysis (Noy and Sklan, 1998b). Intestinal contents may be propelled as proximally as the proventriculus by antiperistalsis for hydrolysis and further mixing (Noy et al., 1996). From embryo d 19 the intestine withdraws into the abdomen and luminal gastrointestinal contents enter the yolk sac lumen via the vitelline diverticulum, with faster emptying to the yolk sac at 19 d compared with 20 d of incubation (Esteban et al., 1991). The presence of material in the lumen of the stomach and intestine may play an important role in stimulating gut function in anticipation of hatching. In poults the relative distribution of yolk-sac nutrients in the gut proximal to Meckel's diverticulum was 33 % at embryo d 27 and 52 % at day of hatch. The yolk stalk remains completely open in the chick until approximately 3 d posthatch after which it narrows due to lymphocyte infiltration (Noy et al., 1996).

Swallowing of Amniotic Fluid during Late Incubation. During the last few days of incubation the embryo begins to rapidly consume the amniotic fluid which passes through the gastrointestinal tract (Moran, 2007). The mixture of amniotic fluid and yolk may also enter the yolk sac and move back to the intestine, as the yolk stalk is permeable and non-selective. Ovalbumin, the most abundant protein in egg white, was detected in both the yolk sac and in the circulation of the embryo during incubation (Sugimoto et al., 1999). The passage and mixing of amniotic fluid and yolk contents in the gastrointestinal tract is associated with some digestion; however, antitrypsin factors originating from the albumin, such as ovomucoid (Sugimoto et al., 1989) inhibit some proteolytic digestion and permit passage of intact proteins from the intestine to the yolk (Baintner and Feher, 1974; Oegema and Jourdian, 1974). The purpose of trypsin inhibitor in the gut at this

juncture may be to protect some important proteinaceous factors such as immunoglobulin A (IgA) from degradation, permitting intact macromolecular transfer during the last stage of incubation, similar to transfer of macromolecules from colostrum in the suckling mammal (Moran, 2007; Pacha, 2000). Delivery of intact IgA to the intestine is important for conferring passive immunity to the bird.

Liver Metabolism during Late Embryogenesis. The movement of albumin-amnion-enzyme mixture from the gut to the yolk sac lumen results in an increase in uptake of VLDL (Moran, 2007). The nutrients extracted from the amniotic fluid are an important source of gluconeogenic precursors that are used for making a store of liver glycogen in preparation for hatching. Gluconeogenic enzymes increase dramatically with development during chick incubation and activity spikes at hatching, at which time the liver has the greatest store of glycogen to fuel the hatching process. The liver also contains the greatest concentration of cholesteryl esters at hatching, with a major decrease in serum cholesterol observed during the first week after hatching due to transport from the liver to other tissues (Castillo et al., 1992; Tarugi et al., 1994). The total lipoprotein content in the blood increased and reached maximal levels during the first few days after hatching, coinciding with the greatest level of production of VLDL and also parallel to the decrease in free serum cholesterol (Castillo et al., 1992).

Tarugi et al. (1994) showed that hepatic accumulation of cholesterol at hatching may be closely linked to hepatic production of proteins apoprotein A and B, which control synthesis of lipoproteins. They showed that the dramatic reduction in hepatic cholesterol in the first 4 d posthatch was accompanied by dramatic increases in apoA-I and apoB, and lipoprotein content in the blood. Interestingly, production of apoA and

apoB was very low prior to hatch. Cholesterol is used for synthesis of bile, cell membranes and for transport of lipids, thus the rapid increase in hepatic clearance after hatch may serve as a mechanism to provide cholesterol for various cellular functions throughout the body in the rapidly growing chick.

Changes in Intestinal Morphology during the Last Week of Incubation. During the last week of incubation villus development sets the framework to prepare chicks for efficient absorption of luminal nutrients (Mamajiwalla et al., 1992; Uni et al., 2003b). At embryo d 15, crude ill-defined villi are visible, and by d 17 two distinct populations of villi are observed, referred to as "large pear-shaped" and "small rocket-shaped". At embryo d 19 budding is detected at the base of the villi and by d 20 a third "wave" of villi development is established, accounting for 30 % of total villi (Uni et al., 2003b). Prior to hatch, crypt invagination has not yet occurred.

Digestive and Absorptive Functions during the Last Week of Incubation. Pancreatic enzyme and intestinal enzyme and transporter expression and activities are also stimulated during late embryogenesis to prepare the chick for immediate consumption of nutrients after hatching. The activities of pancreatic amylase, chymotrypsin and carboxypeptidase from 13 d of incubation to 1 d posthatch increased 9-fold, 24-fold, and 200-fold, respectively (Marchaim and Kulka, 1967). At embryo d 15, expression and activities of intestinal sucrase, maltase, alkaline phosphatase (AP), ATPase and the glucose transporter, SGLT1, are low and demonstrate marked increases to day of hatch (Uni et al., 1998b). Similarly, Chotinsky et al. (2001) observed that sucrase activity increased twofold from embryo d 18 to d 1 posthatch in broilers, although activity of other disaccharidases decreased during this time.

Van et al. (2005) observed a 3.3-fold increase in mRNA expression levels of peptide transporter, PepT1, in turkey intestinal tissue from 5 d before hatch to day of hatch. Chen et al. (2005) observed a 14- to 50-fold increase in intestinal chicken PepT1 mRNA from embryo d 18 to day of hatch, with expression peaking right before hatch.

General Comparative Aspects of Prenatal Nutrition. In mammals, sucrase-isomaltase is low during fetal development and even during suckling and is stimulated at weaning (Pacha, 2000). Chickens on the other hand must be able to handle complex carbohydrates at hatch and thus must turn on slightly different enzymatic machinery to accomplish this feat. Mammals are similar to chickens in the respect that although they extract nutrients primarily from a non-luminal source (placenta) during fetal development, intestinal transporters and enzymes are "turned on" before birth to establish digestive and absorptive functions (Pacha, 2000). Aminopeptidase N expression was not detected until rat embryo d 20 and increased with age (Jardinaud et al., 2004). Rat intestinal PepT1 mRNA and protein was present at fetal d 20, increased at birth, and reached maximal expression levels during d 3 to d 5 (Shen et al., 2001). Similar to the chick, prenatal expression of nutrient transporters allows for the absorption of some nutrients from the swallowed amniotic fluid.

In-Ovo Feeding. In recent years, in-ovo feeding has emerged as a technology to exploit delivery of nutrients to the gastrointestinal tract of the late-term embryo via swallowed amniotic fluid. The rationale behind this idea is that providing nutrition during the last few days of incubation can help establish the framework for ingestion of nutrients posthatch and attenuate problems associated with "hatchling quality phenomenon", in

which some birds may survive the early posthatch period but adjust poorly resulting in sub-optimal overall growth performance (Uni and Ferket, 2004).

In the embryonic chick in-ovo administration of nutrients occurs between embryo d 17 and 18 and is accomplished by injection of a nutrient solution (approximately 1 mL for chicks) into the amnion (Uni and Ferket, 2004). In multiple studies, delivery of different combinations of easily digested carbohydrates (e.g., disaccharides, dextrin), AA (e.g., L-arginine), protein, and β-hydroxy-β-methyl butyrate (HMB; leucine metabolite) to both late-stage turkey and chicken embryos resulted in improved hatchability and mucosal morphological parameters at hatch including enhanced villus surface area, disaccharidase and aminopeptidase activity and jejunal nutrient uptake (Foye et al., 2007; Tako et al., 2004). Up-regulation of digestive enzymes in the small intestine in response to in-ovo feeding suggests that the presence of luminal substrate stimulated intestinal function. Day of hatch chicks that were administered in-ovo nutrients during incubation had greater liver glycogen content, relative yolk size, relative breast muscle size, hatching weight, and intestines that were structurally and functionally similar to a 2-d old chick (Tako et al., 2004; Uni and Ferket, 2004).

In one trial, performance effects were sustained until d 25 (Uni et al., 2005) and in another performance was greater through d 35 (Uni and Ferket, 2004). The proposed mechanism for improved performance in chicks given in-ovo nutrition is that the nutrients provided during late incubation improve the energy status in the chick and direct nutrient utilization away from the developing skeletal muscle (Uni et al., 2005). The administration of HMB in particular has a strong effect on energy utilization due to its ability to promote muscle protein synthesis and inhibit proteolysis. Tako et al. (2004)

observed that HMB alone also had the effect of enhancing villus surface area, most likely through stimulation of cell proliferation and growth. Administration of HMB combined with provision of carbohydrates that can be hydrolyzed to glucose, for hepatic glycogen synthesis to fuel hatching, puts the chick in a more optimal position at hatch. The combination of HMB and free arginine in the in-ovo feeding solution resulted in enhanced circulating levels of IGF-I and IGF-II, as well as glucose-6-phophatase, at hatch, and a more rapid rate of growth in poults (Foye et al., 2006). Thus, the effect of in-ovo nutrients on chick growth may be through an effect of growth factors on carbohydrate metabolism.

Gastrointestinal Development in the Posthatch Chick

During the last few days of incubation the increase in weight of the broiler small intestine is of a greater magnitude than the increase in BW, representing 1 % of total BW at embryo d 17 and 3.5 % on day of hatch (Uni et al., 2003b). Early gastrointestinal development involves growth of the mucosal layer and overall increases in tissue mass, as well as changes in passage time in the small intestine (Sklan and Noy, 2003b). In the posthatch chick passage time is reduced by one-third during the first week (Uni et al., 1995) and by 50 % at 10 d in poults (Noy and Sklan, 1997) increasing the intestinal throughput considerably. During this time the small intestine increases in length, and the allometric increase in BW is accompanied by rapid development of the mucosal layer and digestive and absorptive functions (Sklan and Noy, 2003b; Uni et al., 2003b).

Although most of the digestion and absorption is accomplished in the small intestine, there is some absorptive function in both the paired ceca, located between the ileum and colon, and the colon itself. Both tissues have villi, and in particular, the ceca

contain significant microbial activity and are reported to play a role in absorption of various nutrients (Denbow, 2000). It would be beyond the scope of this review to discuss the ceca and large intestine in great detail, as the focus of this dissertation is nutrient absorption in the small intestine, hence the remainder of this review as it pertains to nutrient digestion and absorption will reflect studies of the small intestine.

Crypt Growth after Hatching. At hatch, crypts are rudimentary and are not well-defined. Only 1 crypt/villus was observable at hatch and each one contained only a few cells (Uni et al., 2000). Posthatch crypt invagination is complete by 48 h and the number of crypts per villus increases and plateaus at 216 h in the duodenum and jejunum and 72 h posthatch in the ileum (Geyra et al., 2001a). The number and size of cells per crypt rapidly increases and declines after 108 h posthatch. Crypt depth was largest in the duodenum and smallest in the ileum (Iji et al., 2001a; Uni et al., 1998b). In 14 d chicks there were 155 cells/crypt which was less than that reported for mice, rats and humans (Falk et al., 1998), but the fraction of those cells undergoing cell division was similar to that reported in rats and humans (Uni et al., 2000). The increase in size and number of crypts posthatch presumably provides more enterocytes to the villi and increases cell renewal rate, and may compensate for the reduction in villus-specific cell proliferation that occurs posthatch (Geyra et al., 2001a).

Villus Growth during the Early Posthatch Period. The intestinal villi undergo rapid morphological changes in the neonate chick. In the early posthatch broiler chick, villus surface area increased rapidly with age (Iji et al., 2001a; Noy and Sklan, 1997) and most rapidly in the duodenum (Geyra et al., 2001a, b; Iji et al., 2001a; Uni et al., 1995). Uni et al. (1998b) found that duodenal villus surface area development was complete by

7 d whereas jejunal and ileal villus size continued to increase through 14 d. In the poult, duodenal and ileal villus length and numbers did not increase after 11 d, whereas jejunal villus length and surface area continued to increase after 11 d. Villus length was greatest in the duodenum at hatch, while villus width was similar in all segments and changed little with age (Geyra et al., 2001a). Enterocyte migration rate was also greater in the duodenum and jejunum. The more rapid rate of maturation of the mucosal layer in the duodenum during the first few days after hatch may be due to the greater proportion of dietary nutrients encountered in the lumen to "stimulate" function, whereas absorption in the upper tract may reduce the amount of nutrients exposed to the brushborder membrane of the distal tract. After 72 h, the total absorptive surface increased dramatically in the jejunum, at a rate that was more than twofold greater than the other segments (Geyra et al., 2001a).

Changes in Goblet Cells at Hatch. Goblet cell function is also altered at hatch to adapt to the rapidly changing luminal environment. Goblet cells are capable of secreting a variety of different mucins and additional post-translational modifications further enhances their diversity and allows the gut to fine-tune the contents of the mucus layer in response to luminal stimuli. The number of goblet cells per unit area increases at hatch and increases most dramatically in the distal small intestine (Uni et al., 2003a). At 18 d of incubation, goblet cells contained almost exclusively acid mucin, whereas after hatch there were similar concentrations of acidic and neutral mucins (Uni et al., 2003a). The ability of acidic mucins to prevent bacterial invasion may explain their dominance during late embryogenesis, as a sort of innate barrier. Goblet cell function at hatch is critical as the chick intestine becomes first exposed to foreign material. The mucus layer plays the

role of an innate barrier to pathogens while also restricting diffusion of luminal nutrients and the thickness of the mucus layer affect its role in both of these capacities.

Enterocyte Development during the Early Posthatch Period. Enterocytes are the most predominant cell species that populate the villi, and their morphology and function change rapidly posthatch to assume the role of efficient nutrient assimilation upon initiation of feed consumption. Enterocytes are non-polar at hatch and lack a defined brushborder membrane (Geyra et al., 2001a). During the first week, enterocytes undergo two distinct stages of development. During the first stage, which occurs within the first 24 h, cells acquire a defined brushborder membrane. The second stage involves hypertrophy, where the cells increase in length (Geyra et al., 2001a). In terms of cell length, the ileum is relatively mature at hatch whereas hypertrophy continues on for several days posthatch in the duodenum and jejunum. In the poult, enterocyte length increased with age after hatch, but width was relatively static, thus numbers of enterocytes per villus increased with increasing villus length (Sklan and Noy, 2003b).

In leghorn chicks, microvilli were longer in the proximal small intestine and length remained fairly stable with age in the duodenum and jejunum while increasing in the distal intestine (Ferrer et al., 1995). Microvillus diameter increased with age in the jejunum and microvillus density increased in the duodenum and ileum. The microvilli maximize absorptive surface area in the small intestine, and changes in both size and density can help account for developmental changes in absorptive surface area.

Intestinal Cell Proliferation at Hatch. At hatch almost all cells along the crypt-villus axis are proliferating (Geyra et al., 2002; Uni et al., 2000). Poultry are unique in that proliferation is not restricted to the crypts but also occurs along the length of the villi

(Noy et al., 2001; Uni et al., 1998a). This could be a mechanism for enhancing absorptive surface area without the time lag associated with cell proliferation and migration from the crypts. This is in contrast to mammals where cell proliferation is restricted to the crypts. For example, in the rat, cell proliferation on the villi ceases shortly before birth (Quaroni, 1985).

The number of proliferating cells decreased from 100 % to less than 10 % on the villi and 60 % in the crypt after 4 d (Uni et al., 2000). The proportion of proliferating cells decreased with age such that the number of proliferating cells representing a proportion of the total cells decreased from over 80 % to 20 % in the villus and increased from 20 % at hatch to 80 % in the crypt at 108 h posthatch (Uni et al., 2000). The decrease in proliferating cells on the villi occurred most slowly in the jejunum (Geyra et al., 2001a).

Assessment of the crypt-villus distribution of a commonly used brushborder marker enzyme, alkaline phosphatase (AP), supported the notion that chickens exhibit ambiguous zones of cell differentiation. Rats showed a gradient of decreased AP activity from the villus tip to the crypt (Traber et al., 1991), while chicken AP exhibited little change along the villi (Uni et al., 1998a). Similarly, in rats, sucrase expression was negligible in crypts and showed a complex pattern of regulation along the villi (Traber, 1990), while in chickens, sucrase and maltase activity were detected in the crypt and increased towards the villus tip (Uni et al., 1998a). Uni et al. (1998a) offered two explanations for the phenomenon of widespread cell proliferation in chickens. Firstly, differentiated cells that express active brushborder proteins may be capable of

proliferating. Secondly, crypt-borne cells that journey up the villus may contain a mixture of differentiated and proliferative cells.

The rate of cell migration in chicks increases with age and peaks at approximately 2 wk posthatch, and concomitant with increases in the length of the villi, the enterocyte life span increases with age (Iji et al., 2001a). During the first week posthatch, cell migration is complete within 96 h of cell birth, whereas in older birds (21 d) the leading enterocytes covered between one-half and one-third of the villus length within 96 h. Imondi and Bird (1966) reported that in the jejunum of chicks 48 h after hatch, it took approximately 48 h for cell turnover, while in the duodenum and ileum, approximately 45 % of the distance of the villi was covered in that time. Uni et al. (2000) reported that cell turnover time was between 3 and 4 d in the young chick.

Comparative Changes in Mucosal Remodeling during Development. Mammals, in contrast to poultry, undergo several phases of intestinal ontogenetic development. Morphogenesis and cytodifferentiation occur before birth and prepare the animal for suckling (Pacha, 2000). The transition at birth from the placenta as a major source of nutrients to colostrum represents a shift in environment that triggers mucosal development, as does the suckling period and transition to weaning. Similar to poultry, non-specific changes in absorptive function that occur during these phases include changes in mucosal surface area, proliferation and migration of enterocytes, changes in plasma membrane composition, and changes in paracellular permeability (Pacha, 2000). Specific changes include changes in the turnover and concentration of transporter proteins, transport affinity constant, and expression of various transporter isoforms (Pacha, 2000). The more complex pattern of ontogenetic changes in intestinal function is

common to mammals. Because the chicken is fed a relatively constant diet after hatching until market age, under normal healthy conditions the small intestine is not subject to the multiple phases of remodeling that occur in a mammal.

Nutrient Digestion during the Early Posthatch Period

Changes in intestinal absorptive surface area, passage time in the gut, and feed intake relate to changes observed in nutrient digestion and absorption. At hatch the chick must shift from the lipid-rich yolk as a source of nutrients to an exogenous carbohydrate and protein-based diet. The ability to utilize certain nutrients is likely to change as available nutrients change and metabolic demands change. The next few sections will focus on nutrient digestion and absorption during the early posthatch period.

Secretion of Pancreatic Enzymes and Bile Acids. Results from multiple studies indicate that pancreatic and intestinal digestive enzymes may be limiting during the early posthatch period (Sell, 1996). Pancreatic function is relatively immature at hatch and enhanced growth and secretion of pancreatic enzymes occurs with ensuing days (Nitsan et al., 1991a; Sklan and Noy, 2000). Nitsan et al. (1991b) observed that pancreatic levels of amylase and lipase activity were greatest at 8 d while trypsin and chymotrypsin were greatest at 11 d posthatch. In the small intestine, lipase was maximal at 4 d, amylase was maximal at 6 d, and trypsin and chymotrypsin were maximal at 11 d, showing that digestion could be a rate-limiting step to nutrient assimilation during the early posthatch period. This is especially problematic in broiler chickens that are selected for rapid growth. Nir et al. (1993) observed that broiler chicks had less small intestinal activity of pancreatic enzymes than layers and suggested that this could limit growth in meat-type chickens.

Secretion of bile acids (Noy and Sklan, 1997) and expression of bile acid transporter in the small intestine (Li et al., 2008) increases during the first few days posthatch and changes little after the first week. During the early posthatch period bile is more limiting than lipase in terms of fat digestion and bile salts have been used with success in the diet to improve fat utilization (Vieira and Moran, 1999). In the poult, similar to the chick, secretion of amylase, trypsin, and lipase increased with age, with amylase and trypsin increasing more dramatically relative to lipase (Sklan and Noy, 2003b). This enhanced secretion of pancreatic enzymes parallels the increases in feed intake and BW. Absorption of fat and protein increased in the ileum with age, indicating that the absorptive capabilities of the ileum are initiated to handle the increased nutrient load in response to increased feed intake.

Intestinal Membrane-Bound Enzyme Expression and Activity

The final stage of nutrient digestion is accomplished by digestive enzymes located on the brushborder membrane of enterocytes. The jejunum and ileum are the major sites of disaccharidase activity, in particular sucrase and isomaltase, which are both part of the alpha-glucosidase complex (Iji et al., 2001b). Activity of aminopeptidase N (APN) was greatest in the ileum of broiler chicks (Iji et al., 2001b). Because most enzymes are secreted as inactive precursors that become activated only after the enterocyte has proceeded up the villus and undergone maturation, enterocytes on longer villi in older birds may secrete enzymes for a longer period of time than enterocytes on shorter villi in young chicks (Iji et al., 2001a). In chick intestines where the glycocalyx was removed, digestive enzyme activity at the membrane surface was not reduced, showing that

nutrients must diffuse through the unstirred water layer in order to have access to brushborder hydrolases (Moran, 1985).

Aminopeptidase N (APN). Aminopeptidase N (APN, α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2, arylamidase, aminopeptidase M, and alanine aminopeptidase) is a member of the M_1 zinc metalloprotease family, and cleaves AA from the N-terminal end of peptides (Mina-Osario, 2008). Aminopeptidase N is fairly specific for peptides containing an N-terminal L-isomer neutral or basic AA (Sanderink et al., 1988; Luciani et al., 1998). Oligopeptides appear to be the preferred substrate, as APN is less effective at cleaving residues from dipeptides (Look et al., 1989). Some substrates include neuropeptides (i.e., somatostatin), vasoactive peptides (i.e., angiotensin III), and cytokines (i.e., interleukin 8; Jardinaud et al., 2004). Aminopeptidase N is unable to cleave N-terminal proline (Jankiewicz and Bielawski, 2003).

APN Structure and Catalysis Mechanism. APN is a type II membrane-bound protein comprised of a single transmembrane domain with an N-terminal intracellular domain and a large carboxyl extracellular domain containing the active site of the enzyme (Olsen et al., 1988). Members of the metallo-aminopeptidase family use conserved residues within the protein at the carboxyl terminus to create a scaffold for binding of one or two metal ions, such as zinc in the case of APN (Lowther and Matthews, 2002). Metalloproteases catalyze cleavage by binding metal ions that increase the reactivity of water, stimulating the nucleophilic attack on the carbonyl carbon of the peptide bond, stabilizing the transition stage (Holz, 2002).

Tissue and Cellular Distribution of APN. Activity of APN is greatest in the small intestine, where it makes up approximately 8% of the protein content of the

brushborder membrane (Delmas et al., 1992). A whole-body investigation of APN expression in rats demonstrated highest labeling of APN in the intestine and kidney of adult rats (Jardinaud et al., 2004). Aminopeptidase N is expressed to a lesser extent in the liver, stomach, lung, fibroblasts, nerve synapses, and colon (Delmas et al., 1992; Jardinaud et al., 2004). Aminopeptidase N activity and protein expression increased from the proximal to distal small intestine in rabbits and chickens (Gal-Garber and Uni, 2000; Kramer et al., 2005), and expression was localized to deep apical tubules (specialized lipid-raft domains) in the brushborder membrane (Hansen et al., 2003; Jardinaud et al., 2004; Kramer et al., 2005). Sihn et al. (2006) observed expression of APN in the small intestine of chick embryos as early as 6 d of incubation and high expression levels thereafter.

Brushborder Membrane Enzyme Activity during Development. Intestinal membrane-bound enzyme expression and activity levels can affect rates of nutrient utilization, especially during the early posthatch period. Intestinal disaccharidase activity increases with age during the first week and may limit assimilation of sugars (Sell et al., 1989; Siddons, 1969). Breed-specific differences in activity of intestinal membrane-bound enzymes could also be a limiting factor to growth of the broiler, similar to observed differences in intestinal activity of pancreatic enzymes. Mahagna and Nir (1996) observed that during the first week posthatch, intestinal disaccharidase activity was greater in broiler chicks, but after the first week, activity was greater in layers.

In two lines of chickens, Arbor Acres (heavy-strain) and Lohman (light-strain), there were distinct differences in morphological development, enzyme secretion and digestion, and passage time in the small intestine (Uni et al., 1995). The heavy-strain

chicks had greater feed intake and more rapid growth, which was accompanied by taller and more dense villi, greater villus volume, a thicker submucosal layer, more rapid increase in the number of enterocytes, longer passage time in the gut, longer intestinal segments, greater enzyme secretion per gram of feed intake at d 4, and greater starch digestion in the first few weeks posthatch. Starch digestion increased 90 to 95 % from 4 to 14 d in the heavy-strain chicks and from 80 to 93 % from 4 to 14 d in the light-strain chicks. While nitrogen digestion increased with age in both strains, fatty acid digestion changed very little.

Intestinal Nutrient Transport

Nutrient uptake in the small intestine is mediated by transporter proteins that bind and relocate nutrients from the lumen to the inside of the cell across the brushborder membrane. The regulation of nutrient transporters has become a hot area of research due to the potential to reduce constraints on growth by improving uptake capacity. Obst and Diamond (1992) suggested that nutrient uptake is matched to intake and may impose a "bottleneck" on availability of nutrients. In particular they commented on the observation that in animals selected for growth, for example in chickens, there is not uptake capacity that exceeds nutrient intake. They suggested a 1:1 ratio for uptake:intake in chickens based on the observation that uptake capacity of glucose, fructose and proline was in excess of resting metabolic rate but was closely matched to total metabolizable energy intake.

While the transporters described in this review are perhaps the best characterized, particularly in mammals where most of the work has been conducted, they do not represent an all-inclusive list of intestinal peptide, AA and sugar transporters. In a

microarray study, we demonstrated the presence and developmental regulation of multiple transporters not yet identified or characterized in the chicken (Li et al., 2008). Hence, there remains much to be learned about the mechanisms of nutrient transport in the small intestine. The following description of transporters pertains to only those evaluated in later chapters of this dissertation.

Peptide Transport in the Small Intestine

Peptide Uptake. Long before the cloning and characterization of the intestinal peptide transporter, PepT1 (Fei et al., 1994), a carrier-mediated mechanism was shown to exist for the uptake of small peptides across the brushborder membrane of the enterocyte. Although the first evidence for peptide transport was provided in the 1950's (Newey and Smyth, 1959), acceptance of AA absorption across the gut wall in the form of peptides was slow to emerge, even as recently as the late 1980's (Webb, 1990). Although it is certain now that AA derived from dietary protein are transported into the enterocyte in the form of di- and tripeptides, there is still much to be learned about the quantitative significance and origin of luminally-derived peptides and the relative post-absorptive use and portal appearance of free AA vs. peptides.

Little or no detection of absorbed intact peptides was reported in dogs (Levenson et al., 1959) and rats (Wiggans and Johnston, 1959). Others have reported that up to 85 % of AA entering the portal blood were in the form of small peptides in rats (Gardner, 1975), Holstein calves (Koeln et al., 1993), lactating Holstein cows (Tagari et al., 2004, 2008), Friesian steers (Seal and Parker, 1996), sheep (Remond et al., 2000), and yak cows (Han et al., 2001a, b). In some of these studies, total appearance of free and peptide AA in the portal vein was greater than the intake of dietary protein, suggesting contributions

from degradation products resulting from tissue protein turnover in the G. I. tract, spleen, and pancreas (Han et al., 2001a). In studies where lower contributions of peptide-bound AA to total portal AA flux were observed (32 %, Remond et al., 2000 and Han et al., 2001b; up to 20 %, Tagari et al., 2004, 2008), the authors attributed the conflicting reports to differences in methodology. Refinements in analytical technique, including chemical deproteinization of plasma followed by physical filtration, result in a more accurate determination of peptide AA concentrations. The more recent reports indicating a lower PDV flux of peptides may represent a more reasonable estimate of this value.

Peptide Uptake Compared with Free AA Uptake. While transporters of free AA exhibit substrate specificity, the peptide transporter can potentially transport all 400 di- and 8,000 tripeptides that result from combining the 20 different dietary AA (Daniel, 2004). In terms of energetic efficiency, two or three AA can be transported into the cell by the peptide transporter for the same expenditure of energy required to transport a single free AA (Daniel, 2004). Individuals suffering from deficiencies in free AA transport were still able to assimilate essential AA, pointing to the possibility that the peptide transporter transports enough dietary AA to compensate for a deficiency in free AA transport (Adibi, 1997).

Transport of AA in the form of peptides was demonstrated to be a faster route of uptake per unit time than their constituent AA in the free form (Adibi and Phillips, 1968). In rats, AA in soy protein hydrolysate (Kodera et al., 2006) or egg white protein hydrolysate (Hara et al., 1984) were absorbed faster into the portal blood after duodenal infusion than those representing an AA mixture or intact protein with the same respective AA composition. Intestinal perfusion of 0, 50, 100, or 200 g/L solutions of intact soy

protein or 0, 100, 200, 300, or 400 g/L solutions of hydrolyzed soy protein caused a load-dependent slowing of intestinal transit in dogs (Zhao et al., 1997). Interestingly, the intact protein slowed transit more effectively than the hydrolysate, supporting the notion that digestion is a rate-limiting step in nutrient assimilation. Furthermore, a greater amount of AA were absorbed in the proximal small intestine when protein was infused as a hydrolysate instead of in the intact form, suggesting that AA as peptides were more readily available for absorption.

The Peptide Transporter, PepT1. Di- and tripeptides are transported into the cell by the peptide transporter, PepT1 (SLC15A1; Fei et al., 1994), a member of the proton oligopeptide cotransporter family (Table 2.1). In addition to transport through PepT1, peptides may also be absorbed through alternate routes including paracellular movement and by cell-penetrating peptides (CPP) that are capable of moving cargo across the plasma membrane (Figure 2.1). The intestinal peptide transporter has been characterized in the chicken (Chen et al., 2002; 2005) in addition to many other types of animals. Characterization and regulation of the peptide transporter has been extensively reviewed (Gilbert et al., 2008).

Mechanism of Peptide Absorption. The peptide transporter, PepT1, is proton-dependent and is a low-affinity, high-capacity transporter (Daniel, 2004). The proton to substrate ratio for neutral and cationic AA transported by PepT1 is one, whereas the ratio for charged anionic AA is two (Steel et al., 1997). The unstirred water layer at the brushborder membrane is an isolated microenvironment free from the influence of the luminal contents and maintains a high extracellular concentration of protons (Adibi, 1997). Following uptake of peptides and H^+ by PepT1, protons are then transported out of

the cell by the Na^+/H^+ exchanger on the brushborder membrane in exchange for Na^+. The Na^+ in turn is taken out of the cell by the Na^+/K^+ ATPase pump on the basolateral membrane where three Na^+ are transported out of the cell in exchange for two K^+, restoring the electrochemical gradient. The electrochemical gradient needed to drive transport and maintain the electronegative cell potential is driven by ion exchangers and pumps in the brushborder and basolateral membranes. The Na^+/K^+ ATPase pump thus supplies the energy needed to drive active transport across the brushborder membrane. Sodium is an important part of the diet of the broiler. In chicks fed a low Na^+ diet (0.07 % Na^+), Na^+/K^+ ATPase activity was lower than in control birds. After 4 d, pancreatic enzyme activity, BW and intestinal growth were depressed as compared with birds that were fed a balanced diet (Sklan and Noy, 2000). Uptake of glucose and methionine was greater in the presence of adequate Na^+ to support co-transport (Noy and Sklan, 1999).

Cellular and Tissue Distribution of PepT1. The PepT1 mRNA and protein are expressed primarily in intestinal and renal epithelial cells (Daniel and Kottra, 2004). Sites with lower levels of PepT1 expression and/or activity include the colon, bile duct epithelium, brain, and liver (Ford et al., 2003; Knutter et al., 2002; Miyamoto et al., 1996). Although almost no dietary peptides or AA reach the colon, there is a large supply of endogenous proteins in the colon, possibly serving as substrates for proteolysis by microflora.

Within the small intestine there are interesting species differences in PepT1 expression. Expression of PepT1 mRNA was detected in greatest quantities in the duodenum, jejunum, and ileum of the chicken, pig, and ruminant, respectively (Chen et al., 1999). These differences may correspond to differences in site of maximal digestion

and absorption. In the chicken, there was considerable expression of PepT1 in the ceca in addition to the small intestine and kidney (Chen et al., 2002). In rats, protein expression was greater in the ileum as compared with the jejunum (Tanaka et al., 1998). Howard et al. (2004) found that rat PepT1 mRNA was equally expressed throughout the length of the small intestine. In the black bear, mRNA abundance of PepT1 was greatest in the mid-region of the intestinal tract (Gilbert et al., 2007). In contrast to most species, ruminants, such as sheep and cattle, express PepT1 mRNA in the stomach, specifically, in the omasum and rumen (Chen et al., 1999; Pan et al., 2001).

Expression of intestinal PepT1 increases with enterocyte maturation. The PepT1 mRNA and protein were restricted to enterocytes between the crypt-villus junction and villus tip with increased expression towards the tip (Freeman et al., 1995; Ogihara et al., 1996; Tanaka et al., 1998). Within the colon, expression was restricted to the surface columnar epithelial cells.

Substrate Specificity of PepT1. Potentially all 400 di- and 8,000 tripeptides can be transported by PepT1. PepT1 also accepts pharmaceutical compounds with structural similarities to peptides called “peptidomimetics” and participates in their absorption which is of major therapeutic value (Brandsch et al., 2004). Water is thought to play an important role in accommodating the broad specificity of PepT1 by shielding the charges of the AA side chains within the substrate binding domain of PepT1. This allows both charged and uncharged substrates to bind at the same domain (Daniel, 2004).

Basolateral Peptide Transporter. The discovery of a peptide transporter in the basolateral membrane of enterocytes (Terada et al., 1999) and the knowledge that some peptides are relatively resistant to hydrolysis, provides at least presumptive evidence that

a carrier-mediated mechanism exists for transport of intact peptides to the bloodstream. Irie et al. (2004) characterized the efflux properties of the basolateral peptide transporter in Caco-2 cells by preloading cells with [^{14}C]-Gly-Sar and sampling the medium on the basolateral side after incubation. Efflux to the basolateral side was not affected by basolateral pH and was saturatable with a K_t of 24.8 ± 6.4 m*M* and V_{max} of 1.61 ± 0.35 nmol/mg protein/min, indicating a low-affinity. The K_t in the efflux direction was five to 10 times greater than in the influx direction, indicative of asymmetry in affinity.

The identity of the basolateral peptide transporter remains elusive. Shepherd et al. (2002) identified a candidate protein in the basolateral membrane of rat jejunum through the use of photoaffinity labeling with [4-azido-D-phe]-L-ala. The labeled candidate protein was extracted from SDS-PAGE gels and analyzed by MALDI-TOF. Interestingly, database searches for the 112 kDa candidate protein with a p*I* 6.5 revealed no similarities to PepT1 or other known proteins, thus establishing that this is a novel protein.

Free AA Transporters

Free AA are transported into the cell by a variety of different AA transporters that vary in substrate specificity and exhibit various ion dependencies and mechanisms for translocating AA (Table 2.2; Figure 2.2). This review will focus on the transporters evaluated in each of the feeding trials within this dissertation. For a detailed review on each of these transporters, the reader is referred to Gilbert (2005).

The Neutral AA Transporter, B^{o}AT. The B^{o}AT transporter (SLC6A19) is a sodium-dependent brushborder membrane neutral AA transporter, and is the major apical glutamine transporter (Avissar et al., 2004; Broer, 2004). Expression is restricted to kidney and small intestine in the mouse (Broer, 2004). Within the kidney mRNA is

localized to the proximal tubule, and within the intestine, mRNA is localized to enterocytes on the villi, with greatest expression towards the tip of the villus. Uptake studies in oocytes revealed a membrane-potential and pH dependent transport, independent of chloride. Uptake of radioactively labeled leucine was significantly inhibited by an excess of all other neutral AA, suggesting that all L- neutral AA are recognized and transported by this protein. Mutations in this system are thought to be responsible for hartnup disorder, an autosomal recessive disorder in which patients exhibit defective neutral AA transport, resulting in neutral hyperaminoaciduria (Seow et al., 2004).

The Excitatory AA Transporter 3 (EAAT3). The EAAT3 transporter (SLC1A1) is specific for anionic AA, including aspartic and glutamic acid (Kanai and Hediger, 2004). To overcome the steep electrochemical gradient of transporting negatively charged AA against their concentration gradient into the enterocyte, several ion exchanges are employed. Three Na^+ and one H^+ are co-transported with the AA in exchange for a K^+. The binding of glutamate to the glutamate transporters is rapid, and is responsible for the quick clearance of glutamate from the synaptic cleft (Kanai and Hediger, 2004). Also characteristic of the high-affinity glutamate transporters is a substrate-gated anion conductance (Kanai and Hediger, 2004).

The EAAT3 transporter is expressed in the apical membrane of kidney proximal tubules, intestinal epithelial cells, and in the neurons of the brain hippocampus, cerebral cortex, olfactory bulb, striatum, superior colliculus and thalamus (Kanai and Hediger, 2004). The EAAT3 protein localized to the brushborder membrane of enterocytes in the crypts and at the base of the villi, a distribution quite different from other nutrient

transporters, suggesting that EAAT3 may play a role in providing fuel to rapidly dividing cells in the crypts, rather than providing glutamate to the rest of the body. Glutamate is the primary oxidative fuel source for enterocytes (Wu, 1998), so the study of the regulation of EAAT3 in the small intestine is of particular interest.

Abundance of EAAT3 mRNA increases towards the distal region of the small intestine (Erickson et al., 1995), and increases from rat postnatal d 4 to d 21 in all segments of the small intestine (Rome et al., 2002). The protein expression was similar in all three segments of the small intestine at all ages.

The $b^{o,+}$AT and rBAT Transporter Complex. The $b^{o,+}$AT (SLC7A9) and rBAT (SLC3A1) proteins heterodimerize inside the cell by disulfide linkages and traffic to the brushborder membrane as a complex, and function as an obligatory exchanger for AA (Palacin and Kanai, 2004; Verrey et al., 2004). Coexpression of rBAT and $b^{o,+}$AT induces a Na^+-independent, high affinity transport of L-cystine, ornithine, and cationic AA, and a lower affinity for neutral AA, occurring by an exchange mechanism (Palacin and Kanai, 2004; Wagner et al., 2001). Luminal cystine and cationic AA are transported because of a high extracellular affinity, while intracellular neutral AA are transported out of the cell to the lumen because of a high intracellular concentration. Using brushborder membrane vesicles isolated from 6 wk-old chickens, Torras-Llort et al. (2001) demonstrated an asymmetry in substrate binding affinity for $b^{o,+}$ transport, with a greater extracellular affinity for arginine compared to intracellular affinity. Chillaron et al. (1996) demonstrated that transport through $b^{o,+}$AT is driven by the transmembrane gradient of neutral AA maintained by an apical unidirectional Na^+-dependent transporter. Thus, neutral AA are transported out of the cell into the lumen by $b^{o,+}$AT/rBAT in

exchange for extracellular cationic AA and cystine. Defects in this system cause an inherited defect, known as cystinuria, a type of aminoaciduria.

Tissue Distribution of $b^{o,+}$ Transporters. The $b^{o,+}$AT and rBAT transporter genes are expressed in the apical membrane of epithelial cells in small intestine, kidney, liver, placenta, lung, heart, adrenal gland, spinal cord, brain and pancreas (Palacin and Kanai, 2004; Verrey et al., 2000; Wagner et al., 2001). In addition to being expressed on the apical membrane of epithelial cells, rBAT was also found within enteroendocrine cells and submucosal neurons (Pickel et al., 1993).

The rBAT mRNA showed decreased expression from proximal to distal small intestine, and an increased gradient of expression in the apical membrane from the crypt-villus junction to the tip of the villus (Rome et al., 2002). There was no protein detected in the crypt cells. Howard et al. (2004) on the other hand, found that rBAT mRNA exhibited equal mRNA abundance throughout the length of the rat small intestine.

Physiological Importance of Obligatory Exchangers. The functions of AA transporters that serve as exchangers are dependent on AA concentrations intra- and extracellularly, ionic concentrations, and the membrane potential (Verrey et al., 2000). The normal molar ratio of AA exchange for these transporters is 1:1, indicating that a net flux of AA can not be achieved. All evidence argues that the glycoprotein-associated AA transporters all function only as obligatory exchangers. However, these obligatory exchangers can cooperate with unidirectional transporters expressed on the same membrane by transporting out AA that are substrates of the unidirectional transporter against the exchanged AA. The concentration gradient of the intracellular recycled AA can then provide a driving force for uphill transport of the exchanged AA.

Thus, obligatory exchangers can increase the AA specificity range for transport across the apical and basolateral membrane by providing recycled substrate for the unidirectional system expressed on both membranes. This also brings up the matter of transport asymmetry and the driving force for transport. Sources of transport asymmetry for the $b^{o,+}$AT transporter includes a high extracellular affinity for cationic AA that drives transport both by a membrane potential and by the concentration gradient of the intracellular neutral AA. In the case of cystine, for which $b^{o,+}$AT exhibits a lower affinity, there is a chemical sink leading to a concentration gradient, in which transported cystine is reduced to two cysteines and a concentration gradient of the exchanged AA is developed.

The Basolateral Cationic AA Transporters, CAT1 and CAT2. Transporters within the CAT family (SLC7) transport cationic AA by deriving energy from a differential *trans*-stimulation by intracellular substrates (Verrey et al., 2004). The CAT proteins play an important role in transporting arginine into pools of cationic AA from which endothelial and inducible nitric oxide synthase can receive substrate (Verrey et al., 2004).

The CAT1 (SLC7A1) is a high-affinity, low-capacity transporter whose expression is widespread throughout the body, except for the liver (Verrey et al., 2004). Transport is pH and Na^+-independent and is sensitive to *trans*-stimulation (Kizhatil and Albritton, 2003). The greatest expression levels of CAT1 have been found in the testis, and to a lesser extent, in the bone marrow, brain, stomach, spleen, kidney, lung, ovary, uterus, large and small intestine, thymus, heart, skeletal muscle, and skin (Deves and Boyd, 1998). Expression of CAT1 is generally localized to the basolateral membrane of

epithelial cells, but is expressed in the plasma membrane and intracellular vesicles in glioblastoma cells (Wolf et al., 2002). Kizhatil and Albritton (2003) found using immunofluorescence microscopy that CAT1 localized as clusters in the basolateral membrane of kidney cells. Different proportions of CAT1 clusters in different microdomains could be indicative of differences in physiological function. CAT1 is the major transporter for system y^+ in most cells, because homozygous CAT1 knockout in mice was lethal 1 d after birth (Kizhatil and Albritton, 2003).

