

SMALL INTESTINAL TRANSPORTERS IN TWO SPECIES OF GALLIFORMES:
MALE AND FEMALE TURKEY (*Meleagris gallopavo*) AND CHICKEN (*Gallus gallus*
domesticus)

by

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ABSTRACT

The objective of the first study was to characterize amino peptidase N (APN), peptide (PepT1), amino acid (ASCT1, b⁰⁺AT, CAT1, EAAT3, LAT1, y⁺LAT2), and sugar transporter expression (GLUT2, GLUT5, SGLT1) in the small intestine of male and female turkeys. Small intestine samples were collected during embryonic development (E21, E24) and DOH. In a separate experiment during post-hatch development (DOH, D7, D14, D21, D28). APN, b⁰⁺AT, PepT1, y⁺LAT2, GLUT5 and SGLT1 were expressed most on DOH. Post-hatch, all genes except GLUT2 and SGLT1 were expressed greater in females than males. SGLT1 was expressed greater in males. Basolateral transporters were expressed more during early development; while there was more expression of brush border transporters EAAT3, GLUT5 and SGLT1 later in development. In chickens, there are alternatively spliced exons of the PepT2 gene that encode proteins with four different N-termini (Variants 5-8). The objective of this study was to characterize the patterns of expression of these PepT2 variants. Brain, kidney, liver and intestine were analyzed at E18 and D7 (n=5). Expression of Variant 5 was most prominent in the brain and variant 6 was most prominent in the kidney. Variant 8 appeared in all tissues on E18 and D7. Variant 7 was only expressed in late embryonic development in the ileum. Results from these studies demonstrate that there are differences in gene expression of nutrient transporters in two agriculturally important avian species from the same order *Galliformes*. These differences can be used to improve feed efficiency and enhance the growth of both species.

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Chapter 1

Introduction

Turkey production is a nearly five-billion dollar industry in the United States. However, progress in turkey genome sequencing is far behind sequencing of other species of agricultural importance (Dalloul et al., 2014). Completion of the chicken genome, an equally important poultry species in the United States was completed in 2004 (Chicken Genome Sequence Consortium, 2004). While both species are members of the order *Galliformes* and both are grown commercially in the United States, from a molecular standpoint much more information is known about the commercial chicken than commercial turkey. This disparity makes for an underutilization of resources that could improve overall nutrition in turkey.

The literature review for this thesis will focus on the morphological and physiological changes that occur in the small intestinal tract during late stage embryonic development (E24) through four weeks of age (D28) in turkey. A basic understanding of embryonic and early post-hatch development, along with information about the gastrointestinal lining as it applies to nutrition will lay the groundwork for the two studies presented in this thesis. While the change is well documented in commercial chicken species, there is little research in turkey—an equally important commercial poultry species in the United States.

As the chick transitions from relying on the lipid-rich yolk to a mainly carbohydrate and protein diet, intestinal changes are characterized by differing expression of nutrient transporters. These transporters are responsible for absorbing oligo-peptides, amino acids, and sugars from the diet. The objective of the turkey study is to examine differences between male and female turkeys, and to characterize expression of nutrient transporters from late embryonic development

to week four. The objective of the second study is to characterize variants of the oligo-peptide transporter PepT2 in chicken.

Chapter 2

Literature Review

Introduction

Galliformes. *Galliformes* is an order of birds belonging to the Class Aves. There are over 250 species belonging to five families: Phasianidae, Odontophoridae, Numididae, Cracidae, and Megapodiidae. Turkeys and chickens are both in the Phasianidae family (Figure 2.1).

Galliformes birds are characterized by their large size, stocky body, small head and short wings. Old World quails (*Coturnix chinensis*) are the smallest member weighing in at about 1 oz., and wild turkeys (*Meleagris gallopavo*) weigh 17-22 pounds as adults (though domesticated commercial turkeys can weigh much more) (Hughes, 2004).

The order *Galliformes* has been largely studied because many of its members are used as dual models for human health and agricultural production (Kaiser et al., 2007). Physiological evidence (Maxwell, 2008) and molecular data (Kaiser et al., 2007) support the conclusion that the chicken ancestor (*Gallus gallus*) and turkey ancestor (*Meleagris gallopavo*) are not as closely related as some of the other members of this order, thus characterizing intestinal transporters for each separate species is important.

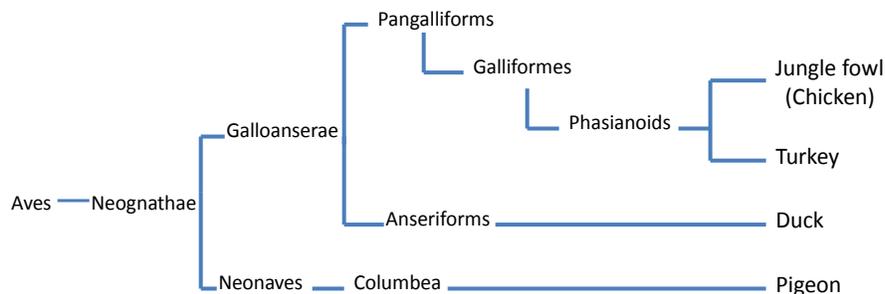


Figure 2.1. Taxa of some agriculturally important poultry species.

Late Embryonic Development

Embryo development in turkey, like chicken, is divided into three stages. The first stage is germ establishment (day 1-8), the second is embryo completion (day 9 to 24), and the third stage is preparation for emergence (day 25-28) (Moran, 2007). During late embryogenesis, the chick receives nutrients from the yolk via the blood stream, and the small intestine. The yolk can continue to provide nutrients until as late as four days after hatch (Noy and Sklan, 1998). During mid-incubation to about 2-3 days before internal pipping, fatty acids from the yolk are the main source of energy via β -oxidation. Because glucose levels are low and oxygen is not limiting, the triacylglycerol is broken down and the glycerol carbon backbone can be made into glucose via gluconeogenesis and fatty acids are oxidized. This process serves to accumulate glycogen in the liver. This is paramount because this stored glycogen is the main glucose source between internal pipping and the first exogenous feeding when oxygen is low.

Hormones also regulate growth of the late stage embryo. Insulin increases during development until internal pipping, and then increases again about 24 hours after the first feeding. Glucagon is the hormone responsible for keeping most avian species in a hyperglycemic state (de Oliveira et al., 2008). A week before hatch glucagon increases, which is necessary because, the liver and muscle are storing glycogen at this time in preparation for hatch. Three days before hatch, both glucagon and insulin are both acting on the liver, however the glucagon is not sensitive to the insulin and thus glucose is both stored and utilized simultaneously (Lu et al., 2007).

Intestinal Morphology

During late stage embryogenesis and immediately post-hatch, the small intestine increases in weight more than total body weight. The largest increase in both chicks and poults is

observed about six to eight days post-hatch (Sklan, 2001; Uni et al., 2003; Lilburn and Loeffler, 2015). This weight increase is also observed in domestic pigeon (*Columba livia*) and though the domestic pigeon is not a member of the order *Galliformes*, these findings further suggest that a great portion of body investment is dedicated to intestinal growth (Dong et al., 2012). Increased body investment to the small intestine is achieved by the internalization of the yolk, which is then used as a fuel source. Body investment provided by the yolk is independent of whether exogenous feed is administered. During hatch, the enterocytes are rounded, but within twenty-four hours after hatch they develop polarity as well as a defined brush border. Within 144 hours in the jejunum and 216 hours in the duodenum hypertrophy occurs. However, in the ileum, polarization, brush border membrane scaffold formation and hypertrophy occur simultaneously, suggesting that enterocytes in the ileum are more mature at hatch than the other two segments (Geyra et al., 2001).

At hatch, the villi are also small and undeveloped and the crypts cannot be detected. After two to three days of continual growth the crypts become well defined (Geyra et al., 2001; Sklan, 2001). Furthermore, the differing segments plateau at different rates, with the duodenum and jejunum continuing to grow after the ileum has reached a constant ratio of crypts per villus. Villus height increases 2-fold 48 hours after hatch, and plateaus first in the duodenum in six to eight days after hatch, but in the jejunum and ileum it takes ten days to reach a plateau. The villus also widens at this time, increasing the surface area for nutrients to be absorbed.

While pancreatic enzymes needed for digestion such as trypsin, amylase, and lipase are present at hatch, their activity is increased with feed intake (Sklan, 2001). Poults have mucosal enzymes capable of breaking down carbohydrates even before a first feeding, indicating the ability to break down carbohydrates before they are introduced in the feed. Mucosal enzymes

become prominent at different times, in different sections of the small intestine, especially in poults. These differences will affect the digestion of feed, and overall body weight gain of the growing bird.

Compared to other species, poults are considered to have an underdeveloped gastrointestinal tract. Turkey poults and Pekin ducklings that hatch from eggs of similar weight, incubation periods, and body weights at hatch develop very differently (Applegate et al., 2005). Aside from the weight of the duodenum, ducklings have a significantly heavier jejunum and ileum and longer small intestinal tract overall from hatch to day 7. Ducklings also have a greater villus height and greater crypt depth than poults on day of hatch to day 7. Because the small intestine is considered the supply organ for growth, especially in the days following post-hatch, it is not unexpected that turkeys weigh significantly less than ducklings on day 7 because their supply organ is slower to grow in both size and surface area.

Diets in Commercial Turkeys

The National Research Council last published guidelines for poultry nutrition in 1994. Although these guidelines are still referenced when formulating a poultry diet, many would argue that they are too outdated. This is partly due to the fact that very little research has been performed about feed efficiency due to the difficulty of quantification and labeling of feed sources. Therefore, outdated methods using purified ingredients that by today's standards are not suitable for modern day commercial procedures are still being considered to formulate diets (Leeson, 2011). Table 2.1 shows how modern day diets have deviated from NRC guidelines. While it is understood that feed formulations are not absolute, it is interesting to see the changes that have occurred.

In comparison to turkey, there has been extensive research in optimizing chicken diets. Improvements range from investigating microbiota to improving feed efficiency (Stanley et al., 2012), to quality comparisons based on genetics (Aggrey et al., 2010). Table 2.2 compares a modern day chicken broiler feed formulation and a few amino acids to the NRC guidelines. Broiler chickens are grown to about 6 weeks of age, thus they reach market weight long before turkeys.

Table 2.1. Sample of feed requirements for turkey.

	Modern Commercial Turkey Diet	NRC Guidelines Turkey
Age (weeks)	0-4 weeks	0-4 weeks
Crude protein %	28	28
Metabolizable energy kcal/kg	2,850	2,800
Dig. Met %	0.56	0.55
Dig. Met + Cys.%	0.96	1.05
Dig. Lys %	1.55	1.6

Data taken from a modern day commercial turkey diet (Leeson, 2011) vs NRC Guidelines for Turkey (1994).

Table 2.2. Sample of feed requirements for chicken broilers.

	Modern Broiler Diet	Modern Broiler Diet	NRC Guidelines	NRC Guidelines
Age	0-15 days	16-27 days	0-3 weeks	3-5 weeks
Crude protein %	22	20	23	20
Metabolizable energy kcal/kg	3,050	3,100	2,300	2,300
Dig. Met %	0.46	0.40	0.50	0.38
Dig. Met. + Cys. %	0.85	0.78	0.90	0.72
Dig. Lys %	1.25	1.18	1.10	1.00

Data taken from a modern day commercial broiler diet (Leeson, 2011) versus NRC Guidelines for Chicken (1994).

Limitations in Turkey Production. Raising commercial turkeys is especially challenging when compared to other species of commercial poultry. Turkeys tend to have microbial or physiological stress that promotes pathogens in the intestine and decreases digestibility. Temperature extremes from moving from brooding farms to growing farms negatively affect nutrient digestibility. Furthermore, flushing (diarrhea) is also a problem possibly due to feed composition (Leeson, 2011). Formulating pre-starter and starter diets is difficult because of the necessity for large amounts of lysine and arginine. These diets need to be comprised of 28% to 30% crude protein (CP). SBM and corn meal are often limiting in methionine and other sulfur-containing amino acids (Lu et al., 2007). Lysine levels for turkeys are very similar to other meat birds, though turkeys are especially responsive to lysine. There is poor efficiency with high-energy diets because amino acid contents do not account for the inevitable decreased feed intake. Turkey hens are raised to 11-15 weeks and toms are raised to 15-22 weeks of age (Leeson, 2011).