Two alternative splice products of CAT2 (SLC7A2), CAT2A and CAT2B, are expressed quite differently (Verrey et al., 2004). The CAT2A, a low-affinity transporter, is most highly expressed in the liver, but is also present in skeletal muscle, cardiomyocytes, cardiac microvascular cells, vascular smooth muscle, and pancreas. In contrast, CAT2B requires induction of expression, usually after cytokine or lipopolysaccharide treatment, and follows the same expression pattern as CAT1. The CAT2B is also induced with the inducible isoform of nitric oxide synthase. Gene knockouts of CAT2B, although apparently normal phenotypically, do exhibit decreased nitric oxide production in peritoneal macrophages, demonstrating that CAT2B is critical for supplying a sufficient substrate for inducible nitric oxide synthase.

The y^+LAT Transporters. The y^+L system transporters (SLC7 family) heterodimerize with the 4F2hC protein to couple the exchange of intracellular cationic AA to the co-transport of extracellular neutral AA and Na^+ into the cell across the basolateral membrane. (Verrey et al., 2004; Wagner et al., 2001). Transport in the absence of Na^+ also occurs where intracellular dibasic AA are exchanged for extracellular dibasic AA. Like rBAT, 4F2hC functions to traffic the complex to the plasma membrane.

The y^+LAT1 (SLC7A7) protein is expressed on the basolateral membrane of epithelial cells in kidney proximal tubule and small intestine, and follows a decrease in expression along the length of the kidney proximal tubule (Wagner et al., 2001). The y^+LAT1 transporter is also expressed in leukocytes, lung, erythrocytes, and placenta. Mutations of y^+LAT1 result in a condition known as lysinuric protein intolerance, in which patients experience poor absorption of dibasic AA, especially lysine, and large amounts are found in the urine (Palacin et al., 2005).

In contrast to the y^+LAT1 protein, y^+LAT2 (SLC7A6) expression is widespread and expressed in both nonepithelial and epithelial tissues (Verrey et al., 2004). It is expressed in brain, small intestine, testis, parotids, heart, kidney, lung, and liver (Wagner et al., 2001). The influx of neutral and cationic AA is very efficient, but the efflux of cationic AA is more efficient than the efflux of neutral AA.

The AA Transporter, LAT1. The system L, LAT transporters (SLC7 family), heterodimerize with the heavy chain 4F2hC protein and mediate the Na^+-independent movement of large, neutral AA across the basolateral membrane (Rajan et al., 2000; Verrey et al., 2004). System L transport is widespread and found in most types of cells. Expression of LAT-1 (SLC7A5) mRNA was found in most tissues including brain, spleen, thymus, heart, lung, blood-brain barrier, leukocytes, placenta, testis, colon and human tumor cell lines (Kanai et al., 1998; Wagner et al., 2001).

The LAT1 transporter has a high affinity for branched chain and aromatic AA, with the affinity being up to 100-fold higher for extracellular AA than for intracellular AA (Verrey et al., 2004). Because of this, the extracellular AA concentration limits the rate of uptake. The heterodimeric complex functions as an exchanger that does not

transport by facilitated diffusion, and uptake is *trans*-stimulated by intracellular AA, with a 1:1 ratio. This complex is proposed to function to equilibrate concentrations of AA across the plasma membrane rather than to cause net uptake. The LAT-1 transporter is also capable of recognizing and transporting D-isomers of leucine, methionine, and phenylalanine (Kanai et al., 1998).

Monosaccharide Transporters

The mechanism of monosaccharide transport in the small intestine is a bit more simplistic than that of AA. Glucose and galactose are transported into the enterocyte by SGLT1 in a Na^+-dependent manner, while fructose is transported by GLUT5 via facilitated diffusion (Ferraris, 2001; Uldry and Thorens, 2004; Wright and Turk, 2004). All three sugars exit the enterocyte via GLUT2 on the basolateral membrane by facilitated diffusion dependent on the transmembrane concentration gradient. The mechanism of glucose transport may vary across avian species. In the sparrow, for example, glucose transport was shown to occur passively, predominately through the paracellular route (Chang et al., 2004).

The Na^+/Glucose Intestinal Transporter, SGLT1. The SGLT1 protein (SLC5A1) was the first cotransporter protein identified in the SLC5 Na^+/glucose cotransport family (Wright and Turk, 2004). It is a high-affinity/low-capacity Na^+/glucose transporter expressed in the intestine and kidney (Ferraris, 2001). The SGLT1 is expressed predominantly in the apical membrane of small intestinal enterocytes, and mediates the cotransport of 2 Na^+ and either D-glucose or D-galactose from the lumen across the brushborder membrane (Bird et al., 1996; Wright et al., 1994). The SGLT1 also functions as a Na^+ uniporter, water channel, urea channel, and cotransporter of water and urea (Loo

et al., 1996; Wright and Turk, 2004). Loo et al. (1996) demonstrated that approximately 260 molecules of water are coupled to each glucose molecule transported by SGLT1, accounting for the absorption of approximately 5 L of water per d in a human. Mutations in the SGLT1 gene have been linked to glucose-galactose malabsorption, a rare autosomal recessive disease (Wright and Turk, 2004).

Tissue and Cellular Distribution of SGLT1. On a cellular level, SGLT1 mRNA was limited to the villi, with no labeling in the crypts (Ramsanahie et al., 2004). Villi were labeled in the mid and upper thirds of the villi, with very little label in the tips (Ramsanahie et al., 2004; Shirazi-Beechey et al., 1994). Immunofluorescence staining revealed the presence of SGLT1 protein on the apical membranes in the jejunum and diffuse staining in the cytoplasm (Ramsanahie et al., 2004). The SGLT1 is predominantly expressed in the small intestine but is also expressed in the plasma membrane of cells in the trachea, kidney, and heart (Wright and Turk, 2004). A 58 kDa non-glycosylated SGLT1 precursor protein was observed in enterocytes leaving the crypt (Shirazi-Beechey et al., 1994). As enterocytes migrate out of the crypt, the expression of this protein declines, while expression of the 75 kDa mature glycosylated SGLT1 appears and begins to increase. Shirazi-Beechey et al. (1994) suggested the presence of a sugar receptor in the crypt cells that stimulate synthesis of the precursor protein in response to the presence of luminal sugars.

The Brushborder Membrane Fructose Transporter, GLUT5. The GLUT5 transporter (SLC2A5) is a Na^+-independent transporter that exclusively transports fructose (Uldry and Thorens, 2004). Garriga et al. (2004) characterized the kinetics of brushborder membrane D-fructose transport in chicken jejunum, and found that fructose

transport is low-affinity, high-capacity, with a V_{max} of 2.40 nmol · (mg prot)$^{-1}$ · s^{-1}, a diffusion constant (K_d) of 25 nl · (mg prot)$^{-1}$ · s^{-1} and a Michaelis constant (K_m) of 29 m*M*, indicating that transport is saturatable and may also occur through simple diffusion. The transport of fructose was Na^+-independent and unaffected by the presence of other sugars, suggesting a transporter with high specificity for fructose.

Expression of GLUT5 is prominent in the mid-region of the small intestine, and is detected at a lesser extent in the kidney, skeletal muscle, adipocyte, microglial cells, and in the blood-brain barrier (Uldry and Thorens, 2004).

The Basolateral Monosaccharide Transporter, GLUT2. The GLUT2 transporter (SLC2A2) mediates the Na^+-independent low-affinity movement of glucose, galactose, mannose, and fructose across the basolateral membrane (Uldry and Thorens, 2004). It also mediates the high-affinity transport of glucosamine. A second low-affinity/high-capacity glucose transporter is proposed to exist on the brushborder membrane of enterocytes (Wright et al., 2003). The GLUT2 has been implicated in the conditional transport of glucose via a translocation to the brushborder membrane (Mithieux, 2005).

The GLUT2 transporter is expressed in the basolateral membrane of intestinal and renal epithelial cells, the sinusoidal membrane of hepatocytes, and the plasma membrane of pancreatic β-cells (Uldry and Thorens, 2004). Garriga et al. (1997) confirmed, using purified jejunum basolateral membrane preparations, that a single GLUT2-type carrier exists, capable of transporting both glucose and fructose, with a higher affinity for glucose, favoring its exit over fructose. The maximal rate of transport (V_{max}) for D-Glc was 2.36 nmol · mg^{-1} · s^{-1} and for D-Fru was 3.79 nmol · mg · s^{-1}. The Michaelis constants were 17.3 mmol/L for D-Glc and 40.4 mmol/L for D-Fru. The transport of both hexoses

was Na^+-independent. In GLUT2 null mice there was no deficiency in glucose absorption, suggesting the presence of an alternate pathway for transport of glucose out of cells (Wright et al., 2003). An alternate pathway for basolateral glucose transport was proposed that involved vesicle trafficking, but further studies are needed to elucidate this pathway (Wright et al., 2003).

Dietary Regulation of Nutrient Transporters

Intestinal nutrient transport is regulated by diet and by substrate concentration in the lumen. Ferraris and Diamond (1989) rationalized the differences observed in transporter regulation among different nutrients. They suggested that transporters for metabolizable nontoxic nutrients, such as sugars, nonessential AA, and short-chain fatty acids should be up-regulated with increasing dietary level and that transporters for essential nutrients, which are toxic in large quantities, such as water-soluble vitamins and minerals should be down-regulated by increasing concentrations of substrates. For AA, the matter is complicated by the fact that AA can be also used as sources of energy. Some AA are more essential to a cell than others, or more toxic. Furthermore, enterocytes express transporters with overlapping substrate specificity and transporters that mediate the movement of both essential and nonessential AA (Karasov et al., 1987). Thus, it becomes difficult to predict whether a transporter should be up-regulated in response to certain AA deficiencies or imbalances.

Substrates can regulate transporters specifically and nonspecifically. Nonspecific regulation involves general changes in mucosal surface area, transcellular electrochemical gradient, paracellular permeability, and plasma membrane lipid composition and fluidity (Ferraris, 1994, 2000). Specific regulation includes changes in

the site density of specific transporters in enterocytes as a result of changes in protein synthesis or degradation rate or an increased insertion of preformed cytoplasmic transporters into the brush border membrane (Ferraris and Diamond, 1989).

Regulation of SGLT1 by Dietary Sugars. The SGLT1 is regulated by luminal sugar contents (Dyer et al., 1997). In lambs, weaning coincides with development of the rumen, which ferments carbohydrates and limits their entry into the small intestine. The onset of weaning (5 wk) also parallels a significant decrease in active glucose transport and SGLT1 protein expression in lamb brushborder membrane vesicles. Lambs maintained on a milk-replacer at 5 wk maintained active transport of glucose and SGLT1 protein expression, demonstrating that luminal sugar contents regulate the expression of SGLT1 (Dyer et al., 1997).

Expression of SGLT1 is regulated by dietary fructose (Goda, 2000). Rats fed a fructose-containing diet showed upregulation of SGLT1 and GLUT5 mRNA (Goda, 2000). SGLT1 expression and activity is also induced by 3-0-methyl α-D-glucopyranoside and methyl α-D-glucopyranoside, demonstrating that metabolizable substrates are not necessary for regulation (Shirazi-Beechey et al., 1994).

Regulation of PepT1 by Dietary Protein Level. In general, peptide transporter expression and activity increases with dietary protein and peptide level. Shiraga et al. (1999) maintained rats on a 20% casein diet for 1 wk. A group of rats were switched to a protein-free diet and others were switched to diets consisting of 50%, 20%, or 5% casein, 20% of a dipeptide, or 10% of a single AA, fed for 3 d. Greater levels of dietary protein were associated with greater expression of PepT1 mRNA and protein. Compared with rats fed the protein-free diet, there was an increase in PepT1 mRNA and protein for rats

fed the Gly-Phe diet and significantly higher PepT1 mRNA for the phenylalanine-fed but not the glycine-fed rats. Results from this study demonstrated that PepT1 may be regulated by specific AA and is very responsive to changes in dietary protein, in particular, quantity and composition.

In chickens, an increase in PepT1 mRNA was observed in the intestine of chickens fed 18 and 24% crude protein (CP) diets with restricted food intake and a decrease in PepT1 mRNA was observed in chickens fed a 12% CP diet (Chen et al., 2005). In chicks fed the 24% CP diet ad-lib, there was lower expression of PepT1 as compared with chicks consuming restricted amounts of the diet. Thus, the increase in PepT1 was probably not due to an increase in CP but instead to the feed restriction.

A high-protein diet (72 vs. 18%) resulted in increased absorption of the dipeptide carnosine in mice (Ferraris et al., 1988) and feeding a protein-free diet for 40 to 84 d resulted in an increased ability of rat jejunum to transport a methionine dipeptide but not free methionine (Lis et al., 1972). It is perhaps counterintuitive that both abundance and lack of substrate will stimulate PepT1. Upregulation by a high-protein diet appears to be a mechanism to take advantage of the abundant resource while upregulation in response to a lack of substrate may be a compensatory mechanism to scavenge AA in the lumen. The magnitude of the response in changes of PepT1 expression and activity will probably be dependent on length of time for a particular dietary manipulation, availability of transportable substrate, AA composition (concentrations of free and peptide-bound), and presence of other components in the lumen that can change digestive and absorptive dynamics (i.e., sugars, vitamins, minerals, fats, etc.).

Effect of Luminal Starvation on Nutrient Transporter Expression. Rayo et al. (1992) observed that chicks subjected to acute (48 h) fasting at 3, 8, or 15 d old, or repetitive fasting (cumulative at all three ages), adapted by increasing both duodenal glucose and tryptophan uptake. Food restriction in rats (8 to 10 g food/d) increased glucose transport (Marciani et al., 1987). In 6-wk old male broiler chickens, SGLT1 mRNA was enhanced in birds that were totally deprived of feed for 4 d as compared to birds given ad-libitum access to feed (Gal-Garber et al., 2000). Diamond et al. (1984) sought to determine the effect of diet history prior to starvation, and found that in fasted mice previously fed a carbohydrate-free diet, glucose uptake increased by 50 to 100%. In fasted mice previously fed a high-carbohydrate diet, glucose uptake decreased.

Short-term starvation in rats increases mRNA and protein expression of PepT1 (Ihara et al., 2000). Rats starved for 4 d exhibited a 179% increase in mRNA and protein expression. Rats that were fed 50% of the intake of controls for 10 d and rats given total parenteral nutrition (TPN) for 10 d exhibited a 161% and 164% increase in PepT1 mRNA, respectively. This dramatic up-regulation occurred in spite of the fact that the mucosal weight decreased in the starved and TPN group by 41 and 50%, respectively. In a different study, PepT1 mRNA and protein increased threefold and the rate of peptide transport in rats increased dramatically after only 1 d of fasting (Thamotharan et al., 1999).

Howard et al. (2004) examined the effects of TPN and administration of glucagon-like peptide (GLP-2) on the mRNA expression of AA transporters and PepT1 in rat small intestine. Total parenteral nutrition for 7 d up-regulated PepT1 mRNA in the distal intestine while proximal (duodenal) mRNA was unchanged. Administration of

GLP-2 inhibited the effect of TPN on mRNA expression of PepT1. It is known that GLP-2 is a trophic factor that maintains cellular protein synthesis during luminal starvation and perhaps infusion of GLP-2 reduced the need for upregulation of PepT1. During luminal starvation, the need to absorb endogenous protein products increases and thus, apical membrane transporters, which in this study included rBAT, EAAT3, ASCT2, and PepT1, were up-regulated in the distal intestine to maximize assimilation of AA (Howard et al., 2004).

Mechanism of Transporter Regulation. Regulation of nutrient transporters by dietary substrate appears to occur by two mechanisms: 1) by increasing mRNA stability and 2) by increasing gene transcription rate (Adibi, 2003). Increased expression and activity of nutrient transporters per mg of tissue in response to reduced luminal nutrient availability may serve as a mechanism to compensate for mucosal atrophy (Ferraris and Carey, 2000). In protein-malnourished rats (5% CP diet), intestinal villi were shorter in comparison with control rats (20 % CP diet). There were older enterocytes on villi in malnourished rats compared with those at the same villus height in well-fed rats. Delayed access to feed for 36 h posthatch resulted in depressed villus height and crypt depth and reduced growth in all segments of the chick small intestine (Uni et al., 1998b). Silva et al. (2007) subjected male broiler chicks to a feed restriction at 30% of ad libitum intake from 7 to 14 d and found that feed restriction decreased the surface area of the tip of the enterocytes in the small intestine at 14 d. Hence, the mechanism leading to a fasting or malnutrition-related increase in nutrient transport may be a combination of increased gene expression and ratio of transporting to non-transporting cells (Ferraris and Carey, 2000).

The mechanism of dietary protein regulation of nutrient transporters may be through a pathway of direct substrate regulation. Shiraga et al. (1999) found elements in the 5` upstream region of rat PepT1 responsive to peptides and free AA. Fei et al. (2000) identified a similar AA responsive element in the promoter region of mouse PepT1. Addition of Gly-Sar to the medium of Caco-2 cells caused a threefold and twofold increase in the expression of human PepT1 mRNA and protein, respectively (Thamotharan et al., 1998). Treatment with brefeldin, an inhibitor of protein transport from the endoplasmic reticulum to the Golgi, abolished transport, showing that increased synthesis and processing by the trans Golgi network accounted for increased expression at the apical membrane. Similar uptake experiments were performed with a naturally occurring dipeptide, Gly-Gln, confirming the physiological relevance of these findings (Walker et al., 1998). Additionally, it is worth noting that free AA uptake may be indirectly regulated by PepT1 activity. Since many AA transporters serve as exchangers, filling of cells with a variety of AA as peptides by PepT1 may be important for net movement of AA. For example, Wenzel et al. (2001) demonstrated that uptake of dipeptides caused stimulation of AA uptake by the $b^{o,+}$ system.

Results from these studies demonstrate that the small intestine readily adapts and undergoes functional alterations in response to sudden changes in the luminal environment. Ferraris and Diamond (1989) described the regulation of nutrient transporters as a way to match uptake capacity to requirements without wasting energy on unnecessary transporters. Thus, the study of regulation of transporters leads to elucidation of mechanisms that can change rates of transport (Ferraris, 2001). As we

explore various dietary regimens in the chicken it is important to understand how the diet influences intestinal function.

Age-Related Changes in Intestinal Nutrient Uptake

Parallel with low levels of digestive enzymes and some transporters at hatch, intestinal uptake of nutrients appears to be relatively under-developed at hatch and increases during the first few days posthatch. Uptake capacity of methionine and glucose was low at hatch in chicks and increased in the duodenum and jejunum between day of hatch and 7 d, and was fairly constant from 7 to 14 d (Noy and Sklan, 1996). The increase in methionine or glucose absorption with age was most accentuated in birds with immediate access to feed at hatch (Noy and Sklan, 2001). In fed-birds, glucose absorption increased from less than 50 % at hatch to 80 % at 4 d, while in birds not given feed for 4 d, absorption increased to only 59 % at 4 d. In birds denied access to feed for only 2 d, the difference was less apparent from fed-birds at 4 d. Methionine absorption followed a similar pattern, increasing from 43 % at hatch to 75 % at 4 d in fed birds, while in birds not given feed for 4 d, absorption was only 59 %. Again, birds denied feed for 2 d showed less of a difference at 4 d. With age, there was little change in K_t but a greater plateau accompanying higher concentrations of substrate, suggestive of increases in production of mucosal transporters. The authors suggested that digestion and absorption are probably not as rate limiting as feed intake in controlling nutrient absorption. Although in a different study, Sklan et al. (2003b) suggested that low expression of nutrient transporters such as SGLT1 at hatch followed by increases in expression, may help account for the observed low uptake of nutrients at hatch.

In contrast to methionine and glucose, uptake of oleic acid was very high at hatch and showed little response to developmental age or access to feed at hatch (Noy and Sklan, 1996; Noy and Sklan, 2001; Sklan, 2003). Poultry do not have a functional lymphatic system, and absorbed lipids generally enter the circulation via the portal vein. In broilers chicks, the rate of intestinal lipid absorption was highest at hatch and changed very little with age, while the rate of metabolism and use of fatty acids for energy decreased with age (based on respiratory quotient). The change in lipid metabolism is also supported by a decrease in concentration of plasma lipoproteins after exhaustion of yolk sac reserves, indicating reduced transport of lipids, resulting in reduced utilization of circulating lipids (Sklan, 2003). Glucose on the other hand, was used for energy at a constant rate during the posthatch period (based on respiratory quotient), while rate of intestinal absorption into circulation increased.

In the poult, similar to the chick, uptake of oleic acid was 78 % at hatch, changed little with age, and was not affected by delayed access to feed (Sklan and Noy, 2003b). In poults, the small intestinal lumen concentrations of triglycerides, phospholipids and cholesteryl esters decreased posthatch, while concentrations of free fatty acids increased (Noy and Sklan, 1998b), suggesting that hydrolysis increased after hatch. Hydrolysis appeared to increase rapidly first in the duodenum followed by the distal segments. Others observed increased intestinal lipase activity as well as increased bile salt production with age in turkey poults (Krogdahl, 1985; Krogdahl and Sell, 1989). The presence of hydrophobic yolk in the small intestine at hatch may help to explain the findings discussed in preceding paragraphs regarding differences in uptake of methionine and glucose versus oleic acid at hatch. The yolk may hinder uptake of hydrophilic

nutrients in the early posthatch period explaining low uptake of glucose and methionine, while not affecting fat absorption (Noy and Sklan, 1999).

Factors Influencing Intestinal Absorptive Function. While absorptive surface area in the small intestine is related to villus and crypt development, it should also be correlated to brushborder membrane surface area and density of membrane-bound nutrient transporters (Geyra et al., 2001a). The nutrient uptake capacity will depend on a variety of factors, including luminal concentrations and availability as well as transporter density and turnover rate. Understanding changes in transporter density involves an evaluation of mRNA stability and post-translational modifications and trafficking of the transporter protein.

Numerous groups have observed that with age, transport per unit tissue decreases, relative abundance of transporters decrease, while total transport capacity is increased (based on total intestinal mass). In broilers, uptake of L-tryptophan (pmol/mg protein/min) in the small intestine (jejunum and ileum) decreased with age but total uptake capacity increased (Iji et al., 2001c). Iji et al. (2001c) also observed that while specific activities of maltase, sucrase, APN and alkaline phosphatase decreased with age in all segments, total activities of the enzymes increased during the first two weeks posthatch. The increase in total enzyme activity was hypothesized to be more related to the accompanying increase in total surface area (greater villus height and intestinal segment length) rather than an increase in the efficiency of individual cells.

Barfull et al. (2002) evaluated ontogenetic changes in glucose uptake in male leghorn chickens at 2 d compared with 5 wk, taking into account glucose concentrations in the lumen, rates of uptake, abundance of glucose transporter, and absorptive surface

area as determined by size of villi and microvilli. They found that the concentration of glucose increased 48 % in the jejunum of 5 wk chickens as compared with 2 d, and that the rate of uptake across brushborder membrane vesicles was 40 % greater in 2 d compared with 5 wk chickens. Relative abundance of SGLT1 protein was 38 % lower in 5 wk compared with 2 d, but the microvilli amplification factor was 27.6 at 2 d compared with 37.4 at 5 wk. Hence, the total surface area represented by villi and microvilli was 11-fold higher in 5 wk birds, compensating for reduced SGLT1 at 5 wk, resulting in an actual 6.6-fold increase in total SGLT1. The greater rate of transport per unit brushborder membrane protein at 2 d may be explained by a greater site density of SGLT1 protein at an earlier age although in a different study Sklan et al. (2003) reported that mRNA abundance of SGLT1 was low in the early posthatch broiler chick. Obst and Diamond (1992) observed that rates for uptake of glucose, fructose and proline either changed little or decreased with age, while total uptake expressed as a product of tissue-specific rates and intestinal mass increased with age for all nutrients.

Mechanisms of Changes in Transporter Activity. Similar to chickens, mammalian postnatal total intestinal transport capacity increases with age parallel to the increase in intestinal mass, while transport of most nutrients decreases in relation to weight (Pacha, 2000). Pacha (2000) offered several explanations for the changes in specific activity of enzymes and transporters with age. The reduction in transport could be due to changes in transporter density, change in predominant isoform, and change in turnover rate. It was further concluded that the decline in activity is probably not the result of dilution in the mucosal surface area at the expense of muscle, but instead is more

likely caused by an increase in the number of immature cells populating the villi, in effect diluting the mature enterocytes.

There is still some uncertainty as to whether regulation of digestive enzymes and nutrient transporters occurs by induction in immature, undifferentiated cells, or whether older cells are capable of being reprogrammed (Ferraris, 2001). Ferraris (2001) proposed a model to describe regulation of SGLT1 and GLUT5 based on findings from multiple studies. For SGLT1, transcription is initiated in crypt cells and as cells migrate up the sides of the villi there is a lag time due to cell migration during which the transporter protein is synthesized and inserted into the membrane. For GLUT5, there is evidence of de novo synthesis of mRNA and protein in mature cells lining the villi, leading to a much shorter lag time (4-8 h vs. 12-24 h for substrate-induced regulation of SGLT1).

Age-Related Changes in Metabolizable Energy of Feed

Changes in expression and activity of digestive enzymes and nutrient transporters as well as observed changes in the intestinal uptake of various nutrients, parallels changes in the metabolizable energy of various feed ingredients. This is of particular importance for dietary formulation in a commercial setting. The standard for formulating diets to meet recommended nutritional parameters is the use of NRC (1994). Unfortunately, although there is an overwhelming amount of evidence showing dramatic changes in nutrient utilization during the first 2 wk posthatch (Batal and Parsons, 2002b), metabolizable energy and AA digestibility (AAD) values for a given ingredient encompass the entire first 3 wk of life of the chick with the underlying assumption that nutrient utilization is relatively constant during this time. In general, nutrient utilization is low during the first week posthatch and rises with age (Sulistiyanto et al., 1999).

The metabolizability of sorghum, maize, starch and dextrin increased with age after hatch (Sulistiyanto et al., 1999). Dextrin, an oligosaccharide that requires less enzymatic machinery for complete hydrolysis to glucose, has a greater true metabolizable energy (corrected for nitrogen) than starch at 1 d posthatch, but not at 3 d, showing that limited enzymatic machinery may be a limiting step in nutrient assimilation. Protein sources also showed age-dependent changes in energy utilization although for casein, an easily digested protein, there was no age dependency, further supporting the idea that easily digested nutrients are better utilized in the early posthatch period while the gastrointestinal tract is still developing. There was no difference in metabolizability of fat during the first few days posthatch.

In a different study, apparent metabolizable energy (corrected for nitrogen; AME_n) and AAD was determined in broiler chicks fed corn-soybean meal, cornstarch-crystalline AA, dextrose-casein, or corn-canola meal based diets (Batal and Parsons, 2002b). The AME_n and AAD increased with age for the corn-soybean meal and corn-canola meal diets. From 0 to 14 d, AME_n of the corn-soybean meal increased from 2,970 to 3,430 kcal/kg DM, while lysine digestibility increased from 78 to 79 %. Interestingly, AME_n and AAD of the starch-AA and dextrose casein diets were high at hatch and changed little with age. Collectively, results from these studies demonstrate that nutrient utilization changes during the first 2 wk posthatch with small changes observed for diets containing easily-digested ingredients. This suggests that partially-hydrolyzed ingredients may benefit chicks during the early posthatch period.

Effect of Nutrient Level in the Diet on Intestinal Nutrient Utilization. Other studies have examined the effect of protein, fat and fiber levels on feed intake and

nutrient utilization in order to evaluate the ability of certain diets to saturate intestinal nutrient uptake or change feed intake (Noy and Sklan, 2002). Since uptake of lipids vs. AA and hexoses is different during the early posthatch period this is of particular interest. In young broiler chicks, increasing levels of cellulose in the diet led to reduced intake and to subsequent reductions in BW and feed efficiency, presumably due to the effect of cellulose on stimulating intestinal "fill" and to the sacrifice of energy density at the expense of higher inclusion levels of fiber. Increased levels of fat in the diet also reduced intake, probably due to the higher energy density of the diet, with a reduction in intake serving to maintain a constant level of caloric intake while not affecting feed efficiency.

When crude protein levels of the diet were raised with concentrations of essential AA held constant, feed intake and efficiency of protein retention was reduced, most likely driven by the limiting AA and the excess of non-limiting AA requiring disposal (Noy and Sklan, 2002). Similarly, Swatson et al. (2002) observed that increasing crude protein levels without AA supplementation and without adjusting the energy to protein ratio, led to poor broiler performance. When dietary crude protein levels were raised (> 230 g/kg) while maintaining a balanced ratio of essential AA to crude protein, broilers showed improved performance that carried over through market age (Sklan and Noy, 2003a). Thus, AA are needed to support maximal protein synthesis in both sufficient quantities and proportions.

Mucosal Metabolism of Dietary Nutrients. Gastrointestinal tract metabolic activity has a tremendous impact on nutrient supply to the rest of the body. The portal-drained viscera (stomach, intestine, pancreas, spleen) represent 1 to 6 % of BW but represent over 35 % of whole-body protein turnover and energy expenditure (Stoll and

Burrin, 2006). There are several fates of an absorbed AA in the enterocyte including protein synthesis, conversion into a different AA or other compound, complete oxidation to carbon dioxide, or transport to the basolateral membrane (Stoll and Burrin, 2006). As much as 95 % of dietary glutamate, glutamine and aspartate is utilized by the gut (Stoll et al., 1998; Wu, 1998). Approximately 50 % of glutamate, glutamine and aspartate carbons that are used by the gut become oxidized to CO_2 via the TCA cycle, while the remaining carbons are accounted for as lactate, alanine and glucose. A substantial portion of dietary threonine (60 %) is used by the gut and is directed towards mucosal protein synthesis, in particular, synthesis of mucins (Stoll et al., 1998, Stoll and Burrin, 2006).

Because of preferential utilization of AA for oxidative fuel and high rates of protein turnover, the profile of AA that are absorbed by the enterocyte may be very different than the profile of AA that enter the portal circulation. In addition to AA, ingested fatty acids and glucose are also metabolized in the chick mucosa, although not nearly to the extent that AA are utilized by the gut (Sklan et al., 1996). Metabolism of [^{14}C]-oleic acid to non-lipid compounds constituted 15 % of the dose of oleic acid to the bird, while CO_2 represented approximately 25 %. Up to 37 % of glucose was metabolized to lactate in a different study, demonstrating substantial nutrient metabolism in the chick mucosa (Reisenfeld et al., 1982).

These results demonstrate that intestinal tissue maintenance and growth use a substantial proportion of dietary nutrients, in particular AA. Whether or not this is truly a "waste" to the animal (e.g., using 60 % of dietary threonine to support mucin synthesis) is debatable. We know that the mucin layer is extremely important as a barrier to mechanical shear and to harmful substances and a potentially harsh luminal environment.

At the same time, diverting such a large proportion of an essential AA that is limiting in protein synthesis in other parts of the body imposes a restriction on the growth potential of the broiler. In our attempts to improve feed efficiency and performance in the chicken, the inter-play between the diet and gastrointestinal mucosal metabolism is an important point of consideration.

Importance of Early Posthatch Nutrition on Growth and Development

In a commercial setting there is a large window of time between birds hatching and their removal from the hatchery. Birds are removed after the optimal number of eggs have hatched and common processing treatments such as de-beaking, vaccination, sexing, and transportation can further increase time until placement, usually averaging 48 h or more. During this period, the chick is without food or water and may suffer from dehydration, weight loss, ketosis, inadequate stimulation of gastrointestinal and immune system development, and ultimately impaired performance that can carry over to market age. This phenomenon was demonstrated in broiler chicks subjected to 24 or 48 h without access to feed after hatch and resulted in significantly lower BWs at 40 d (Nir and Levanon, 1993).

Early nutrition is critical for adequate development of digestive and absorptive functions of the intestinal epithelia. This is obvious in situations where chemically-defined diets are fed containing a mixture of crystalline AA as the source of protein. Because the AA are immediately available for absorption in the upper small intestine and have a relatively short half-life, little substrate is made available to the ileum and colon, resulting in reduced villus height and crypt depth in those regions similar to the

morphology of unfed birds (Batal and Parsons, 2002a). The following sections will discuss the effects of early posthatch nutrition on growth and development.

The Yolk Sac Reserves at Hatch. The yolk sac weight usually represents between 20 and 25 % of the chick BW at hatch and closer to 15 % of the poult BW at hatch (Noy and Sklan, 1998b; Noy et al., 1996) and reserves are exhausted within the first week posthatch. Within the first 2 d posthatch, more than 50 % of the yolk protein and 90% of yolk fat is transferred to the tissues of the neonate chick (Nitsan et al., 1991b). Although poults have a proportionally lower yolk wt., turnover time is similar to chicks, thus the reserves are utilized more rapidly in poults. The difference in relative size of the yolk in poults and chicks could help explain why growth of the poult is less rapid during the first few days after hatching and why mortality is higher in poults (Sklan and Noy, 2003b).

Yolk Sac Reserves as an Energy Source. Although the yolk sac represents a reserve source of nutrients at hatch to support survival of the chick, immediate access to feed is critical for optimal growth performance (Noy and Sklan, 1998a). The residual contents of the yolk have important uses in the growing chick (Dibner et al., 1998). Maternal IgG represents approximately 20 % of the residual yolk protein at hatch and is best used for passive immunity rather than as an AA source. The yolk sac also contains soluble protein antigens that can promote development of the secondary immune organs. The phospholipids and cholesteryl esters represent approximately one-third of residual yolk lipid and are important for building cell membranes, the central nervous system and retina, and residual decosahexanoic acid and arachidonic acid are important in eicosanoid metabolism (Dibner, 1999).

Poults without access to feed showed increased levels of NEFA in the blood showing that lipids from the yolk sac were transported to the peripheral circulation and metabolized for energy (Noy et al., 2001). In chicks and poults without access to feed, T3 levels were lower, indicative of reduced metabolic rate, as this hormone is known to stimulate oxidative metabolism (Noy and Sklan, 2001; Noy et al., 2001). Activity of the Na^+/K^+ ATPase pump in the small intestine of chicks without access to feed for the first 48 h posthatch was low until after initiation of feeding, indicating that secondary and tertiary active transport may have been compromised (Sklan and Noy, 2000).

If the bird is without exogenous nutrient supply, yolk sac reserves may be used to supply energy for maintenance. In the non-fed poult for example, energy used from the yolk sac was calculated to be approximately 4 kcal/d per poult based on utilization of the yolk sac nutrients (1 g yolk fat, 0.7-0.8 yolk protein; Noy et al., 2001). In the chick, fat and protein utilized from the yolk sac reserves in the absence of feed amounted to approximately 5.3 kcal/bird/d (Noy and Sklan, 1999).

Rate of Yolk Sac Utilization. Interestingly, chicks and poults that are supplied with feed immediately posthatch utilize the yolk sac more rapidly and extensively than their non-fed counterparts (Corless and Sell, 1999; Moran and Reinhart, 1980; Noy et al., 1996; Noy and Sklan, 1999; Noy and Sklan, 2001; Sklan and Noy, 2000). In chicks that were not given access to feed, the primary route of yolk utilization was through the circulation, while in fed-birds, most yolk entered through the yolk stalk to the small intestine. This suggests that the presence of luminal substrate stimulates release of yolk via the yolk stalk or that perhaps pressure of material in the gut on the abdominal cavity enhances secretion.

That use of the yolk sac nutrients depends on the nutritional status of the chick demonstrates that its use is the result of conflicting interests (Gonzales et al., 2003). In chicks without access to feed it might seem counterintuitive that the yolk sac is not rapidly utilized as it is the primary energy source available. However, without feed, there is stunted development and growth, which should be accompanied by a lower metabolism reducing the needs of the chick to such that even in the absence of feed its utilization of yolk sac reserves is still lower than in fed birds. Additionally, muscle protein is available to be catabolized as an energy source. Nutrient utilization in the fasted chick will depend on a variety of factors including length of the fasting period. In contrast to previous groups, several others observed that yolk sacs weighed more in fed chicks or that there was no difference in yolk sac utilization between fed and fasted chicks, however, differences in duration of the experimental protocol, water availability, genetic strain of chick used, and time of sampling could explain the discrepancy (Bigot et al., 2003; Gonzalez et al., 2003).

Intestinal Growth. The BW of chicks without access to feed and water at hatch decreased with age but intestinal weight increased by 80 % in the first few days posthatch, while in fed birds the intestinal weights increased by 110 % (Noy and Sklan, 1999). In a later study, the intestinal weight of birds that had no access to feed for 48 h posthatch increased by 60 % in comparison to the twofold increase observed for fed-birds (Sklan and Noy, 2000). It is interesting in these studies that there was preferential growth of the small intestine, emphasizing the importance of gastrointestinal development during the early posthatch period.

Intestinal Cell Proliferation. During the early posthatch period, cells are needed to support intestinal crypt and villus development. In poults that were immediately placed on feed after hatching, amylase activity increased while maltase activity decreased with age (Corless and Sell, 1999). In poults with delayed access to feed (30 to 54 h) there was delayed gastrointestinal development but the decline in maltase activity was much slower than in fed-poults. It was suggested that the more rapid decline in specific activity of maltase with age in fed birds could be the result of more rapid cell proliferation and more immature enterocytes on the villi.

No access to food or water for the first 48 h posthatch led to a decrease in the number of cells per crypt and number of proliferating cells (Geyra et al., 2001b), as well as decreased villus length and area (Geyra et al., 2002). After feeding, these parameters recovered resulting in normal crypts and villi after 6 d. Similarly, in poults that were denied access to feed for the first 48 h posthatch, villus surface area, cell size, and proportion of proliferating cells were all reduced (Noy et al., 2001).

A decrease in cell proliferation and decreased cell migration led to fewer cells per villus and reduced surface area. One important aspect of this effect is that cell hyperplasia results in limited compensatory growth capacity, and delayed access to feed in the first few days posthatch can have effects that carry over to market age (Gonzales et al., 2003). Stunted morphological development is accompanied by lower pancreatic enzyme activity in the small intestine of fasted chicks which did not recover until 7 d posthatch (Sklan and Noy, 2000). Similarly, in deutectomized chicks or chicks held for 36 h posthatch, villus surface area was decreased with reduction still in place even after 11 d (Uni et al., 1998b). Crypt depth was reduced and recovered at 10 d, and microvilli had a “clumped”

appearance. Feed withdrawal for 48 h at later stages in development produced less dramatic effects in terms of villus and crypt development (Geyra et al., 2001b), showing the importance of immediate access to feed at hatch.

Goblet Cell Development. Early access to feed has a profound effect on goblet cells (Uni et al., 2003a). Delayed access to feed for 48 h posthatch resulted in an increase in goblet cell area in the small intestine of broiler chicks. Similarly, 28 d male broiler chicks that were subjected to feed deprivation for 72 h showed an increase in the size of goblet cells by 33 % in the duodenum, 100 % in the jejunum and 66 % in the ileum (Smirnov et al., 2004). Abundance of mucin mRNA and glycoprotein showed similar increases in the duodenum and jejunum with no effect in the ileum. The thickness of the mucus layer was reduced by 22 % in the duodenum, 26 % in the jejunum, and 45 % in the ileum. These findings are very interesting because the thickness of the mucus layer directly influences nutrient digestion as it represents a diffusional barrier to nutrients reaching the brushborder membrane.

The thickness of the mucus layer is the result of a balance between mucin synthesis and secretion, as well as degradation. This study (Smirnov et al., 2004) did not show changes in mucin secretion or that size of the goblet cells was directly related to mucin content in those cells. Several explanations for the findings in this study are 1) feed withdrawal encouraged proliferation of mucolytic bacteria which feed off of the mucus layer and in effect would reduce its thickness or 2) that there was an up-regulation in mucin production and/or down-regulation or impairment in mucin secretion. This would account for the enlargement in goblet cells, resulting in less replenishment to the mucus layer, which could serve to improve nutrient diffusion to the brushborder

membrane. The situation is further complicated by the fact that there are many known mucin genes and only measuring one or two may not paint an accurate overall picture.

Expression of CdxA and B. Expression of CdxA and CdxB, transcription factors that bind DNA regulatory sequences through the homeobox domain, are altered by feeding regimen (Geyra et al., 2002). The transcription factors are instrumental in regulating gut development during embryogenesis and the early posthatch period, and are located exclusively in the gut. Expression of CdxA and CdxB mRNA and/or protein was lower in birds that were denied access to feed and water for 48 h posthatch. The CdxA protein was expressed exclusively on the villi, and is presumably correlated with mature, differentiated enterocytes. Interestingly, the response of CdxA and B to withholding feed lagged behind the response of cell proliferation, further indicating that CdxA plays a role in regulating enterocyte maturation. The Cdx genes may play an important role in regulating the genes associated with the final stages of digestion and nutrient absorption on the brushborder membrane. There is evidence that CdxA binds to the sucrase-isomaltase promoter (Sklan et al., 2003). The spike in expression of CdxA from embryo d 15 to 20 parallels the increase observed for sucrase-isomaltase (Sklan et al., 2003).

Summary

The studies discussed in this review support the idea that nutrient utilization in the broiler chick changes rapidly during the early posthatch period and is influenced by diet composition and feed intake, and by rate of maturation of the gastrointestinal tract. The direct effect of diet on nutrient utilization by the bird is complicated by additional factors including the interactions of dietary components, gut structure and function, and the composition of the intestinal microflora. Furthermore, genetic selection for desirable

growth traits may alter nutrient utilization although the exact mechanisms underlying this observation are poorly understood.

The objective of the following experiments was to understand the dietary and developmental regulation of nutrient transporter gene expression in two lines of broilers that were selected in different nutritional environments and have different growth characteristics. The two lines originate from the same genetic stock but were selected on different diets for more than 10 generations. The rationale behind the following experiments was that selection on the different diets may have led to differences in expression of intestinal nutrient transporters, resulting in differences in growth. In the first trial, a commercial corn-soy diet provided by Aviagen® was fed to chicks for the first 2 wk posthatch and nutrient transporter gene expression was surveyed in the small intestine. In the second trial, the effect of dietary protein quality and feed restriction on nutrient transporter gene expression was evaluated. Birds were fed diets that contained either soybean meal (higher quality) or corn gluten meal (lower quality) as the source of protein in the diet. A select list of transporters was evaluated, based on transporters that were most responsive to developmental stage in the first trial. In the final experiment, the effect of dietary protein composition on transporter gene expression and intestinal morphometrics was evaluated. Three diets were fed that differ in degree of hydrolysis (intact protein vs. partial hydrolysate vs. mixture of free AA) and expression levels of the peptide transporter, AA transporters, and aminopeptidase N were evaluated. This dissertation concludes with a discussion of the implications of these experiments and recommendations for future research.

Table 2.1 The Proton Oligopeptide Cotransporter family (Based on Daniel and Kottra, 2004)

SLC gene name	Protein	Aliases	Substrates	Transport type/coupling ion	Tissue distribution/cellular expression
SLC15A1	PEPT1	Oligopeptide transporter 1, H^+/peptide transporter 1	Di-, and tripeptidesprotons	Cotransport, H^+	Intestine and kidney apical membrane, lysosomal membrane
SLC15A2	PEPT2	Oligopeptide transporter 2, H^+/peptide transporter 2	Di-, and tripeptides protons	Cotransport, H^+	Kidney, lung, brain, mammary gland, bronchial epithelium
SLC15A3	hPTR3	Peptide/histidine transporter 2, human peptide transporter 3, PHT2	Histidine, di- and tripeptidesprotons	Cotransport, H^+	Lung, spleen, thymus, brain, liver, adrenal gland, heart
SLC15A4	PTR4	Peptide/histidine transporter 1, human peptide transporter 4, PHT1	Histidine, di- and tripeptidesprotons	Cotransport, H^+	Brain, retina, placenta

Table 2.2 AA transport systems and families (Based on Palacin and Hediger, 2003; Kanai and Hediger, 2004, and Broer et al., 2004)

SLC gene name	Protein	Aliases	Substrates	Transport type/coupling ion	Tissue distribution/cellular expression
SLC3A1	rBAT	NBAT, D2	Heterodimerizes with light subunit $b^{o,+}$AT to form system $b^{o,+}$	Exchanger	Apical membrane of epithelial cells in kidney and small intestine; also in liver and pancreas.
SLC3A2	4F2hC	CD98hC	Transport systems L, y^+L, Xc- and asc; heterodimerizes with light subunits SLC7A5-8 and SLC7A10-11	Exchanger	Ubiquitous. Basolateral membrane of epithelial cells.
SLC7A1	CAT-1	ATRC1, ERR, REC1	Cationic L-AA	Facilitated transporter, non-obligatory exchanger	Ubiquitous except for liver/plasma and intracellular membranes. Basolateral membrane of epithelial cells.
SLC7A2	CAT-2 (A or B)	TEA, ATRC2	Cationic L-AA	Facilitated transporter	CAT-2A: liver, skeletal muscle, pancreas; CAT-2B: inducible in many cell types.
SLC7A3	CAT-3	ATRC3	Cationic L-AA	Facilitated transporter	Thymus, ovary, testis, brain (neurons) plasma membrane.
SLC7A4	CAT-4		Unknown	Orphan transporter	Brain, testis, placenta/plasma and intracellular membranes.
SLC7A5	LAT1	Assoc. with 4F2hC	Large neutral L-AA, T3, T4, L-DOPA, BCH (system L)	Exchanger	Brain, ovary, testis, placenta, blood-brain barrier, fetal liver, activated lymphocytes, and tumor cells.
SLC7A6	y^+LAT2	Assoc. with 4F2hC	Na^+ indep.: cationic AA; Na^+ depend.: large neutral L-AA (system y^+L)	Exchanger (prefers intracell. cationic AA for extracell. neutral AA plus Na^+	Brain, small intestine, testis, parotid, heart, kidney, lung, liver, basolateral membrane in epithelial cells
SLC7A7	y^+LAT1	Assoc. with 4F2hC	Na^+ indep.: cationic AA; Na^+ depend.: large neutral L-AA (system y^+L)	Exchanger (prefers intracell. cationic AA for extracell. neutral AA plus Na^+	Small intestine, kidney, leukocytes, placenta, lung/basolateral in epithelial cells

SLC gene name	Protein	Aliases	Substrate	Transport type/coupling ion	Tissue distribution/cellular expression
SLC7A8	LAT2	Assoc. with 4F2hC	Neutral L-AA (large and small), BCH (system L)	Exchanger (similar intra- and extra-cellul. selectivities. Lower intracell. apparent affinity.	Small intestine, kidney, brain, placenta, ovary, testis, skeletal muscle/ basolateral membrane in epithelial cells
SLC7A9	$b^{o,+}AT$	Assoc. with rBAT	Cationic AA, large neutral AA (system $b^{o,+)}$	Exchanger, prefers extracell. cationic AA for intracell. neutral AA	Small intestine, kidney, lung, placenta, brain, liver/apical membrane in epithelial cells
SLC7A10	Asc-1	Assoc. with 4F2hC	Small neutral L- and D-AA (Na^+ indep.: system asc)	Exchanger	(Human) brain, heart, placenta, skeletal muscle, and kidney
SLC7A--	Asc-2	Assoc with ?	Small neutral AA (Na^+ indep.: system asc)	Facilitated transporter; exchanger?	(Mouse) kidney, placenta, spleen, lung and skeletal muscle/plasma membrane, non-polarized
SLC1A1	EAAT3	EAAC1, system X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Brain (neurons), intestine, kidney, liver, and heart
SLC1A2	GLT-1	EAAT2, system X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Brain (astrocytes), liver.
SLC1A3	GLAST	EAAT1, system X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Brain (astrocytes), heart, skeletal muscle, placenta
SLC1A4	ASCT1	AAAT, system ASC	L-Ala, L-Ser, L-Thr, L-Cys, and L-Gln	Cotransporter (Na^+), exchanger (AA)	Widespread
SLC1A5	ASCT2	AAAT, system ASC, $hATB^o$	L-Ala, L-Ser, L-Thr, L-Cys, and L-Gln	Cotransporter (Na^+), exchanger (AA)	Lung, skeletal muscle, large intestine, kidney, testis, and adipose tissue
SLC1A6	EAAT4	System X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Cerebellum (Purkinje cells)
SLC1A7	EAAT5	System X^-_{AG}	L-Glu, D/L-Asp	Cotransporter (Na^+, H^+, and K^+)	Retina
SLC6A19	B^oAT1	System B^o	Neutral AA	Cotransporter (Na^+)	Kidney and small intestine, apical membrane

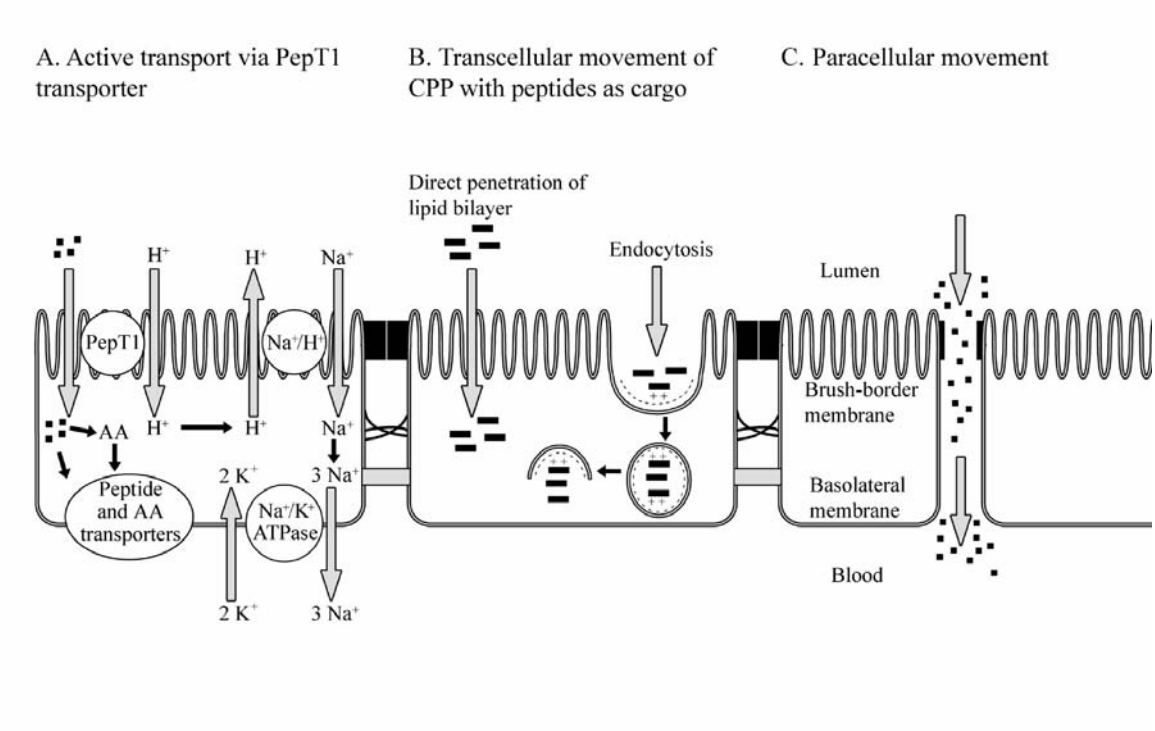

Figure 2.1 Potential routes of peptide uptake in enterocytes. (A) The primary route of di- and tripeptide absorption is through cotransport with H^+ by the peptide transporter, PepT1. (B) Cell-penetrating peptides (CPP) are capable of carrying cargo such as peptides to the inside of cells. (C) Increased permeability of tight junctions permits uptake of peptides via the paracellular route. From Gilbert et al. (2008).

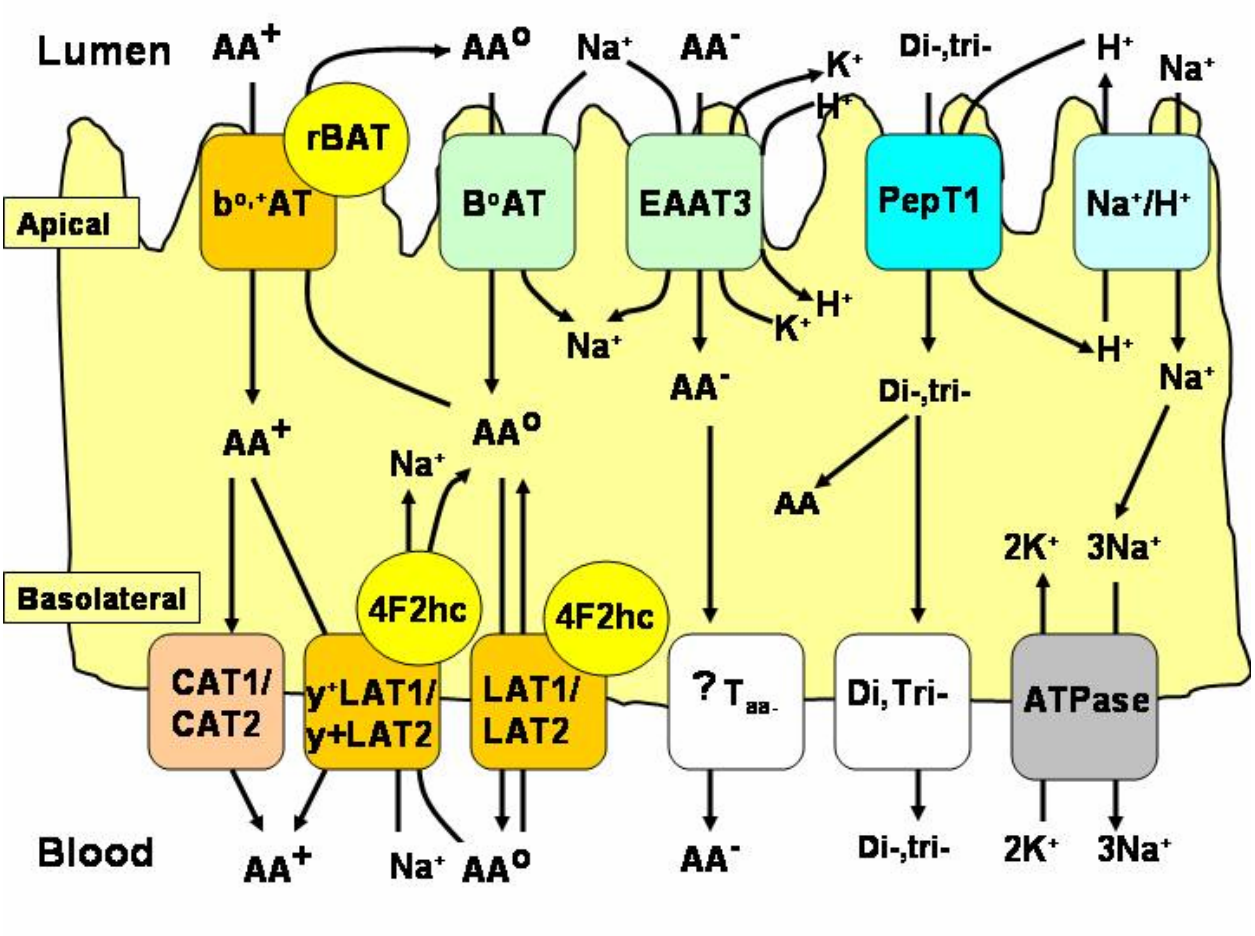

Figure 2.2. Simplified depiction of mechanisms of peptide and AA uptake in the small intestine.

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Chapter III

Developmental Regulation of Nutrient Transporter and Enzyme mRNA Abundance in the Small Intestine of Broilers

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ABSTRACT

The objective of this study was to investigate intestinal nutrient transporter and enzyme mRNA in broilers selected on corn-soybean (Line A) or wheat-based (Line B) diets. We investigated the peptide transporter, PepT1, ten AA transporters (rBAT, $b^{o,+}$AT, $ATB^{o,+}$, CAT1, CAT2, LAT1, y^+LAT1, y^+LAT2, B^oAT and EAAT3), four sugar transporters (SGLT1, SGLT5, GLUT5, and GLUT2), and a digestive enzyme, APN. Intestine was collected at embryo day 18 and 20, day of hatch, and d 1, d 3, d 7 and d 14 posthatch. The mRNA abundance of each gene was assayed using real time PCR and the absolute quantification method. For PepT1, Line B had greater quantities of mRNA compared with Line A ($P = 0.001$), suggesting a greater capacity for absorption of AA as peptides. Levels of PepT1 mRNA were greatest in the duodenum ($P < 0.05$), whereas the abundances of SGLT1, GLUT5 and GLUT2 mRNA were greatest in the jejunum ($P < 0.05$). Abundances of EAAT3, $b^{o,+}$AT, rBAT, B^oAT, LAT1, CAT2, SGLT5 and APN mRNA were greatest in the ileum ($P < 0.05$). Quantities of PepT1, EAAT3, B^oAT, SGLT1, GLUT5, and GLUT2 mRNA increased linearly ($P < 0.01$), while CAT1, CAT2, y^+LAT1, and LAT1 mRNA decreased linearly ($P < 0.05$) with age. Abundance of y^+LAT2 mRNA changed cubically ($P = 0.002$) with peaks of expression at doh and d7, and APN and SGLT5 mRNA changed quadratically ($P = 0.005$) with age. These results provide a comprehensive profile of the temporal and spatial expression of nutrient transporter mRNA in the small intestine of chicks.

Key words: amino acid transporter, broiler, monosaccharide transporter, PepT1

INTRODUCTION

In the small intestine, the final digestion and absorption of dietary nutrients occurs through digestive enzymes and transporter proteins expressed in the apical membrane of enterocytes. Amino acids are transported into the enterocyte as di- or tripeptides by the H^+-dependent peptide transporter 1, PepT1 (Leibach and Ganapathy, 1996; Chen et al., 2002) or as free amino acids by a variety of different amino acid transporters that vary in substrate specificity (Kanai and Hediger, 2004; Palacin and Kanai, 2004; Verrey et al., 2004). Reports from our laboratory suggest that, from a nutritional perspective, peptide transport through PepT1 is important in chickens, cows, pigs and sheep (Matthews et al., 1996; Pan et al., 1997; Chen et al., 1999). Less is known about free amino acid transport in livestock and poultry. Since optimal growth of broilers is dependent upon amino acid supply, studies of genes involved in the process of protein digestion and absorption are important (Gal-Garber and Uni, 2000).

The process of dietary carbohydrate absorption in the small intestine occurs through the action of facilitated and Na^+-dependent transport proteins expressed in the enterocyte (Thorens, 1996). Glucose and galactose are transported into the cell by the Na^+-dependent transporter SGLT1 (Thorens, 1996; Ferraris, 2001; Wright and Turk, 2004), while fructose enters through GLUT5 by facilitated diffusion (Ferraris, 2001; Uldry and Thorens, 2004; Wright and Turk, 2004). The exit of monosaccharides from the cell is mediated by GLUT2 through facilitated diffusion (Ferraris, 2001; Uldry and Thorens, 2004; Wright and Turk, 2004). Absorption of carbohydrates from the intestinal lumen is critical for maintaining energy supplies in animals, and is impacted by luminal digestion, apical membrane digestion, and transport into the enterocyte by SGLT1 (Sklan et al., 2003). Using white Leghorn chickens, Barfull et al. (2002) found that SGLT1 mRNA and protein declined with age after hatch, matching the described

decline in sugar transport activity, while Sklan et al. (2003) observed low mRNA levels of SGLT1 at hatch followed by a slight increase to d 7. Others demonstrated that uptake of glucose was low right after hatch and increased slowly with age suggesting that transport may be a limiting factor in glucose assimilation during the early posthatch period (Sulistiyanto et al., 1999), possibly due to the presence of hydrophobic yolk in the lumen at hatch and low concentrations of luminal sodium (Noy and Sklan, 1999). Understanding sugar transporter gene regulation is important for the elucidation of mechanisms that facilitate changes in rates of nutrient uptake (Ferraris, 2001).

Genetics can also influence nutrient requirements in chickens, including arginine requirements in white leghorn chicks (Nesheim and Hutt, 1962) and protein requirements for egg production in layers (Harms and Waldroup, 1962). It follows that genetic variation in nutrient requirements may be attributed to digestive and absorptive capacities at the intestinal level and post-absorptive utilization of nutrients (NRC, 1975) and these differences may be most significant during the first few days of life. Uni et al. (1995) observed differences in starch digestion between two broiler strains between d 4 and d 14 posthatch. Starch digestion in the heavy (Arbor Acres) chicks was 90 % to 95 % from d 4 to d 14, respectively, whereas in the light (Lohman) chicks it increased from 80 % on d 4 to 93 % on d 14. Nutritional conditions in the early posthatch period may have the greatest impact on overall lifetime performance of chickens (Lilja, 1983; Geyra et al., 2001) and nutrient deficiencies during the first few days after hatch will depress mucosal development (Uni et al., 1998b) and compromise immune function (Casteel et al., 1994). Although intestinal absorption of nutrients may be of minimal significance in ovo, digestive and absorptive functions are established before the onset of exogenous feeding to prepare the chick (Sklan, 2001). In chicks from embryo d 16 to d of hatch there was a 14- to 50-

fold increase in mRNA levels of PepT1 (Chen et al., 2005). Similarly, Uni et al. (2003) observed that intestinal mRNA expression of aminopeptidase, ATPase, maltase and SGLT1 increased 9- to 25-fold from d 15 of incubation to d 19 in chick embryos. Thus, a greater understanding of the influence of both genetics and development on expression of nutrient transporters may facilitate improvements in growth performance through dietary manipulation to take advantage of differential gene expression. Additionally, to reduce the cost of providing protein in the diet and to reduce excess nitrogen excretion into the environment, a greater understanding of amino acid absorption in chickens is needed. The objective of this study was to investigate the developmental regulation of intestinal peptide, amino acid, and monosaccharide transporter mRNA in two genetically selected lines of broiler chicks. This study is the first to report analysis of mRNA abundance of these genes by absolute quantification, and is the most comprehensive study of chicken nutrient transporter mRNA abundance to date.

MATERIALS AND METHODS

Animals and Tissue Collection

The chickens in the present experiment represented two commercial broiler lines, which were genetically selected under different nutritional environments. Line A was developed in a nutritional environment in which corn and soy-based diets and lower relative amino acid concentrations were fed. Line B was fed wheat-based diets and amino acid concentrations that were 15-20% higher than for line A. When fed an approximately 20% crude protein (CP), corn-soy based diet, line A had a higher body weight than line B at 40 days. In contrast, line B attained a higher 40-day body weight compared with line A on a 20% CP, wheat-based diet (Figure 3.1). Both lines have been subjected to balanced selection for improved growth, feed conversion, meat yields, reproduction and general fitness. These lines were chosen for this study

to determine if the difference in protein responsiveness was related to expression of nutrient transporters.

Eggs from each genetic line were obtained from Aviagen (Huntsville, AL). Day of hatch chicks from each genetic line were randomly assigned to heated floor pens with wood shavings. Day of hatch was defined as the birds having cleared the shell on d 21 of incubation. All pens had 24-h lighting and chicks had free access to water. After hatch, birds were given ad libitum access to a corn-soy based diet that was formulated to contain 1,390 kcal/lb ME, 20.0 % CP, 1.17 % total lysine, 0.50 % methionine, 0.85 % TSAA, 0.76 % threonine, 1.33 % arginine, 0.83 % isoleucine, 0.95 % valine, 0.23 % tryptophan, 0.94 % calcium, 0.45 % phosphorus, and 0.21 % sodium. Birds were killed by cervical dislocation at the following time points: embryo d 18 (e18), embryo d 20 (e20), d of hatch (doh; after hatch but before feeding), 1 d posthatch (d1), 3 d posthatch (d3), 7 d posthatch (d7), and 14 d posthatch (d14). Birds were processed in a manner similar to the procedure described by Chen et al. (2005). Body weights of all birds were recorded. For all time points except e18, the intestine was separated into duodenum, jejunum, and ileum. The proximal enlarged loop of the small intestine was taken as the duodenum. The rest of the small intestine was divided by Meckel's diverticulum into proximal and distal portions, corresponding to the jejunum and ileum, respectively. For e18, the entire small intestine was collected due to the difficulty in handling the tissue. Intestinal segments were rinsed with ice-cold phosphate-buffered saline, minced with a razor blade, frozen as aliquots in liquid nitrogen, and stored at –80°C. Sex of the birds was determined by PCR. DNA was isolated from liver samples using the DNeasy Tissue kit (QIAGEN, Valencia, CA). Polymerase chain reaction was performed using liver DNA and primers for tyrosinase (positive control; forward primer: 5'-TCGAGAGGCATAATAATGCATCCA-3', reverse primer: 5'-

AGAGCTTGCTGAGGAAGGAGTG-3'), and primers for the W chromosome (female specific; forward primer: 5'-CTGTGATAGAGACCGCTGTGC-3', reverse primer: 5'-CAACGCTGACACTTCCGATGT-3'), and reactions were electrophoresed on a 1.5 % agarose gel and stained with ethidium bromide. All reaction products contained the 400 bp tyrosinase-specific band and female birds were identified by the presence of the 1,200 bp band corresponding to the W chromosome, whereas male birds lacked this band. Four male birds were selected from each line at each time point to use for real time PCR. All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Real Time PCR

Total RNA was isolated from each tissue sample using the RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. Total RNA was quantified spectrophotometrically at 260/280 nm and stored at -80°C. Real time PCR was used to determine the number of molecules of mRNA present for each gene of interest per nanogram of total RNA starting template. An RNA standard curve for each gene was generated based on modification of the protocol of Fronhoffs et al. (2002). Briefly, chicken-specific cDNA were amplified and sub-cloned into a vector. Total RNA from d7 jejunum and gene-specific primers (Table 3.1) were used to perform reverse transcriptase polymerase chain reaction (RT-PCR). Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Two-step RT-PCR was performed using Promega reagents (Madison, WI) in a PTC-200 Peltier DNA Engine (MJ Research, Reno, NV), following the manufacturer's protocols. The following PCR conditions were used: 95°C for 5 min and 36 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final step of 73°C for 10 min. The PCR products were electrophoresed on a 2 % agarose gel, excised for purification using the QIAquick-Gel Extraction Kit (QIAGEN), and

ligated into the pGEM®-T Easy Vector (Promega). *Escherichia coli* competent cells were transformed using a BTX-Harvard Apparatus ECM Electro Cell Manipulation System (Holliston, MA), and plated out overnight in the presence of 100 μg/mL ampicillin, isopropyl thiogalactoside (IPTG) and X-GAL. The vector DNA was purified from cells containing the vector with the PCR insert using the QIAprep Spin Miniprep kit (QIAGEN). Purified plasmid samples were then sequenced at the Virginia Bioinformatics Institute at Virginia Tech. The HiSpeed Plasmid Midi Kit (QIAGEN) was used to purify and obtain high yields of the plasmid samples following the manufacturer's protocol. Nested primers were designed (Table 1) within cloned chicken cDNA sequences using the Primer Express software, optimized for use with Applied Biosystems Real-Time PCR Systems (Foster City, CA).

To generate a standard curve, plasmids containing amplified chicken cDNA were linearized opposite a T7 or SP6 promoter depending on the orientation of the insert sequence. The Pst I and Sac II restriction enzymes (New England Biolabs, Ipswich, MA) were used to linearize the plasmid for synthesis of RNA from the T7 and SP6 promoters, respectively. Digested DNA were purified using a High Pure PCR Product Purification kit (Roche, Pleasanton, CA), and quantified spectrophotometrically. In vitro transcription was performed on linearized plasmids using the MEGAscript® T7 or SP6 in vitro transcription kit (Ambion, Austin, TX). Samples were treated with DNase I to remove the DNA template and cRNA was precipitated with lithium chloride and quantified using the ribogreen assay (Molecular Probes, Eugene, OR) and a FLUOstar OPTIMA microplate reader (BMG LABTECH, Germany).

The number of molecules per microliter (N) was calculated using the following equation, using a molecular mass constant derived from Avogadro's constant:

$$N = \frac{\text{Concentration of cRNA } (\mu g/\mu L) \quad x \quad (183.5 \times 10^{13})}{\text{cRNA size (bases)}}$$

A dilution series of 10^{11} to 10^4 molecules per microliter was performed in the presence of yeast tRNA at 10 μg/mL. The dilution series for each standard curve was reverse-transcribed in parallel with chick intestinal total RNA samples using the High-Capacity cDNA Archive Kit (Applied Biosystems). Each reverse transcription reaction contained 2,000 ng of RNA at a concentration of 100 ng/μL, and an equal volume of each standard curve dilution cRNA was added to its respective reaction. The cDNA was then diluted 1:30 before addition to PCR reactions.

Real-Time PCR was performed on an Applied Biosystems 7300 instrument (Foster City, CA) with ABI plates using the absolute quantification method. For each 25 μL PCR reaction, 2 μL of the cDNA diluted 1:30, 12.5 μL of SYBR green master mix (Applied Biosystems), 9.5 μL of water, and 0.5 μL of the forward and reverse primer at a 5 μ*M* stock concentration were added. PCR was performed under the following conditions: 50°C for 10 min and 40 cycles of 95°C for 1 min and 60°C for 1 min. A dissociation step consisting of 95°C for 15 sec, 60°C for 30 sec, and 95°C for 15 sec, was performed at the end of each PCR reaction to ensure amplification of a single product.

Statistical Analysis

All data were analyzed using the Proc MIXED procedure of SAS (SAS Institute, Cary, NC). The model included the main effects of genetic line, intestinal segment, age, and all two-way interactions. The three-way interaction was removed from the model as they were determined to be non-significant ($P < 0.05$). Differences among segments were evaluated by the Tukey test for multiple comparisons. The e18 time point was not included in the statistical model. The main effects of age were further tested for linear, quadratic, and cubic responses

using orthogonal contrasts in the MIXED procedure. The contrast coefficient matrix for unequal spacing was generated using Proc IML.

RESULTS

After hatch, birds were given ad libitum access to a 20% CP, corn-soy based diet, as this has been shown to accentuate differences in body weight between the two lines over 42 d. Body weights were not significantly different between genetic lines by d14 ($P = 0.5$; Figure 3.2), although weights of Line A and B birds were starting to diverge with Line A weights greater than Line B weights.

The mRNA abundance for a number of amino acid, peptide and monosaccharide transporters was determined using real time PCR and the absolute quantification method. Thus, the results presented herein represent mRNA and it is unknown at this time if these data parallel expression and activity of the functional proteins. The functions of all genes evaluated in this study are listed in Table 1. The mRNA expression data are summarized in Tables 3.2 and 3.3, with separate rows for main effects of developmental age, intestinal segment, and genetic line, interactions, and orthogonal contrasts across developmental age. If no significant differences were observed between genetic lines, data for Line A and Line B were combined and displayed as a single graph.

At the brush border membrane, the uptake of amino acids is mediated by the action of the peptide transporter PepT1 and a number of different amino acid transporters, which transport neutral, cationic and anionic amino acids. The number of molecules of peptide transporter, PepT1 mRNA per nanogram of total RNA ranged from 700 to 12,000 (Figure 3.3A and B). Abundance of PepT1 mRNA was greater in Line B compared to Line A ($P = 0.0007$). In Line B PepT1 mRNA rose continuously with age, whereas, in Line A mRNA leveled off after d 3. There

was an approximate twofold difference between Lines A and B in concentration of PepT1 mRNA per nanogram of total RNA (3,600 vs. 6,300, respectively). Quantities of PepT1 mRNA were greatest in the duodenum compared with the ileum ($P = 0.04$), while jejunal mRNA was intermediate. Abundance of PepT1 mRNA increased linearly ($P = 0.0001$) from e20 to d14.

The rBAT and $b^{o,+}$AT proteins heterodimerize and serve as Na^+-independent amino acid exchangers on the apical membrane, with a high extracellular affinity for cationic amino acids and a high intracellular affinity for neutral amino acids (Palacin and Kanai, 2004). Molecules of $b^{o,+}$AT mRNA per nanogram of total RNA ranged from 35,000 to 170,000 (Figure 3.4A and B). Neither genetic line nor age of bird influenced abundance of $b^{o,+}$AT mRNA. The $b^{o,+}$AT mRNA quantities were greater in the ileum, compared with the duodenum and jejunum ($P = 0.0001$). There was a line x intestinal segment interaction ($P = 0.04$), where Line A mRNA levels were greatest in the ileum, lowest in the jejunum, and intermediate in the duodenum, whereas in Line B, mRNA levels were also greatest in the ileum, but lowest in the duodenum, and intermediate in the jejunum. There was also an intestinal segment x age interaction ($P = 0.01$). Quantities of mRNA in the ileum increased to d1, whereas mRNA levels decreased to d1 in the duodenum and jejunum, and quantities of mRNA in the ileum increased most dramatically after d3 compared with the duodenum and jejunum. The rBAT mRNA quantities ranged from 5,900 to 57,000 molecules per nanogram of total RNA (Figure 3.5A). Differences observed between genetic lines and among ages were not significant. Quantities of rBAT mRNA were greater in the ileum, compared with the duodenum and jejunum ($P < 0.01$).

The EAAT3 brushborder membrane-bound protein is a Na^+-dependent transporter with a high affinity for anionic amino acids, including aspartate and glutamate (Kanai and Hediger, 2004). Quantities of EAAT3 mRNA molecules per nanogram of total RNA ranged from 1,000 to

12,500 (Figure 3.5B). There was no effect of genetic line on EAAT3 mRNA quantities. Quantities of EAAT3 mRNA were greatest in the ileum, lowest in the duodenum, and intermediate in the jejunum ($P = 0.0001$). Quantities of EAAT3 mRNA increased linearly ($P = 0.001$) from e20 to d14. There was an intestinal segment x age interaction ($P = 0.0001$), where EAAT3 mRNA increased most dramatically after d3 in the ileum, compared with the duodenum and jejunum. Molecules of B^oAT mRNA per nanogram of total RNA ranged from approximately 300 to 11,000 (Figure 3.5C). Quantities of mRNA were not influenced by genetic line. Molecules of B^oAT mRNA were greater in the ileum compared with the duodenum and jejunum ($P < 0.001$). Quantities of B^oAT mRNA increased linearly ($P = 0.0001$) from e20 to d14. There was an intestinal segment x age interaction ($P = 0.002$), where B^oAT mRNA levels increased most dramatically with age in the ileum as compared to the duodenum and jejunum where mRNA remained fairly constant with age.

Amino acid and peptide supply to the enterocyte is dependent on terminal digestion at the brushborder membrane by aminopeptidases, such as aminopeptidase N (APN) which cleaves neutral and basic amino acids from the N-terminal end of peptides (Sanderink et al., 1988). The amount of APN mRNA per nanogram of total RNA ranged from 47,000 to 300,000 molecules (Figure 3.5D). The quantities of APN mRNA were not influenced by genetic line. Quantities of APN mRNA were greatest in the ileum, lowest in the duodenum, and intermediate in the jejunum ($P = 0.0001$). Quantities of APN mRNA increased quadratically ($P = 0.02$) from e20 to d14, with a decline from doh to d1, and subsequent increase in mRNA to d14.

At the basolateral membrane, amino acids are similarly transported out of the enterocyte and into the blood via amino acid transporters with specificity for neutral, anionic and cationic amino acids. The y^+LAT1 and y^+LAT2 proteins mediate the Na^+-dependent transport of neutral amino

acids with high affinity, in exchange for intracellular cationic amino acids, while LAT1 exhibits a high affinity for branched-chain and aromatic amino acids (Verrey et al., 2004). The CAT1 and CAT2 proteins both transport cationic amino acids with high affinity. The number of molecules of LAT1 mRNA per nanogram of total RNA ranged from 40 to 530 (Figure 3.6A). Abundance of LAT1 mRNA was not influenced by genetic line. Abundance of LAT1 was greater in the ileum compared with the duodenum ($P = 0.007$), and levels were intermediate in the jejunum. Quantities of LAT1 mRNA decreased linearly ($P = 0.05$) from e20 to d14. There was an intestinal segment x age interaction ($P = 0.04$), where LAT1 mRNA increased from e20 to doh in the ileum, in contrast to the duodenum and jejunum where mRNA levels did not change, and duodenal mRNA increased after d3 as compared to the jejunum and ileum where mRNA decreased.

The number of molecules of y^+LAT1 mRNA per nanogram of total RNA ranged from 120 to 550 (Figure 3.6B). No significant main effects were observed. The quantities of y^+LAT1 mRNA decreased linearly ($P = 0.003$) from e20 to d14. Abundance of y^+LAT2 mRNA per nanogram of total RNA ranged from 2,700 to 11,000 molecules (Figure 3.6C). There was no effect of genetic line or intestinal segment on y^+LAT2 mRNA abundance. Quantities of y^+LAT2 mRNA changed cubically ($P = 0.002$) with age, with greatest mRNA quantities observed at doh and d7.

The number of molecules of CAT1 mRNA per nanogram of total RNA ranged from 180 to 4,400 molecules (Figure 3.6D). The abundance of CAT1 mRNA was not influenced by genetic line or intestinal segment. The CAT1 mRNA quantities decreased linearly ($P = 0.0001$) from e20 to d14. Quantities of CAT2 mRNA per nanogram of total RNA ranged from 30 to 500 (Figure 3.6E). The quantities of CAT2 mRNA were not influenced by genetic line. Abundance

of CAT2 mRNA was greatest in the ileum, lowest in the duodenum, and intermediate in the jejunum ($P < 0.05$). The quantities of CAT2 mRNA decreased linearly from e20 to d14 ($P = 0.0001$). The Na^+-dependent and Cl^--dependent cationic and neutral amino acid transporter, $ATB^{o,+}$ was also evaluated in this study; however, there was no amplification of the PCR product during real time PCR and thus we concluded that it is most likely not expressed in the chicken small intestine.

In a similar manner to amino acid assimilation, oligosaccharides are broken down into monosaccharides, which are then taken up by sugar transporters located on the brushborder membrane. SGLT1 protein mediates the Na^+-dependent uptake of glucose and galactose across the brushborder membrane (Thorens, 1996) and is considered to be the primary mediator of glucose assimilation in the small intestine (Wright, 1993; Hediger and Rhoads, 1994). A search of the chicken genome revealed two predicted SGLT genes, with one located on chromosome 8 (GenBank accession number XM_422459) and the other on chromosome 15 (GenBank accession number XM_415247). A multiple sequence alignment (http://workbench.sdsc.edu) of amino acids from the two predicted SGLT genes and all 11 members of the human SLC5 gene family predicted that SGLT on chromosome 15 is most similar to human SGLT1, while SGLT on chromosome 8 is most similar to SGLT5, a kidney glucose transporter. Thus, in this paper, the SGLT gene on chromosome 15 will be referred to as SGLT1 and the SGLT gene on chromosome 8 will be referred to as SGLT5.

Quantities of SGLT1 mRNA were greatest in comparison to the other monosaccharide transporters examined in this study, and ranged from 1,200 to 63,300 molecules per nanogram of total RNA (Figure 3.7A). Abundance of SGLT1 mRNA was not influenced by genetic line. Levels of SGLT1 mRNA increased linearly ($P = 0.0001$) from e20 to d14. The quantities of

SGLT1 mRNA were greater in the jejunum, compared with the duodenum and ileum (P = 0.0001). There was an intestinal segment x age interaction (P = 0.007), where SGLT1 mRNA increased most dramatically with age in the jejunum as compared to the duodenum and ileum.

Abundance of SGLT5 mRNA per nanogram of total RNA ranged from 1,200 to 7,300 molecules (Figure 3.7B). Quantities of SGLT5 mRNA changed quadratically (P = 0.005) with time, with mRNA quantities declining to d1 and increasing thereafter. The mRNA was greatest in the ileum, lowest in the duodenum and intermediate in the jejunum (P < 0.01).

Transport of fructose across the brushborder membrane is facilitated by GLUT5 (Uldry and Thorens, 2004). Quantities of GLUT5 mRNA ranged from 300 to 4,300 molecules per nanogram of total RNA (Figure 3.7C). Quantities of GLUT5 were greater in the jejunum and ileum, compared with the duodenum (P = 0.0001). Abundance of GLUT5 mRNA increased linearly (P = 0.0001) from e20 to d14. An intestinal segment x age interaction (P = 0.0006) was observed, in that abundance of GLUT5 mRNA increased more dramatically after doh in the jejunum and ileum, as compared to the duodenum.

The GLUT2 transporter mediates the facilitated transport of glucose, galactose and fructose across the basolateral membrane (Uldry and Thorens, 2004). The number of molecules of GLUT2 mRNA per nanogram of total RNA ranged from 60 to 2,300 (Figure 3.7D). The mRNA levels of GLUT2 were greatest in the jejunum, lowest in the ileum, and intermediate in the duodenum (P = 0.0001). Abundance of GLUT2 mRNA increased linearly (P = 0.0001) from e20 to d14. There was an intestinal segment x age interaction (P = 0.0002), where GLUT2 mRNA increased more dramatically in the jejunum after d3 compared with the duodenum and ileum.

In addition to nutrient transporters and a digestive enzyme, mRNA for a housekeeping gene, GAPDH was evaluated in this study. The housekeeping gene, GAPDH is assumed to be uniformly expressed across cell types, and is commonly used as an internal standard in relative quantification studies to correct for differences in RNA loading amount (Jain et al., 2006). Our results demonstrate that there was a linear decrease ($P = 0.0001$) in GAPDH mRNA quantities from e20 to d14. GAPDH mRNA per nanogram of total RNA ranged from 31,700 to 190,000 (Figure 3.8). Quantities of GAPDH mRNA were greater in the duodenum compared with the ileum ($P = 0.002$) and levels were intermediate in the jejunum. Abundance of GAPDH mRNA was not influenced by genetic line.

DISCUSSION

Chicken lifetime performance is greatly influenced by diet quality and nutrient availability during the early posthatch period (Lilja, 1983; Geyra et al., 2001). The growth of chicks is dependent on the ability of the intestine to assimilate nutrients efficiently as the bird shifts from the lipid-rich yolk as the sole source of energy to a carbohydrate- and protein-based diet (Sklan, 2001). Because the developmental regulation of nutrient transporter mRNA has not been thoroughly evaluated in chickens, in this study we compared developmental changes in mRNA abundance in two genetically selected lines of broilers. A greater understanding of the capacity of the intestine to assimilate amino acids and glucose is critical to formulating diets that best accommodate the profile of digestive enzymes and nutrient transporters expressed in the small intestine at any point in time. Amino acids are required for many life processes including protein synthesis and for energy, yet little is known about the process of amino acid transport in the chicken intestine. Broilers are raised on diets consisting of approximately 60% carbohydrates

(Gal-Garber et al., 2000), thus, an understanding of intestinal sugar transport is also critical to improving efficiency of growth.

At the brushborder membrane, a number of proteases and peptidases break down proteins into peptides and free amino acids for uptake into cells by peptide and amino acid transporters. Peptide transport is one of the major routes of amino acid assimilation by the enterocyte and is H^+-dependent (Chen et al., 2002). Interestingly, in our study, PepT1 was the only gene for which mRNA abundance was influenced by genetic line. Line B expressed approximately twofold higher levels of mRNA than Line A. If protein expression data parallels these findings, it is possible that Line B may have a greater capacity to assimilate amino acids in the form of di- and tripeptides. If this is the case, a diet that optimizes availability of amino acids as small peptides may improve growth performance and feed efficiency in Line B broilers. Based on evidence from numerous studies, Daniel (2004) suggested that peptides are absorbed faster and more efficiently than free amino acids in the small intestine. Chen et al. (2005) found that chicken PepT1 mRNA levels varied with both dietary protein level and developmental stage. In this study, PepT1 mRNA increased linearly with time to d14. Similarly, Chen et al. (2005) observed a linear increase in cPepT1 with age, suggesting an importance in peptide transport in the posthatch chick. We found that mRNA for PepT1 was expressed at greatest levels in the duodenum, in agreement with previous studies (Chen et al., 2002), while the putative brushborder membrane-expressed amino acid transporters were expressed at greatest levels in the ileum. It is likely that the spatial expression pattern of these genes corresponds to their transport kinetics, with PepT1 exhibiting a greater V_{max} and capacity to absorb large amounts of substrate entering the proximal intestine (Daniel, 2004), while the free amino acid transporters, with higher affinities and lower transport capacities (Kanai and Hediger, 2004; Palacin and

Kanai, 2004; Verrey et al., 2004) extract remaining unabsorbed amino acids from the lumen of the distal intestine, before they are excreted.

Aminopeptidase N is a digestive enzyme that cleaves amino acids from the N-terminal end of peptides, and is fairly specific for peptides with an N-terminal neutral or basic amino acid (Sanderink et al., 1988), which then generates substrate for the amino acid transport systems. Abundance of APN mRNA was greater than all transporter genes examined. Maintaining high expression levels of APN mRNA throughout the small intestine would provide a constant supply of substrates for the amino acid transporters.

The B^oAT is a Na^+-dependent neutral amino acid transporter expressed on the apical membrane. This transporter was recently cloned in mammals (Broer et al., 2004), and in this study, a search of the chicken genome using a mammalian consensus sequence revealed a similarity to a predicted sequence given the gene name "X transporter 2" (GenBank accession number: XM_419056). Quantities of B^oAT mRNA were of a similar magnitude to the quantities of mRNA observed for EAAT3 and PepT1. Quantities of EAAT3 mRNA increased with age and were greatest in the ileum of the small intestine. With the rapid rate of intestinal growth as a proportion of total body mass (Sklan, 2001), and the use of glutamate as an oxidative fuel source in the intestine (Wu, 1998; Nissim, 1999), increasing amounts of energy are needed to meet the needs of the rapidly growing chick. Thus, it is not surprising that mRNA expression of the glutamate transporter, EAAT3 increased over threefold from e20 to d14 in our study. Rome et al. (2002) found that levels of EAAT3 mRNA increased from rat postnatal d 4 to d 21 all along the small intestine. The reported cellular distribution of EAAT3 also supports its role in providing energy to intestinal cells and stimulating cellular proliferation, with protein expression localized to the crypts and lower villi and decreased abundance towards the villus tip (Rome et al., 2002;

Iwanaga et al., 2005). Both groups also reported an increased gradient of expression from proximal to distal intestine, similar to our findings, suggesting increased capacity for glutamate absorption in the distal small intestine. For three of the amino acid transporters, $b^{o,+}$AT, EAAT3, and B^{o}AT, there was an interaction of age x segment with ileal mRNA increasing most dramatically with age in comparison to the duodenum and jejunum, suggesting that the ileum is an important site for free amino acid assimilation in the growing chick.