A recent study comparing Pekin duck and turkey intestinal morphology indicated that poults weighed significantly less than ducklings on day 3 post-hatch. The small intestine of the turkey is not as developed at hatch and does not develop as quickly as the ducklings. This was determined by measuring villi height, crypt depth, and length of the duodenum, and jejunum-ileum segments of the small intestine (Applegate et al., 2005). Although turkey and Peking duck are not members of the same order, both are commercially produced. Thus, if turkey intestine is not as developed as other production birds early post-hatch, it might explain why they are prone to so many problems early on. A better understanding of the limitations this under-development poses may increase the survival of a young poult.

Advances in Turkey Production. There have been many advances in turkey production in the last four decades. Havenstein et al. (2007) performed a study comparing 1966 turkeys fed diets formulated in 1966 with modern day turkeys fed diets formulated in 2003. Growth rate to market age has doubled, and body weights of toms and hens have been increasing 208 and 140 grams/year, respectively. Furthermore, feed efficiency was 20% better in 2003 in a modern turkey fed a modern diet than a 1966 turkey fed a 1966 diet.

Limitations in Nutrient Profiles. Obtaining accurate quantification of nutrient profiles at different stages of development is not easy. A compounding problem is also poor quality control of nutrient requirements such as amino acids and assessing digestibility of a certain ingredient is near impossible. Factoring in the variability of different plants, different ambient temperatures, and other factors, nutrient requirements are a lax guideline at best. Protein and energy are often the most expensive part of a poultry diet. It is impossible to quantify both of these with any type of accuracy or precision in real time (Leeson, 2011). With advances in molecular biology, it may become pertinent and necessary to examine the gastrointestinal tract's ability to absorb nutrients rather than the digestibility of a certain nutrient as digestibility does not translate into absorption capacity.

Nutrient Transporters

Intestinal transporters are members of the solute carriers (SLC) family of genes. Currently, there are 400 transporter genes identified belonging to 47 families (Schlessinger et al., 2010). Transporters are defined as gatekeepers of the cells and allow influx or efflux of solutes through passive, active, exchange, coupled and ion-channel transport. Typically, each transporter has a large domain that spans the membrane with ten to fourteen α -helices. Transporters absorb oligo-peptides, amino acids, and sugar, and can also absorb nucleic acids, inorganic ions, and

pharmaceuticals. Passive transport allows solutes into the cell down their electrochemical gradient. Active transport utilizes ion/solute gradients across membranes utilizing either ATP hydrolysis or ATP binding. Transporters that act as exchangers or utilize coupled transport move one solute into the cell while simultaneously moving another solute out of the cell. Ion channels act similarly to passive transporters, where the solutes move down their electrochemical gradients (Hediger et al., 2004).

SLCs are usually located on one of the two plasma membranes of enterocytes. A transporter located at the brush border membrane faces the intestinal lumen and transports nutrients from the lumen into the enterocyte. A transporter located at the basolateral membrane is responsible for transport of nutrients to or from the bloodstream (Figure 2.2)

Because there are so many transporter genes, 12 genes representing the larger categories; peptidases, oligo-peptide transporters, amino acid transporters and sugar transporters were chosen for this research. These include aminopeptidase N (APN), peptide transporters 1 and 2 (PepT1 and PepT2), excitatory amino acid transporter 3 (EAAT3), alanine, serine, cysteine and threonine transporter 1 (ASCT1), cationic amino acid transporter 1 (CAT1), large neutral amino acid transporter 1 (LAT1), y^+ L-amino acid transporter 2 (y^+ LAT2), cationic and large amino acid exchanger ($b^{0,+}$ AT). The sugar transporters include; sodium glucose transporter 1 (SGLT1), glucose transporter 2 (GLUT2) and glucose transporter 5 (GLUT5). These transporters are further described in Table 2.3.

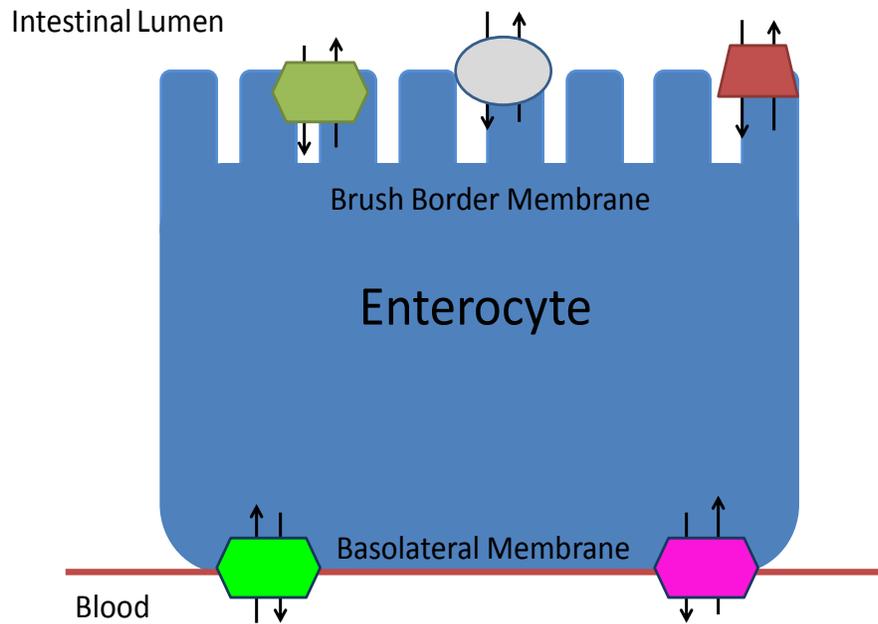


Figure 2.2. A simplified diagram of an enterocyte

The enterocyte is a polarized intestinal cell that has two membranes. The brush border membrane is a microvilli, which serves to increase surface area. Nutrients are transported either to or from the intestinal lumen. The basolateral membrane transports nutrients to or from the bloodstream.

Table 2.3. List of nutrient transporters

Gene	Name	Location	Accession #	Function
APN ⁶	Aminopeptidase N	BB	XM_003209631	N-terminal peptidase with affinity to cleave non-polar amino acids (Ala, Met, Leu, Tyr)
EAAT3 ¹	Excitatory amino acid transporter 3 (SLC1A1)	BB	XM_010725627	Na ⁺ /K ⁺ exchange dependent transporting mainly glutamate
ASCT1 ¹	Alanine, serine, cysteine and threonine transporter 1 (SLC1A4)	BL	XM_010706509	Na ⁺ -dependent exchanger of neutral amino acids (mainly Ala, Ser, Cys, Thr)
SGLT1 ³	Sodium glucose transporter- 1 (SLC5A1)	BB	XM_003211023	Na ⁺ -coupled glucose and galactose transporter
GLUT2 ²	Glucose transporter 2 (SLC2A2)	BL	XM_010716927	Facilitated fructose, galactose, mannose and glucose transporter
GLUT5 ²	Glucose transporter 5 (SLC2A5)	BB	XM_010722761	Facilitated fructose transporter
CAT1 ⁴	Cationic amino acid transporter 1 (SLC7A1)	BL	XM_003203401	Cationic amino acid transporter
LAT1 ⁴	Large neutral amino acid transporter 1 (SLC7A5)	BL	NM_001030579	Large, neutral amino acid exchanger
y ⁺ LAT2 ⁴	y ⁺ L amino acid transporter 2 (SLC7A6)	BL	XM_010717824	Na ⁺ -independent cationic/Na ⁺ -dependent large amino acid exchanger
b ^{0,+} AT ⁴	Solute carrier family 7, member 9 (SLC7A9)	BB	XM_010717754	Cationic and large amino acid exchanger
PepT1 ⁵	Peptide transporter 1 (SLC15A1)	BB	NM_001303166	H ⁺ -dependent di- and tri- peptide transporter
PepT2 ⁵	Peptide transporter 2 (SLC15A2)	BB	XM_004942923	H ⁺ -dependent di- and tri- peptide transporter

¹(Kanai et al., 2013), ²(Mueckler and Thorens, 2013), ³(Wright, 2013), ⁴(Fotiadis et al., 2013), ⁵(Smith et al., 2013), ⁶(Mane et al., 2010)

Abbreviations: BL (Basolateral), BB (Brush Border)

Brush Border Peptidase. Aminopeptidase N (APN) is characterized as a low affinity enzyme that has a preference for cleaving Ala, Met, Leu, and Tyr at the N-terminus of peptides (Mane et al., 2010). APN is active within a wide pH range, but functions best between pH 6 and 7.5 and is inhibited by heavy metal ions except for Mn^{2+} (Jamadar et al., 2003). In two lines of broiler chickens, APN expression was highest in the ileum, lowest in the duodenum and intermediate in the jejunum. Furthermore, overall APN increased from E20 to D14 (Gilbert et al., 2007). There was no difference in embryonic APN expression between Cobb and Leghorn breeds, however in Ross broilers, there was greater expression in the duodenum than in the midgut (jejunum and ileum). Overall, APN expression increased during late embryonic development (Speier et al., 2012; Miska et al., 2014). During post-hatch development, expression of APN peaked around D14 in the small intestine (Miska et al., 2015). APN mRNA abundance was greater than any nutrient transporter for all studies (Gilbert et al., 2007; Speier et al., 2012; Miska et al., 2014; 2015). APN in pigeon demonstrated a similar pattern where there was an overall increase from E12 to D14. Expression was greatest in the ileum compared to duodenum and jejunum (Dong et al., 2012).

In summary, embryonic APN expression patterns differ between different lines of chicken. There was no change in expression in the intestinal segments of Cobb and Leghorn, while Ross embryos expressed APN more in the duodenum than in the jejunum and ileum. Most breeds showed peak APN expression in late embryonic development. APN in post-hatch broilers and pigeons increased from late embryonic development to about two weeks of age in all tissues. Broilers and pigeon have greatest expression in the ileum compared to duodenum and jejunum.

Brush Border Amino Acid Transporters. The enterocyte is capable of absorbing free amino acids into the cell from the intestinal lumen. The brush border transporters characterized

in this thesis come from two families: SLC1 and SLC7. The SLC1 family has 7 members, which are responsible for high-affinity glutamate and neutral amino acid transport. The SLC7 family is responsible for cationic and glycoprotein-associated amino acid transport (Hediger et al., 2004).

Excitatory amino acid transporter 3 (EAAT3, SLC1A1) is a brush border membrane exchanger that is dependent on inward Na^+ electrochemical potential and the outward K^+ potential in order to transport glutamate against its concentration gradient inside the cell (Kanai et al., 2004). Glutamate is a major fuel source of the enterocyte. Mice fed high protein diets expressed EAAT3 more than mice fed low protein diets, indicating that the enterocyte can upregulate glutamate intake in the presence of high protein content in the intestinal lumen (Erickson et al., 1995). In pigs, exogenous glutamate is the primary source of glutathione synthesis in the intestinal cell (Reeds et al., 1997). Histology analysis of intestinal cells showed that EAAT3 is located in the upper regions of the crypts and on the basal half of villi. This localization indicated that EAAT3 was transporting glutamate well before the enterocyte was fully differentiated and therefore supported the idea that the enterocyte relied on glutamate as a main energy source (Iwanaga et al., 2005).

In chicken broilers EAAT3 linearly increased from E20 to D14 and the greatest expression was found in the ileum, with the lowest in the duodenum, and the jejunum was intermediate (Gilbert et al., 2007). There was no difference between embryonic intestinal EAAT3 expression in Cobb and Leghorn breeds, but Ross broilers expressed more EAAT3 in the midgut (jejunum and ileum) than the duodenum (Speier et al., 2012; Miska et al., 2014). In both Cobb and Leghorn, expression of EAAT3 increased with embryonic age (Speier et al., 2012), but in Ross broilers expression decreased with embryonic age (Miska et al., 2014). During

post-hatch overall expression of EAAT3 in the small intestine increased with age (Miska et al., 2015).