Expression of the amino acid transporters on the basolateral membrane differed from the amino acid transporters on the brush border membrane. CAT1, CAT2, y^{+}LAT1, and LAT1 are basolaterally expressed in mammalian epithelial cells (Verrey et al., 2004; Dave et al., 2004; Kizhatil and Albritton, 2003), and in this study mRNA quantities were highest at e18 and decreased with age, suggesting an importance for these transporters in the chick embryo. While the chick relies on yolk for nourishment during embryological development, nutrients are obtained in the intestine through the basolateral surface from the bloodstream until exogenous feeding at hatch. In contrast, mRNA quantities of y^{+}LAT2 were lowest at e18 and increased with age, suggesting an important role in the posthatch chick. The Na^{+}-dependent and Cl^{-}-dependent cationic and neutral amino acid transporter, $ATB^{o,+}$ was also evaluated in this study; however, we were unable to detect $ATB^{o,+}$ mRNA and concluded that it is most likely not expressed in the chicken small intestine.

Expression of monosaccharide transporters also differed temporally and spatially in the chick small intestine. The mRNA for SGLT1 was expressed from 6 to 29-fold greater than the other three monosaccharide transporters, indicating that SGLT1 is the major pathway for glucose assimilation in the small intestine. Obst and Diamond (1992) observed a dramatic increase in glucose uptake 14 d posthatch, and in our study, we observed an approximate ninefold increase

in SGLT1 mRNA from e18 to day of hatch, followed by a twofold increase to d14, suggesting that the capacity for glucose uptake increases with age in chickens. Uni et al. (1998b) observed that broiler mucosal sucrase and maltase activities were lower in the duodenum than in the jejunum and ileum, with activities increasing to adult levels from day of hatch to d 2 posthatch in all segments of the small intestine. After d 2 activities declined to d 4 after which levels gradually rose to d 11 in the jejunum, and activities plateaued after d 4 in the duodenum and ileum. We observed that SGLT1 mRNA was greater in the jejunum and ileum as compared to the duodenum, matching the gradient of disaccharidase activity in broiler small intestine, but we also observed a continual increase in mRNA expression of SGLT1 from day of hatch to d 14 posthatch. It may be that digestion at the intestinal level is the limiting factor to assimilation of glucose, and not expression of the transporter per se.

For GLUT2, GLUT5 and SGLT1, there was an interaction of age x intestinal segment, with mRNA levels increasing most dramatically with age in the jejunum, suggesting that the jejunum is the primary site of sugar assimilation in the chicken intestine. Garriga et al. (2002) found that apical Na^+-dependent D-glucose transport was reduced in the ileum, compared to the duodenum and jejunum, also paralleling protein expression of SGLT1. This is in agreement with our results, where we observed greater mRNA levels of SGLT1 in the jejunum and duodenum. Interestingly, in our study, SGLT5 mRNA was greatest in the ileum, in contrast to highest expression in the jejunum observed for the other sugar transporters. It is possible that SGLT5 may have a higher affinity for substrate, and thus serves to transport glucose at the distal intestine while SGLT1 transports the majority of dietary glucose in the proximal intestine. Also, while expression of SGLT1, GLUT2, and GLUT5 mRNA increased linearly from e20 to d14, SGLT5 mRNA changed quadratically with lowest expression at d1 and a subsequent increase to

d14. To date, SGLT1 is the only intestinal transporter reported in chickens that is known to be responsible for the uptake of glucose (Wright and Turk, 2004). That mRNA for SGLT5, a transporter not yet reported to be present in the intestine, was present and exhibited a regional pattern of expression different from SGLT1, suggests a unique role for this transporter in the uptake of glucose. Zhao et al. (2005) reported that bovine SGLT5 mRNA was found predominantly in bovine kidney, and was undetectable in bovine mammary gland, liver, lung, and small intestine.

Quantities of GLUT5 mRNA increased with age. The diet offered to chicks lacked fructose; therefore, it is surprising that GLUT5 mRNA was upregulated with age despite the lack of luminal substrate. In rabbits, fructose uptake increases during the last week of gestation (Phillips et al., 1990) and after birth, fructose transport decreases, and then increases again after weaning with the introduction of fructose-containing diets, demonstrating substrate-dependent induction (Buddington and Diamond, 1990). Quantities of GLUT2 mRNA increased with age but represented the lowest quantities compared to the other sugar transporters.

Expression of nutrient transporters and aminopeptidase showed different developmental patterns. In general, brushborder membrane transporter mRNA increased with age while basolateral membrane transporter mRNA decreased with age. Transporter expression changed dramatically between e18 and doh. The expression of nutrient transporters and digestive enzymes is induced before hatch to prepare the chick for exogenous feeding (Sklan, 2001). We observed an approximate three- to fivefold increase in mRNA for APN (56,000 to 147,500 molecules), y^+LAT2 (3,000 to 8,500 molecules), $b^{o,+}$AT (43,000 to 106,300), EAAT3 (1,600 to 4,300 molecules), rBAT (7,200 to 36,200 molecules) and PepT1 (750 to 3,600 molecules) and a seven- to ninefold increase in mRNA for GLUT2 (70 to 490 molecules), B^oAT (500 to 4,000

molecules), and SGLT1 (1,500 to 14,400 molecules) from e18 to doh. Birds sampled at day of hatch had not yet received exogenous feed, suggesting that the rise in mRNA levels was genetically hardwired to prepare the chick for a carbohydrate and protein-based diet at hatch, and was not induced by dietary substrate. Moreno et al. (1996) observed uptake of α-methyl-D-glucoside, a non-metabolizable glucose analogue in chick small intestine 2 d before hatch and an increase in V_{max} to 1 d posthatch, and suggested that genotypic programs of development influence changes in the absorptive capabilities of the intestine before hatch. Uni et al. (2003) observed a 9- to 25-fold increase in aminopeptidase, ATPase, maltase and SGLT1 mRNA abundance from d 15 of incubation to d 19 in chick embryos, and a subsequent decline to hatch. Chen et al. (2005) found that PepT1 mRNA increased 14- to 50-fold from embryo day 16 to day of hatch in mixed sex Cobb chicks.

Induction of gene expression of intestinal digestive enzymes and nutrient transporters prenatally also occurs in mammals. In a review of regulation of sugar transport, Ferraris (2001) described the appearance of intestinal GLUT5, GLUT2 and SGLT1 mRNA in the developing rat. At early gestation GLUT2 and SGLT1 mRNA are detected, while GLUT5 is not detected in significant amounts until weaning. Phillips et al. (1990) described a threefold increase in the active transport of glucose and galactose during the final week of gestation of rabbits. Thus, it is clear that in both avian and mammalian species, mechanisms exist to prepare the developing animal for nutrient uptake posthatch or postnatally. Buddington and Diamond (1990) suggested that in the newly born rabbit, intestinal uptake rates of glucose, galactose, proline, leucine, lysine and methione are at levels 2 to 2.5 times higher than maximum adult values, and that levels declined until weaning. The authors hypothesized that this may be partially explained by the occurrence of transport along the entire crypt-villus axis at birth. The suckling of milk induces

mucosal hypertrophy and establishment of a well-defined crypt-villus gradient of expression, resulting in a dilution of mature enterocytes with immature cells, with localization restricted to the mid-to-upper villus. Interestingly, in the chicken small intestine, in contrast to mammals, cell proliferation is not restricted to the crypts, but occurs along the entire length of the villus during the first few days posthatch (Uni et al., 1998a). Future studies should attempt to correlate changes in cell proliferation and development of the mucosal layer with changes in nutrient transporter mRNA and protein quantities, site-specific localization, and transport activity in the chicken.

In summary, we utilized the method of absolute quantification by real time PCR to quantify mRNA abundance for 10 amino acid transporters, a peptide transporter, four monosaccharide transporters, a digestive enzyme, and a housekeeping gene. Our study represents a comprehensive profile of the mRNA abundance of nutrient transporters and an aminopeptidase in the small intestine of broilers. Transporter mRNA levels ranged from as low as 100 molecules per nanogram of total RNA for CAT1 to as high as 100,000 molecules for $b^{o,+}AT$. Of the genes examined in this study, the mRNA levels for the digestive enzyme, APN, were the highest at 200,000 molecules per nanogram of total RNA. We evaluated the temporal and spatial distribution of these genes in the small intestine of two genetically selected lines of broiler chicks. Developmentally, mRNA for PepT1, the brushborder membrane-associated amino acid transporters, and the sugar transporters increased with age from e18 to d14, while the basolateral amino acid transporters decreased with age. This may be associated with the consumption of feed reducing the need for basolateral uptake of nutrients from the bloodstream. In terms of spatial distribution, PepT1 was expressed at greatest levels in the duodenum, the sugar transporters were expressed at greatest levels in the jejunum, and the amino acid transporters in

the ileum. This suggests differences in absorptive capacity along the length of the small intestine. Future studies will involve manipulation of the diet to exploit differences in transporter gene expression in order to better meet the nutritional requirements of the growing chick.

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Table 3.1. Primers used for cloning chicken cDNA[a] and real time PCR[b].

Gene[c]	GenBank ID	Description/gene function	Cloning cDNA, sense/antisense	Real time PCR, sense/antisense
PepT1	NM_204365	oligopeptide transporter	ACTGTCAATCCAATCCTG/ GACAGTCACGTTCTGAAGA	CCCCTGAGGAGGATCACTGTTGGCAGTT/ CAAAAGAGCAGCAGCAACGA
APN	NM_204861	aminopeptidase N, digestive enzyme	GAATACGCGCTCGAGAAAAC/ CTTGTTGCCAATGGAGGAGT	AATACGCGCTCGAGAAAACC/ AGCGGGTACGCCGTGTT
rBAT	XM_426125	heavy chain corresponding to the $b^{o,+}$ transport system	CTCCCAACAACTGGGTGAGT/ TTCACTTGTGGCTCATCTCG	CCCGCCGTTCAACAAGAG/ AATTAAATCCATCGACTCCTTTGC
$b^{o,+}AT$	XM_414130	Na^+-independent cationic and zwiterionic amino acid transporter	AGGTTGTGATCCACCTCCAG/ TTTTGGGTTTTTCCTTGTGC	CAGTAGTGAATTCTCTGAGTGTGAAGCT/ GCAATGATTGCCACAACTACCA
EAAT3	XM_424930	excitatory amino acid transporter 3, Na^+, H^+, K^+ depend.	GGAGTTGCTGCTTTGGATTC/ AGGCCTGGACGAGATTTTCT	TGCTGCTTTGGATTCCAGTGT/ AGCATGACTGTAGTGCAGAAGTAATATAT
y^+LAT1	XM_418326	Na^+-indep. cationic & Na^+-depend. neutral amino acid	AATGTGAAGTGGGGAACTCG/ CACCCTGCGTAGGAGAAGAG	CAGAAAACCTCAGAGCTCCCTTT/ TGAGTACAGAGCCAGCGCAAT
y^+LAT2	NM_001005832	Na^+-indep. cationic & Na^+-depend. neutral amino acid	GAGAATTCACAGCCCTTCCA/ CAGGCTTATCCCGTTTAGCA	GCCCTGTCAGTAAATCAGACAAGA/ TTCAGTTGCATTGTGTTTTGGTT
GAPDH	NM_204305	glyceraldehyde-3-phosphate dehydrogenase	AGTCGGAGTCAACGGATTTG/ ACAGTGCCCTTGAAGTGTCC	GCCGTCCTCTCTGGCAAAG/ TGTAAACCATGTAGTTCA
LAT1	CD217821	Na^+-independ. branched-chain and aromatic amino acid transporter	TCACGGCAGTGAACTGCTAC/ CCATCCTCCATAGGCAAAAA	GATTGCAACGGGTGATGTGA/ CCCCACACCCACTTTTGTTT
$ATB^{0,+}$	XM_426267	Na^+- and Cl^--dependent neutral and cationic amino acid	TGGGAAACAGTCCAACATCA/ TCCCAGGACAGAGAAAATGG	GTGGACTTGTTGCTTTGTCTTCA/ GAGTTTGTTACACAAAACAAAAATAGC
CAT1	XM_417116	Na^+-independent cationic amino acid transporter	TCTCCTTGGCTCCATGTTTC/ CAGGAGGGTCCCAATAGACA	CAAGAGGAAAACTCCAGTAATTGCA/ AAGTCGAAGAGGAAGGCCATAA
CAT2	XM_420685	Na^+-independent cationic amino acid transporter	TCTGTGATGGCAGGTCTCTG/ CCACTCCAGGCTCTTGCTAC	TGCTCGCGTTCCCAAGA/ GGCCCACAGTTCACCAACAG
B^oAT	XM_419056	Na^+-dependent neutral amino acid transporter	AATGGGACAACAAGGCTCAG/ CAAGATGAAGCAGGGGGATA	GGGTTTTGTGTTGGCTTAGGAA/ TCCATGGCTCTGGCAGAGAT
SGLT1	XM_415247	Na^+-dependent glucose and galactose transporter	CATCTTCCGAGATGCTGTCA/ CAGGTATCCGCACATCACAC	GCCATGGCCAGGGCTTA/ CAATAACCTGATCTGTGCACCAGTA

SGLT5	XM_422459	glucose transporter	TGGGAGCTTTGGTCCTTATG/ AAAGAGCCAGCACAGAGAGG	ATACCCAAGGTCATAGTCCCAAAC/ TGGGTCCCTGAACAAATGAAA
GLUT2	Z22932	Na^+-independent glucose, galactose and fructose transporter	CCGCAGAAGGTGATAGAAGC/ ACACAGTGGGGTCCTCAAAG	CACACTATGGGCGCATGCT/ ATTGTCCCTGGAGGTGTTGGTG
GLUT5	XM_417596	Na^+-independent fructose transporter	GTCCTTGGGAAGGAGAATCC/ TCCAATAGCATGTCCGATGA	TTGCTGGCTTTGGGTTGTG/ GGAGGTTGAGGGCCAAAGTC

[a]Primers designed by Primer3 software (program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

[b]Primers designed by Primer Express software (Applied Biosystems, Foster City, CA)

Table 3.2. Developmental regulation of peptide and amino acid transporter mRNA in the small intestine of broiler chicks[a]

Item		Gene							
		PepT1	$b^{o,+}AT$	rBAT	EAAT3	B^oAT	LAT1	y^+LAT1	y^+LAT2
		Molecules of mRNA per ng total RNA							
Line	A	3,600	96,900	29,600	4,000	4,300	193	289	6,500
	B	6,300	81,800	28,900	4,200	4,800	185	266	6,200
	SEM	520	7,708	4,036	333	719	24	25	852
	P-value	0.0007	0.1729	0.8965	0.5940	0.5997	0.8185	0.5216	0.8132
Segment	Duodenu	5,300[c]	78,700[e]	26,000[h]	1,800[e]	3,700[h]	166[h]	271	6,400
	Jejunum	5,200[c,d]	75,000[e]	27,600[h]	3,800[f]	3,800[h]	182[h]	268	6,600
	Ileum	4,400[d]	114,400[f]	34,200[i]	6,700[g]	6,200[i]	220[i]	294	6,000
	SEM	424	6,901	3,082	301	601	20	26	662
	P-value	0.0311	0.0001	0.0002	0.0001	0.0001	0.0073	0.6898	0.4527
Age	E20	1,400	81,600	20,000	2,000	1,300	214	349	5,600
	DOH	3,600	106,300	36,200	4,300	4,000	229	304	8,500
	D1	4,900	83,800	31,500	3,500	4,700	101	194	7,800
	D3	5,800	74,900	24,800	3,600	3,600	229	197	4,600
	D7	6,400	97,000	31,500	5,000	6,000	171	333	6,500
	D14	7,500	92,600	31,600	6,200	7,700	192	288	4,900
	SEM	901	13,350	6,991	578	1,245	42	44	1,475
	P-value	0.0004	0.6031	0.6317	0.0003	0.0225	0.2646	0.0615	0.3664
		Age Response *P*-value							
Contrast	Linear	0.0001	0.8597	0.1198	0.0012	0.0001	0.0447	0.0028	0.8048
	Quadratic	0.2050	0.0847	0.0621	0.0382	0.2503	0.0723	0.2611	0.1472
	Cubic	0.0204	0.3470	0.0993	0.6234	0.6910	0.2465	0.3882	0.0020
		Interaction *P*-value							
Interaction[b]	L*S	0.6008	0.0413	0.0823	0.9388	0.4797	0.7460	0.8658	0.4692
	L*A	0.0637	0.8374	0.9015	0.3374	0.6380	0.8275	0.7471	0.6629
	S*A	0.0524	0.0112	0.3793	0.0001	0.0018	0.0390	0.6893	0.4245

[a]All means expressed as molecules of mRNA per nanogram of total RNA. Means represent the main effects, interactions, and contrasts for data displayed in the figures.

[b]For the interactions, L, S, and A represent the main effects of line, segment and age, respectively.

[c,d,]Within a column, means without a common superscript differ ($P < 0.05$).

[e,f,g]Within a column, means without a common superscript differ ($P = 0.0001$).

[h,i]Within a column, means without a common superscript differ ($P < 0.01$).

Table 3.3. Developmental regulation of amino acid and sugar transporter and enzyme mRNA in the small intestine of broiler chicks[a].

Item		Gene[b] CAT1	CAT2	APN	GAPDH	SGLT1	SGLT5	GLUT5	GLUT2
		Molecules of mRNA per ng total RNA							
	A	569	100	157,000	87,500	17,300	3,300	1,300	566
Line	B	557	98	141,800	82,700	17,800	2,700	1,600	652
	SEM	111	8	14,408	12,555	2,301	254	157	68
	P-value	0.9370	0.8177	0.4582	0.7893	0.8663	0.0818	0.2032	0.3766
	Duodenum	499	74[c]	88,200[f]	96,200[f]	13,900[f]	1,800[i]	900[f]	600[f]
	Jejunum	565	99[d]	148,300[g]	87,000[i,j]	23,400[g]	3,200[j]	1,800[g]	900[g]
Segment	Ileum	625	124[e]	211,900[h]	72,000[j]	15,300[f]	4,000[k]	1,500[g]	300[h]
	SEM	90	8	12,981	9,700	1,895	233	136	63
	P-value	0.2381	0.0001	0.0001	0.0024	0.0001	0.0001	0.0001	0.0001
	E20	944	176	78,500	118,500	4,400	2,300	400	100
	DOH	1,040	134	147,500	120,000	14,400	3,900	600	500
	D1	309	64	124,800	85,400	16,300	2,200	1,200	700
	D3	508	53	162,700	73,300	17,700	2,900	1,500	400
Age	D7	272	87	187,000	63,800	20,700	3,000	2,300	900
	D14	305	81	196,200	49,600	31,600	3,700	2,500	1000
	SEM	193	15	24,956	21,745	3,986	440	273	118
	P-value	0.0176	0.0222	0.0222	0.1453	0.0001	0.0001	0.0001	0.0001
		Age Response *P*-value							
Contrast	Linear	0.0001	0.0001	0.0012	0.0001	0.0001	0.4895	0.0001	0.0001
	Quadratic	0.1316	0.9563	0.0199	0.7109	0.3105	0.0049	0.4192	0.2621
	Cubic	0.0184	0.1105	0.1270	0.0096	0.2352	0.6151	0.0001	0.5883
		Interaction *P*-value							
	L*S	0.2705	0.9627	0.5182	0.3057	0.6556	0.5218	0.0839	0.6636
Interaction[b]	L*A	0.7524	0.1659	0.8861	0.9098	0.1471	0.7936	0.5043	0.0549
	S*A	0.6765	0.2693	0.1620	0.3923	0.0073	0.0873	0.0006	0.0002

[a]All means expressed as molecules of mRNA per nanogram of total RNA. Means represent the main effects, interactions, and contrasts for data displayed in the figures.

[b]For the interactions, L, S, and A represent the main effects of line, segment, and age, respectively.

[c,d,e] Within a column, means without a common superscript differ ($P < 0.05$).

[f,g,h]Within a column, means without a common superscript differ ($P < 0.0001$).

[i,j,k]Within a column, means without a common superscript differ ($P < 0.01$).

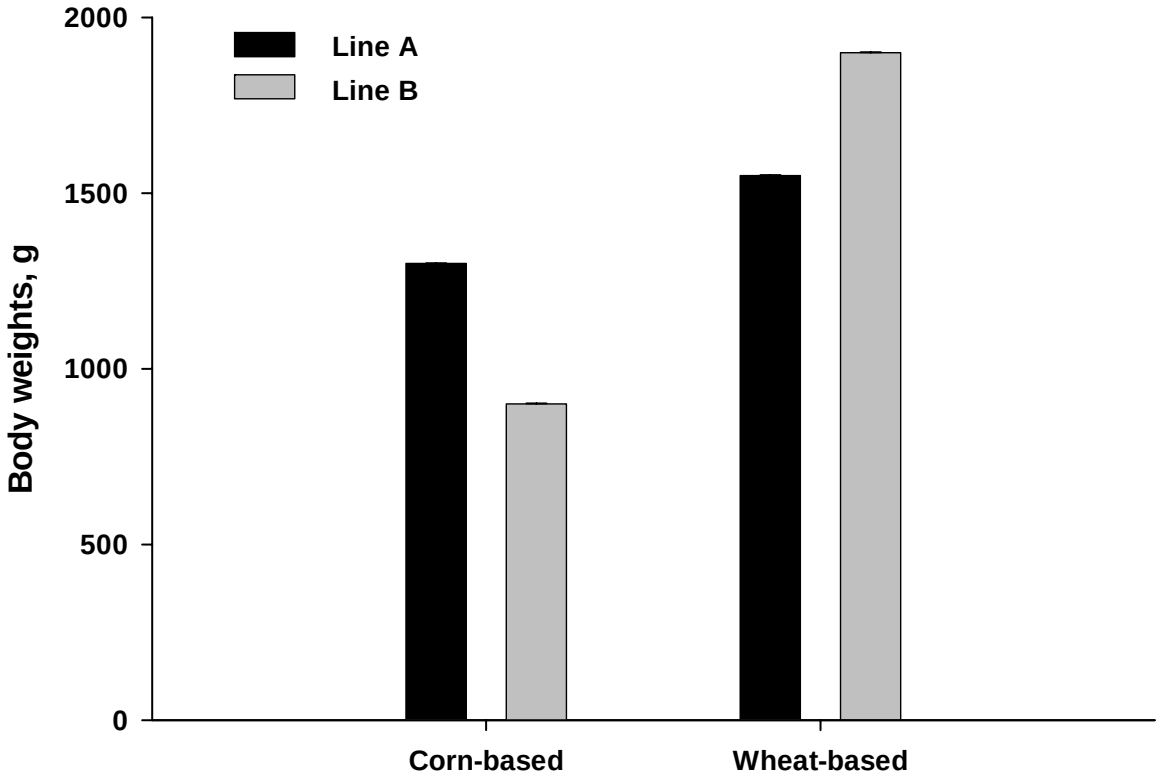

FIGURE 3.1 Body weights at 40 d for mixed-sex birds from Line A and B fed either a corn-based or wheat-based diet.

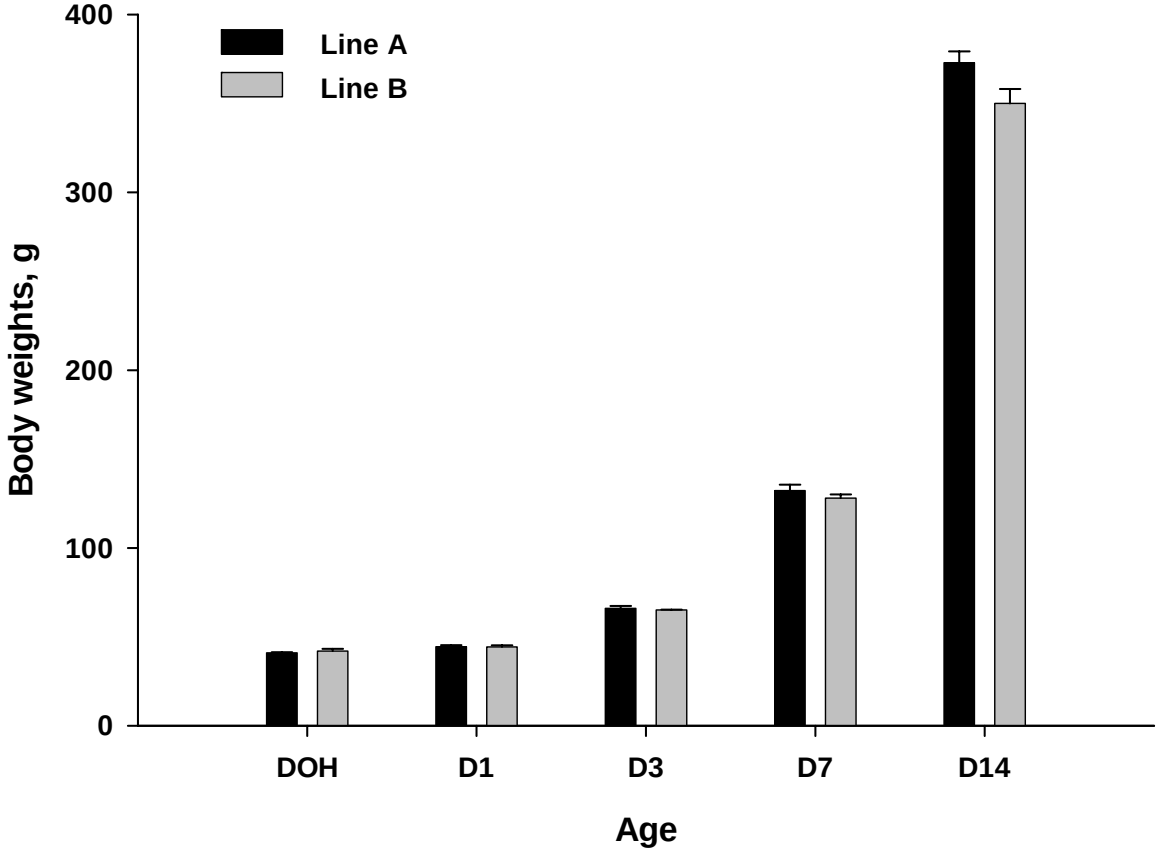

FIGURE 3.2 Bird body weights from day of hatch to d 14 posthatch. Means represent body weights (g) ± SEM from 35 mixed-sex birds. Four male birds were randomly selected from both genetic lines at each time point for the experiment.

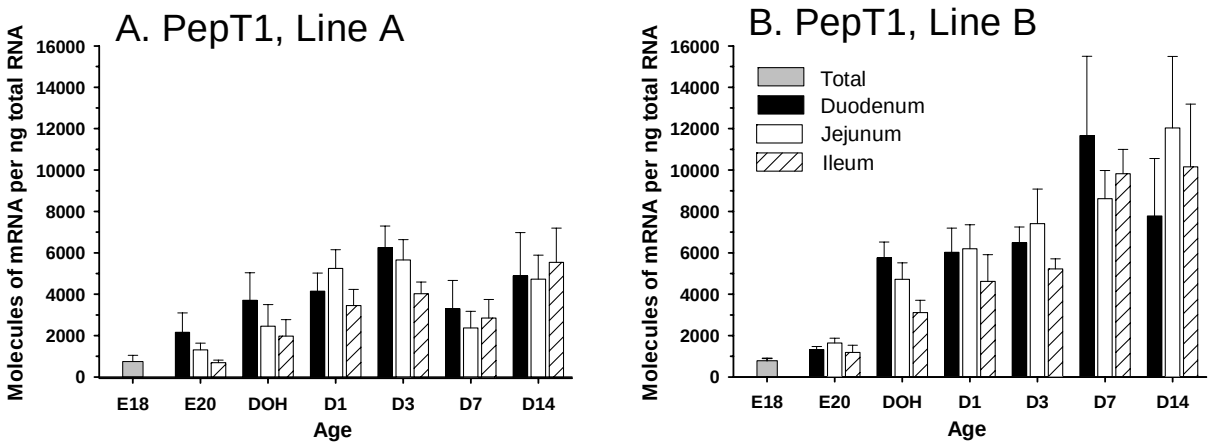

FIGURE 3.3 The number of PepT1 mRNA molecules per nanogram of total RNA in Line A (A) and Line B (B) chicks was determined by real time PCR. Quantities represent the means of four male birds ± SEM at embryo d 18 (e18) and 20 (e20), day of hatch (doh; after hatch but before feeding), and d 1 (d1), d 3 (d3), d 7 (d7) and d 14 (d14) after hatch. The statistical model did not include the e18 timepoint. There was a main effect of genetic line ($P = 0.0007$), intestinal segment ($P = 0.031$) and linear effect ($P = 0.0001$) with age.

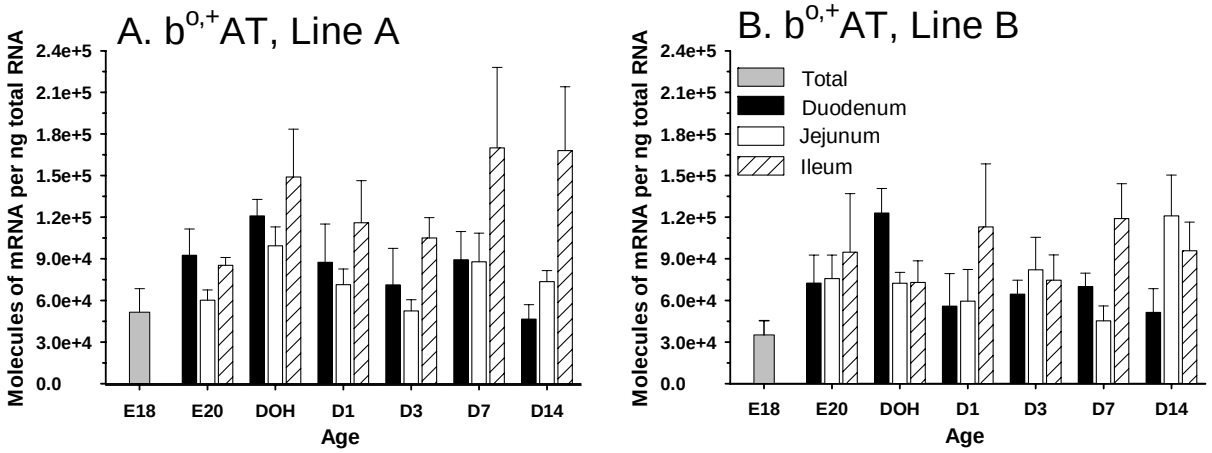

FIGURE 3.4 The number of $b^{0,+}AT$ mRNA molecules per nanogram of total RNA in Line A (A) and Line B (B) chicks was determined by real time PCR. Quantities represent the means of four male birds ± SEM at embryo d 18 (e18) and 20 (e20), day of hatch (doh; after hatch but before feeding), and d 1 (d1), d 3 (d3), d 7 (d7) and d 14 (d14) after hatch. The statistical model did not include the e18 timepoint. There was a main effect of intestinal segment ($P = 0.0001$), a genetic line x intestinal segment interaction ($P = 0.041$), and intestinal segment x age interaction ($P = 0.011$).

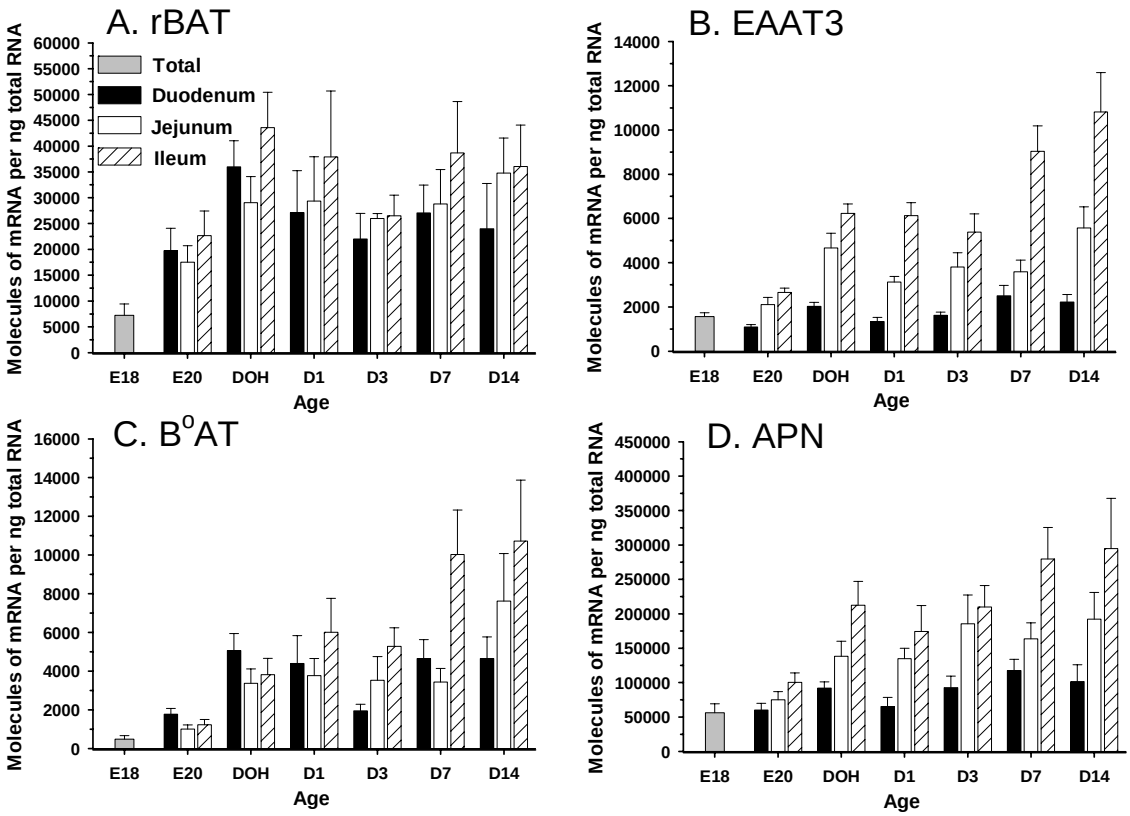

FIGURE 3.5 Real time PCR analysis of brushborder membrane amino acid transporter and enzyme mRNA in the small intestine of two genetically selected lines of broiler chicks. The number of rBAT (A), EAAT3 (B), B^oAT (C), and APN (D) mRNA molecules per nanogram of total RNA. Quantities represent the means of four male Line A and Line B birds (n = 8) ± SEM at embryo d 18 (e18) and 20 (e20), day of hatch (doh; after hatch but before feeding), and d 1 (d1), d 3 (d3), d 7 (d7) and d 14 (d14) after hatch. The statistical model did not include the e18 timepoint. There was a main effect of intestinal segment ($P < 0.001$) on rBAT, EAAT3, B^oAT, and APN mRNA and a linear increase ($P < 0.01$) with age on EAAT3, B^oAT and APN mRNA. There was an intestinal segment x age interaction ($P < 0.05$) for EAAT3 and B^oAT mRNA.

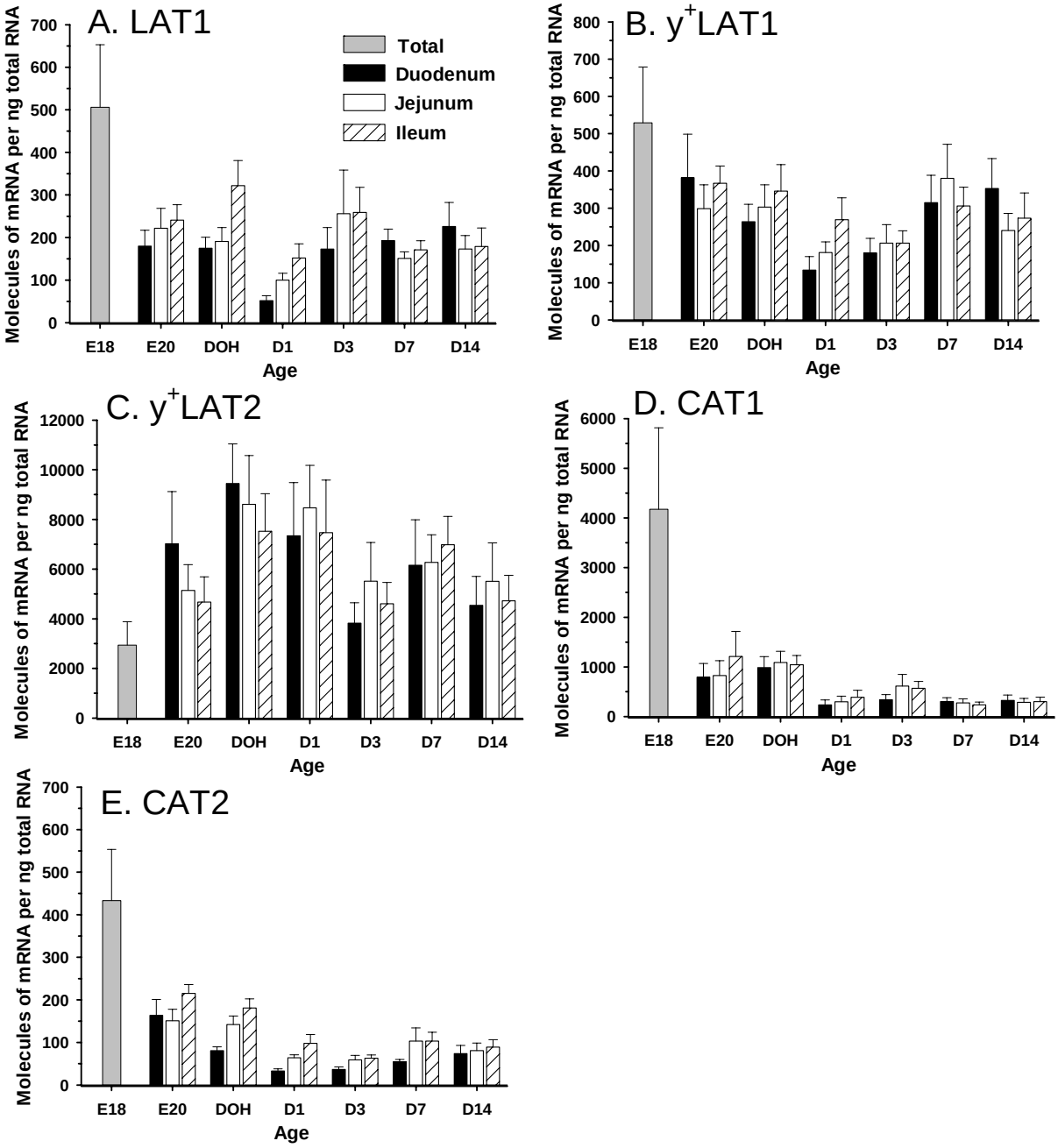

FIGURE 3.6 Real time PCR analysis of basolateral amino acid transporter mRNA in the small intestine of genetically selected lines of broiler chicks. The number of LAT1 (A), y^+LAT1 (B), y^+LAT2 (C), CAT1 (D) and CAT2 (E) mRNA molecules per nanogram of total RNA.

Quantities represent the means of four male Line A and Line B birds (n = 8) ± SEM at embryo d 18 (e18) and 20 (e20), day of hatch (doh; after hatch but before feeding), and d 1 (d1), d 3 (d3), d 7 (d7) and d 14 (d14) after hatch. The statistical model did not include the e18 timepoint. There was a linear decline ($P < 0.05$) with age of y^+LAT1, CAT1, CAT2 and LAT1 mRNA. There was a cubic effect ($P = 0.002$) with age on y^+LAT2 mRNA. There was a main effect of intestinal segment ($P < 0.01$) on CAT2 and LAT1 mRNA. There was an interaction of age x segment ($P = 0.04$) on LAT1 mRNA.

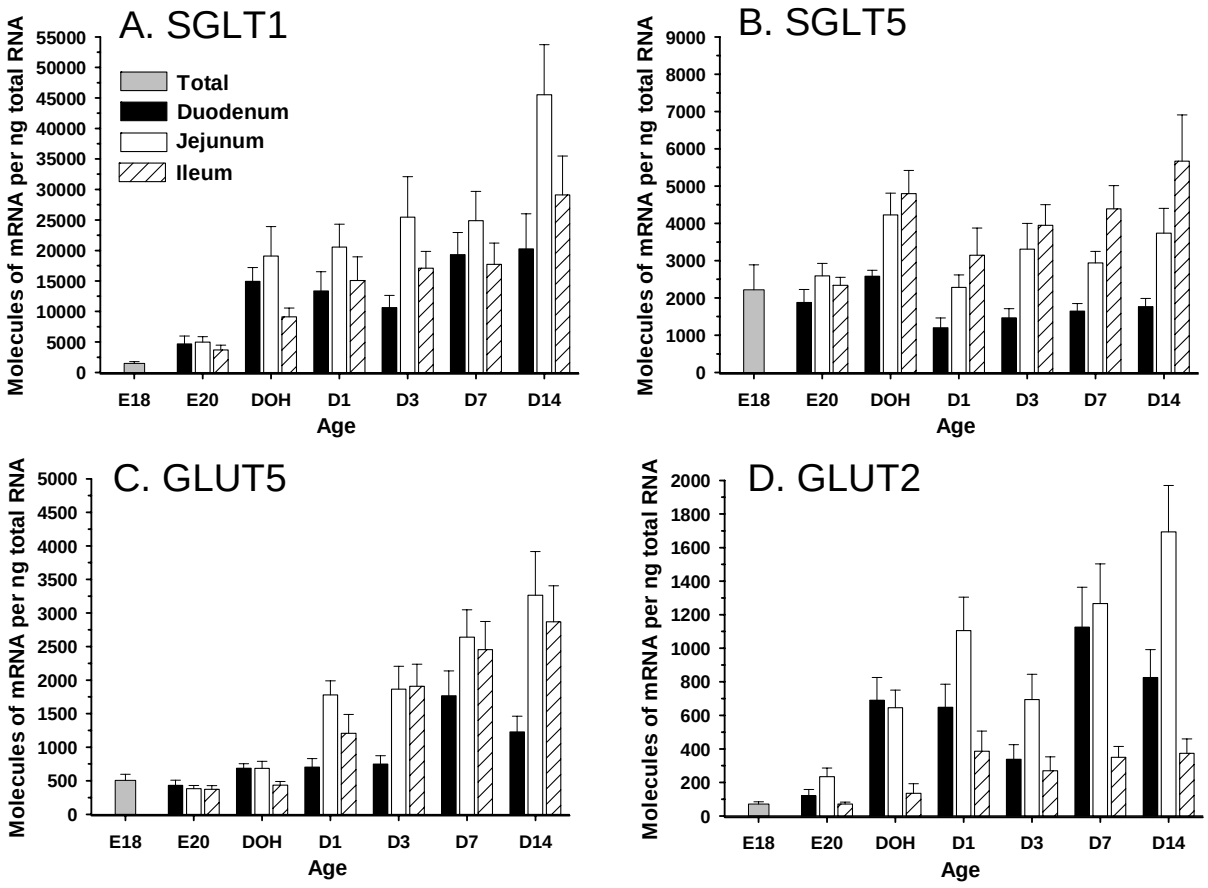

FIGURE 3.7 Real time PCR analysis of monosaccharide transporter mRNA in the small intestine of two genetically selected lines of broiler chicks. The number of SGLT1 (A), SGLT5 (B), GLUT5 (C), and GLUT2 (D) mRNA molecules per nanogram of total RNA. Quantities represent the means of four male Line A and Line B birds (n = 8) ± SEM at embryo d 18 (e18) and 20 (e20), day of hatch (doh; after hatch but before feeding), and d 1 (d1), d 3 (d3), d 7 (d7) and d 14 (d14) after hatch. The statistical model did not include the e18 timepoint. There was a main effect of intestinal segment ($P = 0.0001$) on SGLT1, SGLT5, GLUT5 and GLUT2 mRNA., a linear effect ($P = 0.0001$) with age on SGLT1, GLUT5 and GLUT2 and a quadratic effect ($P =$

0.005) with age on SGLT5. There was an intestinal segment x age interaction ($P < 0.01$) on SGLT1, GLUT5 and GLUT2.

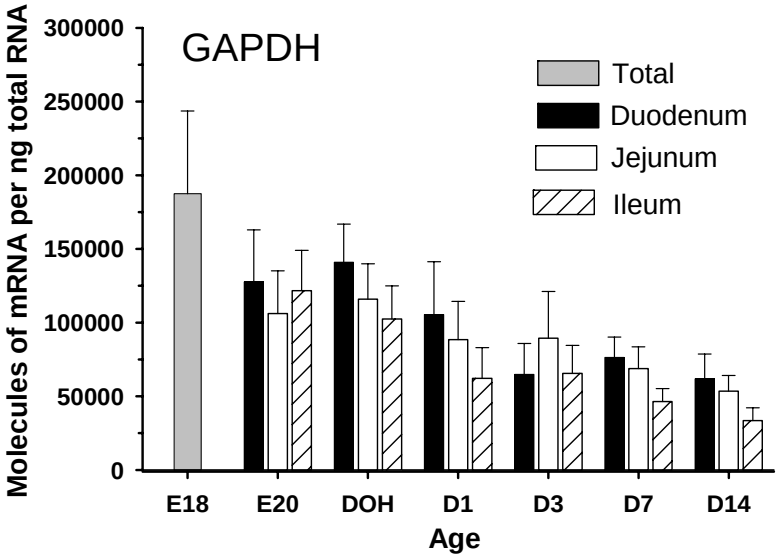

FIGURE 3.8 The number of GAPDH mRNA molecules per nanogram of total RNA was determined by real time PCR. Quantities represent the means of four male Line A and Line B birds (n = 8) ± SEM at embryo d 18 (e18) and 20 (e20), day of hatch (doh; after hatch but before feeding), and d 1 (d1), d 3 (d3), d 7 (d7) and d 14 (d14) after hatch. The statistical model did not include the e18 timepoint. There was a main effect of intestinal segment ($P = 0.002$) and a linear effect ($P = 0.0001$) with age.

Chapter IV

Dietary Protein Quality and Feed Restriction Influence Abundance of Nutrient Transporter mRNA in the Small Intestine of Broiler Chicks

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ABSTRACT

The objective of this study was to evaluate the effect of dietary protein quality on intestinal peptide transporter (PepT1), amino acid transporter ($b^{o,+}$AT, EAAT3, y^{+}LAT2, and CAT2), glucose transporter (SGLT1 and GLUT2), and digestive enzyme (APN) mRNA abundance in two lines of broilers (A and B). At day of hatch (doh), chicks from both lines were randomly assigned to corn-based diets containing 24% crude protein (CP) with either soybean meal (SBM) or corn gluten meal (CGM) as the supplemental protein source. Chicks were given unlimited access to feed and water. Groups of chicks from both lines were also assigned to the SBM diet at a quantity restricted to that consumed by the CGM group (SBM-RT). Intestinal transporter and enzyme mRNA abundance was assayed by real time PCR using the absolute quantification method. Abundance of PepT1, EAAT3, and GLUT2 mRNA was greater in Line B ($P < 0.03$), while APN and SGLT1 were greater in Line A ($P < 0.04$). When feed intake was equal (CGM vs restricted SBM), a greater abundance of PepT1 and $b^{o,+}$AT mRNA was associated with the higher quality SBM ($P < 0.04$), while a greater abundance of EAAT3 and GLUT2 mRNA was associated with the lower quality CGM ($P < 0.01$). When feed intake was restricted (SBM vs SBM-RT), a greater abundance of PepT1 mRNA was associated with the restricted intake ($P < 0.04$). These data demonstrate that both dietary protein quality

and feed restriction influence expression of nutrient transporter mRNA in the small intestine of broiler chicks.

KEY WORDS: • *Amino acid transporter* • *Broiler* • *Feed restriction* • *Pept1* • *Protein quality*

INTRODUCTION

Intestinal nutrient absorption is mediated by transporter proteins expressed on enterocytes. Amino acids are transported into the cell in their free form by a variety of transporters that vary in specificity (1-3) or as di- and tripeptides by the peptide transporter, PepT1 (4). In addition to humans and laboratory species, peptide transport through PepT1 is nutritionally important in agricultural species, such as chickens, cows, pigs and sheep (5-7); however, the free amino acid transporters have not been as well-studied in these species. Amino acid transporters have varying substrate specificities. For example transporters were characterized that are specific for basic amino acids (rBAT and $b^{o,+}$AT, CAT1 and 2, y^{+}LAT1 and 2), neutral amino acids (B^{o}AT, LAT1), and anionic amino acids (EAAT3), and most of these transporters exhibit some substrate overlap, and various ion dependencies and mechanisms for movement of amino acids across the cell membrane (8-13). The absorption of glucose is mediated by the Na^{+}-dependent transporter SGLT1 at the brushborder membrane (14) and exit of glucose across the basolateral membrane is mediated by the facilitated transporter GLUT2 (15).

The dietary regulation of nutrient transporters has been studied extensively, especially with regard to the influence of fasting and starvation. Ferraris and Diamond (16) described the regulation of nutrient transporters as a way to match uptake capacity to requirements without wasting energy on unnecessary transporters. Thus, the study of

regulation of transporters leads to elucidation of mechanisms that can change rates of transport (17). The objective of this experiment was to determine the influence of dietary protein quality and feed restriction on peptide, amino acid, and monosaccharide transporter and aminopeptidase mRNA abundance in the small intestine of two genetically-selected lines of broilers. Chicks were fed diets containing either a higher-quality protein source (soybean meal) or lower-quality protein source (corn gluten meal) as the sole supplemental protein during the first 2 wk.

MATERIALS AND METHODS

Animals and Tissue Collection. The chickens in the present experiment represent two commercial broiler lines that were genetically selected under different nutritional environments. Line A was developed on a corn and soy-based diet whereas Line B was developed on a wheat-based diet with amino acid concentrations that were 15-20% higher than for line A. The lines share a common origin but have been separated for at least 30 generations, and have been selected in the different nutritional environments for more than 10 generations. Both lines have been subjected to balanced selection for improved growth, feed conversion, meat yields, reproduction and general fitness. Additional information on these lines has been previously described (18). These lines were chosen for this study because they respond differently to dietary AA concentrations.

Eggs from both genetic lines were obtained from Aviagen (Huntsville, AL). A total of 40 d of hatch chicks from both genetic lines (total 80) were sampled for intestinal tissue before consumption of feed. The remaining chicks (n = 810; 405/genetic line) were randomly assigned to one of six heated floor pens with wood shavings. All pens had 24-h lighting and chicks had free access to water. Birds in a pen were randomly assigned to

diets containing either soybean meal (SBM) or corn gluten meal (CGM) as the supplemental protein source (Table 4.1). A comparison of essential amino acid compositions of the two experimental diets to NRC requirements (19), and a comparison of non-essential amino acid compositions are shown (Table 4.2). Additionally, to separate the effect of feed intake from protein quality, chicks from both lines were also randomly assigned to the diet containing SBM at a quantity restricted to that consumed by chicks fed the CGM diet the previous 8 h (SBM-RT). Feed intake data were collected at three time intervals each day (0700, 1500, and 2300). Birds were killed by cervical dislocation at the following time points: d of hatch (doh; after hatch but before feeding), 1 d (d1), 3 d (d3), 7 d (d7), and 14 d (d14) posthatch. At d1, d3 and d7, 40, 29, and 29 chicks were removed from each pen for sampling, respectively. At d14, the remaining birds were sampled in each pen. Birds and intestinal samples were processed in a manner similar to the procedure described by Chen et al. (20). The intestine was separated into duodenum, jejunum, and ileum. Digesta was squeezed out of the intestine, and segments were rinsed three times in phosphate-buffered saline (PBS; NaH_2PO_4: 1.47 m*M*, Na_2HPO_4: 8.09 m*M*, and NaCl: 145 m*M*). Whole segments were minced using a razor blade, mixed thoroughly, and frozen as aliquots at -80°C. Sex of the birds was determined by PCR as previously described (18). A total of five males were randomly selected from each group for RNA isolation. Remaining bird samples were used for other laboratory analyses. All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Real Time PCR. Total RNA was isolated from each tissue sample using the RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. Total RNA was quantified

spectrophotometrically at 260/280 nm and stored at -80°C. Real time PCR and the absolute quantification method was used to determine the number of molecules of mRNA present for each gene of interest per nanogram of total RNA starting template (18). An RNA standard curve for each gene was generated based on modification of the protocol of Fronhoffs et al. (21). Total RNA from d7 jejunum and gene-specific primers (see 18 for list of primers) were used to perform reverse transcriptase polymerase chain reaction (RT-PCR). Two-step RT-PCR followed by subcloning into the pGEM®-T Easy Vector (Promega) was performed as previously described (18). Purified plasmid samples were sequenced at the Virginia Bioinformatics Institute at Virginia Tech. Nested primers were designed (see 18 for list) within cloned chicken cDNA sequences using the Primer Express software, optimized for use with Applied Biosystems Real-Time PCR Systems (Foster City, CA).

Beta actin primers were designed in a similar manner for cloning (forward primer: 5`-GTCCACCTTCCAGCAGATGT-3`, reverse primer: 5`-AGTCAAGCGCCAAAAGAAAA-3`) and real time PCR (forward: 5`-GTCCACCGCAAATGCTTCTAA-3', reverse: 5`-TGCGCATTTATGGGTTTTGTT-3`).

Standard curves were made as previously described (18). Briefly, plasmids containing amplified chicken cDNA were linearized opposite a T7 or SP6 promoter depending on the orientation of the insert sequence. In vitro transcription was performed on linearized plasmids using the MEGAscript® T7 or SP6 in vitro transcription kit (Ambion, Austin, TX) and cRNA was precipitated with lithium chloride and quantified using the ribogreen assay (Molecular Probes, Eugene, OR) and a FLUOstar OPTIMA

microplate reader (BMG LABTECH, Germany). A dilution series of 10^{11} to 10^{4} molecules per microliter was performed in the presence of yeast tRNA at 10 mg/L.

Each reverse transcription reaction contained 2,000 ng of RNA at a concentration of 100 ng/μL, or an equal volume of a dilution series of cRNA (High-Capacity cDNA Archive Kit; Applied Biosystems). The cDNA was diluted 1:30 before addition to PCR reactions that contained primers and SYBR green master mix (Applied Biosystems). PCR was performed under the following conditions: 50°C for 10 min and 40 cycles of 95°C for 1 min and 60°C for 1 min using an Applied Biosystems real time PCR 7300 system. A dissociation step consisting of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s was performed at the end of each PCR reaction to verify amplification of a single product.

Statistical Analysis. All data were analyzed using the Proc MIXED procedure of SAS (SAS Institute, Cary, NC). The model included the main effects of genetic line, intestinal segment, age, diet, and all two-way interactions. Differences among segments and diets were evaluated by Tukey's test for multiple comparisons. Differences were considered significant at $P < 0.05$. The doh and d1 timepoints were not included in this model as the feeding restriction was not imposed until after intestinal sampling on d1.

RESULTS

Feed intakes and body weights. Feed intake rose with age in Line A and Line B chicks with free access to feed containing SBM as the supplemental protein, with a mean consumption of 31 $g \cdot bird^{-1} \cdot d^{-1}$; approximately 32 g/d for Line A chicks and 30 g/d for Line B chicks (Figure 4.1). In contrast, in all chicks with free access to the CGM diet and in chicks fed the SBM diet at an intake restricted to that consumed by the chicks fed CGM, there was a lower intake throughout the course of the feeding trial, with a mean

intake of 7.8 g · bird^{-1} · d^{-1}); 8.7 g/d for Line A chicks and 6.9 g/d for Line B chicks. This represents an approximate fourfold increase in feed intake in birds consuming SBM as compared with birds consuming CGM. Reflective of the differences in intake are differences in body and intestinal weight across the different dietary groups. There were interactions of age x diet for BW, total intestinal weight, relative intestinal weight, and segmental weight (Table 4.3; Figure 4.2; $P = 0.0001$). Intestinal weights showed a more rapid increase with age for the chicks with ad-libitum access to SBM as compared with feed-restricted chicks (SBM-RT), and more rapid increases with age for chicks consuming SBM-RT, as compared with chicks consuming equal amounts of CGM (Table 4.3; Figure 4.2). Chicks consuming SBM ad libitum were heaviest in terms of BW, total intestinal weight, and individual segmental weights, chicks consuming CGM were lightest, and chicks consuming restricted quantities of SBM had intermediate intestinal, segmental and BW ($P = 0.0001$; Table 4.3). Although Line A chicks had greater total intestinal, jejunal, ileal and BW than Line B chicks ($P = 0.0001$), when total intestine was expressed relative to BW there was no significant difference. Intestinal mass expressed relative to BW was greater in chicks consuming ad-libitum quantities of SBM as compared with feed-restricted chicks ($P = 0.0001$), and was greater in chicks consuming restricted amounts of SBM as compared with chicks that consumed CGM ($P = 0.0001$).

Effects of genetic line and diet on mRNA abundance. The mRNA abundance for a peptide transporter PepT1, four amino acid transporters ($b^{o,+}$AT, EAAT3, y^{+}LAT2, and CAT2), two glucose transporters (SGLT1 and GLUT2), a digestive enzyme (APN) and two housekeeping genes (beta actin and GAPDH) was determined using real time PCR and the absolute quantification method. Thus, the results presented herein represent

mRNA and it is unknown at this time if these data parallel expression and activity of the functional proteins. The mRNA expression data are summarized in Table 4.4, with separate rows for main effects of genetic line, diet, intestinal segment, developmental age, and two-way interactions. Only significant interactions are expressed graphically. Effects of genetic line, age, diet, and intestinal segment were observed for most of the genes; however, for brevity, only effects involving genetic line, diet, and age will be discussed. To facilitate a more meaningful discussion, only two dietary comparisons, the effect of feed restriction (SBM vs. SBM-RT) or protein quality (SBM-RT vs. CGM), will be discussed.

Multiple main effects of genetic line were observed in this study (Table 4.4). Abundance of PepT1, EAAT3, and GLUT2 mRNA was greater in Line B compared with Line A chicks ($P < 0.03$). Abundance of APN and SGLT1 mRNA was greater in Line A chicks ($P < 0.04$). The dietary response of $b^{o,+}AT$, EAAT3, $y^{+}LAT2$, APN, and SGLT1 mRNA was differentially regulated by the two genetic lines. Line A chicks varied little in expression of $b^{o,+}AT$ (Figure 4.3A), $y^{+}LAT2$ (Figure 4.3B), EAAT3 (Figure 4.3C), and APN (Figure 4.3D) when fed SBM ad-libitum or SBM at restricted quantities; however, Line B chicks showed greater expression of these genes in feed-restricted chicks compared with chicks given ad-libitum amounts of SBM ($P = 0.0003$). For the glucose transporter, SGLT1, mRNA abundance was greater in ad-libitum fed chicks than in feed-restricted chicks within Line A, but in Line B chicks there was no difference between the two groups consuming SBM ($P = 0.0001$; Figure 4.3E).

There was reduced expression of $b^{o,+}AT$ ($P = 0.0003$; Figure 4.3A) and $y^{+}LAT2$ ($P = 0.0001$; Figure 4.3B) in Line A chicks consuming CGM as compared with chicks

consuming restricted quantities of SBM, whereas, in Line B chicks, there was no difference between the dietary groups. Abundance of EAAT3 mRNA was greater in chicks consuming CGM as compared with chicks consuming equal amounts of SBM, in both lines, however, the difference was more accentuated in Line B chicks ($P = 0.005$; Figure 4.3C). In Line A chicks, mRNA abundance of APN was lower in chicks consuming the CGM diet, in contrast to Line B chicks where mRNA was greater in chicks consuming the CGM diet compared with chicks that consumed equal quantities of the SBM diet (P = 0.0001; Figure 4.3D). In Line A chicks there was also a lesser quantity of SGLT1 mRNA in response to the low-quality protein CGM, whereas in Line B chicks, there was a dramatic increase in SGLT1 mRNA in chicks consuming CGM compared with chicks consuming equal amounts of the SBM diet ($P = 0.0001$; Figure 4.3E).

The peptide transporter, PepT1 was the only gene influenced by feed restriction with greater expression in the feed-restricted chicks compared with chicks given free access to the diet ($P < 0.03$; Table 4.4)**.** Abundance of PepT1 and $b^{o,+}AT$ mRNA were both influenced by dietary protein quality, with greater expression in the chicks that consumed the diet containing the higher-quality protein, SBM, as compared with chicks that consumed CGM ($P < 0.04$). The abundance of EAAT3 and GLUT2 mRNA was greater in chicks that consumed CGM, compared with chicks consuming an equal amount of SBM ($P < 0.01$).

Feed restriction and protein quality influenced the age response of PepT1, $b^{o,+}AT$, EAAT3, y^+LAT2, CAT2, APN, and SGLT1 ($P < 0.01$). In the small intestine of chicks that ate restricted quantities of SBM, expression of PepT1 (Figure 4.4A), $b^{o,+}AT$ (Figure 4.4B), EAAT3 (Figure 4.4C), y^+LAT2 (Figure 4.4D), and CAT2 (Figure 4.4E), mRNA

increased with age of the chicken, from D3 to D14, whereas, in chicks with ad-libitum consumption of the feed, mRNA levels increased less dramatically (CAT2), decreased (PepT1), or remained relatively unchanged ($b^{o,+}$AT, EAAT3, y^{+}LAT2, and APN). Quantities of APN mRNA remained unchanged in response to restricted feeding (Figure 4.4F). Protein quality influenced the age-response of transporters and APN in a similar manner. In contrast to the general upregulation in gene expression observed in the feed-restricted chicks consuming SBM from D3 to D14, the chicks consuming CGM showed decreased expression of PepT1, $b^{o,+}$AT, EAAT3, y^{+}LAT2, CAT2, APN, and SGLT1 from d3 to d7, followed by an increase to d14 ($P < 0.01$; Figure 4.4). Quantities of SGLT1 mRNA remained unchanged with age in response to both ad-lib and restricted consumption of the SBM diet, but changed in response the CGM diet (Figure 4.4G). There was also a highly significant diet x intestinal segment interaction on expression of 9 of the 10 genes evaluated in this study but not described in this paper (Appendix B; Figure B.1).

Expression of two housekeeping genes, GAPDH and beta actin, was evaluated in this study, and both were found to be influenced by genetic line, age, and dietary treatment. Beta actin was expressed in greater quantities in Line A chicks compared with Line B chicks (Table 4.4; $P = 0.0021$). Expression between lines of both housekeeping genes differed according to dietary treatment ($P < 0.03$). In Line A chicks, GAPDH mRNA abundance decreased in response to feed restriction, while in Line B chicks, the two groups did not differ (Figure 4.5A). In response to protein quality, there was a more accentuated difference in GAPDH expression between the Line B SBM-RT and CGM groups (Figure 4.5A). Beta actin also displayed a differential pattern of expression across

dietary groups among the two genetic lines, with Line B chicks expressing greater quantities of mRNA when fed the CGM diet as compared with chicks fed an equal amount of SBM (Figure 4.5B). Expression of GAPDH was greater in the chicks consuming CGM, compared with the chicks consuming equal amounts of SBM (P = 0.002). There was an interaction of age x diet on both GAPDH (P = 0.0001) and beta actin (P = 0.005) mRNA quantities. The decrease in mRNA abundance of GAPDH with age was less pronounced in feed-restricted chicks, as compared with ad-libitum fed chicks (Figure 4.5C), and for beta actin, there was a less pronounced decrease in feed-restricted chicks from D3 to D7, and a more pronounced increase in expression from D7 to D14, relative to ad-libitum fed chicks (Figure 4.5D). In response to dietary protein quality, both GAPDH and beta actin decreased from D3 to D7, and increased from D7 to D14, in chicks that consumed the lower-quality protein, CGM. Thus, beta actin and GAPDH mRNA levels did not remain constant and were deemed unsuitable as internal controls for relative quantification.

DISCUSSION

Protein quality is reflected by the balance of amino acids required for maximal growth and protein synthesis and the limiting amino acids present (22). Additionally physical properties of a protein (i.e., other chemical consituents) may also contribute to the availability of amino acids to the animal. It should be pointed out, that in our study these physical differences in the two protein sources may contribute to differences in digestion and absorption at the intestinal level. Both SBM and CGM are deficient in essential AA and contain differences in concentrations of specific AA, AA ratios and other physical properties. However, these two protein sources represent a good model for

evaluating the effect of a single protein source on nutrient transporter gene expression in the small intestine. Consumption of a diet with protein that contains an imbalance of essential amino acids and amino acid levels lower than the nutritional requirements leads to metabolic disturbances and reduced feed intake (23). Lower intake observed in birds that consumed the CGM diet (25 % of SBM) may be related to a deficiency in essential AA, and to the metabolic cost associated with removal of amino acids that are in excess of the most limiting for protein synthesis. Differences in body and intestinal weight were reflective of differences in intake and protein quality, with greatest weights and rates of growth in chicks that consumed ad-libitum quantities of SBM, and the least growth in chicks that consumed CGM. Greater body and intestinal weights of chicks with restricted intake of SBM as compared with chicks that consumed CGM illustrates that SBM was a better dietary protein source than CGM. As expected, there was a difference between genetic lines, with chicks from Line A heavier than Line B. Total intestinal weight and the individual weights of the jejunum and ileum were greater in Line A chicks, but when intestinal weight was expressed relative to BW, there was no difference. This indicates that per unit body mass, intestines were developing at the same rate in both lines of broilers.

Effect of genetic line on transporter expression. The rationale for comparing the two genetically-selected lines of broilers was to evaluate the idea that differential growth responses to varying amino acid concentrations in the diet is correlated with differences in the profile of nutrient transporters expressed in the small intestine. We previously reported that out of 15 genes evaluated (one peptide transporter, nine amino acid transporters, a digestive enzyme, and four sugar transporters), only one was influenced by

genetic line, with Line B expressing more PepT1 mRNA than Line A (18). In that experiment, a standard commercial starter diet (20% CP, corn-soy) was fed, with no dietary treatment. In the present experiment, it is clear that there is a differential response to dietary perturbations, with multiple effects of genetic line and interactions of line and diet. We find that genetic selection has changed expression of intestinal nutrient transporters but that these differences are masked when birds are fed a balanced diet and accentuated when fed deficient diets. We found, in the present experiment, an effect of genetic line for five transporter or digestive enzyme genes. We observed greater expression of EAAT3, GLUT2, and similar to the previous study, PepT1 mRNA, in Line B chicks. Expression of APN and SGLT1 mRNA was greater in Line A chicks. Although the implications of these differences are unknown, they clearly suggest that genetic selection based on nutrition has robust effects on gene expression of nutrient transporters. Future experiments will involve exploitations of these differences through the diet in attempts to drive changes in growth performance and intestinal development.

Effect of feed restriction on transporter expression. Of the genes evaluated in this study, PepT1 was the only one for which there was greater expression in the feed-restricted birds compared to birds with ad libitum consumption of SBM. As a low-affinity high-capacity transporter that is considered to be a faster and more energetically efficient means of assimilating amino acids (24-27), increased expression of PepT1 in response to feed restriction may serve as a way to conserve energy while maximizing amino acid uptake in spite of protein and energy deficiency.

There was an interaction of age x diet for seven genes associated with digestion or transport, including PepT1, $b^{o,+}AT$, EAAT3, $y^{+}LAT2$, CAT2, APN and SGLT1. There

was a similar response to feed restriction with age, in which there was upregulation in expression, which occurred as early as d7 for some genes (PepT1, EAAT3, y^+LAT2, and CAT2), and at d14 for others ($b^{o,+}$AT). Regardless of whether expression increased with age in chicks fed SBM ad libitum (CAT2 and EAAT3), the response to restriction with age was of a greater magnitude.

We observed little response of APN, GLUT2 and SGLT1 mRNA to feed restriction. Susbilla et al. (28) observed increased activities of amino- and dipeptidases in the small intestines of broiler chicks that were feed-restricted to 40% of the control intake from 5 to 11 d of age. In leghorn chicks feed-restricted (54% of control intake) from 12 to 17 d, duodenal aminopeptidase-N activity was greater than in control or refed birds (29). Food restriction in rats (8-10 g food/d; 38) and protein-energy restriction in postnatal rabbits (31) increased glucose transport. In 6-wk old male broiler chickens, SGLT1 mRNA was enhanced in birds that were totally deprived of feed for 4 d as compared to birds with ad libitum intake (32).

Feed-restriction, fasting or other sources of malnutrition result in reduced intestinal absorptive surface area, which may explain why expression of nutrient transporters increased with age in response to feed restriction. Delayed access to feed for 36 h posthatch resulted in depressed villus height and crypt depth, and depressed growth in all intestinal segments (33). Silva et al. (34) subjected male broiler chicks to feed restriction (30% of ad libitum) and found that feed restriction decreased the surface area of the tip of the enterocytes in the small intestine at 14 d. Thus, the mechanism leading to a fasting or malnutrition-related increase in nutrient transport may be a combination of increased gene expression and ratio of transporting to non-transporting cells (35).

Effect of dietary protein quality on transporter expression. In the present study we found four transporter genes to be expressed differentially due to the effect of protein quality. The peptide transporter, PepT1, and amino acid transporter, $b^{o,+}AT$ were expressed at greater quantities in chicks that consumed restricted quantities of SBM, as compared to chicks that consumed CGM. Quantities of EAAT3 and GLUT2 were greater in chicks that consumed CGM. Dietary protein level and feeding restriction influenced PepT1 expression in a previous study. Chen et al. (20) reported that, by feeding diets to broiler chicks containing 12, 18 or 24 % CP at intakes restricted to that consumed by birds fed the 12 % CP diet, greater levels of CP in the diet were associated with greater expression of PepT1 during the first 35 d posthatch. Chen et al. (20) found additionally, that in the group of birds fed the 24 % CP diet ad-lib (40 % greater intake), mRNA abundance was lower than that observed for the feed–restricted group, demonstrating that feed restriction up-regulated PepT1 expression, similar to what we observed in the present study.

Karasov et al. (36) described the relationship between dietary amino acid levels and intestinal amino acid uptake. Amino acids can be used as sources of energy, some amino acids are more essential to a cell than others, or more toxic, and to further complicate matters, enterocytes express transporters with overlapping substrate specificity and transporters that mediate the movement of both essential and nonessential amino acids. Thus, it becomes difficult to predict whether a transporter should be upregulated in response to certain amino acid deficiencies or imbalances.

Kilberg et al. (37) described regulation of gene expression in response to reduced amino acid availability. The amino acid response (AAR) pathway detects and acts in

response to an amino acid deficiency, increasing translation of activating transcription factor (ATF) 4. This pathway is thought to be cell-type specific, with an insufficient amount of an amino acid essential to a particular cell, triggering pathway activation, and subsequent changes in RNA splicing, chromatin remodeling, nuclear RNA export, mRNA stabilization, and translational control. Stein et al. (38) observed that amino acids given as dietary supplements to mice were potent inducers of transporters, differing in their ability to regulate the same amino acid transporter, with a discrepancy between amino acids that were inducers and those that were transporter substrates. Shiraga et al. (39) found elements in the 5` upstream region of rat PepT1 responsive to peptides and free amino acids. Hence, nutrient transporter genes are likely to be responsive to changes in dietary protein.

The age-related response in gene expression to dietary protein quality showed an interesting pattern. For PepT1, $b^{o,+}$AT, EAAT3, y^{+}LAT2, CAT2, APN, SGLT1, beta actin and GAPDH there was an identical age-related pattern of expression in chicks consuming the CGM diet, in which mRNA abundance decreased from d3 to d7, and then rose sharply from d7 to d14. This pattern may be related to depletion of yolk sac reserves, which occurs between week 1 and week 2 (40). Because of the severe imbalance of amino acids in the diet, the bird experiences metabolic disturbances (41, 42, 43). In general it is hypothesized that depressed intake is related to a conservation of energy, as there is a metabolic cost associated with disposing of excess amino acids. Along those same lines, the cost associated with upregulating the nutrient transporters may outweigh the benefits to be derived from assimilating those amino acids, especially if the overlap in

substrate specificity and potential coordinate regulation prevents them from better controlling the balance of amino acids entering the cell.

Expression of mRNA for GAPDH and beta actin, two commonly used housekeeping genes, showed significant interactions of age x diet, line x diet, and diet x segment, similar to the digestive enzyme and nutrient transporters. The age response for chicks consuming CGM was similar for all genes, suggesting that this is a generalized effect. As GAPDH and beta actin are proteins involved in vital cellular processes including glucose metabolism and cystoskeletal structure and movement, respectively, it is not surprising that they are also influenced by diet. These data demonstrate that correcting gene expression to a housekeeping gene is in some cases inappropriate and should be exercised with caution.

In conclusion, our data demonstrate that dietary protein quality and feed restriction do influence mRNA abundance of peptide, amino acid, and sugar transporters, and a digestive enzyme in the small intestine of two genetically-selected lines of broilers. The influence of protein quality and feed restriction is dependent on age. In response to feed restriction, expression of peptide and amino acid transporters increased at d7, whereas, in response to ad-libitum intake of a diet containing the low-quality protein, CGM, expression of transporters decreased at d7 and then increased at d14. In general, the response to feed restriction and to the lower quality protein was greater in Line B chicks, although for those genes Line A chicks expressed greater overall quantities. A greater understanding of how dietary protein and genetic selection regulates nutrient transporter gene expression will allow for the discovery of ways to potentially manipulate

regulatory processes and exploit differences to enhance nutrient uptake, and improve nutrient utilization in chickens.

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Table 4.1. Ingredient and chemical composition of the experimental diets.

	Diet	
Ingredient (%)	SBM[1]	CGM[2]
Ground corn	39.0	39.0
CGM	0.00	34.2
SBM	46.66	0.00
Cellulose	0.55	16.34
Corn starch	3.93	0.00
Soybean oil	6.00	6.00
Dicalcium phosphate	1.61	2.24
Calcium carbonate	1.47	1.42
Sodium chloride	0.50	0.50
Vitamin-mineral mix[3]	0.16	0.16
Choline chloride	0.07	0.07
BMD-50[4]	0.05	0.05
Crude protein, %[5]	24.00	24.00
Metabolizable energy, kJ/g	12.61	12.61

[1] SBM = soybean meal

[2] CGM = corn gluten meal

[3] Layer/Starter/Breeder PMX® vitamin-mineral premix provided by Akey. Guaranteed analysis (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; vitamin A, 4,409,171.076 IU; Vitamin D_3, 1,410,934.744 ICU; 13,227.513 IU; d-biotin, 88.183 mg

[4] BMD-50 was provided by Alpharma, Inc. The addition of BMD-50 provided 1 g bacitracin activity/kg diet.

[5]Experimentally determined value.

Table 4.2. Amino acid composition (%) of experimental diets in comparison to NRC requirements for broilers[1]

	Diet		
Amino acids	SBM[2] (% of diet)	CGM[3] (% of diet)	NRC requirements
Methionine	0.40	0.65	0.50
Arginine	1.73	0.84	1.25
Threonine	0.99	0.90	0.80
Tryptophan	0.35	0.14	0.20
Histidine	0.65	0.55	0.35
Isoleucine	1.39	1.20	0.80
Lysine	1.57	0.47	1.10
Leucine	2.11	4.07	1.20
Valine	1.34	1.24	0.90
Phenylalanine	1.24	1.65	0.72
Methionine + Cystine	0.77	1.10	0.90
Cystine	0.37	0.45	--------
Tyrosine	0.80	1.27	--------
Alanine	1.41	2.24	--------
Aspartic acid	3.35	1.79	--------
Glutamic acid	5.51	5.81	--------
Glycine	1.23	0.74	--------
Proline	1.69	2.63	--------
Serine	1.51	1.32	--------

[1] Nutrient Requirements of Poultry: Ninth Revised Edition, 1994 (19)

[2] SBM = soybean meal, tabular values

[3] CGM = corn gluten meal, tabular values

Table 4.3. Intestinal weight, BW, and relative intestinal weight[1] in two lines of broiler chicks subjected to feeding restriction and diets differing in protein quality from day of hatch to day 14.

Item		Duodenum (g)	Jejunum (g)	Ileum (g)	Total intestine (g)	BW (g)	(Intestinal weight/BW)x100
	A	2.32	3.11	2.06	7.48	124.31	6.02
Line	B	2.25	2.78	1.76	6.79	117.57	5.94
(n=5)	SEM	0.06	0.05	0.03	0.12	1.5	0.07
	P-value	0.40	0.0001	0.0001	0.0001	0.002	0.42
	SBM	3.69[a]	5.16[a]	3.38[a]	12.24[a]	200.16[a]	6.53[a]
	SBM-RT	1.88[b]	2.30[b]	1.44[b]	5.63[b]	96.87[b]	5.99[b]
Diet[2]	CGM	1.27[c]	1.38[c]	0.899[c]	3.55[c]	65.78[c]	5.41[c]
(n=10)	SEM	0.07	0.06	0.04	0.14	1.84	0.08
	P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Age	D3	1.34	1.57	1.06	3.96	63.77	6.09
(n=10)	D7	2.26	2.80	1.74	6.81	101.06	6.49
	D14	3.25	4.47	2.93	10.65	197.99	5.35
	SEM	0.07	0.06	0.04	0.14	1.83	0.08
	P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
----------Interaction *P*-value----------							

	A x D	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Interaction[3]	L x D	0.87	0.02	0.34	0.28	0.36	0.67

[1] Relative intestinal weight represents the intestinal weight expressed as a percentage of BW.

[2] Within a column: [a,b,c]difference in superscript represents differences between SBM (ad-libitum consumption of soybean meal), SBM-RT (restricted intake of soybean meal), and CGM (ad-libitum consumption of corn gluten meal; $P = 0.0001$)

[3] For the interactions, A, D, and L represent the main effects of age, diet, and genetic line, respectively.

Table 4.4. Dietary regulation of nutrient transporter, enzyme, and housekeeping gene mRNA in the small intestine of broiler chicks[1]

		Gene									
Item		PepT1	$b^{o,+}AT$	EAAT3	y^+LAT2	CAT2	APN	SGLT1	GLUT2	GAPDH	Beta-actin
		Molecules of mRNA per ng total RNA									
	A	10330	157270	6010	3320	90	519330	37260	1050	99470	1474610
Line	B	12810	148350	6790	3290	80	462470	33340	1300	98770	1256250
(n=5)	SEM	770	5723	239	118	3	16860	1269	60	4631	48538
	P-value	0.03	0.27	0.02	0.83	0.22	0.02	0.03	0.003	0.92	0.002
	SBM	10280^{b}	$152020^{a,b}$	5220^{b}	3290	$80^{b,c}$	475200	35430	$1160^{a,b}$	$102330^{a,b}$	1413370
	SBM-RT	13910^{a}	168500^{a}	6040^{b}	3540	$90^{a,c}$	509880	32640	1010^{b}	83330^{b}	1322780
Diet[2]	CGM	10520^{b}	137920^{b}	7930^{a}	3080	100^{a}	487600	37820	1360^{a}	111700^{a}	1360140
(n=10)	SEM	943	7009	292	144	4	20651	1555	73	5672	59447
	P-value	0.01	0.01	0.0001	0.09	0.002	0.49	0.07	0.004	0.003	0.56
	Duodenum	13230^{d}	131230^{d}	3240^{d}	3030^{d}	50^{d}	297290^{d}	33840^{d}	1340^{d}	105520	1182870^{d}
	Jejunum	11950^{d}	105810^{e}	5720^{e}	$3320^{d,e}$	70^{e}	479610^{e}	43800^{e}	1530^{e}	97550	1425490^{e}
Segment[3]	Ileum	9530^{e}	221400^{f}	10220^{f}	3570^{e}	140^{f}	695790^{f}	28260^{f}	660^{f}	94290	1487930^{e}
(n=10)	SEM	630	6399	293	110	3	18606	1348	56	5460	40814
	P-value	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001	0.0001	0.0001	0.31	0.0001

Age	D3	11740	146890	5300	3060	80	487990	33330	1020	104900	1413720
(n=10)	D7	10650	122650	5420	3020	80	405320	29910	1050	79260	1046840
	D14	12330	188900	8460	3830	100	579380	42660	1460	113190	1635720
	SEM	943	7009	293	144	4	20651	1555	73	5672	59447
	P-value	0.45	0.0001	0.0001	0.0001	0.0019	0.0001	0.0001	0.0001	0.0002	0.0001
----------Interaction *P*-value----------											
	A x D	0.0053	0.0001	0.0001	0.0001	0.0014	0.0001	0.0001	0.83	0.0001	0.006
Interaction[4]	L x D	0.20	0.0003	0.005	0.0001	0.17	0.0001	0.0001	0.34	0.002	0.03
	D x S	0.01	0.0007	0.002	0.0004	0.0001	0.0001	0.15	0.0001	0.007	0.0002

[1]All means expressed as molecules of mRNA per nanogram of total RNA.

[2] Within a column: [a,b,c]difference in superscript represents differences between SBM (ad-libitum consumption of soybean meal), SBM-RT (restricted intake of soybean meal), and CGM (ad-libitum consumption of corn gluten meal; PepT1: $P < 0.04$; $b^{o,+}AT$: $P = 0.01$; EAAT3: $P = 0.0001$; CAT2: $P = 0.001$; GLUT2: $P = 0.003$; GAPDH: $P = 0.002$).

[3] Within a column: [d,e,f]difference in superscript represents difference between intestinal segments (PepT1, EAAT3, $y^{+}LAT2$, CAT2, APN, Beta actin: $P = 0.0001$; $b^{o,+}AT$ and SGLT1: $P < 0.01$; GLUT2: $P < 0.02$).

[4] For the interactions, A, D, L, and S, represent the main effects of age, diet, genetic line, and intestinal segment, respectively.

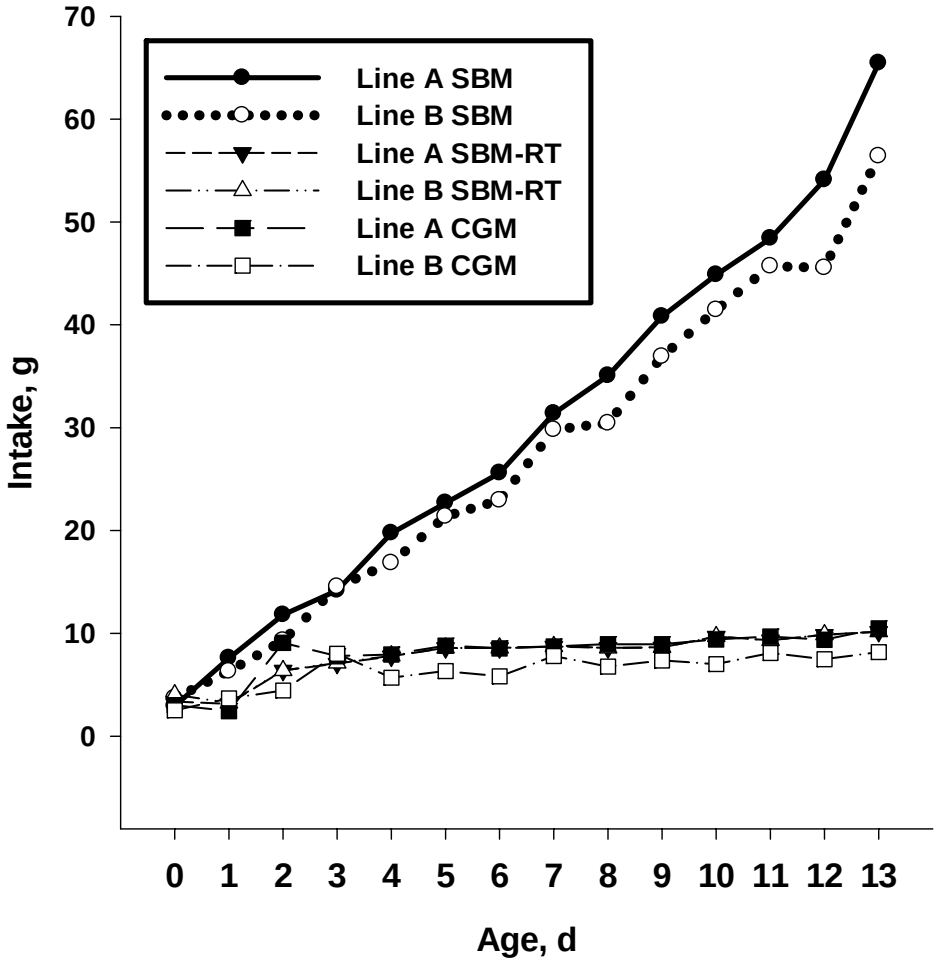

FIGURE 4.1 Pen feed intakes from doh to d13 for two genetically selected lines of broiler chicks fed diets that differed in the source of protein. Lines represent feed intake per bird (calculated based on total pen feed intake divided by the number of chicks) for mixed-sex chicks from both lines and each dietary group (SBM = ad-libitum intake of soybean meal; SBM-RT = restricted intake of soybean meal; CGM = ad-libitum intake of corn gluten meal) from doh through d13.