The brush border transporter $b^{0,+}$ AT (SLC7A9) is responsible for transporting mainly cysteine (Leclerc et al., 2002) and other cationic amino acids (Hediger et al., 2004). In humans, $b^{0,+}$ AT mutations are associated with a genetic disease cystinuria. Patients with cystinuria have decreased cysteine and basic amino acid absorption into the small intestine and the kidney. Increased levels of cysteine and basic amino acids led to crystallization in the urinary tract (Leclerc et al., 2002).

In broiler chickens, $b^{0,+}$ AT is not expressed differently from E20 to D14; however, expression is highest in the ileum compared to the other segments of the small intestine (Gilbert et al., 2007). Expression of $b^{0,+}$ AT increased with embryonic development for broilers (Zeng et al., 2011; Miska et al., 2014). During embryonic development, Ross broilers expressed more $b^{0,+}$ AT in duodenum than in the jejunum and ileum (Miska et al., 2014). During post-hatch, $b^{0,+}$ AT expression increased around two weeks of age in the duodenum and ileum, and remained largely unchanged in the jejunum (Miska et al., 2015).

In summary, embryonic EAAT3 expression changed between different breeds of chicken. There was no change in EAAT3 expression in the intestinal segments of Cobb and Leghorn, while Ross embryos expressed EAAT3 more in the jejunum and ileum than duodenum. During embryonic development of Ross broilers, $b^{0,+}$ AT was expressed the greatest in the duodenum. In Cobb and Leghorn embryos, EAAT3 increased and peaked at E15 and E17 respectively, while in Ross broilers EAAT3 declined from mid-incubation to E20. Embryonic $b^{0,+}$ AT does not change in broilers. Post-hatch EAAT3, like embryonic EAAT3, expression patterns were variable. One group of broilers demonstrated increased expression from E20 to D14 with the most expression

in the ileum, while another group had varied expression in the three segments. Post-hatch the most expression of b⁰⁺AT was in the ileum. Overall b⁰⁺AT post-hatch expression increased with development in most tissues for differing broiler breeds. One exception was b⁰⁺AT expression in the jejunum of Ross broilers where it remained the same.

Brush Border Peptide Transporters. The SLC15 family has four members responsible for oligo-peptide co-transport with H⁺ (Hediger et al., 2004). Two members of this family include PepT1 and PepT2. PepT1 (SLC15A1) is a H⁺-dependent low affinity/high capacity di- and tri-peptide transporter that uses an electrochemical proton gradient that allows for a higher intracellular concentration of oligo-peptides than extracellular concentration (Spanier, 2014). PepT1 can transport a large variety of oligo-peptides including neutral, cationic and anionic amino acids. Chicken and turkey PepT1 share 94.8% amino acid identity within the transmembrane domains of the transporter, and 88% similarity within the extracellular loop between domains 9 and 10. cPepT1 and tPepT1 share 60% identity with human PepT1 (Van et al., 2005). In chickens, PepT1 increased linearly with development from E20 to D14. Furthermore, mRNA expression was highest in the duodenum, lowest in the ileum and intermediate in the jejunum (Gilbert et al., 2007). During E15 to E21, Ross broilers expressed more PepT1 in the duodenum than in the jejunum and ileum and expression increased with age (Miska et al., 2014). Post-hatch, PepT1 expression increased with age in all intestinal segments (Miska et al., 2015).

PepT1 is present mostly in the gastrointestinal tract of layers compared to other tissues. In all segments expression was highest during late embryogenesis to DOH (Zwarycz and Wong, 2013). Expression of PepT1 was not different between embryonic Cobb and Leghorn small intestine and expression increased with embryonic age (Speier et al., 2012).

In turkeys, there was no difference in expression between embryonic male and female chicks, and embryonic expression increased with age (Van et al., 2005). Turkey PepT1 has a lower affinity but higher transport velocity for the dipeptide Met-Met than that of chicken. Interestingly, turkeys have a higher requirement for methionine than chickens according to the 1994 NRC guidelines (Van et al., 2005). Dong et al. (2012) studied developmental expression of PepT1 in pigeons where PepT1 mRNA levels increased linearly from E12 to D14. Expression was highest in the duodenum, lowest in the ileum and intermediate in the jejunum.

PepT2 (SLC15A2) is a high affinity/low capacity transporter responsible for transporting di- and tri- peptides across the brush border membrane (Smith et al., 2013). PepT2 shares a 50% amino acid identity and 70% similarity with PepT1 and also shares many of the same substrates. In chickens, PepT2 was expressed the most in the kidney and brain. Within the kidney, there was an increase in expression from E20 to D10. Expression was greatest in the brain than any other tissue studied; however, there was no change between E18 and D14. In the intestine, expression was greatest at E18 and decreased in the duodenum by E20 and in the jejunum by D3. In the ileum, expression was greatest at D1. Finally in the liver, PepT2 expression decreased during late embryonic development to D1 (Zwarycz and Wong, 2013).

In summary, PepT1 is a low affinity/high capacity transporter. In embryonic chickens, turkeys, and pigeons, expression of PepT1 increased with age. Duodenum is the location of highest expression in embryonic and post-hatch chickens. Ross broiler chickens express more PepT1 in the duodenum than in the jejunum and ileum. During post-hatch overall PepT1 expression increases with age. In contrast, PepT2 is a high affinity/low capacity transporter expressed mainly in brain and kidney.

Brush Border Sugar Transporters. The enterocyte is capable of transporting sugars from the intestinal lumen into the cell. Two families responsible for sugar absorption include SLC2 and SLC5. The SLC2 family is responsible for facilitative transport of glucose and has 14 members. The SLC5 family has 8 members responsible for co-transporting sodium to the outside of the cell and glucose inside the cell (Hediger et al., 2004).

GLUT5 (SLC2A5) is a brush border transporter that is responsible for facilitated fructose transport (Douard and Ferraris, 2008). In broiler chickens, mRNA abundance of GLUT5 increased linearly from E20 to D14 with greatest amounts observed in the jejunum and ileum compared to the duodenum (Gilbert et al., 2007). The small intestine of Cobb embryos expressed GLUT5 almost 2-fold more than Leghorn embryos of the same age (Speier et al., 2012).

SGLT1 (SLC5A1) is a Na⁺/glucose co-transporter located on the brush border membrane. Embryonic chicken SGLT1 increased with age (Uni et al., 2003). Broiler chicken SGLT1 expression increased linearly from E20 to D14. Expression was greater in the jejunum than in the duodenum and ileum (Gilbert et al., 2007). The small intestine of Cobb embryos expressed SGLT1 more than Leghorn embryos of the same age, though expression increased with age in both breeds (Speier et al., 2012). In pigeons, SGLT1 increased linearly with age (E12 to D14). There was greater expression in the jejunum and ileum than in the duodenum (Dong et al., 2012).

In summary, broiler chickens express both GLUT5 and SGLT1 more than layer chickens. However, sugar transporter expression for both breeds increased during late embryonic development. Post-hatch, expression increased with age in broilers and pigeons and both species have highest expression in the jejunum than in other small intestinal segments.

Basolateral Membrane Amino Acid Transporters. The enterocyte can also absorb free amino acids from the blood stream. Absorption is especially critical during embryonic

development when the chick is not digesting amino acids from exogenous sources. The SLC1, SLC2 and SLC7 families include not only brush border transporters, but also basolateral transporters.

ASCT1 (SLC1A4) is a basolateral membrane transporter that is dependent on the Na⁺ electrochemical gradient present inside the cell to exchange mainly neutral amino acids such as Ala, Ser, Cys. and Thr (Kanai and Hediger, 2004).

CAT1 (SLC7A1) is a Na⁺- and pH independent transporter that is located on the basolateral membrane and has a high affinity for cationic amino acids. (Closs et al., 2006). In broiler chickens, CAT1 mRNA decreased linearly from E20 to D14 and there was no difference between intestinal segments (Gilbert et al., 2007). The small intestine of Cobb embryos expressed more CAT1 than Leghorn embryos of the same age. Expression of CAT1 decreased during late embryonic development (Speier et al., 2012). In a separate study in embryonic Ross broilers, there was more expression of CAT1 in the duodenum than in the jejunum and ileum and expression decreased with age (Miska et al., 2014). In two other lines of broilers, expression of CAT1 increased with embryonic development to DOH (Zeng et al., 2011). Post-hatch, overall expression of CAT1 did not change in the small intestine (Miska et al., 2015).

LAT1 (SLC7A5) transports neutral amino acids with large branched or aromatic side chains and is a basolateral Na⁺-independent transporter (Kanai et al., 1998). In broiler chickens, LAT1 mRNA expression was greatest in the ileum, intermediate in the jejunum, and least in the duodenum. Quantities decreased linearly from E20 to D14 (Gilbert et al., 2007). Embryonic expression in Ross broilers was higher in the duodenum than in the midgut (jejunum and ileum). LAT1 expression decreased during late embryonic development (Miska et al., 2014).

The y⁺L amino acid transporter 2 (y⁺LAT2, SLC7A6) is an amino acid exchanger that transporters cationic amino acids through the basolateral membrane in exchange for neutral amino acids and sodium ions (Fotiadis et al., 2013). In broiler chickens, y⁺LAT2 was not expressed differently in the small intestinal segments (Gilbert et al., 2007). Embryonic expression in Ross broilers was higher in the midgut (jejunum and ileum) than the duodenum and overall expression of y⁺LAT2 increased with embryonic age (Zeng et al., 2011; Miska et al., 2014). Post-hatch, y⁺LAT2 increased with age in the duodenum and ileum, and expression did not change in the jejunum (Miska et al., 2015).

In summary, the highest embryonic expression of basolateral membrane amino acid transporters was usually in the ileum. Embryonic expression of basolateral membrane transporters CAT1, and y⁺LAT2 increased in some breeds during embryonic development. However, overall post-hatch expression of all but y⁺LAT2 decreased with age and the highest expression was observed in the ileum.

Basolateral Membrane Sugar Transporters. Intestinal cells can also transport sugar to and from the bloodstream. The SLC2 family also has members that reside on the basolateral membrane. GLUT2 (SLC2A2) is primarily a basolateral membrane transporter, although in certain conditions can move to the brush border membrane. When GLUT2 is on the basolateral membrane, it is responsible for facilitated transport of fructose, mannose, galactose, and glucose (Kellett et al., 2008).

In chickens, GLUT2 expression increases linearly from E20 to D14. GLUT2 mRNA had greatest expression in the jejunum, least in the ileum and intermediate in the duodenum (Gilbert et al., 2007). In pigeons, GLUT2 expression increased with age. The highest mRNA levels were

present in the jejunum and ileum compared to the duodenum, with the most marked increase with age present in the jejunum (Dong et al., 2012).

Summary. A healthy, well developed gastrointestinal tract is paramount to success and productivity of commercial poultry. During late embryogenesis the intestine is rapidly expanding and increasing surface area in order to prepare for absorption of nutrients. Young turkeys are not as developed as some other commercial species, even those in different taxa. Underdevelopment of the poults at hatch, coupled with turkey production challenges soon after hatch, indicate a need to improve feed efficiency to accommodate all of these challenges. Continued discoveries of intestinal transporter proteins and their targets allow for utilization of feedstuffs leading to increased feed conversion and shortened times for birds to reach market size.