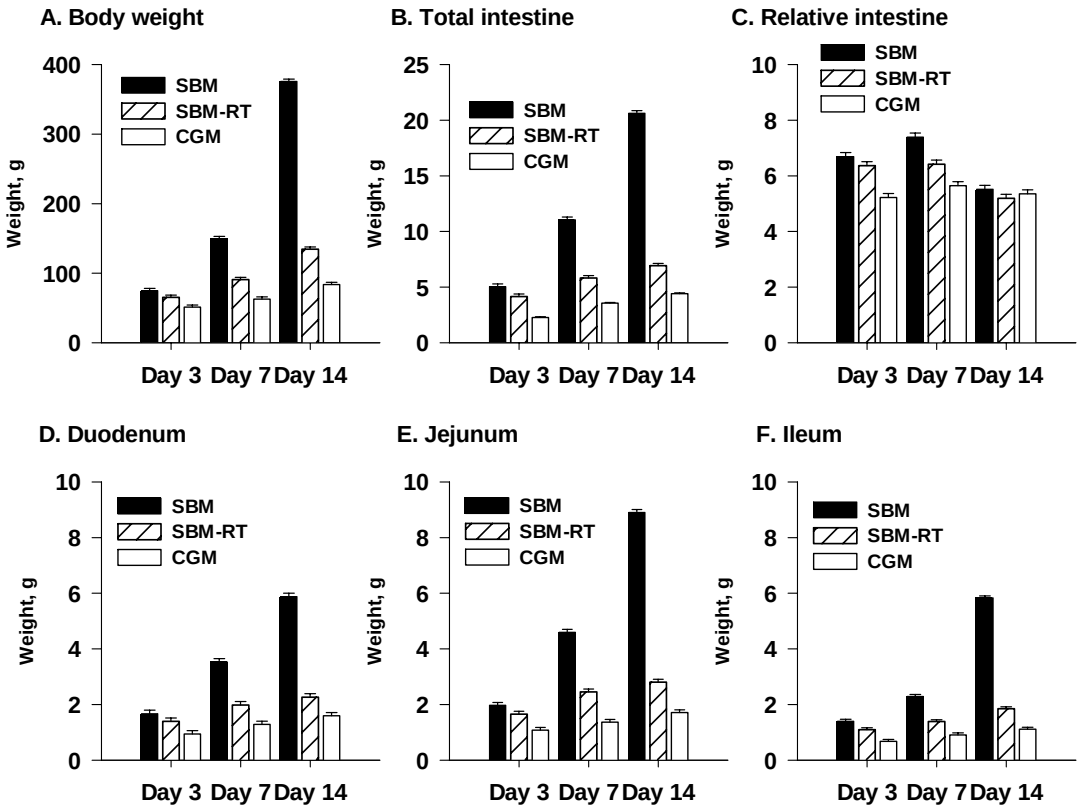

FIGURE 4.2 Intestinal and BW of male chicks (n = 10; five from Lines A and B) from two genetic lines at d3, d7, and d14 in chicks fed soybean meal ad-libitum (SBM), restricted quantities of soybean meal (SBM-RT), and corn gluten meal (CGM). Data for weights of A) body, B) total intestine, C) relative intestine, D) duodenum, E) jejunum, and F) ileum represent means of ten male chicks (five from both lines) ± SEM. Relative intestine weights represent total intestinal weight expressed as a percentage of BW. There was an interaction of diet x age for duodenum, jejunum, ileum, intestine, body, and relative intestinal weight ($P = 0.0001$).

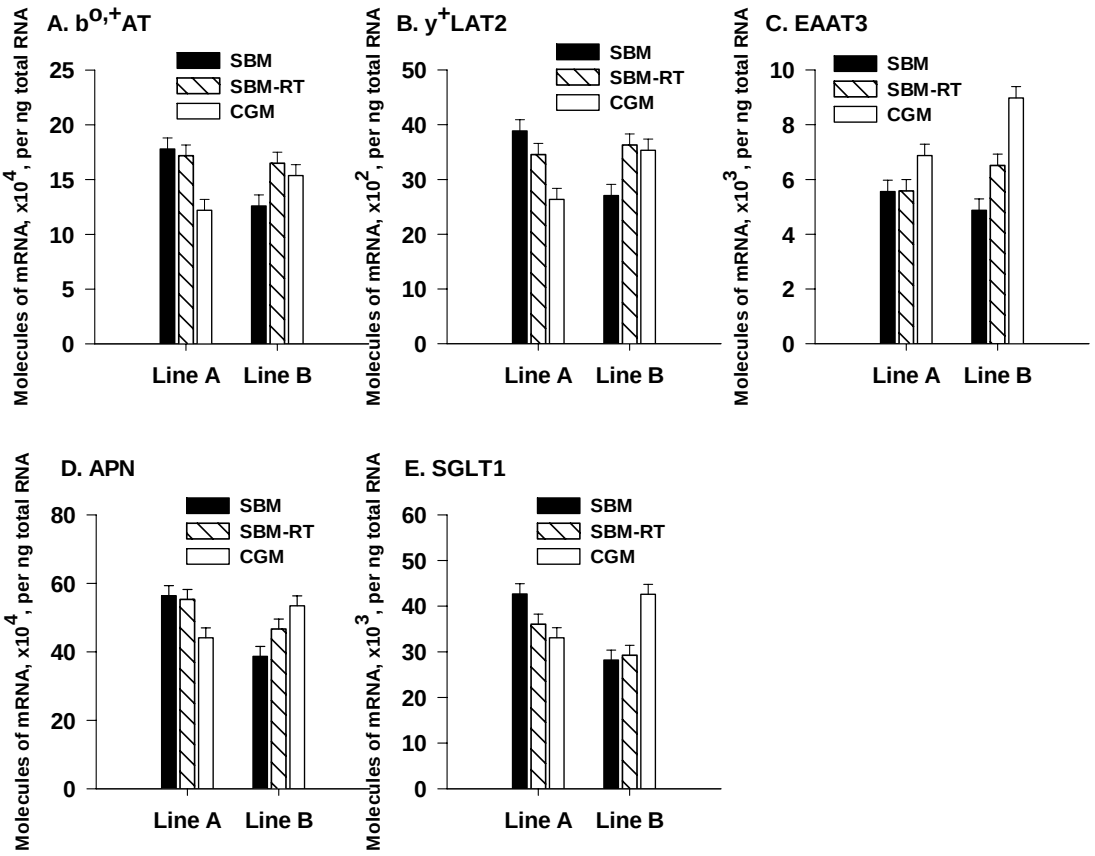

FIGURE 4.3 Effect of genetic line and diet on transporter and enzyme mRNA abundance in two genetically selected lines of broilers fed diets that differed in the source of protein from doh to d14. The number of A) $b^{o,+}AT$, B) $y^{+}LAT2$, C) EAAT3, D) APN, and E) SGLT1 mRNA molecules per nanogram of total RNA. Data represent the means of male chickens (n = 5) from Line A or Line B ± SEM in SBM (ad-libitum intake of soybean meal), SBM-RT (restricted intake of soybean meal), and CGM (ad-libitum intake of corn gluten meal) birds at d3, d7, and d14. There was an interaction of genetic line x diet for all five genes ($P < 0.05$).

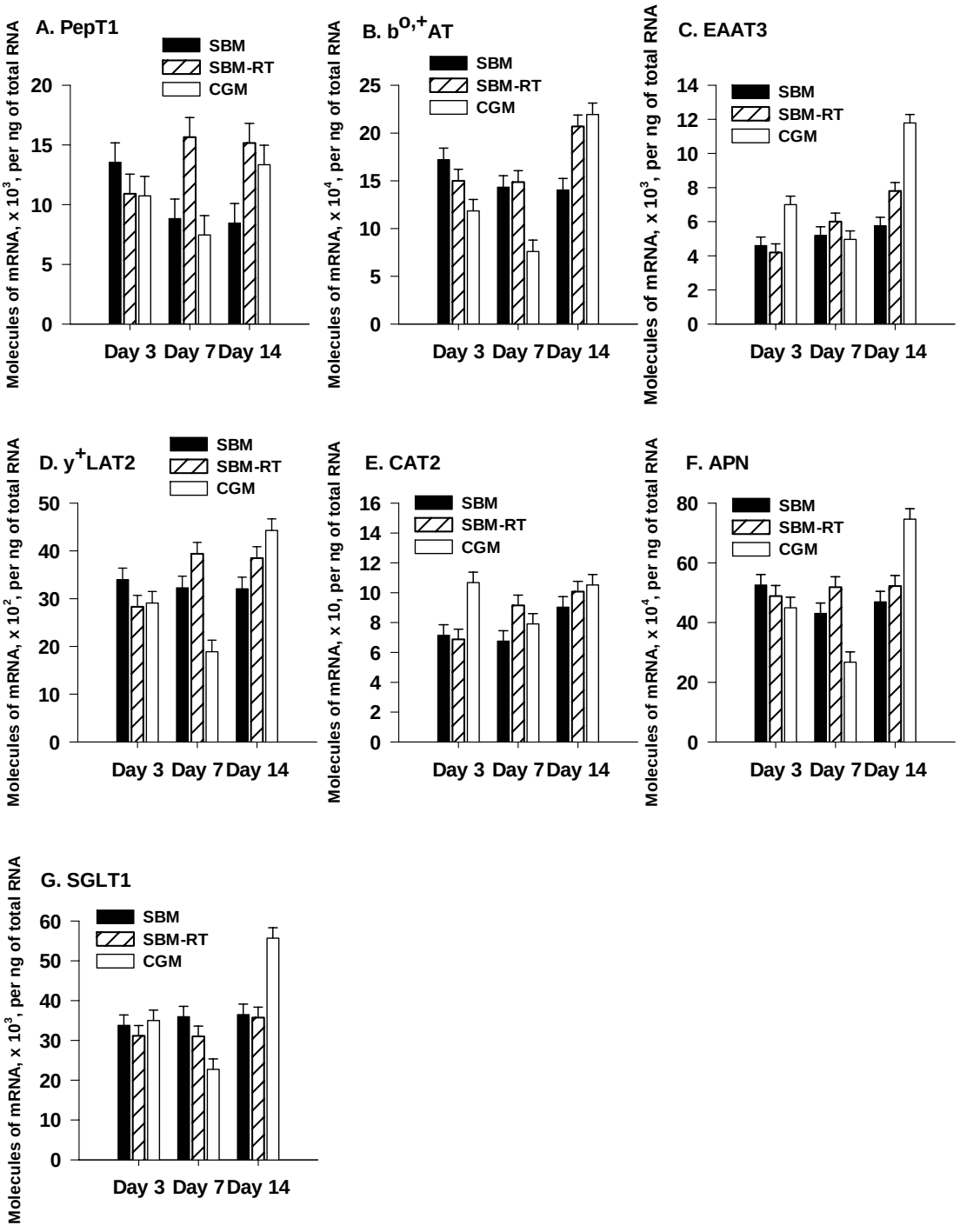

FIGURE 4.4 Effect of age and diet on transporter and enzyme mRNA abundance in two genetically selected lines of broilers fed diets that differ in the source of protein from doh to d14. The number of A) PepT1, B) $b^{o,+}AT$, C) EAAT3, D) $y^{+}LAT2$, E) CAT2, F) APN, and G) SGLT1 mRNA molecules per nanogram of total RNA. Data represent the means of male birds (n = 10;

five from both genetic lines) ± SEM in SBM (ad-libitum intake of soybean meal), SBM-RT (restricted intake of soybean meal), and CGM (ad-libitum intake of corn gluten meal) birds at d3, d7, and d14. There was an interaction of age x diet for all seven genes ($P < 0.01$).

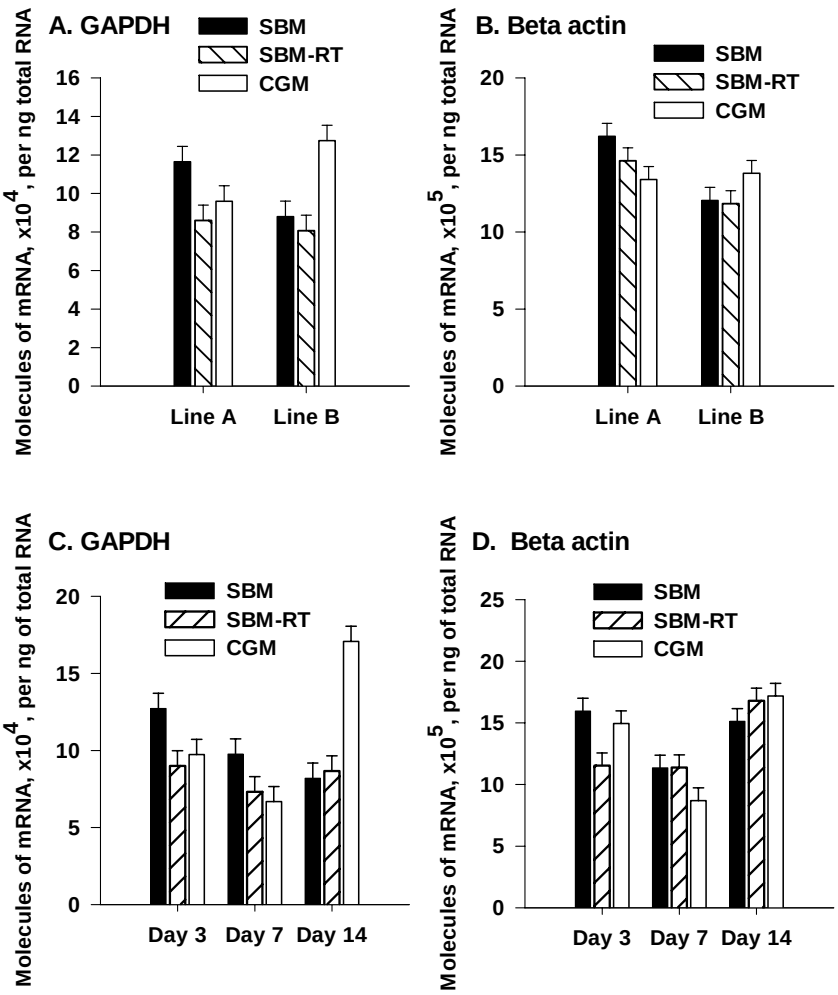

FIGURE 4.5 Effect of genetic line, age and diet on housekeeping gene mRNA abundance in two genetically selected lines of broilers fed diets that differ in the source of protein from doh to d14. The number of GAPDH (A), and beta actin (B), mRNA molecules per nanogram of total RNA in Line A and B chicks are shown in the two upper graphs. Data represent the means of male birds (n = 5) from Line A or Line B ± SEM in SBM (ad-libitum intake of soybean meal), SBM-RT (restricted intake of soybean meal), and CGM (ad-libitum intake of corn gluten meal). There was an interaction of line x diet for both genes ($P < 0.03$). Quantities of GAPDH (C) and beta actin (D) mRNA in birds at d3, d7, and d14 are shown in the two lower graphs. Quantities

represent the means of male chicks (n = 10; five from both genetic lines) ± SEM in SBM, SBM-RT, and CGM birds at d3, d7, and d14. There was an interaction of age x diet for both genes ($P < 0.01$).

Chapter V

Dietary Protein Composition Influences Abundance of Peptide and AA Transporter mRNA in the Small Intestine of Two Lines of Broiler Chicks

To be submitted to Journal of Nutrition. Appendices are not in the submitted manuscript.

Abstract

The objective of this study was to determine the effect of dietary protein composition on mRNA abundance of a peptide transporter (PepT1), AA transporters ($b^{o,+}$AT, EAAT3, y^+LAT2, LAT1, and CAT1), and a digestive enzyme (APN). From d 8 to d 15 posthatch, birds from 2 lines (A and B) were fed equal amounts of one of three diets (24% CP). Dietary protein sources included whey protein concentrate (whey), a partial whey hydrolysate (hydro), or a mixture of free amino acids (AA) identical to the composition of whey. Quantities of mRNA were assayed by real-time PCR in the small intestine of males at days 8, 9, 11, 13, and 15. Expression of all genes except LAT1 was greater ($P < 0.05$) in Line B compared with A. Abundance of PepT1, EAAT3, y^+LAT2, CAT1, $b^{o,+}$AT, and APN mRNA varied little across diets in Line A but for CAT1 mRNA was greatest ($P = 0.005$) in Line A birds that consumed the AA diet. Expression of these genes was greatest ($P < 0.006$) in Line B birds consuming the hydro diet. A greater ($P < 0.05$) age response of $b^{o,+}$AT, EAAT3, CAT1, and APN mRNA was observed in birds consuming the hydro or AA diets relative to whey diet. There was a greater villus-height to crypt-depth ratio ($P < 0.05$) in Line B birds on the hydro diet as compared with Line A. Line B birds were heavier ($P = 0.03$) than Line A, but BW did not differ among

treatments. In conclusion, consumption of a peptide-based protein source resulted in a general upregulation of gene expression in Line B birds.

KEY WORDS: • *Amino acid transporter* • *Broiler* • *Hydrolysate* • *Pept1* • *Whey*

Introduction

The first 2 wk posthatch represent a period of rapid intestinal growth and development in the commercial broiler chick. During this time, activities of digestive enzymes and nutrient transporters rise and dramatic morphological changes occur, including increases in the number and proliferation rate of enterocytes, widening and lengthening of the villi, and deepening of crypts (1, 2, 3, 4, 5). Digestive enzymes are thought to be the rate limiting step for utilization of nitrogen from dietary protein during the early posthatch period. Nitrogen digestibility increases during the first 2 wk posthatch, but from immediately posthatch onwards there is a high capacity for AA absorption and digestion of easily digested proteins (6, 7). The presence of luminal substrate is thought to provide the major stimulus for development of the intestinal mucosal layer, and as such, the type of feed and molecular form of nutrients in the intestine may dramatically influence growth and development.

Amino acids may be absorbed in the gut in their free form or as small peptides. The intestinal peptide transporter, PepT1, has been well characterized in multiple species and to date is the only peptide transporter known to be expressed in enterocytes (8, 9, 10). The PepT1 is proton dependent and has broad substrate specificity, potentially able to transport all di- and tripeptides derived from dietary protein. Amino acids in their free form are transported into enterocytes by AA transporters that have various ion

dependencies and show a narrower substrate specificity and higher affinity than PepT1 (11, 12, 13, 14).

Peptide and AA transporters are regulated by a variety of factors, including intestinal development and dietary protein. Expression of PepT1 and AA transporters are detected before hatch and change with age in chickens (15, 16). Increasing levels of dietary protein and feed restriction were associated with greater expression of PepT1 mRNA in chickens (15). Consumption of a higher quality protein source, soybean meal, as compared to consumption of equal quantities of a lower quality protein source, corn gluten meal, resulted in differences in expression of PepT1 and four AA transporters in broilers during the first 2 wk posthatch (17). Gilbert et al. (17) also observed that feed restriction for 2 wk resulted in an upregulation of transporter mRNA. While studies evaluated the effects of varying the quantity and source of protein and/or AA concentrations in the diet there has been no attempt to examine the effect of completely replacing dietary protein with either peptides or free AA on gene expression of peptide and AA transporters.

We observed in two independent studies that PepT1 mRNA abundance was influenced by genetic line. We found that Line B expressed greater quantities of PepT1 mRNA compared with Line A chicks under a variety of dietary conditions (16, 17), suggesting that they have a greater capacity to absorb AA as peptides. In this study, we fed chicks from both genetic lines one of three diets that differed only in composition of the protein source. In one diet, protein was supplied by whey protein concentrate (Lacprodan 80, Arla Foods). In the second diet, whey protein hydrolysate (Lacprodan 3065, Arla Foods) was used, in which most of the AA were present as oligopeptides

(~95%). In the third diet, a mixture of crystalline AA was used that matched the composition of the whey protein, and hence, should be immediately available for absorption by the free AA transporters. Whey was chosen for this study because it is a very high quality protein source and both the protein and hydrolysate were commercially available and similar in AA composition. The objective of this experiment was to determine in both lines of broilers the influence of dietary protein composition on gene expression of peptide and AA transporters, and a digestive enzyme, aminopeptidase N (APN), which plays a role in maintaining the balance between peptides and AA (18).

Materials and Methods

Feeding trial and tissue collection. All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech. Lines A and B are 2 commercial broiler lines that have been selected under different nutritional conditions. Detailed information on these lines has been published (16, 17). Briefly, both lines originate from the same genetic stock; however, for at least 10 generations Line A was selected on a corn-based diet while line B was selected on a wheat-based diet and higher AA concentrations than for Line A. Over the years this has led to different growth responses in the two lines.

We obtained sexed Line A and Line B day of hatch chicks from Aviagen. Chicks were transported from the hatchery in Alabama to the poultry farm at Virginia Tech in Blacksburg under thermo-neutral conditions. Chicks were placed in heated floor pens with pine shavings and were given free access to drinking water and ad libitum access to a commercial corn-soy based diet that has been described previously (16). Birds were fed

a standard diet for the first week posthatch to allow ample time for the transition to floor pens and feed consumption. At d 8, 17 male and 12 female birds from both lines were killed by cervical dislocation and sampled for intestinal tissue as previously described (15) and the remaining chicks were randomly assigned to one of three experimental diets (Table 5.1). Diets were formulated to be similar with the exception of dietary protein composition. The whey hydrolysate was chosen because its AA composition is similar to the whey protein and most of its AA (~ 95 %) are in the form of oligopeptides (Table 5.2). Diets were supplemented with L-arginine at the recommended ideal ratio to lysine (105%; 19), because preliminary trials using diets not supplemented with L-arginine resulted in poor performance (Appendix C, Figures C.1 and C.2). Diets were formulated to meet or exceed recommended vitamin and mineral concentrations for chemically-defined diets fed to chicks (20).

Pen weights were similar before consumption of experimental diets. Feed intake of all diets was restricted to the amount consumed the least during the previous 8 h (Line B female, AA). Body weights were recorded daily and feed consumption was measured at three intervals (0700, 1500, and 2300) daily. At days 9, 11, 13 and 15, 17 males and 12 females from each line and each diet were killed and sampled for intestinal tissue. For each sampling day intestine was divided into duodenum, jejunum, and ileum. Intestinal segments were squeezed and rinsed in ice-cold PBS (NaH_2PO_4, 1.47 m*M*; Na_2HPO_4, 8.09 m*M*; NaCl, 145 m*M*). Rinsed segments were weighed, minced with a razor blade and mixed. Aliquots were frozen at -80°C. Five males were selected from each group for RNA isolation. Sex of birds was confirmed by PCR as described previously (16). Remaining birds were used for other analyses.

Brushborder membrane isolation. Brushborder membrane isolation was performed on 1 g aliquots of tissue collected as previously described, based on modification of the protocols described by Xiao (21), Kessler et al. (22) and Coleto et al. (23). Frozen tissue (1 g) was added to 10 mL ice-cold homogenization buffer (100 m*M* mannitol, 2 m*M* HEPES/Tris, pH 7.4, 0.1 m*M* PMSF) and homogenized with a Janke and Kunkel Ultra-Turrax T25 homogenizer at full speed for four 30 sec periods, and ice-cold homogenization buffer was added to achieve a 5 % homogenate. Two mL of homogenate were removed and stored at -80°C for subsequent protein and marker enzyme assays. The remaining homogenate was centrifuged at 500 × *g* for 12 min and pellets were discarded. A 1 *M* $MgCl_2$ solution was added to the remaining supernatant to a final concentration of 10 m*M*, followed by occasional gentle mixing for 20 min. The suspension was centrifuged at 3,000 × *g* for 15 min and the supernatant was removed and centrifuged again at 3,000 × *g* for 15 min. The supernatant was transferred to a tube and centrifuged at 30,000 × *g* for 30 min. The resultant pellet was resuspended in 20 mL buffer (100 m*M* mannitol, 2 m*M* HEPES/Tris, pH 7.4, 1 m*M* $MgSO_4$, and 0.1 m*M* PMSF). The suspension was homogenized for 1 min and centrifuged at 30,000 × *g* for 30 min. The final pellet containing purified brushborder membrane vesicles was resuspended in buffer (300 m*M* mannitol, 20 m*M* HEPES/Tris, pH 7.4, and 0.1 m*M* $MgSO_4$). The suspension was homogenized by passing it through a 25-gauge needle 15 times. The resulting suspension was aliquoted and stored at -80 ^{o}C. Protein concentrations were determined using the protein 2-D Quant kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol with bovine serum albumin serving as a standard.

Sucrase Activity Assays. Sucrase activity was used as a marker for enrichment of brushborder membrane (24). The brushborder membrane enrichment factor was calculated as a ratio of the sucrase activity per mg protein in the brushborder membrane fraction divided by the sucrase activity per mg protein in the whole intestinal homogenates. Sucrase activity was assayed as previously described Dahlquist (25).

Western blot analysis. All d 8 samples and only jejunum from d 9 to d 15 were used for western blot analysis. Twenty micrograms of brushborder membrane protein (10 µL at 2 µg/µL) were added to an equal volume of protein sample buffer (1 % SDS, 50 m*M* Tris-Cl, pH 7.0, 20 % glycerol, 0.2 *M* DTT, 0.01 mg/mL bromophenol blue), vortexed, and incubated for 15 min at room temperature. Samples were loaded on to a 7.5 % polyacrylamide gel containing 0.1 % SDS. A pre-stained molecular weight standard was loaded in the first well of each gel (Pierce® 3-color protein molecular weight marker mix). Duplicate samples were run on separate gels and samples from each group were randomly assigned to a gel. Gels were run at 200 V for 2 hr and then equilibrated for 15 min in cold towbin transfer buffer (0.025 *M* Tris, 0.192 *M* glycine, 0.1 % SDS, 20 % methanol). Immun-blot PVDF membranes (Bio-Rad, Hercules, CA) were pre-wetted in methanol and placed in transfer buffer for 10 min prior to transfer. Transfer sandwiches were set up for a semi-dry transfer using the Owl semi-dry transfer apparatus (Thermo Fisher Scientific; Rochester, NY). Transfers were performed at 2 mA per square cm of membrane for 1 hr at constant current. Following transfer, membranes were cut at specific locations on the molecular weight marker and allowed to air-dry on a piece of

filter paper for 3 hr. Membranes were stored in sealed bags at 4°C. Membranes were pre-wet in methanol and blocked simultaneously in 5 % non-fat dried milk (NFDM; Kroger brand) in TBST (30 m*M* Tris, 200 m*M* NaCl, 0.1 % Tween-20) for 4 hr. After blocking solution was poured off, primary antibodies diluted in 1 % NFDM in TBST were added. A multiplex western was performed with anti-PepT1 and an internal standard, anti-beta actin, were added together to the membranes. The PepT1 antibody was raised in a rabbit using a peptide (CQIKQDPDLHGKESEA, > 95 % purity) directed against the C-terminus of the chicken PepT1 protein (9) by EZ-BioLabs (Westfield, IN). The PepT1 antibody was diluted 1:500. The beta actin antibody was a mouse anti-human beta actin antiserum (Abcam, catalog # ab8226) and was diluted 1:2,000. Membranes were incubated in the primary antibody mixture overnight at 4°C on a rocker. Antibody solutions were poured off and membranes were washed 2 x 5 min and 3 x 10 min in TBST at room temperature on a rocker. The secondary antibody solution consisted of goat anti-mouse IgG1 (Bethyl laboratories, catalog # A90-105P) and goat anti-rabbit IgG-Fc (Bethyl laboratories, catalog # A120-111P), both diluted at 1:30,000 in 1% NFDM in TBST. Membranes were incubated in the secondary antibody mixture for 1 hr at room temperature on a rocker. After secondary antibody was poured off, membranes were washed 2 x 5 min and 3 x 10 min in TBST at room temperature on a rocker. Following the last wash, membranes (two at a time) were incubated in detection substrate (GE Healthcare, ECL Plus) for 5 min (4 mL per membrane). Chemiluminescence was imaged on two membranes simultaneously using a Chemi-Doc imager (Bio-Rad) with an exposure time of 20 sec. Bands were quantified by densitometric analysis using Quantity One software (Bio-Rad).

Prior to conducting the multiplex westerns, numerous tests were performed to ensure 1) that bands were specific to the antiserum and not expressed when mixed with only pre-immune serum (Appendix C, Figure C.3), 2) that bands were specific to expected locations of expression by evaluating expression in a negative control, liver, in comparison with intestinal protein (Appendix C, Figure C.4) and that 3) bands were of expected size. Total protein isolated from intestinal samples was also tested in comparison with brushborder membrane protein, and was found to be inappropriate, due to low detectability of PepT1 in samples not enriched for the brushborder membrane.

Histological analysis. Five male birds from each group were used for histology at d 15. Approximately 1 cm sections were dissected out of the tract at the mid-region of each segment and were rinsed as described above and fixed in neutral buffered 10 % formalin. Vials were incubated at 4°C overnight on a rocker. The following day, formalin was poured off and sections were subjected to three 30 min washes in PBS. After the final wash, sections were set in 70 % ethanol and sent to HISTO Scientific Research Laboratories (Mount Jackson, VA) for embedding, 5 μm sectioning, and hematoxylin and eosin staining. For each sample, images were collected using a Nikon Eclipse 50 i microscope and Infinity 1 camera. Morphometric measurements were performed using SigmaScan Pro 5.0 software (SPSS Scientific). Images were calibrated using 2 pt. rescaling based on the number of pixels equal to 1 mm, obtained using a micrometer. For each section, 12 well-oriented villi and 12 crypts were chosen. Villus height was defined as the distance from the tip of the villus to the crypt-villus junction. Villus width was defined as the distance from one side of the villus to the other midway up the length.

Crypt depth was defined as the distance from the crypt-villus junction to the base of the crypt. A representative example of all three measurements is shown (Appendix C; Figure C.5). The villus height to crypt depth ratio (VCR) was calculated based on distance measurements.

Real time PCR analysis. Total RNA was isolated from frozen tissue aliquots using the RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. Total RNA was quantified at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and integrity was verified by gel electrophoresis. Total RNA was stored at -80°C. An RNA standard curve was made for each gene of interest based on modification of a protocol described by Fronhoffs et al (21). Primers used for cloning were described previously (16, 17). Total RNA from jejunum and gene-specific primers were used to perform RT-PCR followed by subcloning into the pGEM-T Easy vector (Promega). The cloning procedure was performed as already described (16). Purified plasmid cDNA samples were sequenced at the Virginia Bioinformatics Institute at Virginia Tech. Gene-specific standard curves were generated as previously described (16). Briefly, plasmids containing amplified chicken cDNA were linearized opposite a T7 or SP6 promoter depending on the orientation of the insert sequence. In vitro transcription reactions were performed on linearized plasmids using the MEGAscript T7 or SP6 in vitro transcription kit (Ambion) and cRNA was precipitated with lithium chloride and quantified using the ribogreen assay (Molecular Probes) and a FLUOstar OPTIMA microplate reader (BMG LABTECH). A dilution series of 10^{11} to 10^{4} molecules/μL was performed in the presence of yeast tRNA at 10 mg/L.

For real time PCR, nested primers were designed (see ref. 16, 17 for complete list) within cloned chicken cDNA sequences using Primer Express software (Applied Biosystems). Total RNA samples and RNA standard curves were reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). Each RT reaction contained 2,000 ng of RNA at a concentration of 100 ng/μL or an equal volume of the dilution series of cRNA. The cDNA was diluted 1:30 before addition to the PCR that contained primers (5 μ*M*) and SYBR green master mix (Applied Biosystems). PCR was performed under the following conditions: 50°C for 10 min and 40 cycles of 95°C for 1 min and 60°C for 1 min using an Applied Biosystems Real-Time PCR 7300 system. A dissociation step consisting of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s was performed at the end of each PCR to verify amplification of a single product.

Statistical Analysis. Data were analyzed using the PROC MIXED procedure of SAS (SAS Institute). The model for BW and intestinal segment weight data included the main effects of age, genetic line, diet, and all 2-way interactions. Morphometric measurement data were subjected to a log base 10 transformation prior to statistical analysis to obtain normally distributed data. Morphometry data are presented in the results as non-transformed data. The model included the main effects of genetic line and diet, and the 2-way interaction. The model for gene expression data included the main effects of age, genetic line, diet, intestinal segment, and all 2-way interactions. Significant segmental and dietary differences were further evaluated by Tukey's test for multiple comparisons. The d8 timepoint was not included in this model because consumption of the experimental diets was not initiated until after intestinal sampling on d8. Quantities of

PepT1 protein were expressed as a ratio to beta actin. Raw beta actin data were also analyzed separately to confirm invariance of expression levels. Relative protein expression data were analyzed using the PROC MIXED function of SAS and the model included the main effects of age, line, diet, and two-way interactions. Differences were considered significant at $P < 0.05$.

RESULTS

Growth performance and feed intake. Body weights did not differ across dietary treatments ($P = 0.4$), although a main effect of genetic line on BW was observed where Line B birds were heavier than Line A (Table 5.3; $P = 0.02$). The line difference was accentuated with age (Figure 5.5A; $P = 0.02$). Pen feed intakes were controlled to be equal to the dietary group with the lowest consumption (Line B female AA) and increased with age to intakes of approximately 36 g/bird for d14. Weights of intestinal segments were affected by both genetic line and diet (Table 5.3). Duodenum, both as an individual weight and as a percentage of BW, was greater ($P < 0.01$) in Line B birds compared with A and line-specific expression changed with time (Figure 5.1B). Ileum weight was greater ($P = 0.02$) in Line B, although when expressed as a percentage of BW there was no difference. Jejunum and ileum, both as individual weights and as percentages of body weight, were affected by diet. Jejunum was largest ($P < 0.03$) in birds that consumed the AA diet, intermediate in those that consumed the hydro diet, and smallest in birds that consumed the whey diet. Ileum was largest ($P < 0.03$) in the birds that consumed the AA or hydro diet, in comparison with birds that consumed the whey

diet. Relative jejunal weight changed differently with age depending on the dietary group (Figure 5.1C).

Influence of dietary protein composition on intestinal morphology.

Morphometric measurements were performed on cross-sections of the duodenum, jejunum, and ileum of birds at d 15 of the trial. There were no main effects of genetic line on villus height, villus width, crypt depth, or VCR (Table 5.4). Dietary protein composition influenced villus width and VCR. Villus width and VCR was greater ($P < 0.02$) in birds consuming the hydro diet as compared with birds that consumed the AA diet. Villus height, crypt depth and VCR were greatest ($P < 0.03$) in the duodenum, lowest in the ileum, and intermediate in the jejunum. Villus width was greater ($P < 0.03$) in the duodenum as compared with the ileum. There was an interaction of genetic line x diet for villus height, crypt depth and VCR (Figure 5.2; $P < 0.05$). In birds consuming the whey diet there was little difference between genetic lines for these parameters. In birds consuming the AA diet, villus height was greater in Line A birds compared with B, while for crypt depth there was little difference between genetic lines, resulting in a slightly higher VCR for Line A birds consuming AA compared with B. In birds that were given the hydro diet, there was little difference in villus height between the two genetic lines; however, Line A birds had deeper crypts, resulting in a greater VCR in Line B birds consuming the hydro diet.

Influence of genetic line and dietary protein composition on expression of transporters and APN. Real-time PCR and the absolute quantification method were

used to determine mRNA abundance of PepT1, AA transporters, APN and a housekeeping gene, beta actin. Results are summarized in Table 5.5 with separate rows for main effects of genetic line, diet, intestinal segment, age, and all two-way interactions. Only the interactions are expressed graphically.

For all genes except LAT1, mRNA quantities were greater ($P < 0.05$) in Line B birds as compared with Line A (Table 5.5). Age influenced the response in genetic lines. Abundance of PepT1 mRNA (Figure 5.3A) varied little with age in Line A birds, while in Line B birds expression increased with age to d13 followed by a slight decline to d15 ($P = 0.005$). For EAAT3 (Figure 5.3B), y^+LAT2 (Figure 5.3C), CAT1 (Figure 5.3D), LAT1 (Figure 5.3E), and beta actin (Figure 5.3F) mRNA quantities increased with age from d9 to d15 in Line A birds ($P < 0.05$). In Line B birds, expression peaked at d13 for EAAT3 and at d11 for LAT1, y^+LAT2, and beta actin. For CAT1, expression was greatest at d9 and d11 ($P < 0.05$).

For all genes except y^+LAT2 there was a main effect of dietary protein composition on mRNA abundance (Table 5.5). Abundance of $b^{o,+}$AT, EAAT3, APN, and beta actin mRNA in birds consuming the hydro diet was greater than in birds that consumed the whey ($P < 0.01$) or AA diet ($P < 0.02$). Expression of PepT1 in birds that were fed the hydro diet was greater ($P = 0.008$) as compared to birds that consumed AA diet, while for CAT1 and LAT1 expression in the birds fed the hydro diet was greater ($P < 0.02$) than in the whey diet-fed group.

Dietary protein composition also influenced gene expression between the two genetic lines. Expression of PepT1 (Figure 5.4A), $b^{o,+}$AT (Figure 5.4B), EAAT3 (Figure 5.4C), y^+LAT2 (Figure 5.4D), CAT1 (Figure 5.4E), APN (Figure 5.4F), and beta actin

(Figure 5.4G) varied little among dietary treatments in Line A birds ($P < 0.006$). In Line B birds, PepT1, $b^{o,+}AT$, EAAT3, CAT1, $y^{+}LAT2$, APN, and beta actin showed a similar expression pattern where mRNA was greatest in the birds consuming the hydro diet compared with the other two diets ($P < 0.006$).

Influence of intestinal segment and diet on expression of AA transporters. For all genes except for $y^{+}LAT2$ there was a main effect of intestinal segment on gene expression. Abundance of PepT1 mRNA was greater ($P < 0.03$) in the duodenum and jejunum as compared with ileum, whereas for $b^{o,+}AT$, EAAT3, CAT1, and APN, mRNA quantities were greatest ($P < 0.03$) in the ileum of the small intestine. Quantities of LAT1 and beta actin mRNA were greater ($P < 0.03$) in the jejunum and ileum as compared with the duodenum. The two brushborder membrane AA transporters, $b^{o,+}AT$ (Figure 5.5A) and EAAT3 (Figure 5.5B), showed a similar effect of diet on their spatial pattern of gene expression in the small intestine ($P < 0.03$). Expression of both genes increased from proximal to distal small intestine and this proximal to distal gradient was accentuated in birds consuming the hydro diet.

Influence of age and dietary protein composition on expression of transporters and APN. All genes evaluated except for LAT1 showed a main effect of age on mRNA abundance ($P < 0.006$). In general, expression was greatest at d9 or d11 and plateaued. There was a different age-specific pattern of gene expression of $b^{o,+}AT$ (Figure 5.6A), EAAT3 (Figure 5.6B), CAT1 (Figure 5.6C), APN (Figure 5.6D), and beta actin (Figure 5.6E) in birds consuming the different diets. Quantities of $b^{o,+}AT$, EAAT3,

CAT1, APN, and beta actin mRNA in birds consuming the whey diet were greatest at d11, decreased to d13 and plateaued ($P < 0.05$). Expression of EAAT3 peaked at d13 in birds consuming the hydro diet ($P = 0.01$), while expression of $b^{o,+}AT$ and APN steadily increased with age in birds consuming the hydro diet ($P < 0.05$). Abundance of CAT1 mRNA decreased from d9 to d13 and maintained constant levels to d15 in birds consuming the hydro diet ($P = 0.003$). Beta actin mRNA quantities were greatest at d11 and d15 in birds consuming the hydro diet. Expression of $b^{o,+}AT$, EAAT3, APN, and beta actin in birds consuming the AA diet peaked at d13 ($P < 0.05$) while CAT1 mRNA was greatest at d15 in birds consuming the AA diet ($P = 0.003$).

Western blot analysis. To compare mRNA abundance of PepT1 with expression of the PepT1 protein, western blots were performed on brushborder membrane vesicles with an anti-chicken PepT1 antibody. The relative enrichment of the brushborder membrane was 25 ± 10.3. Two prominent bands were detected with the anti-chicken PepT1 antibody using chicken brushborder membrane protein (Figures 5.7 and 5.8). The lower band was the expected size of chicken PepT1 at approximately 80 kDa, and the upper band was approximately 90-95 kDa. The molecular weight of the upper band is suggestive of post-translational modifications, such as glycosylation, shown to exist for mammalian PepT1 (8). A number of N-linked glycosylation sites are predicted for the chicken PepT1 protein (9), making this a reasonable assumption.

No significant differences in expression of PepT1 protein were detected from d 9 to d 15 for either the upper (90-95 kDa) or lower (80 kDa) PepT1 band (Figure 5.7). This is in contrast to the genetic line effect observed at the mRNA level for PepT1. When

jejunum was analyzed alone at the mRNA level there was no other significant effect. In other words, the only difference between the mRNA and protein data was the genetic line effect observed at the mRNA level.

On d 8 of the trial, the upper (90-95 kDa) PepT1 band was expressed greatest in the duodenum ($P < 0.05$), in comparison to the other segments, similar to the results observed at the mRNA level (Figure 5.8). Expression of the upper PepT1 band was greater in Line B chicks ($P < 0.05$) compared with A, also corroborating mRNA abundance. There were no significant differences detected in the lower (80 kDa) PepT1 band.

Discussion

Our objective was to determine the effect of dietary protein composition on peptide and AA transporter gene expression in two genetically-selected lines of broilers that differ in intestinal expression of the peptide transporter, PepT1. We observed in prior studies that expression of PepT1 is influenced by both diet and genetic line (15, 16, 17). Others have demonstrated that peptide transport is a faster and more energetically efficient route of AA absorption compared with free AA uptake (27, 28, 29). Collectively, results from these studies suggest that peptide uptake may be very important from a nutritional standpoint, although feeding studies involving inclusion of peptides as a source of AA in animal diets are limited.

Although nutrient digestibility values were not reported in this study, it is likely that AA digestibility was different across diets. In chicks fed corn-soybean meal or corn-canola meal diets, digestibility of essential AA increased with age from hatch to 21 d (7).

In chicks fed a dextrose-casein or starch-crystalline AA diet, AA digestibility was not affected by age and values for essential AA were greater in these diets compared with corn-soybean meal or corn-canola meal at all time-points. The authors indicated that during the first 2 wk posthatch the digestive and absorptive capacities of the chick are still developing and that digestibility values for casein and the AA diet were high because of a high capacity for AA absorption and ability to digest easily-digested proteins. Batal and Parsons (30) also observed that chicks fed a crystalline AA-based diet for the first week or 3 wk posthatch consumed less compared with chicks offered a standard corn-soybean meal diet. Furthermore, Batal and Parsons (30) observed in the gut of chicks fed the crystalline AA for the first 7 or 21 d reduced villus height and crypt depth, particularly in the distal region. The lower intake and high AA digestibility of the AA diet could be due to less luminal stimulation in the gut, with little substrate entering the lower small intestine and colon. In our study, feed intake was equalized across treatments by restricting intake to the group that consumed the least, which was females consuming the crystalline AA diet. Thus, intake was not a factor in this study. We did not observe a significant dietary main effect on villus height or crypt depth, but did observe an effect on villus width.