Chapter 3

Expression of Small Intestinal Nutrient Transporters in Embryonic and Post-hatch

Turkeys

ABSTRACT

Nutrients are absorbed in the small intestine through a variety of transporter proteins, a process that has not been as well characterized in turkeys as in chickens. The objective of this study was to characterize amino acid and carbohydrate transporter gene expression in the small intestine of male and female turkeys. Duodenum, jejunum, and ileum were collected during embryonic development (E21, E24 and DOH) and in a separate experiment during post-hatch development (DOH, D7, D14, D21, D28). Real-time PCR was used to determine expression and ANOVA was used for statistical analysis of mRNA abundance of aminopeptidase N (APN), oligo-peptide transporter 1 (PepT1), 6 amino acid (ASCT1, b⁰⁺AT, CAT1, EAAT3, LAT1, y⁺LAT2) and 3 sugar transporters (GLUT2, GLUT5, SGLT1). APN, b⁰⁺AT, PepT1, y⁺LAT2, GLUT5 and SGLT1 showed increased expression from E21 and E24 to DOH. Post-hatch, all genes except GLUT2 and SGLT1 were expressed greater in females than males. GLUT2 was expressed the same in males as females and SGLT1 was expressed greater in males than females. All basolateral membrane transporters were expressed greater during early development and then decreased with age; while EAAT3, GLUT5 and SGLT1 showed increased expression later in development. EAAT3 showed a 2-fold change from DOH to D14. This new knowledge can be used to not only better formulate turkey diets to accommodate increased glutamate transport, but to also customize nutrition for both sexes.

Key words: nutrient transporters, turkey

INTRODUCTION

The transition from the yolk to the intestinal lumen as the main source of nutrients for a chick is well documented (Noy and Sklan, 1998; Moran, 2007). Thus, understanding developmental changes in transporter expression in the intestine is of interest to better utilize nutrients in feedstuffs in poultry. Transporters are located at both the brush border membrane of intestinal enterocytes for transport of nutrients from the intestinal lumen into the cell and at the basolateral membrane for transport of nutrients into or out of the bloodstream.

Nutrient transporters belong to the solute carrier gene family. Amino acids are transported either as free amino acids through a variety of neutral, anionic, or cationic amino acids or as short peptides through peptide transporters. For example, the alanine, serine, cysteine and threonine transporter (ASCT1) and excitatory amino acid transporter- 3 (EAAT3) are members of the SLC1 family, which transport mainly neutral and anionic amino acids, respectively. Solute carrier family 7 members include $b^{0,+}AT$, cationic (CAT1), large amino acid (LAT1), and y^+L amino acid (y^+LAT2) transporters. The peptide transporter PepT1 is a member of the SLC15 family, which transports small oligo-peptides. Carbohydrate transporters are also members of the SLC gene family. The sodium-glucose co-transporter SGLT1 belongs to the SLC5 family and the facilitated sugar transporters GLUT2 and GLUT5 are members of the SLC2 family and transport glucose, galactose, mannose and fructose.

The developmental expression of nutrient transporters has been profiled during the embryonic and post-hatch periods in chickens (Gilbert et al., 2007; Zeng et al., 2011; Speier et al., 2012; Zwarycz et al., 2013; Miska et al., 2014; 2015) and pigeons (Dong et al., 2012). Amino acid transporters are generally expressed greater in the distal than the proximal part of the small intestine, whereas the sugar transporters are expressed greater in the jejunum than the

duodenum and ileum. Many brush border membrane transporters showed increased expression with developmental age, whereas basolateral membrane transporters showed decreased expression. In turkeys, the expression patterns of a peptide (PepT1) and sugar (SGLT4) transporter have been examined in the duodenum of embryos from embryonic day 20 (E20) to DOH (de Oliveira et al., 2008). The objective of this study was to provide a comprehensive profile of the expression of a digestive enzyme, one peptide transporter, six amino acid, and three sugar transporters during late embryogenesis and during the first 4 weeks post-hatch in turkeys.

MATERIALS AND METHODS

Birds and Tissue Collection.

For the pre-hatch experiment, 100 fertile turkey eggs were obtained from AgForte (Harrisonburg, VA). Eggs were incubated at 37.5 °C and 55% humidity with rocking every 45 minutes. Most turkeys hatched early on E27. On embryonic day 21 (E21), E24 and DOH jejunum was collected from 6 males and 6 females, which were visually sexed. Jejunum was sampled due to the difficulty of collecting individual intestinal segments. All samples were stored at -80°C until RNA extraction.

For the post-hatch experiment, 50 male and 50 female DOH turkey poults (AgForte, Harrisonburg, VA) were housed separately on large floor pens with fresh wood shavings and provided with starter commercial poult feed and water ad libitum (Kim et al., unpublished). On DOH and days 7, 14, 21 and 28 post-hatch, duodenum, jejunum, and ileum segments were collected from 6 males and 6 females and stored in *RNAlater* (Life Technologies, Grand Island, NY).

The sex of the birds was further verified using PCR. Genomic DNA was extracted using the manufacturer's protocol for the Quick-gDNA™ kit (Zymo Research, Irvine, CA) and quantified using a Nanodrop (Thermo Scientific, Wilmington, DE). PCR reactions contained 12.5 µL of AccustartII PCR SuperMix (Quanta Biosciences, Gaithersburg, MD), 1 µL of each of the four primers (5 nM), 7.5 µL of DEPC treated-H₂O, and 1 µL of DNA (1 ng/µL). Two primers for the W Chromosome (5'-GGGTGTAACATGAGAAGAAC-3' and 5'-GCACAGATGGAGACAAAAGC-3') (Kalina et al., 2012) and two primers for the autosomal gene *PepT1* (5'-TTGTCTCCCTGTCCATTGTCTATAC-3' and 5'-

GTTCTTCAAAGTATCCCCACCAAA-3') were used. The PCR conditions were: 94 °C for 10 minutes followed by 30 cycles of 94 °C for 30 seconds, 54 °C for 20 seconds, and 72 °C for 40 seconds. The PCR products were separated on a 1% agarose gel. Male turkeys lacking the W chromosome only exhibited the PepT1 band of 384 bp. Female turkeys exhibited two bands of 384 bp and 565 bp corresponding to PepT1 and the W chromosome, respectively.

RNA Extraction and Relative qPCR

Total RNA was extracted from the embryonic (E21, E24, and DOH) jejunal samples as per the Direct-zol™ RNA Miniprep (Zymo Research) protocol and from the post-hatch samples (DOH, D7, D14, D21, and D28) using RNeasy Mini Spin Columns (Qiagen, Valencia, CA) with the use of the QIAcube (Qiagen) according to the manufacturer's instructions. The RNA samples were quantified using a Nanodrop 1000 (Thermo Scientific) and then diluted to 200 ng/μL. The genes analyzed included aminopeptidase N (APN), the neutral (ASCT1, LAT1, y⁺LAT2), Na⁺-independent (b⁰⁺AT), anionic (EAAT3), and cationic (CAT1) amino acid transporters, the peptide transporter (PepT1) and the facilitated (GLUT2 and GLUT5) and Na⁺-dependent (SGLT1) sugar transporters. cDNA was synthesized from 2 μg of total RNA using a High Capacity Reverse Transcription cDNA kit (Applied Biosystems, Foster City, CA). The cDNA samples were diluted 1:30 for qPCR. Each qPCR reaction contained 5 μL of Fast SYBR Green Mastermix (Applied Biosystems), 1 μL of diluted cDNA, 1 μL of forward primer (5 ng/μL), 1 μL of reverse primer (5 ng/μL) and 2 μL of water and run in a 7500 Fast Real-time PCR Machine (Applied Biosystems). The primers for each of the 11 genes plus 18S rRNA are listed in Table 3.1.

Fold change was calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Expression of rRNA served as the reference gene to calculate ΔC_t . For the embryonic samples,

the average ΔCt of the jejunum of males at DOH was used as the calibrator to calculate $\Delta\Delta\text{Ct}$; whereas for the post hatch samples the average ΔCt of the duodenum of males at DOH was used as the calibrator. Statistical analysis was performed using JMP Pro 11 software. All outliers were removed using Grubb's test for outliers. Post-hatch tissues were analyzed separately from embryonic tissues using ANOVA with a full factorial with Age, Sex, and Tissue as the main effects and an $\alpha=0.05$ level of significance. Tukey's test was performed on all of the significant interactions. The embryonic samples were analyzed in a similar way with Age and Sex as the main effects.

Table 1. Real-time PCR primers for turkey nutrient transporters and aminopeptidase

Gene	Name	Accession #	Function	PCR Primers forward/reverse
APN ⁶	Aminopeptidase N	XM_003209631	N-terminal peptidase	TGCGGGTGCGATGGA/ CGTTGTCATAGAGCAGCGAGTT
ASCT1 ¹	SLC1A4	XM_010706509	Neutral aa exchanger	AGGAAACGTCGCCTTTGGT/ GCTGTACTGGTTGTGTTGGAGACT
b ⁰⁺ AT ⁴	SLC7A9	XM_010717754	Cationic and large aa exchanger	TCCTTACCTTATGGAGGCCTTTG/ GCAGGCTTGCCCAAGAAAA
CAT1 ⁴	SLC7A1	XM_003203401	Cationic aa transporter	TGGCCTTTCTCTTCGACTTGA/ CCAGGAGGGTCCCAATAGACA
EAAT3 ¹	SLC1A1	XM_010725627	Glu, Asp exchanger	CCCAACGCTTGGACTTGTC/ CAGCTGGCAGGCCAACA
GLUT2 ²	SLC2A2	XM_010716927	Facilitated glucose transporter	TTTTTCGAGAGAGCCGGTGTT/ TCACCACTCCAACGCCAAT
GLUT5 ²	SLC2A5	XM_010722761	Facilitated sugar transporter	CAACTCTCCAGCCCCCTACA/ GGAGACTCCGTGCCTGTTGA
LAT1 ⁴	SLC7A5	NM_001030579	Large, neutral aa exchanger	AAGGCCCATCAAGGTGAACA/ AACAAAGCAAGCCAGGATGAAG
PepT1 ⁵	SLC15A1	NM_001303166	di-/tri- peptide transporter	TTTGACCAGCAGGGATCGA/ CAAAGTCCCCATCCATTGTTG
SGLT1 ³	SLC5A1	XM_003211023	glucose and galactose transporter	GGGACAGTAGGTGGATTCTTTCTG/ CACCAATCGGCCACCAA
y ⁺ LAT2 ⁴	SLC7A6	XM_010717824	cationic/ large aa exchanger	TCTGCCTTGTTCTTATTCTGGTT/ TGGGTTTTTGATCTCCTCAGTCA
rRNA	18S Ribosomal RNA	DQ018752.1	reference gene	CCGTCGTAGTTCCGACCATAA/ GCGGGTCATGGGAATAACG

¹(Kanai, et al., 2013), ²(Mueckler and Thorens, 2013), ³(Wright, 2013), ⁴(Fotiadis et al., 2013), ⁵(Smith et al., 2013), ⁶(Mane et al., 2010)

Abbreviations: aa (amino acid)

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

Relative Quantification of Chicken and Turkey

The chicken samples used for this experiment were Ross A hybrids from Gilbert et al. (2007). Male chicken intestinal samples (duodenum, jejunum, and ileum) on DOH, D7, and D14 were compared to male turkey intestinal samples (duodenum, jejunum, and ileum) for those same time points (n=4). Real-time PCR was performed on a 7500 Fast Real-time PCR machine (Applied Biosystems) using EAAT3 primers (5'-AATGCACTGAATGAAGCTACAATGA-3'/ 5'-CCAGCAATTAAAAACACAATACCAA-3') and GAPDH primers (5'-GCCGTCCTCTCTGGCAAAG-3'/ 5'-TGTA AACCATGTAGTTCA-3') that anneal to the cDNA of both species. A total of 1 μ L of the 1:30 diluted cDNA was added to a 10 μ L total PCR reaction containing 1 μ L each of forward and reverse primers, 5 μ L of SYBR green (Applied Biosystems), and 2 μ L of DEPC water. The chicken and turkey samples were compared using the $\Delta\Delta C_t$ method. GAPDH was the reference gene and the calibrator was turkey duodenum on DOH. Results were analyzed using JMP11.0 software with ANOVA full factorial with Species, Age, and Tissue as the main effects and an $\alpha=0.05$ level of significance. Tukey's test was performed on all of the significant interactions.

RESULTS

The expression of one aminopeptidase and 10 nutrient transporters was assayed in two separate experiments. One experiment examined embryonic expression in the jejunum from E21 until day of hatch. The second experiment examined post-hatch expression in duodenum, jejunum, and ileum on DOH, D7, D14, D21, and D28 post-hatch.