To our knowledge, results from feeding hydrolysates or peptide-based diets to poultry have not been reported. Because of the relevance to clinical nutrition there have been multiple reports of feeding hydrolysates and AA-based diets to rats. When casein or whey-based diets were compared with their respective hydrolysates, there was no difference in nitrogen digestibility, but nitrogen retention was greater in rats fed the hydrolysate diets (31). The difference in absorption rate of AA from a peptide-based diet

versus the intact protein may influence subsequent utilization of AA for protein synthesis. When a peptide-based diet was compared with a free AA-based diet, there was a higher energy conversion efficiency, protein efficiency ratio, nitrogen protein utilization and biological value reported for rats fed the peptide-based diet, while there was a higher apparent digestibility reported for rats fed the free AA-based diet (32). There was greater plasma total and essential AA in rats consuming the peptide diet, while there was greater plasma urea in rats fed the AA diet, suggesting that rats consuming the AA diet were using the AA for energy rather than protein deposition. In their discussion of the findings, Boza et al. (2000) pointed out several key factors that can affect utilization of dietary AA including insolubility of free AA, absorption or interference of the molecular form of AA with nutrient transporters, availability of AA in the free or peptide-bound form to gut microflora, effect of the form of AA on osmolarity, and effect of rate of AA absorption on liver AA oxidation.

Based on these findings, we expected to find effects of diet on gene expression of AA and peptide transporters in this study. Although we observed multiple effects of diet on peptide and AA transporter mRNA in this study, no differences in BW were observed. This may in part be due to the fact that diets were fed for only 1 wk (d8 to d15) and this may not have been enough time to see differences in gene expression translate into differences in growth performance. A commercial diet was fed for the first week posthatch to provide an adequate stimulus for gastrointestinal development, as others have demonstrated that crystalline amino-acid based diets fed early in life to broilers may hinder growth and development of the gut (30). In this study intakes were controlled to be equal across diets and increased with age, resulting in corresponding increases in BW. In

this study we observed a similar upregulation in expression of all genes evaluated in Line B birds that consumed the hydro diet, including expression of a housekeeping gene beta actin. In Line B birds, consumption of a peptide-based diet may have led to an overall enhancement in cellular metabolism. A greater absorption of peptides could had led to a substrate-induced up-regulation of PepT1, providing even more AA to the cell. Those AA could in turn serve a variety of functions in the cell which could result in an increase in cell metabolism and enhanced expression of genes in the enterocyte.

Effect of dietary protein composition on intestinal histology. The first two weeks posthatch represent a time of dramatic changes in gastrointestinal development with the most dramatic changes occurring during the first 24 h (5, 33). Changes include cell proliferation, enterocyte hypertrophy and changes in morphology, crypt invagination, and increased villus surface area, with the rate of these changes being segment-specific. All of these changes would be expected to influence digestive and absorptive functions in the gut, and we would anticipate that changes in luminal stimulus would affect intestinal morphology as well. Diet has a profound impact on gastrointestinal development and delayed access to feed stunts mucosal development in broiler chicks (3). We did not observe an effect of genetic line on villus height, villus width, crypt depth, or VCR on d 15 of this study. We did, however, observe several main effects of diet and several line x diet interactions. Villi were wider in the birds on the hydro and whey diets, as compared with birds fed the AA diet. In birds fed the AA diet, a mixture of crystalline AA was used to provide AA to the diet, with a much different availability in the intestine. A change in villus width may be a reflection of differences in the gut microflora. Immediate

absorption of AA by the intestine provides less substrate for the bacteria. Lamina propria is the largest determinant of villus width, and is populated with fibroblasts, endothelial cells, lymphocytes, macrophages and IgA-secreting plasma cells. In germ-free animals, there is delayed development of the lamina propria characterized by less germinal centers and IgA-secreting cells (34). Thus villus width can provide some insight into the intestinal immune status of the animal.

There was no difference in villus height between Line A and B birds consuming the hydro diet, but crypt depths were shorter in Line B birds consuming the hydro diet. This resulted in a larger villus height to crypt depth ratio in Line B birds that were fed the hydro diet. This may indicate that more mature enterocytes inhabited the villi of these birds, with less cell recruitment and hence less energy expenditure needed to maintain the absorptive function. More mature enterocytes surrounding the villi implies that there is a greater absorptive surface. This combined with less energy needed to maintain cells on the villi, potentially allows for more nutrients to be allocated to different regions of the body to support growth.

Effect of genetic line on peptide and AA transporter mRNA. Consistent with what we observed in previous studies (16, 17) PepT1 mRNA was greater in Line B birds. Expression was also greater in Line B for $b^{o,+}$AT, EAAT3, y^{+}LAT2, CAT1, APN and beta actin. The magnitude of difference was greatest for PepT1 where Line B birds had 56 % greater intestinal expression than Line A birds, while for the other genes the difference was between 9 and 39 %. The genetic line difference in PepT1 expression is the only consistent difference that we have observed in nutrient transporters between

lines A and B, and suggests that the process of genetic selection has altered expression of the peptide transporter.

Both genetic line and diet influenced the age-specific response in transporter expression. For the brushborder transporters PepT1 and EAAT3, the difference in expression between lines was most accentuated at d13, while for the basolateral AA transporters LAT1, y^+LAT2 and CAT1, the difference manifested itself earlier (d9 or d11; Figure 5.3). There were also diet-specific age differences observed in this study (Figure 5.4). For genes showing an age x diet interaction, expression in the whey diet was greatest at d11 and decreased afterwards. In birds consuming either the hydro or AA diet, expression of the same genes with the exception of CAT1 increased to d13 and remained at similar levels to d15. For CAT1, expression in birds consuming the hydro diet decreased with age and in birds consuming the AA diet it varied little with age. These data suggest that expression of PepT1 and the AA transporters examined in this study were more responsive to the diets containing greater quantities of absorbable AA, and both age and genetic line influenced this response.

Effect of intestinal segment and diet on AA transporter mRNA. For both of the brushborder membrane AA transporters, $b^{o,+}$AT and EAAT3, mRNA abundance was greatest in the ileum and interestingly, the difference between the ileum and other two segments was most accentuated in birds that consumed the hydro diet (Figure 5.5). This may relate to the presence of more available AA in the ileum of birds consuming the hydro diet, as the presence of more peptides entering the duodenum would also provide more substrate for brushborder peptidases such as APN, in turn yielding more substrate

for brushborder transporters. In the whey and AA diet, less and more free AA relative to the hydro diet, respectively, would in principle be available to the duodenum. In birds consuming the whey diet, this may lead to a slower release of free AA, reducing the need for up-regulation in the ileum, while in birds consuming the AA diet much of the AA absorption may be complete by the time the digesta reaches the ileum, reducing the need for up-regulation of AA transporters in the distal small intestine. This segment x diet interaction was not observed for the basolateral AA transporters, which are unlikely to respond in the same manner to luminal AA.

Effect of dietary protein composition on genetic line-specific expression of transporter mRNA. For all genes evaluated in this study with the exception of LAT1, mRNA abundance was greatest in Line B birds consuming the hydro diet (Figure 6). This was a very curious observation as we had predicted that only PepT1 would be significantly influenced by the hydro diet in Line B birds. In Line A birds there was little difference in expression of these genes among the three dietary groups, however, in Line B chicks expression was greatest in the hydro group. Because we knew that Line B chicks express greater quantities of PepT1 compared to Line A chicks, we hypothesized that expression of PepT1 in Line B birds would be more responsive to a peptide-based diet. Since Line B birds expressed more PepT1 at the start of the study, they may have had a greater capacity to absorb peptides from the hydro diet, which in turn have the ability to induce expression of PepT1. Elements were identified in the 5` upstream region of PepT1 responsive to peptides and free AA (35, 36). Addition of dipeptides, Gly-Sar or Gly-Gln to the medium of Caco-2 cells caused an increase in the expression of PepT1

mRNA and protein (37, 38). Uptake of dipeptides also caused stimulation of AA uptake by the $b^{o,+}$ system (39), suggesting that free AA uptake may also be regulated by PepT1 activity. This may have relevance to the present study where expression of AA transporters and APN were also greatest in Line B chicks consuming the hydro diet. If Line B birds were able to absorb more peptides from the hydro diet, this may have resulted in a general up-regulation of other genes in the enterocyte.

We also evaluated expression of a housekeeping gene, beta actin, in response to dietary protein composition. The age-specific response of beta actin was influenced by both genetic line and dietary protein composition, and we observed a similar interaction of line x diet as seen for PepT1 and the AA transporters. As alluded to earlier, the response of Line B birds to the hydro diet may have led to a generalized upregulation of genes not observed in Line A birds. That expression of a housekeeping gene changed in response to various factors including age, genetic line, and dietary protein, suggests that it may not be appropriate for use as an internal control in relative quantification studies.

Although there are multiple studies that describe the effect of fasting, feed restriction, and dietary protein quantity and quality on expression of the peptide transporter, PepT1, very few studies have attempted to determine how feeding a peptide-based diet influences expression of PepT1 and growth performance in an animal model. There have been numerous feeding studies conducted using hydrolysates in fish diets, with improvements observed in survival and growth of larvae (40-43). There have been some feeding trials conducted in pigs using porcine intestinal hydrolysate as a supplementary source of protein in the diet, with improved growth observed in weanling

pigs (44-45). This is the first study to evaluate the effect of feeding a hydrolysate on gene expression of peptide and AA transporters in chickens.

Protein expression of PepT1 was evaluated using brushborder membrane samples from only jejunum from d 9 to d 15. There was no effect of diet or genetic line on PepT1 protein abundance. The effects observed at the mRNA level represent all three intestinal segments, and when jejunal mRNA data were analyzed separately, there was also no effect of protein composition. Hence, without the protein data from other segments in the small intestine, the findings are tentative. It should be noted that mRNA used for real-time PCR was extracted from total intestinal tissue, while protein used for western blot analysis was purified brushborder membrane vesicles. This does not tell us the levels of PepT1 protein that may be retained in vesicles inside the cell after synthesis, modification and export from the Golgi. Hence, findings should be interpreted carefully.

In conclusion, we demonstrated that genes associated with the digestion and absorption of AA are influenced by dietary protein composition. In particular, Line B chicks, which express greater quantities of PepT1, are more responsive to the peptide-based diet. This has the very interesting implication that genetic selection can indeed alter expression levels of nutrient transporters in response to dietary protein composition. The practical nutritional implications are unknown at this time because differences in growth performance were not observed in this study. Diets were fed for only 1 wk which was most likely insufficient time for differences in growth to become apparent. Future studies will include grow-out trials to evaluate how a hydrolysate diet affects BW of Line B at market age, and further investigation of transporter expression at the protein level.

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protein for early-weaned pigs. J Anim Sci. 2000;78:318-327.

Table 5.1 Composition (as-fed basis) of the whey, hydrolysate, and AA-based diets.

	Diet[1]		
Ingredient (%)	Whey	Hydrolysate	AA
Corn starch	50.09	50.09	50.09
Whey protein concentrate[2]	26.02	----------	----------
Whey hydrolysate[3]	----------	25.57	----------
AA mixture[4]	----------	----------	28.53
Soybean oil	6.00	6.00	6.00
Sucrose	5.00	5.00	5.00
Milk fat	----------	2.04	2.10
Powdered cellulose	4.57	2.94	----------
Mineral premix[5]	5.37	5.37	5.37
Vitamin premix[6]	0.20	0.20	0.20
Choline chloride	0.17	0.17	0.17
BMD-50[7]	0.05	0.05	0.05
DL-α-Tocopheryl acetate	0.002	0.002	0.002
L-arginine	2.53	2.56	2.53

[1]The CP values for the whey and hydrolysate diets were formulated to be 24 %. The CP values for all diets were experimentally determined and are as follows: Whey diet, 24 %; Hydrolysate, 25 %; AA diet 27 %.

[2]Lacprodan® DI-8090 whey protein concentrate, Arla Foods, Denmark.

[3]Lacprodan® DI-3065 whey protein hydrolysate, Arla Foods, Denmark.

[4]AA mixture (% of the diet): glutamic acid, 4.61; L-arginine, 3.38; L-lysine hydrochloride, 3.04; L-asparagine, 2.95; L-leucine, 2.80; L-threonine, 1.96; L-isoleucine, 1.76, L-valine, 1.64; L-proline, 1.61; L-serine, 1.46; L-alanine, 1.34; L-phenylalanine, 0.84; L-tyrosine, 0.84; L-histidine, 0.64; L-cystine, 0.62; DL-methionine, 0.57; glycine, 0.50; L-tryptophan, 0.47

[5]Provided the following per kilogram of diet: $Ca_3(P0_4)_2$, 28.0 g; K_2HPO_4, 9.0 g; NaCl, 8.89 g; $MgSO_4 \cdot 7H_20$, 3.5 g; $ZnCO_3$, 0.10 g; $CaCO_3$, 3.0 g; $MnSO_4 \cdot H_20$, 0.65 g; $FeSO_4 \cdot 7H_20$, 0.42 g; KI, 40 mg; $CuSO_4 \cdot 5H_20$, 20 mg; $Na_2MoO_4 \cdot 2H_20$, 9 mg; H_3BO_3, 9 mg; $CoSO_4 \cdot 7H_20$, 1 mg; Na_2SeO_3, 0.22 mg

[6]Provided the following per kilogram of diet: thiamin-HCl, 20 mg; niacin, 50 mg; riboflavin, 10 mg; D-Ca-pantothenate, 30 mg; vitamin B_{12}, 0.04 mg; pyridoxine-HCl, 6 mg; D-biotin, 0.6 mg; folic acid, 4 mg; menadione dimethylpyrimidinol bisulfate, 2 mg; cholecalciferol, 15 µg; retinyl acetate, 1,789 µg; ascorbic acid, 250 mg

[7]BMD-50 provided by Alpharma, Inc. The addition of BMD-50 provides 1 g bacitracin activity/kg diet.

Table 5.2 Molecular weight distribution of peptides in the whey hydrolysate.

GPC 214 nm[1]	Whey Hydrolysate[2]
MW (Da)	% of hydrolysate
< 175	< 5
< 375	18.5
375-750	40.4
750-1,250	24.2
1,250-2,500	14.6
> 2,500	2.4
Maximum MW	6,000 Da

[1]Based on gel permeation chromatography elution profile monitored by UV at 214 nm.
[2]Lacprodan® DI-3065 (Arla Foods)

Table 5.3 Effect of dietary protein composition on BW and intestinal segment weights in two lines of broilers from day 9 to 15 posthatch[1].

		BW	Duodenum	Relative Duodenum	Jejunum	Relative jejunum	Ileum	Relative Ileum
	A	223.2	4.07	1.87	5.77	2.65	3.93	1.81
Line	B	231.0	4.35	1.96	5.88	2.63	4.11	1.84
(n=5)	SEM	2.5	0.05	0.03	0.07	0.02	0.06	0.02
	P-value	0.02	0.0002	0.005	0.20	0.70	0.02	0.20
	Whey	225.7	4.10	1.88	5.51[c]	2.53[c]	3.79[b]	1.74[b]
	Hydro	225.1	4.21	1.93	5.81[b]	2.64[b]	4.11[a]	1.88[a]
Diet[2]	AA	230.5	4.31	1.94	6.15[a]	2.75[a]	4.15[a]	1.86[a]
(n=10)	SEM	3.0	0.06	0.03	0.08	0.03	0.07	0.03
	P-value	0.40	0.08	0.30	0.0001	0.0001	0.0002	0.0001
Age	D9	163.3	3.65	2.25	4.95	3.05	3.53	2.17
(n=10)	D11	196.6	3.94	2.03	5.37	2.75	3.71	1.90
	D13	256.5	4.50	1.76	6.31	2.46	4.20	1.64
	D15	292.0	4.73	1.64	6.67	2.30	4.62	1.59
	SEM	3.5	0.07	0.03	0.09	0.03	0.08	0.03
	P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
		----------Interaction *P*-value----------						
	A x L	0.02	0.50	0.0005	0.50	0.09	0.70	0.10
	A x D	0.30	0.30	0.50	0.05	0.04	0.30	0.40
Interaction[3]	L x D	0.50	0.30	0.60	0.60	0.40	0.20	0.10

[1]BW, duodenum, jejunum, and ileum expressed as weight (g). Relative duodenum, relative jejunum, and relative ileum expressed as individual segment weight as a percentage of total BW.
[2]Means in a column without a common letter differ, $P < 0.03$ (Tukey's test)
[3]For the interactions, A, L, and D, represent the main effects of age, genetic line, and diet, respectively.

Table 5.4 Effect of dietary protein composition on intestinal morphometry in two lines of broiler chicks at day 15 posthatch[1].

		Villus height	Villus width	Crypt depth	VCR[1]
		Distance, mm			
	A	1.93	0.27	0.32	6.07
Line	B	1.85	0.28	0.30	6.26
(n=5)	SEM	0.04	0.01	0.01	0.16
	P-value	0.15	0.30	0.08	0.42
	Whey	1.85	0.27^{ab}	0.29	6.31^{ab}
	Hydro	1.98	0.30^{a}	0.31	6.48^{a}
Diet[2]	AA	1.84	0.26^{b}	0.32	5.71^{b}
(n=10)	SEM	0.05	0.01	0.01	0.20
	P-value	0.06	0.02	0.18	0.02
	Duodenum	2.55^{d}	0.30^{d}	0.37^{d}	7.04^{d}
	Jejunum	1.80^{e}	0.28^{de}	0.30^{e}	6.09^{e}
Segment[3]	Ileum	1.32^{f}	0.26^{e}	0.26^{f}	5.36^{f}
(n=10)	SEM	0.05	0.01	0.01	0.20
	P-value	0.0001	0.02	0.0001	0.0001
	Interaction *P*-value				
Interaction[4]	L x D	0.04	0.92	0.04	0.0002
	D x S	0.50	0.76	0.87	0.26

[1]Distance measurements expressed as distance (mm). Villus height to crypt depth (VCR) expressed as ratio.
[2]Means in a column without a common letter differ, $P < 0.02$ (Tukey's test)
[3]Means in a column without a common letter differ, $P < 0.03$ (Tukey's test)
[4]For the interactions, L, D, and S represent the main effects of genetic line, diet, and intestinal segment, respectively.

Table 5.5 Effect of dietary protein composition on mRNA abundance of peptide and AA transporters, a digestive enzyme, and a housekeeping gene[1].

		Gene							
Item		PepT1	$b^{o,+}AT$	EAAT3	y^+LAT2	CAT1	LAT1	APN	Beta actin
		Molecules of mRNA per ng total RNA							
	A	7,640	127,610	5,020	5,775	465	525	261,320	693,180
Line	B	11,940	151,180	6,400	8,380	645	560	284,545	815,500
(n=5)	SEM	345	5,170	220	350	20	20	8,235	25,800
	P-value	0.0001	0.001	0.0001	0.0001	0.0001	0.20	0.04	0.0009
	Whey	9,500[a,b]	132,580[b]	5,150[b]	6,405	480[b]	495[b]	251,525[b]	675,340[b]
	Hydro	10,845[a]	161,800[a]	6,930[a]	7,875	600[a]	585[a]	303,905[a]	849,450[a]
Diet[2]	AA	9,030[b]	123,800[b]	5,045[b]	6,950	580[a]	540[a,b]	263,360[b]	738,220[b]
(n=10)	SEM	425	6,335	270	430	25	20	10,090	31,600
	P-value	0.008	0.0001	0.0001	0.05	0.003	0.02	0.0007	0.0005
	Duodenum	11,065[d]	120,320[e]	2,620[f]	6,470	470[e]	490[e]	168,130[f]	611,425[e]
	Jejunum	10,035[d]	112,250[e]	4,430[e]	7,350	545[e]	555[d,e]	247,420[e]	848,990[d]
Segment[3]	Ileum	8,275[e]	185,615[d]	10,075[d]	7,410	650[d]	575[d]	403,245[d]	802,600[d]
(n=10)	SEM	425	6,335	270	430	25	20	10,090	31,600
	P-value	0.0001	0.0001	0.0001	0.20	0.0001	0.03	0.0001	0.0001

Age	D9	8,285	98,471	4,175	5,585	580	530	227,150	601,380
(n=10)	D11	11,035	157,355	5,830	7,900	620	555	278,650	888,970
	D13	10,120	155,050	6,845	7,840	475	500	308,070	780,580
	D15	9,730	146,705	5,985	6,980	540	580	277,855	746,430
	SEM	490	7,315	310	500	30	25	11,650	36,490
	P-value	0.001	0.0001	0.0001	0.003	0.005	0.10	0.0001	0.0001
		----------Interaction *P*-value----------							
Interaction[4]	A x L	0.005	0.20	0.04	0.005	0.0001	0.0001	0.60	0.0002
	A x D	0.08	0.01	0.01	0.10	0.003	0.10	0.04	0.0001
	L x D	0.005	0.005	0.003	0.004	0.005	0.40	0.005	0.0003
	D x S	0.90	0.02	0.0005	0.70	0.80	0.70	0.50	0.80

[1]All means expressed as molecules of mRNA per nanogram of total RNA
[2]Means in a column without a common letter differ, $P < 0.04$ (Tukey's test)
[3]Means in a column without a common letter differ, $P < 0.03$ (Tukey's test)
[4] For the interactions, A, D, L, and S, represent the main effects of age, diet, genetic line, and intestinal segment, respectively.

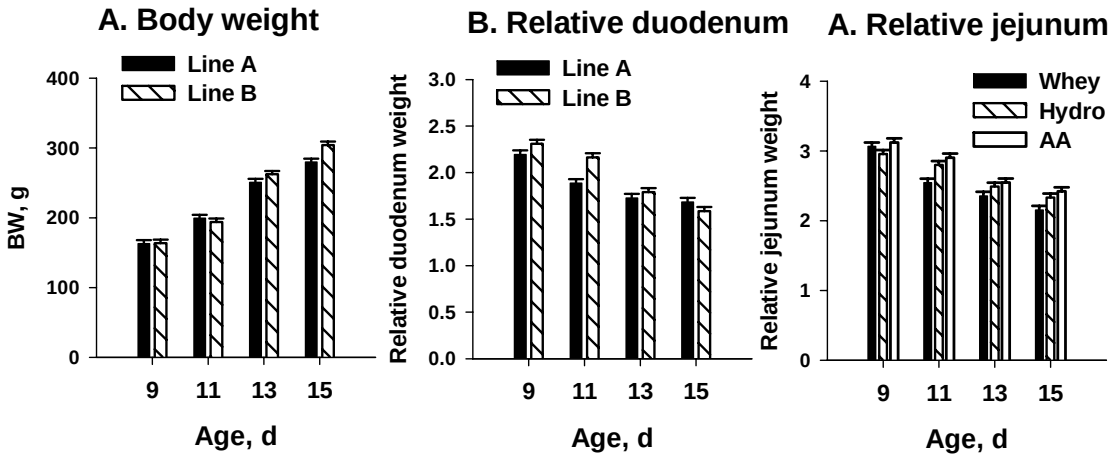


Figure 5.1 Body and intestinal weights of male birds from two genetic lines at days 9, 11, 13 and 15 posthatch in birds fed whey protein concentrate (whey), a partial whey hydrolysate (hydro), or a mixture of AA identical to the composition of whey (AA). Bars on the graph represent means of male birds ± SEM. Relative weights are expressed as the individual segment weight as a percentage of total BW. There was an interaction of age x genetic line for BW and relative duodenum ($P < 0.03$) and an interaction of age x diet for relative jejunum ($P < 0.05$).

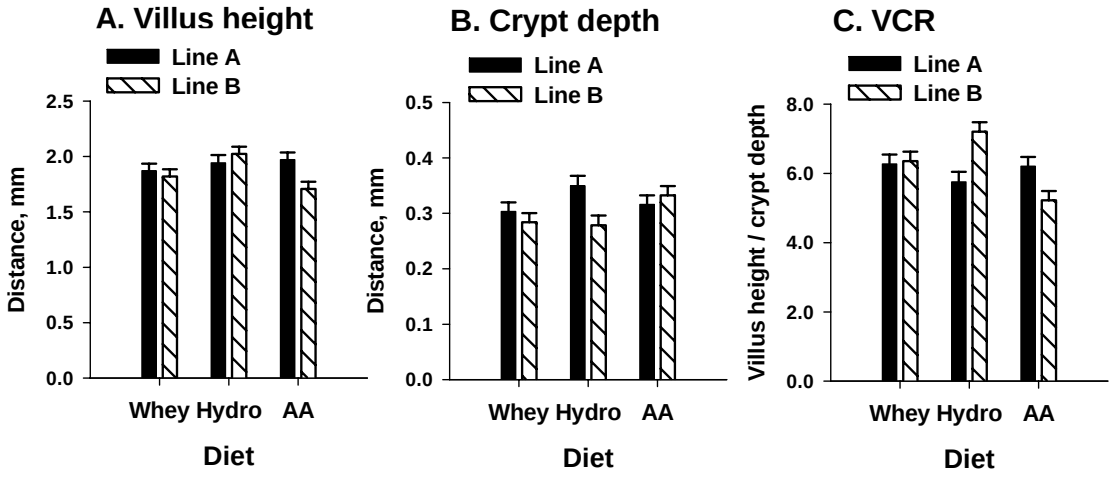


Figure 5.2 Effect of genetic line and dietary protein composition on intestinal morphometry. Line x diet interaction ($P < 0.05$) for A) villus height, B) crypt depth, and C) villus height to crypt depth ratio (VCR) in broilers fed whey protein concentrate (whey), a partial whey hydrolysate (hydro), or a mixture of AA identical to the composition of whey (AA) from d 9 to 15 posthatch. Bars represent the mean of male birds (n = 5) ± SEM.

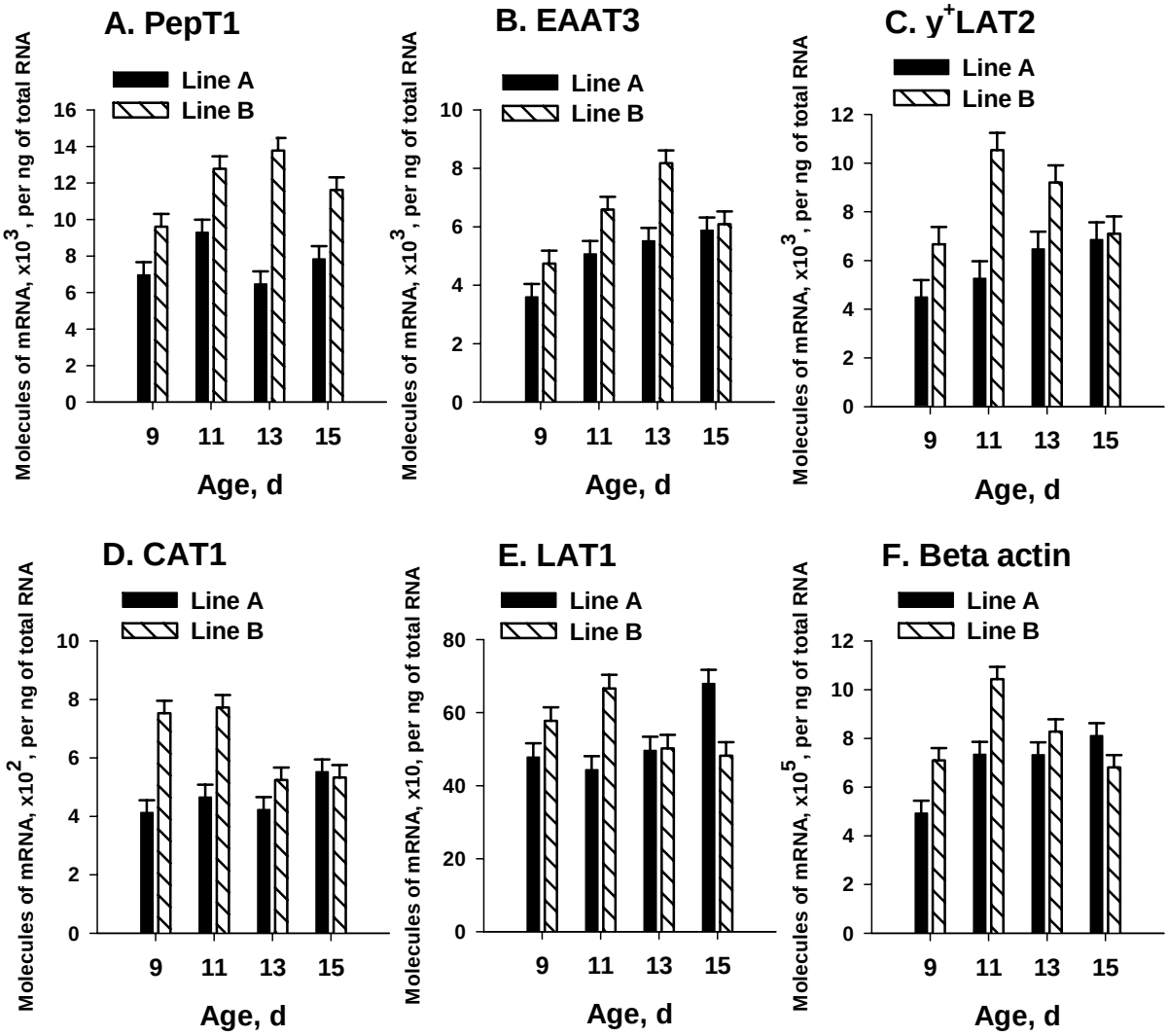
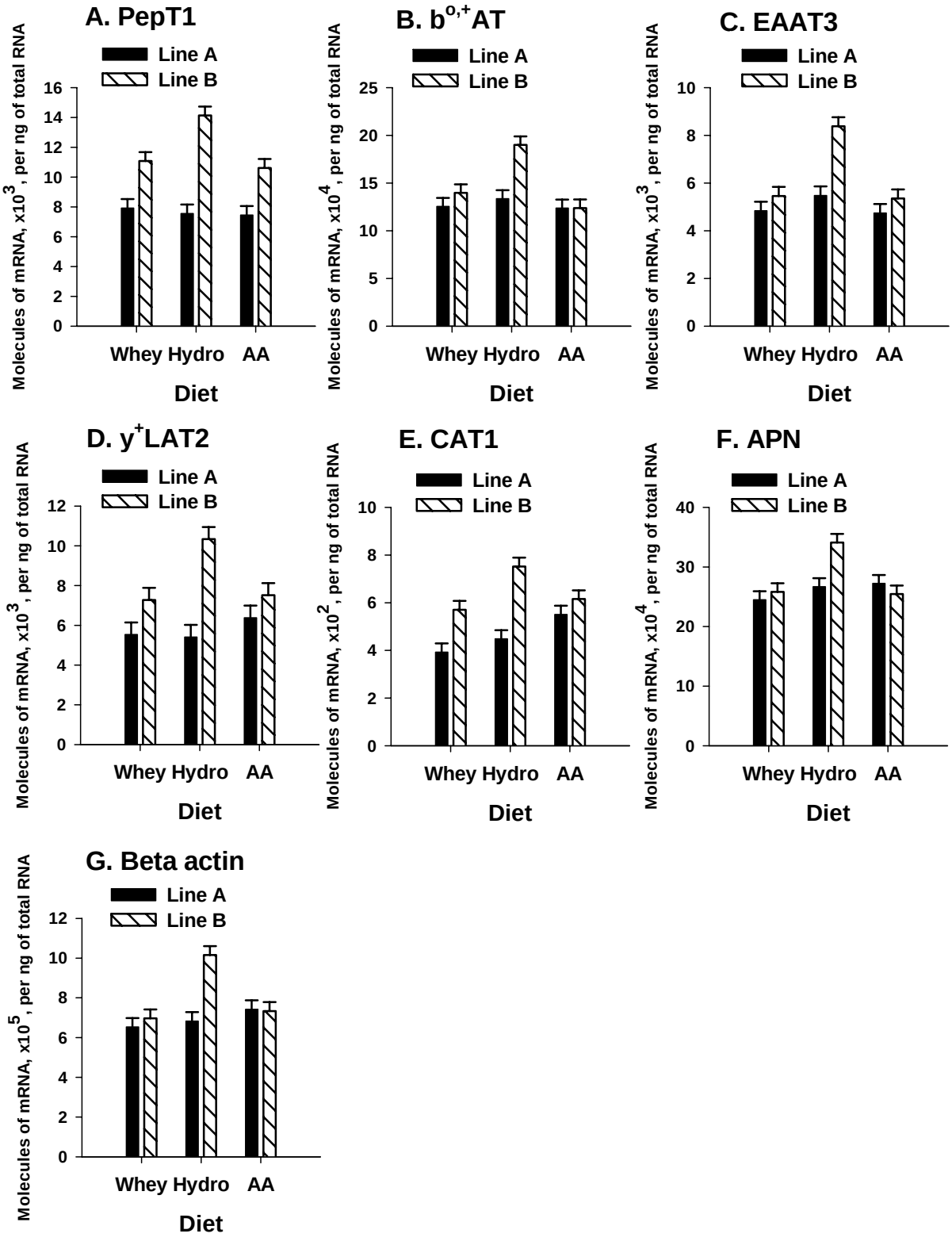


Figure 5.3 Effect of age on peptide and AA transporter and aminopeptidase N (APN) mRNA abundance in two lines of broilers from d 9 to 15 posthatch. The number of A) PepT1, B) EAAT3, C) y^+LAT2, D) CAT1, E) LAT1, and F) beta actin mRNA molecules per nanogram of total RNA. Bars represent the mean of male birds (n = 15; 5 from each dietary group) from Line A or Line B ± SEM. There was an interaction of genetic line by age for all 6 genes ($P < 0.05$).

Figure 5.4 Effect of diet on peptide and AA transporter and aminopeptidase N (APN) mRNA abundance in two lines of broilers. The number of A) PepT1, B) $b^{o,+}AT$, C) EAAT3, D) $y^{+}LAT1$, E) CAT1, F) APN, and G) beta actin mRNA molecules per nanogram of total RNA. Bars represent the mean of male birds (n = 5) from Line A or Line B ± SEM that consumed whey (whey protein concentrate), hydro (whey hydrolysate), and AA (AA mixture identical to the composition of whey) from d 9 to15 posthatch. There was an interaction of genetic line x diet for all 7 genes ($P < 0.006$).


A. PepT1
Line A
Line B
Molecules of mRNA, x10^3, per ng of total RNA
0
2
4
6
8
10
12
14
16
Whey Hydro
AA
Diet
B. b^o,+AT
Line A
Line B
Molecules of mRNA, x10^4, per ng of total RNA
0
5
10
15
20
25
Whey Hydro
AA
Diet
C. EAAT3
Line A
Line B
Molecules of mRNA, x10^3, per ng of total RNA
0
2
4
6
8
10
Whey Hydro
AA
Diet
D. y^+LAT2
Line A
Line B
Molecules of mRNA, x10^3, per ng of total RNA
0
2
4
6
8
10
12
Whey Hydro
AA
Diet
E. CAT1
Line A
Line B
Molecules of mRNA, x10^2, per ng of total RNA
0
2
4
6
8
10
Whey Hydro
AA
Diet
F. APN
Line A
Line B
Molecules of mRNA, x10^4, per ng of total RNA
0
10
20
30
40
Whey Hydro
AA
Diet
G. Beta actin
Line A
Line B
Molecules of mRNA, x10^5, per ng of total RNA
0
2
4
6
8
10
12
Whey Hydro
AA
Diet

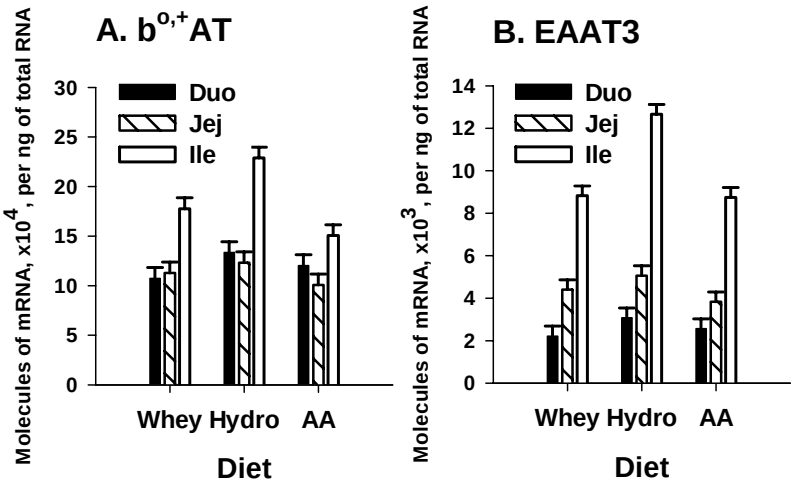


Figure 5.5 Effect of dietary protein composition on spatial pattern of AA transporter mRNA abundance in broilers. The number of A) $b^{o,+}AT$ and B) EAAT3 mRNA molecules per nanogram of total RNA. Bars represent the mean of male birds (n = 10; 5 Line A and B) ± SEM in the duodenum, jejunum, and ileum of birds that consumed whey (whey protein concentrate), hydro (whey hydrolysate), and AA (AA mixture identical to the composition of whey) from d 9 to 15 posthatch. There was an interaction of intestinal segment x diet for both genes ($P < 0.03$).

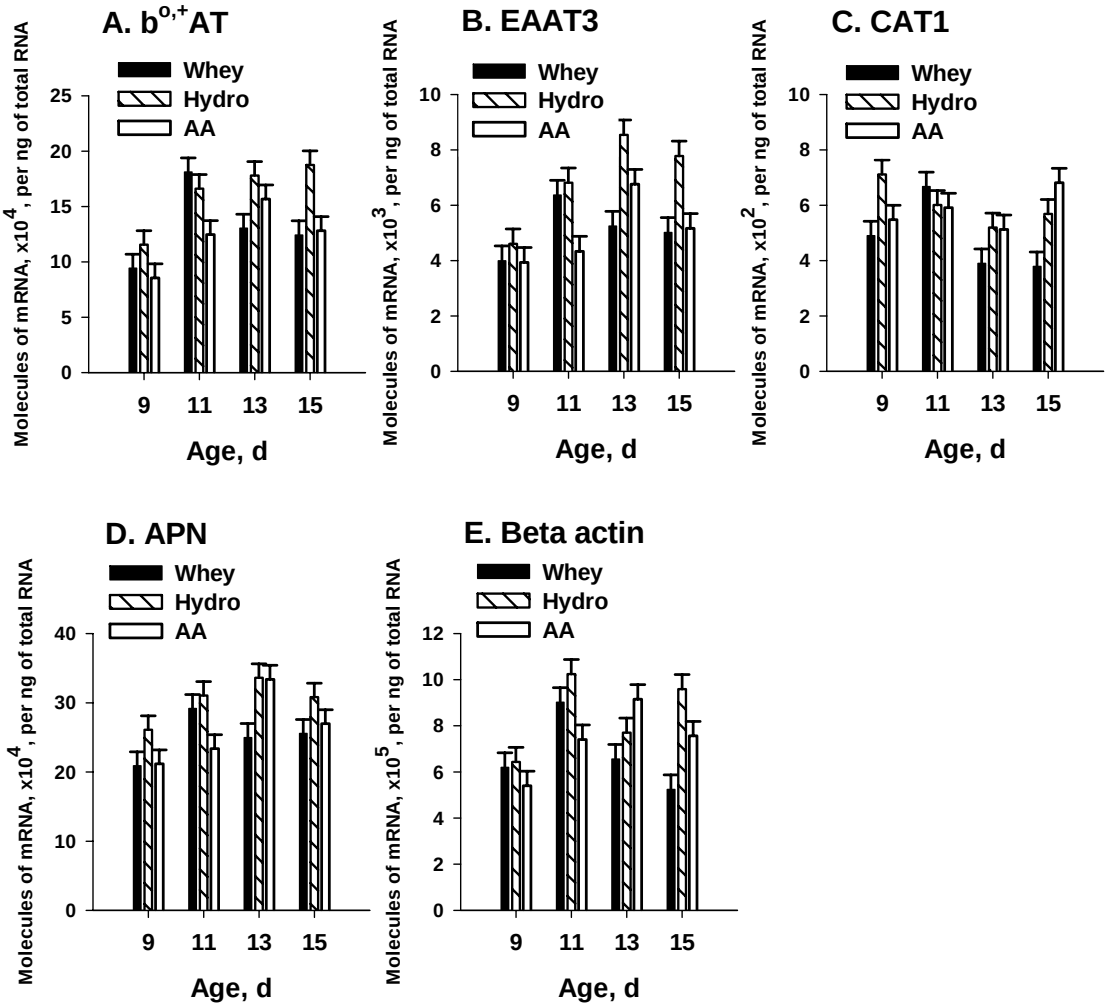


Figure 5.6 Effect of dietary protein composition on the age-related change in mRNA abundance of AA transporters and a digestive enzyme in broilers. The number of A) $b^{0,+}AT$, B) EAAT3, C) CAT1, D) APN, and E) beta actin mRNA molecules per nanogram of total RNA. Bars represent the mean of male birds (n = 10; 5 Line A and B) ± SEM that consumed whey (whey protein concentrate), hydro (whey hydrolysate), and AA (AA mixture identical to the composition of whey) from d 9 to 15 posthatch. There was an interaction of age x diet for all 5 genes ($P < 0.05$).

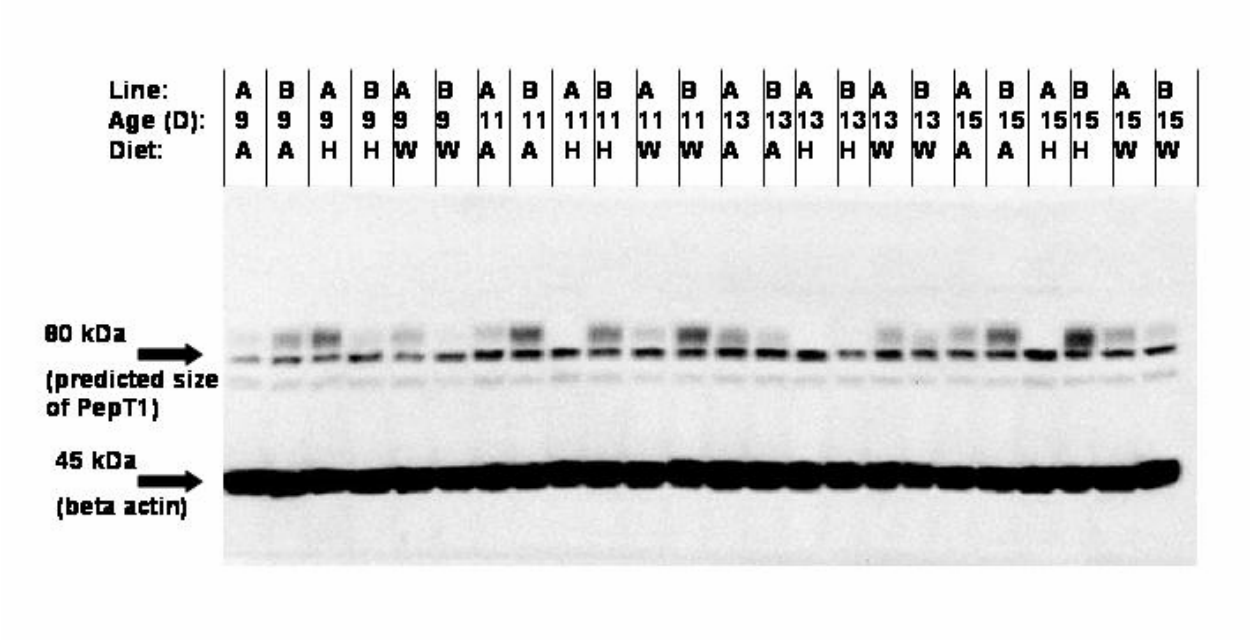


Figure 5.7 Western blot analysis of PepT1. Gels were loaded with 20 µg of chicken intestinal brushborder membrane on to 7.5 % polyacrylamide gels. Membranes were probed simultaneously with a polyclonal rabbit α-chicken PepT1 and monoclonal mouse α-human beta actin antibody. Shown is a representative western blot. Each well represents a jejunum sample from one out of five birds representing each group. Line: A or B, Age: D 9, 11, 13 or 15, and Diet: A (AA), H (Hydro), or W (Whey).