For all genes examined during embryogenesis, there were no differences between males and females (Table 3.2). APN, the amino acid transporter b^{0+} AT, peptide transporter PepT1 and glucose transporters SGLT1 and GLUT5 showed increased expression on DOH compared to both E21 and E24, whereas EAAT3 showed no change. There was a sex x age interaction for EAAT3 (Figure 3.1), where expression in male turkeys was greater than female turkeys at E21 but the same at E24 and DOH. At the basolateral membrane, the amino acid transporters ASCT1 and CAT1 were downregulated between E21 and DOH, and LAT1 was unchanged. y^+ LAT2 and GLUT2 were upregulated between E21 and DOH and between E24 and DOH, respectively.

Table 3.2. Embryonic turkey nutrient transporter gene expression

Gene:	APN	b^{0,+}AT	EAAT3	PepT1	GLUT5	SGLT1	ASCT1	CAT1	LAT1	y⁺LAT2	GLUT2
Attribute:	Brush Border						Basolateral				
Sex											
Male	0.49	0.71	2.14	0.37	0.63	0.69	1.55	2.42	1.32	0.66	0.93
Female	0.49	0.75	1.43	0.62	0.80	0.71	1.15	1.95	1.22	0.63	1.01
SEM	0.06	0.09	0.32	0.15	0.17	0.09	0.24	0.26	0.14	0.07	0.12
<i>p-value</i>	0.99	0.73	0.12	0.24	0.49	0.84	0.25	0.21	0.61	0.75	0.64
Age											
E21	0.19 ^b	0.45 ^b	2.18	0.04 ^b	0.26 ^b	0.21 ^b	1.96 ^a	3.04 ^a	1.32	0.45 ^b	0.91 ^{ab}
E24	0.18 ^b	0.53 ^b	1.68	0.06 ^b	0.44 ^b	0.21 ^b	1.34 ^{ab}	2.33 ^a	1.33	0.35 ^b	0.71 ^b
DOH	1.08 ^a	1.22 ^a	1.50	1.16 ^a	1.44 ^a	1.68 ^a	0.77 ^b	1.19 ^b	1.16	1.12 ^a	1.28 ^a
SEM	0.08	0.11	0.39	0.15	0.20	0.11	0.48	0.32	0.17	0.08	0.15
<i>p-value</i>	<0.0001	<0.0001	0.43	<0.0001	0.0005	<0.0001	0.029	0.0010	0.69	<0.0001	0.04
Interaction											
S x A	0.83	0.17	0.03	0.29	0.65	0.84	0.35	0.27	0.39	0.18	0.33

^{a-b} within a column indicates significant difference (p<0.05)

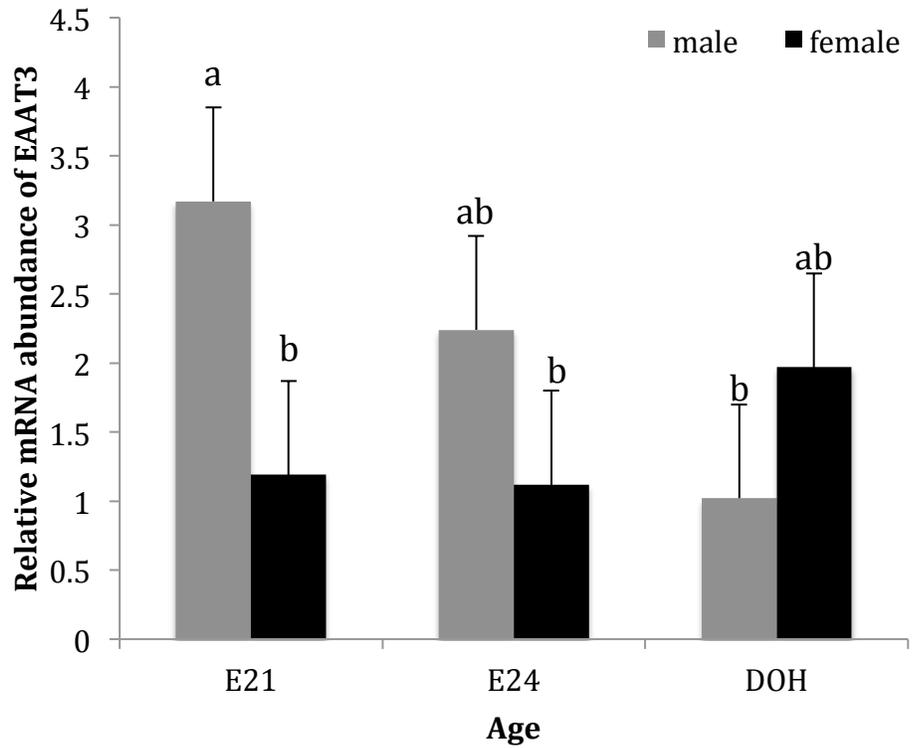


Figure 3.1. Embryonic EAAT3 interaction between age and sex.

Data are relative mRNA abundance of EAAT3 means + SEM on E21, E24 and DOH in mal and females (n=6).

Bars for each gene with a different letter (a-b) are significantly different ($p < 0.05$) when anal with Tukey's test.

Post-hatch, APN and the transporters showed sex-, tissue, and development-specific expression. Nine genes (APN, ASCT-1, b⁰⁺AT, CAT1 EAAT3, LAT1, PepT1, y⁺LAT2 and GLUT5) showed greater expression in females than males (Table 3.3). Only SGLT1 was expressed greater in males than females. In general, APN and the amino acid transporters showed greater expression in the distal than the proximal intestine. APN and EAAT3 showed greater expression in the ileum than the duodenum and jejunum; LAT1 and y⁺LAT2 showed greater expression in the jejunum and ileum than the duodenum and b⁰⁺AT showed greater expression in the ileum than the jejunum. In contrast, the sugar transporters GLUT2 and SGLT1 were expressed greatest in the jejunum and GLUT5 was expressed greatest in the duodenum. Some transporters showed decreased expression after DOH (CAT1, y⁺LAT2, and GLUT2); while others showed increased expression (EAAT3, GLUT5 and SGLT1).

Table 3.3. Post-hatch turkey nutrient transporter gene expression

Gene:	APN	b ^{0,+} AT	EAAT3	PepT1	GLUT5	SGLT1	ASCT1	CAT1	LAT1	y+LAT2	GLUT2
Attribute:	<i>Brush Border</i>						<i>Basolateral</i>				
Sex											
Male	3.70 ^b	1.01 ^b	36.02 ^b	1.13 ^b	2.80 ^b	3.17 ^a	0.98 ^b	1.22 ^b	1.15 ^b	1.36 ^b	1.58
Female	6.11 ^a	1.54 ^a	58.28 ^a	2.16 ^a	4.34 ^a	1.90 ^b	1.82 ^a	3.23 ^a	2.02 ^a	2.02 ^a	1.91
SEM	0.42	0.12	4.63	0.11	0.29	0.24	0.15	0.42	0.15	0.11	0.19
<i>p-value</i>	<0.0001	0.0025	0.0009	<0.0001	0.0002	0.0003	0.0001	0.0008	<0.0001	<0.0001	0.2174
Tissue											
D	3.43 ^b	1.26 ^{ab}	4.14 ^b	1.78	5.72 ^a	1.89 ^b	1.24	1.85	0.89 ^b	1.45 ^b	1.85 ^b
J	3.83 ^b	0.94 ^b	22.84 ^b	1.73	3.34 ^b	3.26 ^a	1.61	2.27	1.66 ^a	1.60 ^{ab}	2.76 ^a
I	7.45 ^a	1.63 ^a	114.48 ^a	1.44	1.60 ^c	2.45 ^{ab}	1.35	2.56	2.23 ^a	2.01 ^a	0.63 ^c
SEM	0.52	0.15	5.67	0.14	0.35	0.30	0.19	0.51	0.18	0.14	0.24
<i>p-value</i>	<0.0001	0.0061	<0.0001	0.1555	<0.0001	0.0058	0.3296	0.6079	<0.0001	0.0121	<0.0001
Age											
DOH	3.52	1.45	33.46 ^{bc}	1.80	1.73 ^b	1.26 ^b	2.10 ^a	4.98 ^a	1.94 ^b	2.31 ^a	2.56 ^a
D7	5.98	1.08	37.60 ^c	1.53	4.09 ^a	2.03 ^{ab}	1.55 ^{ab}	2.14 ^b	2.95 ^a	1.51 ^b	0.88 ^b
D14	4.95	1.20	67.20 ^a	1.56	4.34 ^a	3.32 ^a	1.01 ^b	1.39 ^b	1.19 ^{bc}	1.76 ^{ab}	1.83 ^{ab}
D21	4.51	1.30	51.06 ^{abc}	1.73	4.33 ^a	3.18 ^a	1.21 ^{ab}	1.30 ^b	0.80 ^c	1.33 ^b	1.83 ^{ab}
D28	5.55	1.35	56.42 ^{ab}	1.63	3.37 ^{ab}	2.87 ^a	1.13 ^b	1.32 ^b	1.06 ^{bc}	1.52 ^b	1.62 ^{ab}
SEM	0.67	0.19	7.31	0.18	0.46	0.39	0.24	0.66	0.23	0.17	0.30
<i>p-value</i>	0.0846	0.7043	0.0008	0.8001	0.0002	0.0008	0.0108	0.0002	<0.0001	0.0011	0.0041
Interaction											
SxT	0.054	0.5561	0.1999	0.5976	0.0124	0.0722	0.7055	0.7322	0.0605	0.5191	0.6797
TxA	0.0241	0.1438	0.0002	0.1388	<0.0001	0.0488	0.3615	0.9381	0.0443	0.1658	0.0422
SxA	0.6263	0.1118	0.7686	0.4878	0.0499	0.2231	0.7676	0.1385	0.0498	0.6956	0.4640
SxTxA	0.2577	0.9462	0.8144	0.2300	0.2079	0.3778	0.2389	0.9989	0.8338	0.9159	0.6680

^{a-c} within a column indicates significant difference (p<0.05)

For APN and the amino acid transporters EAAT3 and LAT1, there were tissue x age interactions. Expression of APN (Figure 3.2) and EAAT3 (Figure 3.3) in the ileum was greater than the duodenum and jejunum at D28 and at D14, D21, and D28, respectively. Expression of LAT1 (Figure 3.4) was greater in the ileum and jejunum than the duodenum at D7. Furthermore, there was a sex x age interaction for LAT1. LAT1 expression peaked at D7 in females but was unchanged in males (Figure 3.5).

For the sugar transporters, expression of GLUT2 and SGLT1 showed tissue x age interactions. GLUT2 was expressed greater in jejunum than ileum at DOH (Figure 3.6) and SGLT1 was expressed greater in jejunum than duodenum at D28 (Figure 3.7). For the fructose transporter GLUT5, there was a tissue x age interaction (Figure 3.8). GLUT5 was expressed most in the duodenum at day 7 than jejunum and ileum at any other time. GLUT5 also had a sex x tissue interaction where GLUT5 was higher in females than males in the duodenum and jejunum, but there was no difference between the sexes in ileum (Figure 3.9). GLUT5 showed a sex x age interaction (Figure 3.10). Females expressed more GLUT5 than males on D21.

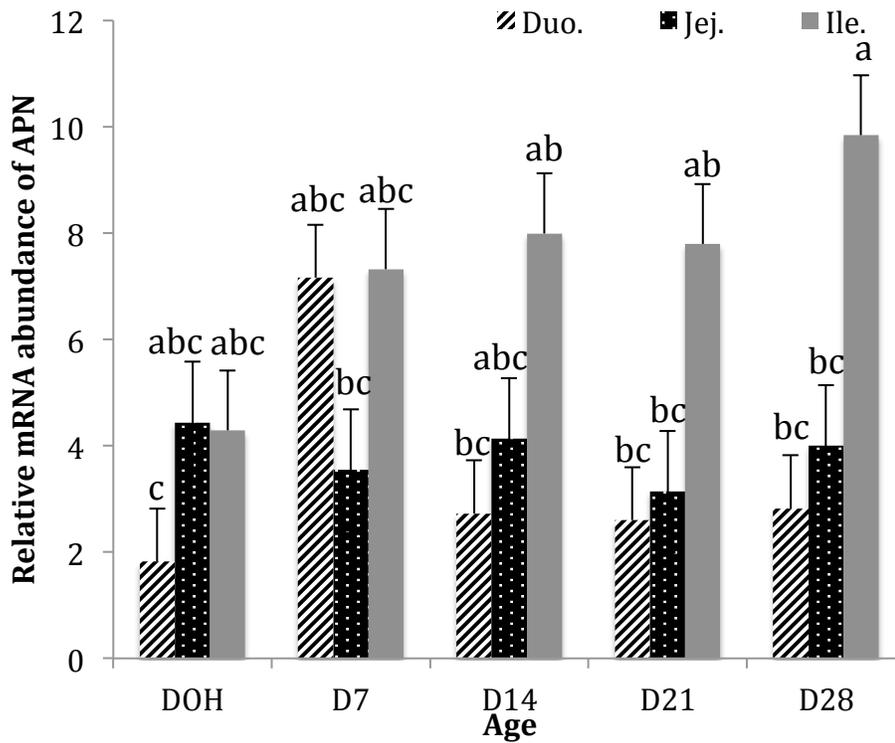


Figure 3.2. APN interaction of tissue and age.

Data are relative mRNA abundance of APN means + SEM on DOH, D7, D14, D21 and D28 in duodeunum, jejunum and ileum (n=12).

Bars for each gene with a different letter (a-c) are significantly different ($p < 0.05$) when Tukey's test was performed.

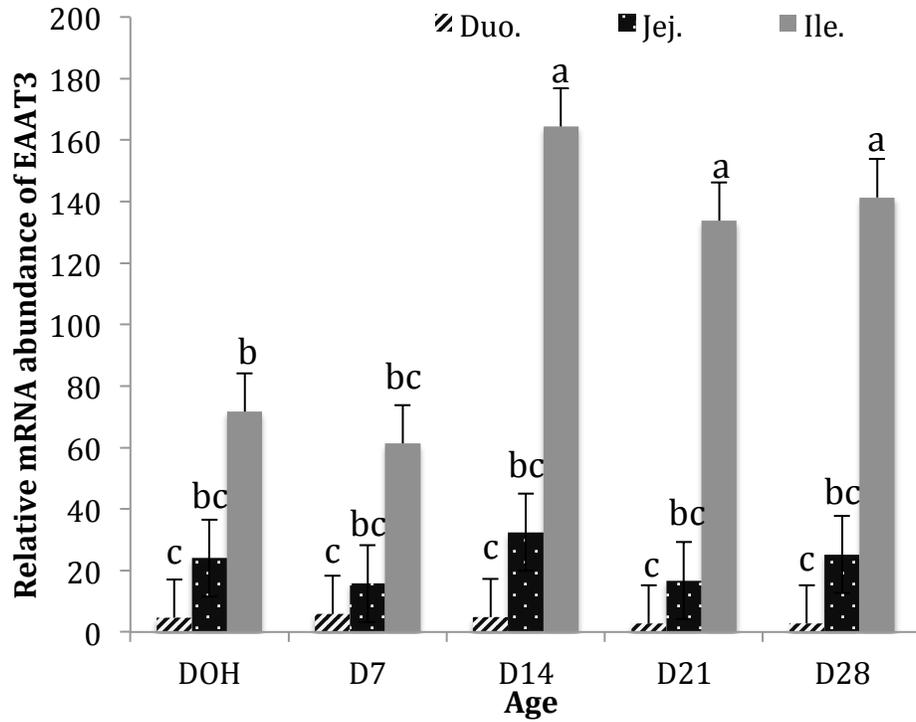


Figure 3.3. EAAT3 interaction of tissue and age.

Data are relative mRNA abundance of EAAT3 means + SEM on DOH, D7, D14, D21, and D28 in duodenum, jejunum and ileum (n=12).

Bars for each gene with a different letter (a-c) are significantly different ($p < 0.05$) when Tukey's test was performed.

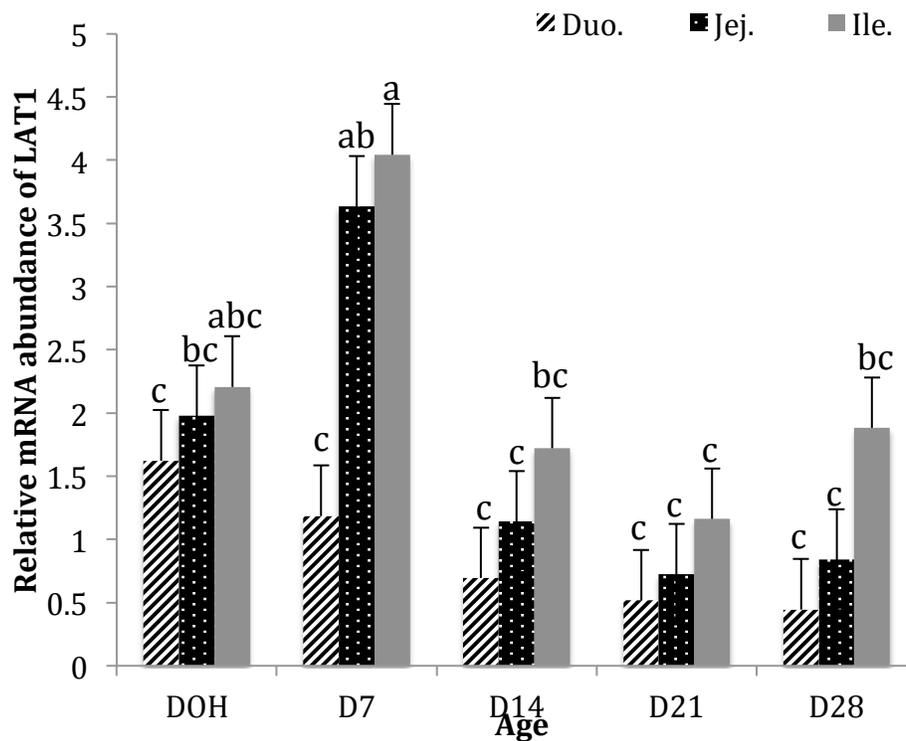


Figure 3.4. LAT1 Interaction of tissue and age.

Data are relative mRNA abundance of LAT1 means + SEM on DOH, D7, D14, D21, and D28 in the duodenum, jejunum and ileum (n=12).

Bars for each gene with a different letter (a-c) are significantly different ($p < 0.05$) when Tukey's test was performed.

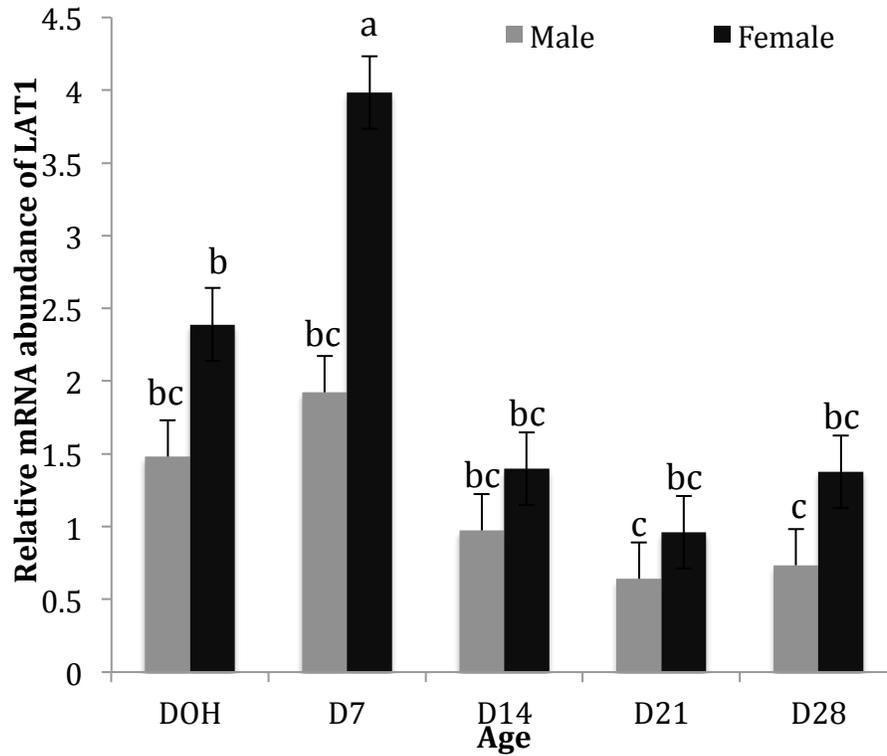


Figure 3.5. LAT1 interactions of sex and age.

Data are relative mRNA abundance of LAT1 means + SEM on DOH, D7, D14, D21, and D28 in males and females (n=6).

Bars for each gene with a different letter (a-c) are significantly different ($p < 0.05$) when Tukey's test was performed.

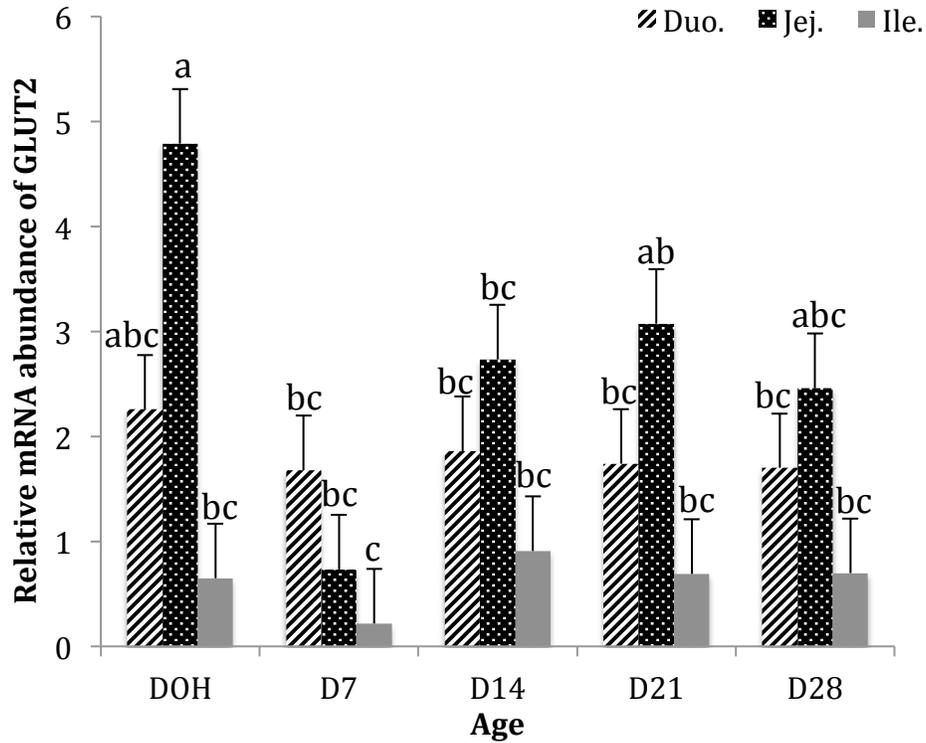


Figure 3.6. GLUT2 interaction of tissue and age.

Data are relative mRNA abundance of GLUT2 means + SEM on DOH, D7, D14, D21, and D28 in the duodenum, jejunum, and ileum (n=12).

Bars for each gene with a different letter (a-c) are significantly different ($p < 0.05$) when Tukey's test was performed.