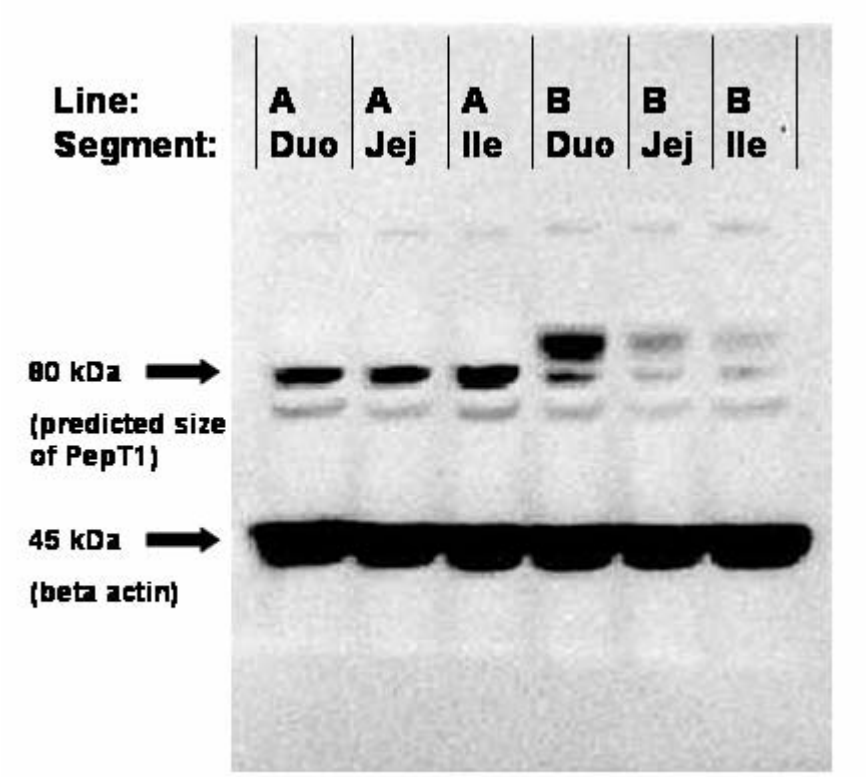


Figure 5.8. Western blot analysis of PepT1 at d 8. Gels were loaded with 20 µg of chicken intestinal brushborder membrane on to 7.5 % polyacrylamide gels and subjected to electrophoresis followed by semi-dry electroblotting on to PVDF membrane. Membranes were probed simultaneously with a polyclonal rabbit α-chicken PepT1 and monoclonal mouse α-human beta actin antibody. Shown is a representative western blot. From left to right are one out of five birds from each line at D 8. Line: A or B, Segment: Duo (duodenum), Jej (jejunum) or Ile (ileum).

Chapter VI

EPILOGUE

There is still much to be learned about the physiological processes associated with digestion and absorption in the small intestine of the chicken. Years of intense selection for desired performance traits has pushed the digestive and absorptive capacities of the chicken gut to increase performance. It is unknown if we have reached a "bottleneck" in terms of digestion and absorptive capacities. The ability to make improvements in nutrient utilization, feed efficiency, and carcass traits at market age will require a strategic re-thinking of how we formulate the diet. In particular, dietary protein is expensive, and there are many natural proteins to be experimented with and used to refine the protein content of the diet. Dietary peptides as a source of AA represent a relatively unstudied area in animal research. In humans however, peptides are commonly used in enteral feeding formulas, infant formulas, and body-building supplements to name a few.

The studies presented in this dissertation compared two lines of broilers that originated from the same genetic stock but were selected on different diets for at least 10 generations. While we found an interesting difference in expression of PepT1 that remained consistent through all of our feeding trials, I do not think we answered the question of what contributes to the difference in growth between the two lines. The difference we detected in PepT1 should be pursued. The diets that we used for the third feeding trial (whey or hydrolysate-based diets) should be further evaluated by performing grow-out trials with the two lines. In our first feeding trial, there was no significant difference in body weight between the two lines. In the second trial where there were nutritional challenges associated with feed restriction and protein quality, Line A birds were heavier. In the third trial, where we fed a high-quality whey-based diet for the

second week posthatch, Line B birds were significantly heavier. So, line difference varies with diet, and perhaps nutritional challenge accentuates performance in Line A birds, while a more AA-dense diet accentuates Line B performance.

Since Line B was selected on a wheat-based diet, I have hypothesized several explanations for how their gut may have evolved under such conditions. A wheat-based diet will be particularly high in NSP which are known to increase viscosity in the gut. Viscosity has numerous detrimental effects in the gut such as reduced digestion, greater diffusional barrier to the brushborder membrane, mechanical shear in the gut, and a shift in environment that could be more conducive to bacterial attachment. So perhaps a more economical way of absorbing AA is needed to maximize assimilation in spite of the challenges associated with viscosity. Increased expression of PepT1, known to be a more energetically-efficient way of absorbing AA, is one possibility, and could explain why greater PepT1 expression in Line B birds was seen. It would be interesting to measure the mucus layer in Line B birds, to see if there is a difference as a result of selection on a wheat-based diet.

Based on the information discussed in the preceding paragraph, I think that future studies should continue to attempt to take advantage of high expression levels of PepT1 in Line B birds. As I mentioned before, a grow-out trial feeding the peptide-based diet would be very interesting. Future studies should also evaluate other ways in which selection may have altered nutrient utilization in Line B birds including digestion, mucin dynamics as mentioned above, and post-absorptive utilization, such as an evaluation of skeletal muscle protein synthesis.

It is my hope that the research presented in this dissertation will lead to studies aimed at evaluating the use of peptides as an AA source or supplement in animal diets. I have shown that the peptide transporter is influenced by genetic selection, developmental stage, and diet, and an

interaction of all of these factors. This plasticity in expression is an attractive target for dietary manipulation. There has been an interest in the peptide transporter, PepT1, by many research groups around the world, due to its enormous pharmacological significance as a transporter that improves the bioavailability of many drugs that are designed to have a peptide-like structure. The broad specificity of PepT1 makes it appealing as a drug target but also makes the task of designing highly-available dietary AA supplements a daunting task. As discussed in our review of the peptide transporter, results published from our lab and others indicate that the affinity for di- and tripeptides can vary depending on the composition of the peptide, as well as presence of other potential substrates or inhibitors in the extracellular milieu. Transporter-substrate interactions and determinants of substrate affinity are related to the three-dimensional structure of the transporter. Structure and function go hand-in-hand, and without having a complete understanding of the structural features of the transporter, we are greatly limited in our ability to target it with substrates. While there have been many studies that use site-directed mutagenesis to determine the functional importance of individual AA residues in the protein, and attempts have been made to predict its three-dimensional structure using homology modeling, there is still much to be learned about how the protein folds and interacts with the cell membrane during normal physiological conditions.

While x-ray crystallography has improved and the number of structures of membrane-bound proteins solved by this method has increased dramatically in recent years, the number of membrane proteins in the protein database is still severely under-represented. The close association of the membrane protein with the lipid-bilayer makes it very difficult to express and purify the protein and many times, interactions with the detergent lead to aggregation and precipitation. While homology modeling can be successful and accurate in many cases, the

quality of this method depends on the starting template used. Since the number of membrane proteins that have been solved is relatively few, and most of these proteins are bacterial proteins not closely related to the peptide transporter, this method is very limiting. Thus, I propose that until the structure of PepT1 is solved, we will still be limited in our ability to predict how well a mixture of peptides will be transported by the protein.

This leads me to discuss the problems associated with generating a mixture of peptides. There are still limitations in analytical procedures for determining peptide profiles. While we can determine the size of peptides and relative proportion of molecular weight fractions in a hydrolysate, we are still unable to determine the exact composition of each peptide in the mixture. The procedure of synthesizing a mixture of peptides, which would have a known composition, is extremely expensive. At the moment this is not yet economically feasible for supplementing livestock diets. Alternatively, a protein hydrolysate could be economically produced using a multitude of enzymes with varying specificities. Thus, the composition of the peptide mixture can vary dramatically depending on the cocktail of enzymes used, pH conditions, length of hydrolysis, etc. So, in addition to improvements in technology for characterizing peptide profiles and even perhaps synthesizing peptides, there should be more studies conducted that evaluate various hydrolysis procedures for different proteins, and experimentation with different inclusion levels in chicken diets.

I think that we also need a greater understanding of the digestion events that occur between swallowing and absorption in the small intestine. Even if we know the exact composition and quantity of nutrients that contribute to animal intake, we still do not know what proportion and composition of nutrients reaches the brushborder membrane. Stomach acid, digestive enzymes, microbial activity, some absorption proximal to the small intestine, and

ability to traverse the unstirred water layer are examples of factors that influence the composition of the nutrients that reaches the brushborder membrane.

The use of hydrolysates in animal diets is further complicated by the phenomenon of bio-active peptides. Many known bio-active peptides (anti-hypertensive, anti-oxidant, anti-microbial) are released upon hydrolysis from a variety of proteins. In particular, whey protein is known to contain a variety of bio-active peptides, making it popular as a "nutraceutical". Thus, when we feed a hydrolysate, we are not only supplying a source of AA to the cell, we are potentially influencing cell metabolism and function through the introduction of regulatory peptides. This continues to be an exciting area of research with much promise in improving animal health.

The destination of AA that are absorbed in the form of peptides is still a subject of controversy. We know that peptides are absorbed into the cell. In fact, PepT1 may not be the only route of uptake. Cell-penetrating peptides and paracellular uptake are accepted as common routes of nutrient uptake. We know that once inside the cell there are a variety of fates, as discussed in the literature review. There is evidence for the presence of a basolateral membrane peptide transporter, and for absorption of hydrolysis-resistant peptides. Peptides are detected in the circulation and diet affects these concentrations. In mammary cell culture models, AA from peptides are more readily incorporated into proteins than if only free AA are provided in the media. We still do not know the exact origin or final destination of the peptides detected in the blood because of the complex metabolism and physiology of cells throughout the body. We think that the peptides detected in the blood may be of nutritional significance to the rest of the body. Some have suggested that the concept of clearance by erythrocytes undermines the importance of blood-borne peptides, however the only published papers on the topic indicate that there is negligible clearance of peptides by red blood cells. A way of accurately tracking digestion and

absorption and the route of resultant products through the enterocyte and potentially into the portal circulation is needed.

The peptide transporter is expressed on the brushborder membrane of enterocytes that line the intestinal villi. When we collect intestinal tissue for laboratory analysis, we are collecting a mixture of cells that may or may not be representative of the expression profile of the enterocyte. Others have demonstrated with laser capture microdissection to analyze individual enterocytes, that whole tissue and individual enterocyte expression patterns were highly correlated, providing good support for our method of determining relative changes in expression using whole tissue samples. In terms of dietary regulation of transporter expression though, I think there is a complex inter-play between diet and remodeling of the mucosal layer. Differences in tissue mass, and proportions of certain components of tissue mass such as connective tissue, may provide a skewed picture of the overall tissue transport capacity. More studies should attempt to correlate changes in gene expression with crypt-villus distribution of transporters and changes in villus height and crypt depth, along with changes in length and thickness of the intestinal segment.

I mentioned that there should be more attempts to evaluate the crypt-villus distribution of transporter expression. I think that the question still remains about the site of enhanced or repressed transcription, and whether mature enterocytes are capable of being reprogrammed. In chickens, this situation is further complicated by the fact that proliferating cells are detected in both the crypts and all along the length of the villus, in sharp contrast to mammals. The origin of villus-specific proliferating cells is still not known and requires further evaluation.

The study of dietary regulation of nutrient transporters is further complicated by the issue of intestinal tissue maintenance. The intestine uses a substantial proportion of dietary AA for

energy and protein synthesis. In one sense, this is logical because the intestine is one of the first organs to process dietary nutrients, thus, the health and function of this tissue is of critical importance in order to supply nutrients to the rest of the body. In another sense, however, some of the oxygen consumption and protein turnover seems unnecessary. A case in point is the use of approximately 60 % of dietary threonine for sythesis of mucins that make up the mucus layer. While the mucus layer is extremely important in protecting the intestinal epithelium, the question arises of "what is too much?". Similarly, it can be argued that a low villus-height to crypt-depth ratio is too energy consuming. It implies that there is more cell proliferation and recruitment to the intestinal villi, which requires energy. Additionally, the presence of less mature enterocytes on the villi might result in less efficient digestion and absorption. Hence, a smaller crypt to support a longer villus is more energy efficient to the animal.

The importance of host-microbial interactions in the small intestine can not be over-emphasized. While the intestinal microflora was not a component of my research, it is one of the most critical determinants in gut health and nutrient availability to the animal. The microbes compete for nutrients, make nutrients available, can inhibit nutrient digestion (e.g., deconjugation of bile acids), induce an immune response by the host, and generate metabolites (SFCA, amines) that are trophic factors in the gut. It is beyond the scope of this discussion to describe these processes and interactions in great detail. I wanted to mention, however, that a greater understanding of nutrient availability will only be facilitated with a more complete evaluation of the interactions between the luminal dietary components, the intestinal microflora, and the gut mucosal layer. Hence, there should be more synergy in animal nutrition research projects. In addition to the molecular biology component that I performed, more information

could be gained by characterizing the intestinal microflora, examining digestibility, and evaluating the intestinal immune system in concert.

I think that in-ovo nutrition is an area of research with infinite possibilities. Since learning of its discovery and eagerly staying updated on its progress, I believe that peptides could be a focus of research on improving in-ovo feeding solutions. This should go hand-in-hand with more studies aimed at learning more about chicken embryological development, specifically the development of digestive and absorptive processes in the gut. Additionally, the yolk sac membrane is an enticing area of research. During development, it represents the limiting step in delivery of nutrients between the yolk and embryo. Results from recent studies have shown the presence of nutrient transporters in the yolk sac membrane, thus, a greater understanding of nutrient absorption across the yolk sac membrane could be instrumental in improving the energy status of the chick at hatch.

There is still much to be learned about how transporters are regulated at the transcriptional and post-translational level. I mention these two levels of regulation because they are of particular interest to me. There should be more studies aimed at characterizing transcription factor binding sites on the PepT1 promoter. There are a multitude of computer programs that predict the location of these sites, but studies that prove that the protein-DNA interactions exist are lacking. As an example, it was recently shown that PPARα, a nuclear receptor up-regulated during periods of fasting, was suggested to be a factor that contributes to the up-regulation of PepT1 during fasting. Future studies should determine the intermediary factor between PPARα and PepT1 that mediates this interaction.

In terms of post-translational modifications there are multiple predicted sites on the PepT1 protein for N-linked glycosylation, and protein kinase phosphorylation. These types of

modifications could provide important clues about the role of PepT1 in cell-signaling and as a nutrient receptor. Also, after translation, how much of PepT1 is actually recruited to the BBM, and how much is retained in vesicles inside the cell? When we see an increase in expression of a protein, is it due to increased mRNA stability, increased protein synthesis, increased trafficking, or decreased protein degradation, or a combination of these events?

We still do not really know what the effect of a lack of PepT1 is on the body. There are numerous cases of deficiencies in free AA absorption in humans, but interestingly, patients are not deficient in the AA, suggesting that another transporter, such as PepT1, can compensate for the deficiency. The only reported cases of PepT1 gene-knockouts in mice evaluate effects on renal reabsorption. A project aimed at evaluating the effect of a lack of expression and over-expression of PepT1 in chickens on intestinal function is currently in progress in our lab and should provide some insight as to the functional importance of PepT1 in the small intestine.

Mechanisms for intestinal transport are known to vary across avian species with specific advantages and disadvantages. These mechanisms have probably evolved as strategies to accommodate specific niches in nature. For example, both glucose and peptide transport is passive in song birds. No peptide transporter was detected in the small intestine of sparrows and the authors determined that the majority of transport occurred through the paracellular route. While this is an energetically efficient means of absorbing nutrients, the disadvantage of this route is that a relatively permeable and non-selective epithelium is more vulnerable to toxins and infection. This is a compromise worth making in an animal where survival is the top priority. In the modern meat-type chicken, rapid growth and muscle development in a short period of time results in potential constraints on growth by limitations in active transport mechanisms in the

gut. The mechanisms that lead to improved permeability of tight junctions in the normal physiological state of a bird like a sparrow, is unknown.

In summary, the field of intestinal nutrient transporter regulation is one with exciting and industry-applicable possibilities. Results from my research have demonstrated that expression of nutrient transporters is responsive to genetic strain of the bird, age of the bird, and various dietary manipulations. In particular, the peptide transporter is an attractive target for future dietary manipulations. Future studies should continue to evaluate the use of peptides as a source of AA in the chicken diet and determine the peptide compositions that best complement the digestive and absorptive characteristics of the chicken gut.

Appendix A

Table A.1. Intestinal morphology of Line A and B chicks fed a commercial corn-soy diet from d of hatch to 14 ds[1].

		Tall villus height	Tall villus width	Short villus height	Short villus width	Crypt depth
		Distance, mm				
	A	0.58	0.11	0.45	0.11	0.09
Line	B	0.58	0.11	0.46	0.11	0.09
(n=4)	SEM	0.01	0.003	0.01	0.004	0.004
	P-value	0.65	0.47	0.39	0.71	0.63
	DOH	0.28	0.06	0.19	0.05	0.06
	D1	0.39	0.08	0.26	0.07	0.05
Age	D3	0.54	0.10	0.44	0.10	0.10
(n=8)	D7	0.72	0.13	0.55	0.13	0.14
	D14	0.98	0.17	0.84	0.18	0.13
	SEM	0.17	0.004	0.02	0.007	0.006
	P-value	0.0001	0.0001	0.0001	0.0001	0.0001
	Duodenum	0.85^{a}	0.11^{a}	0.66^{a}	0.12^{a}	0.11^{a}
	Jejunum	0.53^{b}	0.11^{ab}	0.42^{b}	0.11^{ab}	0.09^{ab}
Segment[2]	Ileum	0.36^{c}	0.10^{b}	0.29^{c}	0.09^{b}	0.08^{b}
(n=8)	SEM	0.13	0.004	0.02	0.005	0.005
	P-value	0.0001	0.0006	0.0001	0.002	0.0001
----------Interaction *P*-value----------						
Interaction[3]	L x A	0.21	0.55	0.81	0.75	0.64
	L x S	0.89	0.88	0.17	0.79	0.63

[1]Distance measurements expressed as distance (mm). Data were subjected to a log base 10 transformation prior to statistical analysis. Data are presented as non-transformed values. Villi

were classified as either “tall” or “short” and measurements were taken accordingly. There was no significant main effect or interaction involving genetic line as the independent variable.

[2]Means in a column without a common letter differ, $P < 0.02$ (Tukey’s test)

[3]For the interactions, A, L and S, represent the main effects of age, genetic line, and segment, respectively.

Appendix B

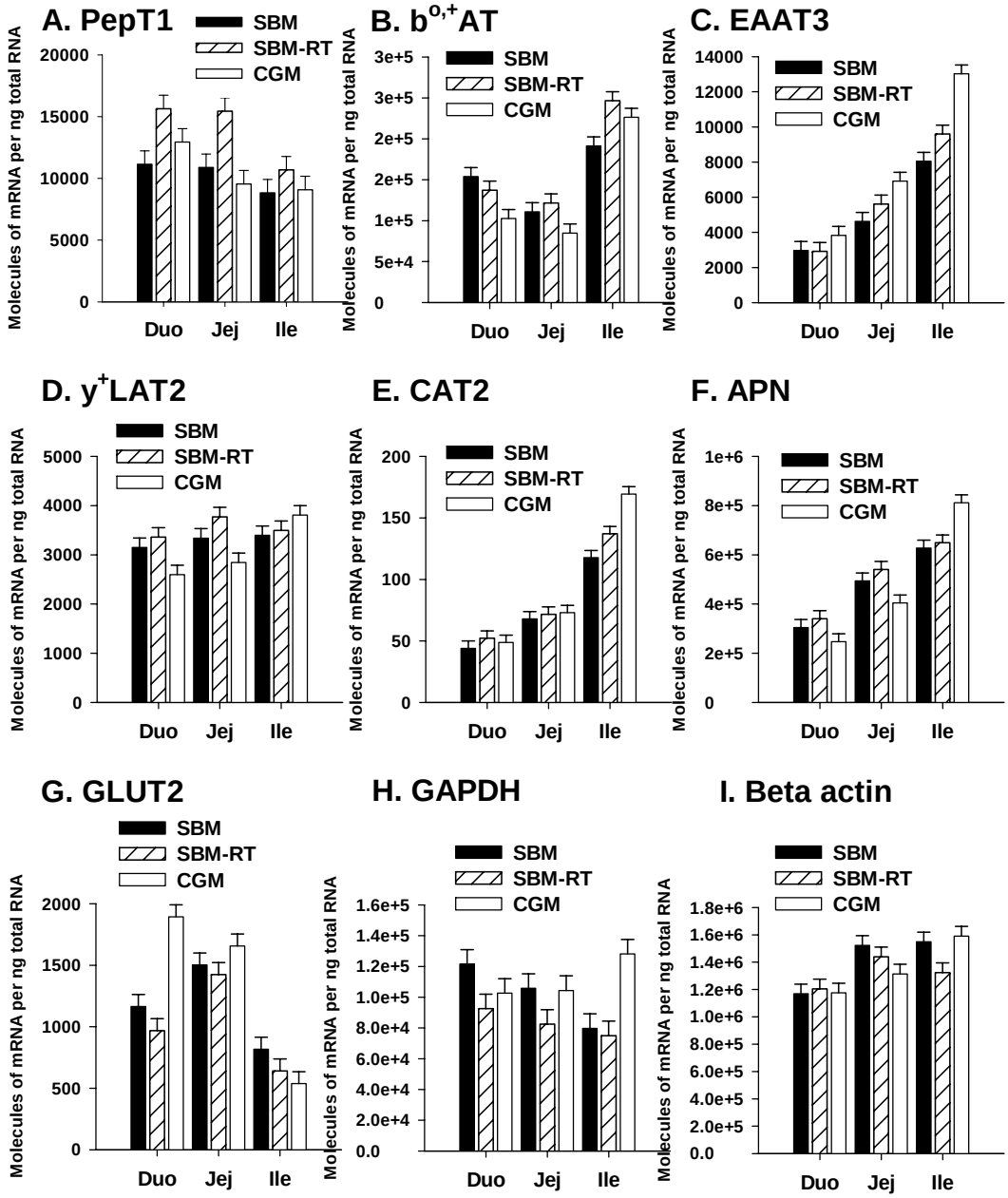


FIGURE B.1 Influence of intestinal segment and diet on mRNA abundance of nutrient transporters, APN, GAPDH and beta actin. The figure represents the number of molecules of mRNA per nanogram of total RNA for A) PepT1, B) $b^{o,+}$AT, C) EAAT3, D) y^{+}LAT2, E) CAT2,

F) APN, G) GLUT2, H) GAPDH, and I) beta actin in the three segments of the small intestine (Duo = duodenum, Jej = jejunum, Ile = ileum) of broiler chicks subjected to feed restriction and consumption of diets differing in protein quality. Diets contained either soybean meal (SBM; higher quality) or corn gluten meal (CGM; lower quality) as the protein source in the diet. In addition to ad-lib consumption of both diets, there were also groups of birds that were fed the SBM diet at an intake restricted to that of birds fed the CGM (SBM-RT). In the chicks consuming SBM, PepT1 mRNA changed very little from proximal to distal intestine, in contrast to the chicks consuming restricted quantities of SBM, where mRNA quantities were greater in the duodenum and jejunum, as compared to the ileum ($P = 0.077$). While PepT1 mRNA was lowest in the ileum in the chicks consuming restricted amounts of SBM, in the chicks consuming CGM mRNA was greatest in the duodenum, with little difference between the jejunum and ileum ($P = 0.077$).

The increase in expression of the amino acid transporters, $b^{o,+}$AT, EAAT3, and CAT2, in the distal intestine was more pronounced in feed-restricted birds as compared to chicks fed the SBM diet ad-libitum ($P < 0.003$). In chicks with ad-libitum intake of SBM, y^{+}LAT2 mRNA levels were slightly greater in the distal intestine, while in chicks with restricted intake, there was slightly higher expression in the mid-region of the intestine. In response to feed restriction, jejunal expression of GLUT2 mRNA was greater as compared with expression in chicks that consumed the SBM diet ad-lib (P = 0.0001). In chicks consuming either ad-libitum or restricted amounts of SBM, GAPDH mRNA levels decreased from proximal to distal intestine, with the decrease more accentuated in free-fed chicks ($P = 0.007$). In the chicks with free access to the SBM diet, ileal beta actin mRNA levels were greater than in chicks that consumed the SBM diet ad-lib ($P = 0.0002$).

APPENDIX B

In response to dietary protein quality, many of the genes showed a similar pattern of expression. In particular, the increase from proximal to distal intestine in EAAT3, y^+LAT2, CAT2, APN, GAPDH, and beta actin, expression was more pronounced in chicks that consumed CGM as compared to the chicks consuming restricted quantities of SBM ($P < 0.003$). In chicks consuming the CGM diet, there was decreasing abundance of GLUT2 mRNA from duodenum to ileum, and intermediate abundance in the mid-region, in contrast to the mid-region dominated expression observed in the feed-restricted chicks consuming SBM ($P = 0.0001$).

Appendix C

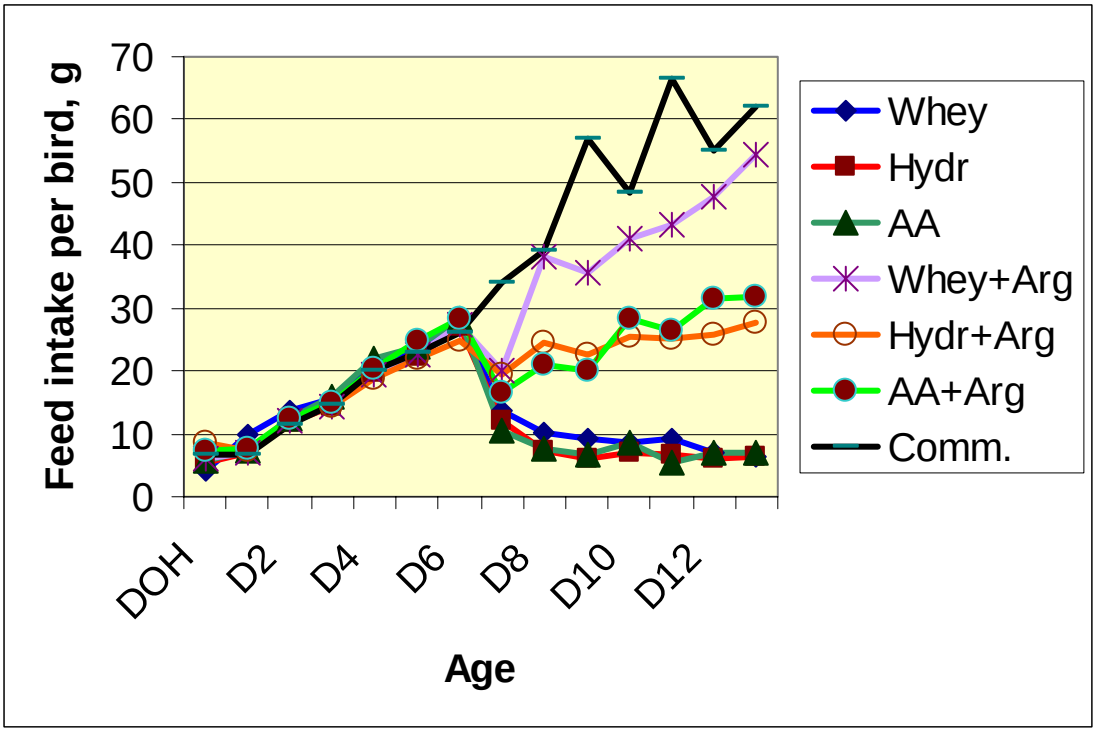


Figure C.1 Feed intake per bird for Cobb-Cobb broiler chicks fed one of seven diets from d 7 to d 14 posthatch. Day of hatch chicks were obtained from George's Hatchery in Harrisonburg, VA. Birds were given a standard corn-soy diet for the first week ad-lib. At d 7 birds were assigned to one of 7 groups: 1) Whey diet (whey protein and no supplemental arginine), 2) Hydr diet (whey hydrolysate and no supplemental arginine), 3) AA diet (AA mixture similar to whey with no supplemental arginine), 4) Whey + supplemental arginine, 5) Hydr + supplemental arginine, 6) AA + supplemental arginine, and 7) Comm. (corn-soy diet for duration of trial). Intake was greatest in birds that remained on the commercial diet. The diets supplemented with arginine had lower intakes that increased with age. Birds that consumed the diets not supplemented with arginine ate less each day to an amount that plateaued after 10 days.

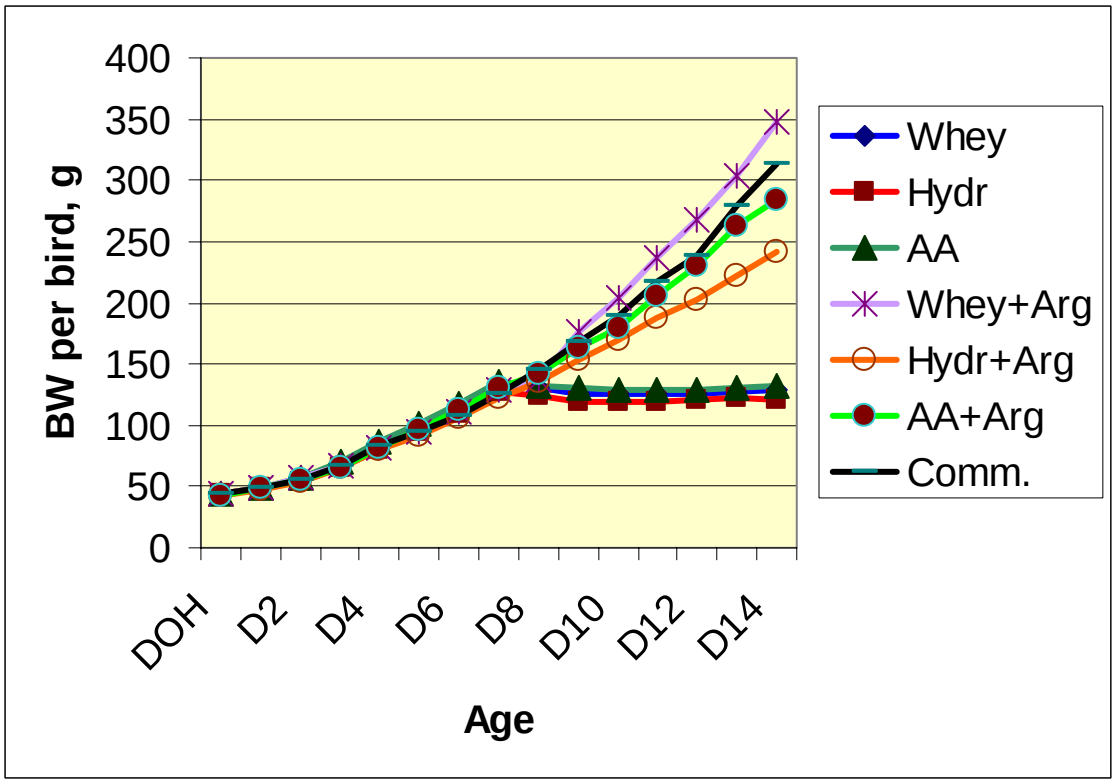


Figure C.2 Body weights for Cobb-Cobb broiler chicks fed one of 7 diets from d 7 to d 14 posthatch. Day of hatch chicks were obtained from George's Hatchery in Harrisonburg, VA. Birds were given a standard corn-soy diet for the first week ad-lib. At day 7 birds were assigned to one of 7 groups: 1) Whey diet (whey protein and no supplemental arginine), 2) Hydr diet (whey hydrolysate and no supplemental arginine), 3) AA diet (AA mixture similar to whey with no supplemental arginine), 4) Whey + supplemental arginine, 5) Hydr + supplemental arginine, 6) AA + supplemental arginine, and 7) Comm. (corn-soy diet for duration of trial). Body weights increased linearly in the birds fed either the commercial diet or one of the three diets supplemented with arginine. Body weights plateaued for birds that consumed diets not supplemented with arginine.

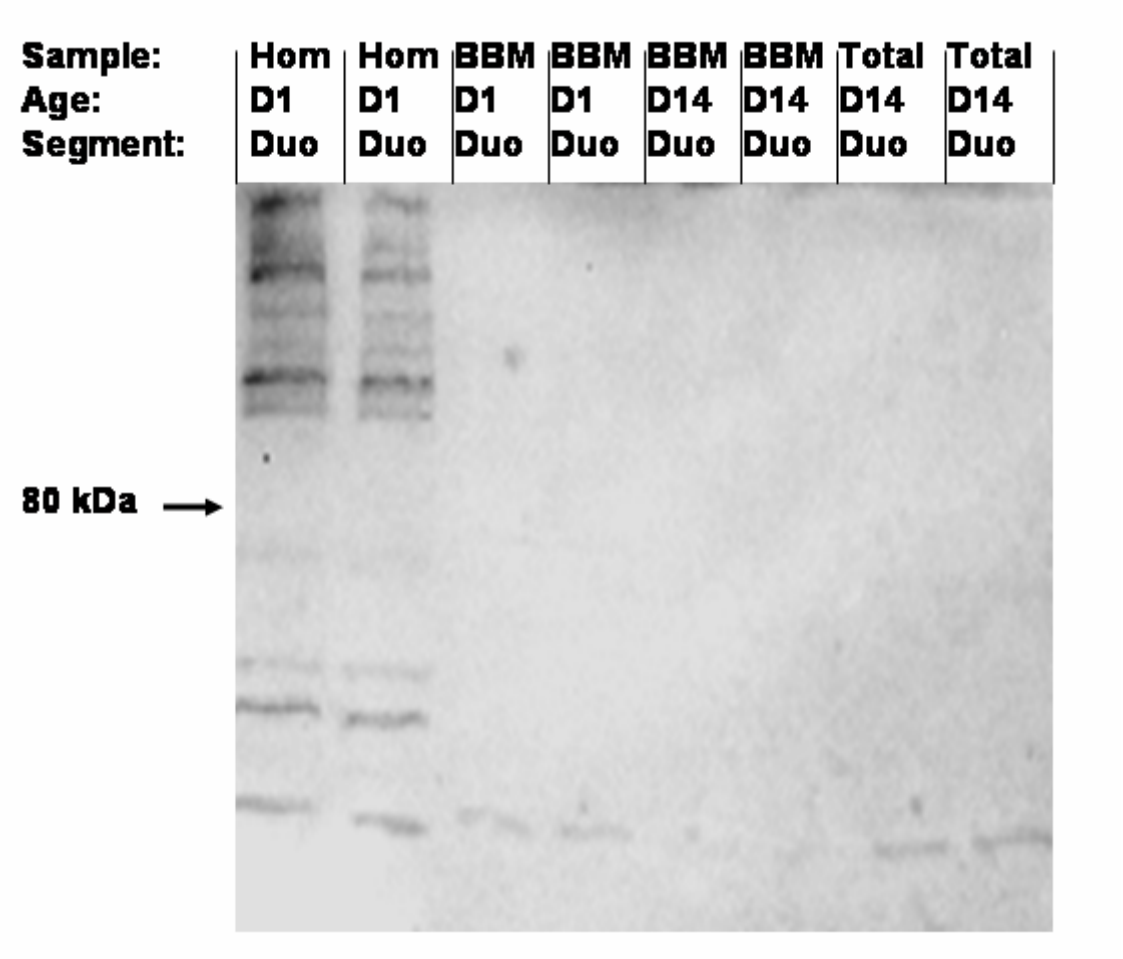


Figure C.3 Western blot analysis of various chicken intestinal protein extracts with rabbit pre-immune sera diluted at 1:500 in 1 % non-fat dried milk in TBST. Hom = homogenate of total intestinal tissue, BBM = brushborder membrane vesicles, Total = total protein extract of total intestinal tissue, D1 = Day 1 posthatch, D14 = Day 14 posthatch. Thirty micrograms of protein were loaded into each well.

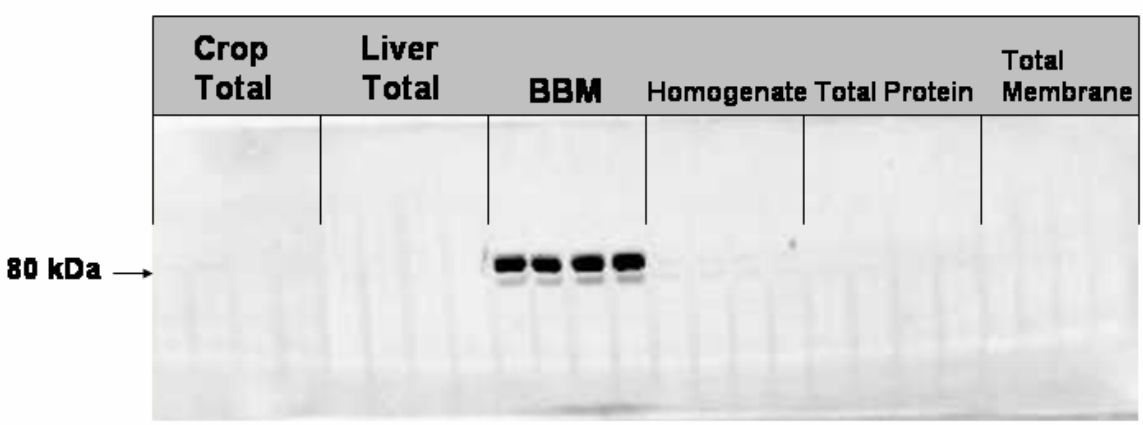


Figure C.4 Western blot analysis of various chicken tissues using a polyclonal rabbit anti-chicken PepT1 antibody diluted at 1:500 in 1 % non-fat dried milk in TBST. Twenty micrograms of protein were loaded into each well except for "Crop Total" where 40 micrograms were loaded. Crop, Liver, and Total Protein wells represent total protein extracted from crop, liver, and duodenum, respectively. Total protein is from duodenum of Line B at Day 13. The BBM = brushborder membrane vesicles from duodenum of Line A at Day 1, and homogenate represents homogenate collected from the beginning of the BBM procedure (from same sample as used to make BBM in BBM wells). Total membrane represents total membranes (brushborder + all other membranes) isolated from duodenum of Line B at Day 13.

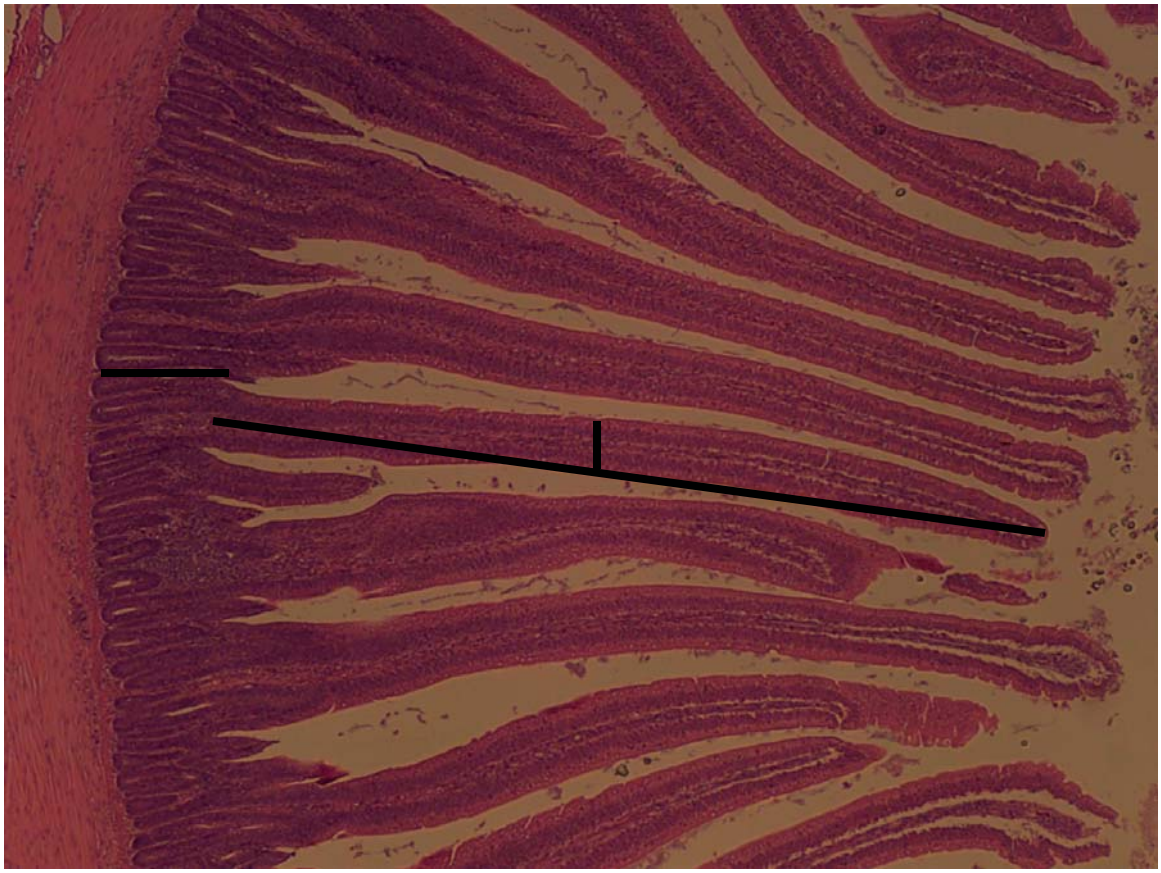


Figure C.5 Example of morphometric measurements taken on a picture of a cross-section of chicken small intestine. Shown are examples of villus height, villus width and crypt depth.