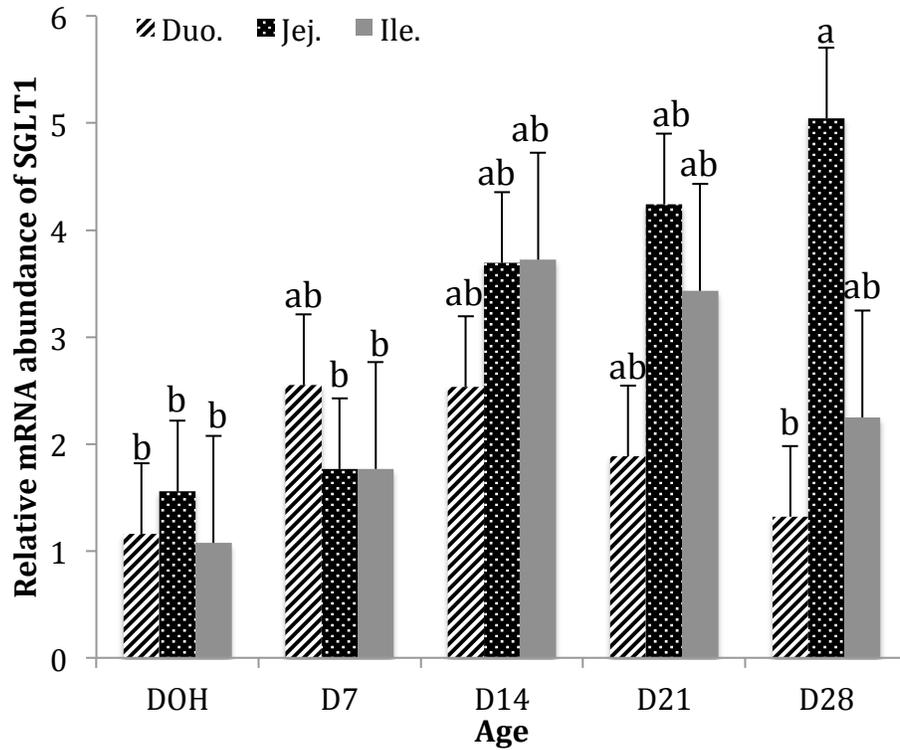


Figure 3.7. SGLT1 interaction of tissue and age.

Data are relative mRNA abundance of SGLT1 means + SEM on DOH, D7, D14, D21, and D28 in the duodenum, jejunum and ileum (n=12).

Bars for each gene with a different letter (a-b) are significantly different ($p < 0.05$) when Tukey's test was performed.

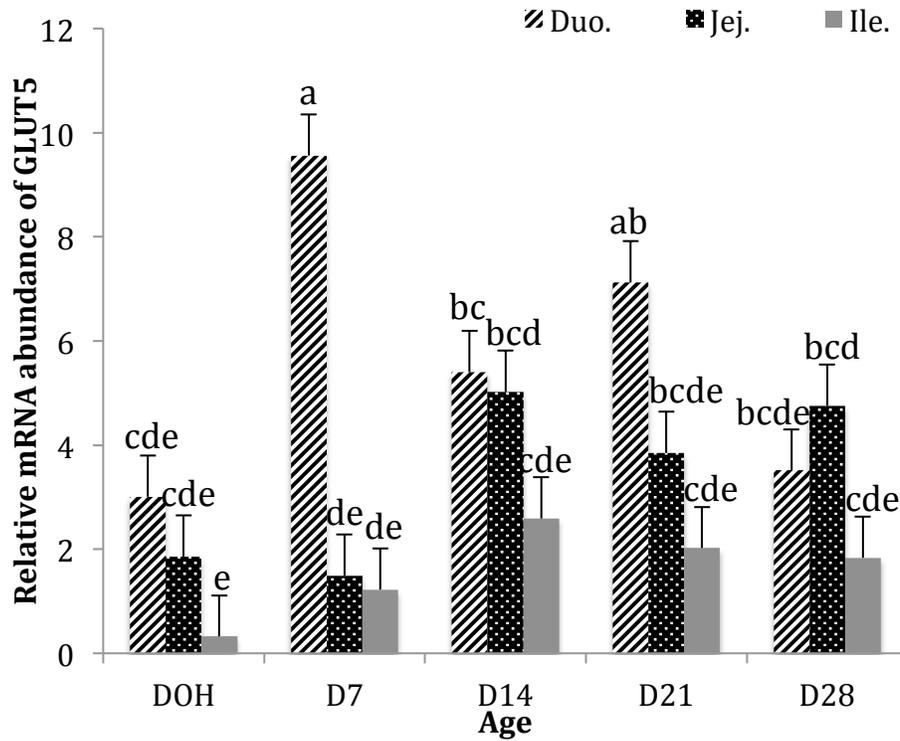


Figure 3.8. GLUT5 interaction of tissue and age.

Data are relative mRNA abundance of GLUT5 means + SEM on DOH, D7, D14, D21, and D28 in the duodenum, jejunum, and ileum (n=12).

Bars for each gene with a different letter (a-e) are significantly different ($p < 0.05$) when Tukey's test was performed.

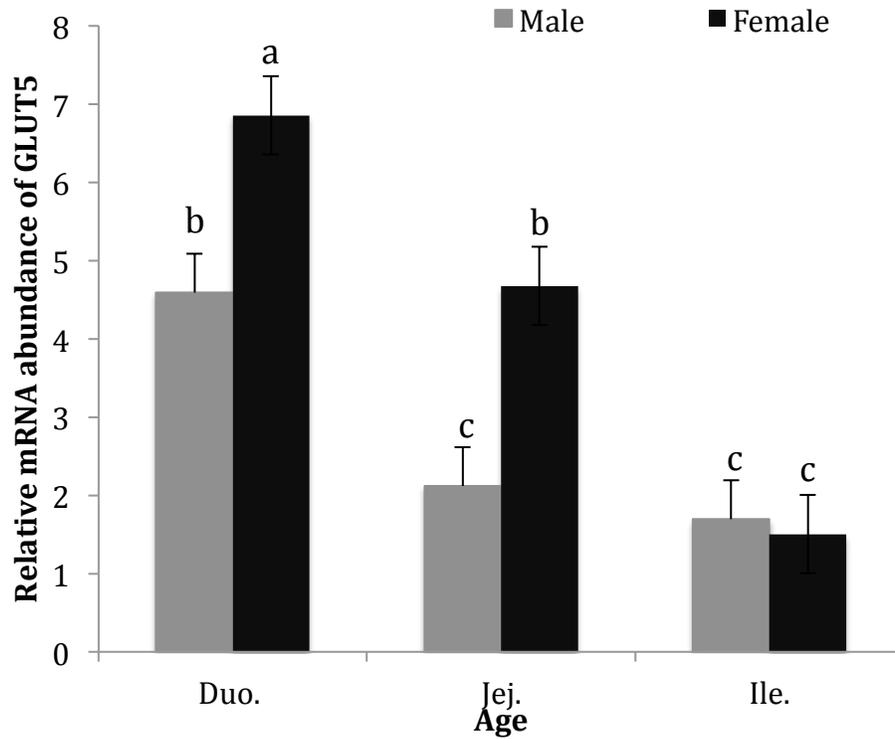


Figure 3.9. GLUT5 interactions of sex and tissue.

Data are relative mRNA abundance of GLUT5 means + SEM in the duodenum, jejunum, and ileum in males and females (n=6).

Bars for each gene with a different letter (a-b) are significantly different ($p < 0.05$) when Tukey's test was performed.

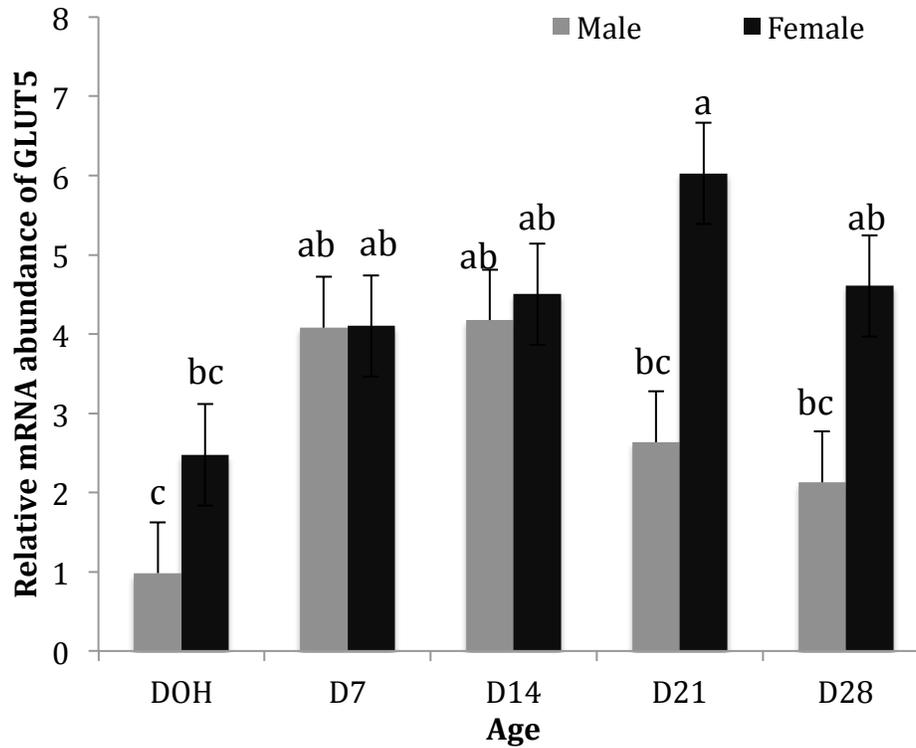


Figure 3.10. GLUT5 interaction of sex and age.

Data are relative mRNA abundance of GLUT5 means + SEM on DOH, D7, D14, D21, and D28 in males and females (n=6).

Bars for each gene with a different letter (a-c) are significantly different ($p < 0.05$) when Tukey's test was performed.

Relative quantification of turkey EAAT3 and chicken EAAT3 showed that turkey expressed more EAAT3 overall. There was more expression in the ileum compared to the duodenum and jejunum and there was most expression of EAAT3 in the ileum (Table 3.4). The 3-way interaction between species, tissue and age showed that expression of EAAT3 was higher on D14 in turkey than any other tissue and time point for both species (Figure 3.11). There was a 4-fold difference between chicken ileum on D14 and turkey ileum on D14.

Table 3.4. Relative quantification of turkey and chicken EAAT3.

Gene: EAAT3	
Attribute:	
Species	
Chicken	353.61 ^b
Turkey	683.28 ^a
SEM	42.2
<i>p-value</i>	<0.0001*
Tissue	
D	65.24 ^b
J	166.65 ^b
I	1323.45 ^a
SEM	51.68
<i>p-value</i>	<0.0001*
Age	
DOH	294.43 ^b
D7	323.87 ^b
D14	937.04 ^a
SEM	51.86
<i>p-value</i>	<0.0001*
Interaction p-value	
S x T	<0.0001*
S x A	<0.0001*
T x A	<0.0001*
S x T x A	<0.0001*

^{a-b} within a column indicates significant difference ($p < 0.05$)

* indicates significance ($p < .05$)

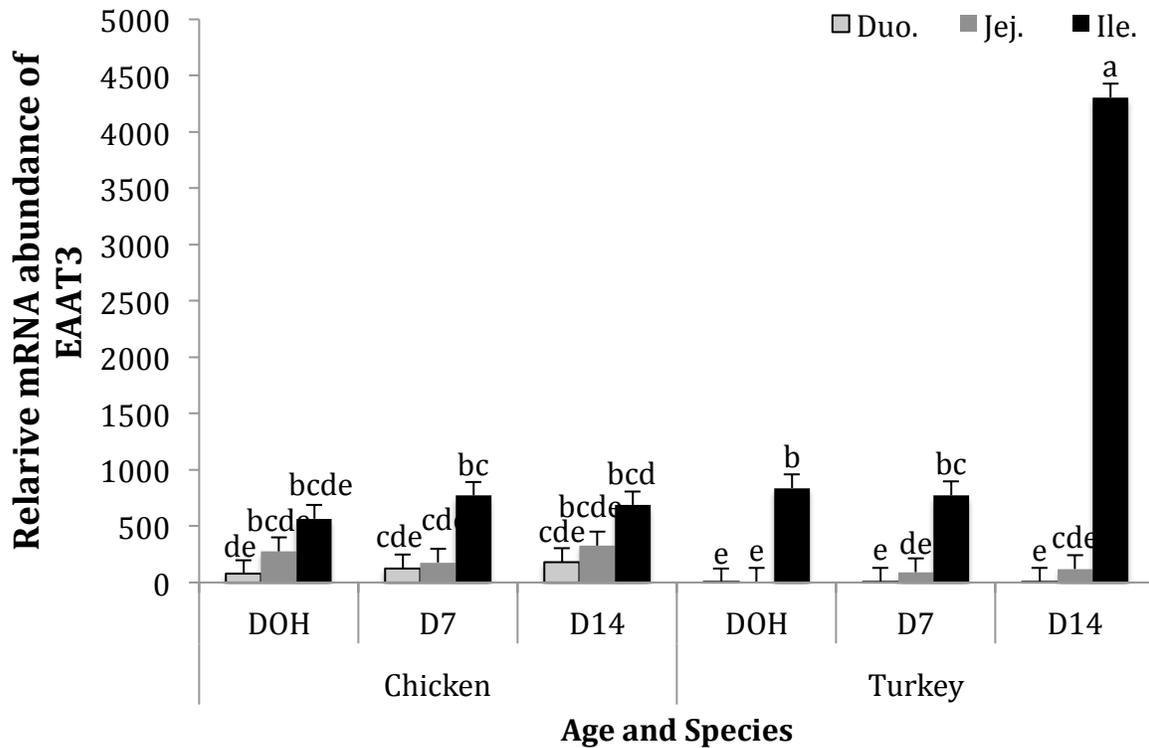


Figure 3.11. EAAT3 chicken and turkey species, age, tissue interaction.

Data are relative mRNA abundance of EAAT3 means + SEM in chicken and turkey on DOH, D7, and D14 in the duodenum, jejunum and ileum (n=4).

Bars for each gene with a different letter (a-e) are significantly different ($p < 0.05$) when a Tukey's test was performed.

DISCUSSION

During late embryogenesis, the yolk decreases in size and the small intestine becomes more developed. These morphological changes cause the embryo that previously obtained energy from the yolk via the blood stream to prepare for absorbing nutrients from the intestinal lumen. These morphological changes cause expression changes of different amino acid, small oligo-peptide, and sugar transporters. Characterization of nutrient transporters will allow for better feed formulations and optimization of turkey diets by catering to what transporters are being expressed (Havenstein et al., 2007; de Oliveira et al., 2008).

Embryonic Transporter Expression

There was no difference in male and female embryonic expression of nutrient transporters in turkey. Zeng et al. (2011) showed similar results for cationic and neutral amino acid transporters in broiler embryos. During embryonic development brush border transporters (APN, b⁰⁺AT, PepT1, GLUT2, GLUT5 and SGLT1) were expressed more on DOH than on E21 or E24. As the embryo prepares to hatch, the seroamniotic connection ruptures allowing oral consumption of the albumin. While digestion at this stage is still limited due to antitrypsin factors, some absorption is possible while in transit through the duodenum and jejunum (Moran, 2007). A similar study in pigeon (*Columba livia*) reported that PepT1, SGLT1, GLUT2, and APN also increased expression as the embryo approached hatch, with the highest expression on DOH (Dong et al., 2012).

Post-Hatch Transporter Expression

There is limited research concerning sexual dimorphism of nutrient transporters in *Galliformes*. In the post-hatch study, female turkey expression of the digestive enzyme APN and 8 transporters ASCT1, b⁰⁺AT, CAT1, EAAT3, LAT1, PepT1, y⁺LAT2 and GLUT5, was higher

than male expression. Interestingly, SGLT1 was the only transporter that was expressed greater in males than females. These results are surprising as the official nutrient requirements for both male and female growing turkeys are the same (1994), but the data suggest that male and female turkeys assimilate nutrients differently. In both male and female chickens during the first two weeks of age, 10% of body weight gain for both sexes is from the growth of the intestine (Plavnik et al., 1982). It is possible that females have a greater need for amino acids and sugar than males or that females utilize feed less efficiently.

Expression of most of the amino acid transporters was highest in the ileum. Expression of the sugar transporters studied was highest in the jejunum. Amino acid and monosaccharide absorption occurs in the distal portion of the small intestine of chicken (Gilbert et al, 2007; Li et al., 2008) and pigeon (Dong et al., 2012) and turkey nutrient transporter expression follows a similar pattern.

PepT1 expression is highest in the duodenum and jejunum in chicken and pigeon while there is no difference across all three small intestinal segments in turkey. Furthermore, PepT1 expression did not increase with age like it did in both chicken and male pigeon (Gilbert et al., 2007; Li et al., 2008; Dong et al., 2012). When chickens were on restricted feed, PepT1 expression increased (Gilbert et al., 2008). PepT1 expression in turkey did not change with age, suggesting that adequate levels of protein were present in the intestinal lumen. Alternatively, young turkeys may not have the capacity to absorb di- and tri- peptides like other avian species, especially considering how under-developed their small intestines are at hatch (Applegate et al., 2005).

Expression of brush border membrane transporters EAAT3 and SGLT1 was the greatest from D14 to D28. Expression of brush border sugar transporter SGLT1 increased from D7 to

D28. All the basolateral membrane transporters, were expressed more on DOH or D7 versus later in development. The expression patterns of turkey nutrient transporters coincide with morphological changes occurring in the small intestine from late embryogenesis to post-hatch. The data reflect that brush border membrane expression increases with development because the poult is still or was very recently absorbing its nutrients from the blood via the basolateral transporters. As the transition to nutritional uptake from the intestinal lumen is complete, the reliance of nutrients from the blood decreases (De Oliveira et al., 2008; Moran, 2007). Chicken also demonstrated the same pattern, while data are only available for a few sugar transporters in pigeon (SGLT1 and GLUT2), they also appeared to utilize transporters in a similar way (Speier et al., 2012; Gilbert et al., 2007; Dong et al., 2012).

Glutamate is a major metabolic substrate in the enterocyte. EAAT3 is responsible for transporting glutamate and its expression in small intestinal cells increases in the presence of a high protein diet (Erickson et al., 1995). In turkey, glutamate is the most digestible amino acid in birds fed a soybean meal (SBM) diet. Chicken and ducks do not demonstrate this increased digestibility for glutamate (Kluth and Rodehutsord, 2006).

The marked increase in EAAT3 on D14 was not observed in chicken (Gilbert et al., 2007). When chicken and turkey intestinal samples were analyzed using the same primers EAAT3 expression was lowest in the duodenum, intermediate in the jejunum, and highest in the ileum for both species at all time points. However, in turkey ileum on D14 there was a nearly 4-fold difference compared to chicken ileum on D14. This increase suggests that turkeys have an increased absorption capacity for glutamate in the ileum.

The turkeys in this study hatched 1 day early (E27), suggesting that samples taken on E21 and E24 might have been more developed than a turkey embryo that was incubated for the full

28 days. While thermal manipulation has been shown to decrease metabolic requirements, excessive heat over the entire incubation period causes teratogenic problems in both turkey and broiler chicks (Piestun et al., 2015). With the increasing knowledge emerging about the genetics, nutrition, and metabolism of commercially grown poultry, there is an opportunity to better formulate turkey diets to meet the need of both males and females and to possibly increase feed conversion and body weight gain with knowledge emerging about amino acid, oligo-peptide, and sugar transporters, namely EAAT3.

Chapter 4

Characterization of Chicken Intestinal Transporter PepT2 mRNA Variants

ABSTRACT

Peptide transporter 2 (PepT2) is a member of the solute carrier family of genes (SLC15A2) and is a low capacity/high affinity transporter responsible for cellular uptake of di- and tri- peptides. In chickens, there is alternative splicing of the first exons that generate PepT2 variants. Two mRNA variants of the PepT2 gene in chickens (variants 5 and 6) have been previously identified. Further bioinformatics analysis revealed two additional variants: 7 and 8. PepT2 variants have not been previously reported in any mammalian or avian species. The four chicken PepT2 variants all contain exons 2-23 but differ in the organization of upstream exons and thus encode different N-termini of the encoded PepT2 proteins. Variant 5 contains exon 1 and variant 8 contains exon 1C, spliced to exon 2. Variants 6 and 7 contain exon 1A and exon 1B, respectively, spliced to an internal splice site in exon 1. In all cases the alternatively spliced mRNAs contain an in-frame methionine start codon. The objective of this study was to determine the tissue- and development-specific patterns of expression of these PepT2 variants. Brain, kidney, liver and intestine samples at embryonic day 18 and day 7 post-hatch were analyzed (n=5). Expression of Variant 5 was most prominent in the brain and variant 6 was most prominent in the kidney, whereas variant 8 appeared in all tissues on E18 and D7. Variant 7 was only expressed in late embryonic development in the ileum. In summary, PepT2 variants have been observed only in chicken and show both tissue- and development-specific expression.

Introduction

Solute carriers (SLC) are primarily defined as genes that encode passive transporters, ion coupled transporters and exchangers. As of 2009, approximately 400 solute carriers have been identified in humans (Schlessinger et al., 2010). These can be divided into 47 families. This classification is based on a similar shared substrate and a 20-25% sequence identity to the other members of that family. The SLC15 family includes peptide transporter 1 (PepT1), peptide transporter 2 (PepT2), peptide/histidine transporter 2 (PHT2), and peptide/histidine transporter 1 (PHT1). PepT1 is a low affinity-high capacity proton coupled transporter located on the brush border membrane of intestinal enterocytes and in the kidney. PepT2 is a high affinity-low capacity proton coupled transporter located in the kidney, lung, brain, mammary gland, and brush border membrane of intestinal enterocytes. Both PepT1 and PepT2 have been widely studied to characterize their uptake of not only peptides but also certain peptidomimetics. PHT1 and PHT2 are proton coupled transporters capable of transporting free histidine. PHT2 is located in the lung, spleen, thymus, brain, liver and heart while PHT1 is located in the brain, retina, and the placenta (Smith et al., 2013).

PepT2 is predicted to have twelve transmembrane domains with both the N- and C-termini facing the cytosol. PepT2 acts as a high affinity/low capacity transporter capable of transporting most di- and tri-peptides from the 20 proteinogenic amino acids. PepT2 is expressed in many tissues, but mainly the kidney, brain and small intestine (Kottra et al., 2004; Lu and Klaassen, 2006). In chickens, PepT2 is mainly expressed in the brain and kidney, with lower expression in the intestine and fetal liver (Zwarycz and Wong, 2013). The chicken and mammalian PepT2 genes have 23 exons in common. However, due to alternative splicing there are additional exons in the chicken PepT2 gene.

Alternative splicing occurs in the eukaryotic genome because protein coding regions called exons are interrupted by non-coding regions called introns. During mRNA synthesis, the spliceosome removes the introns and joins the exons together to form the complete, uninterrupted, protein coding mRNA molecule. During an alternative splicing event, the spliceosome joins two differing portions of the RNA together, leading to a different encoded protein (Matlin et al., 2005). There are many different kinds of alternative splicing events. Mutually exclusive, internal acceptor, retained intron, and alternative promoter are four of the more common splicing events (Breitbart, 1987; Sammeth et al., 2008). The internal acceptor and alternative promoter are diagrammed in Figure 4.1. The objective of this study was to determine if the PepT2 variants are expressed in a tissue- and development-specific manner.

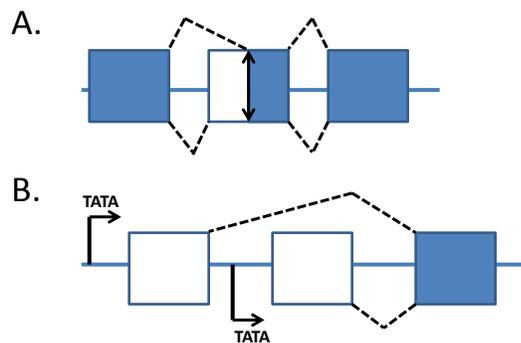


Figure 4.1. Diagram of possible alternative splicing events of PepT2.

A. Internal Acceptor Splicing B. Alternative Promoter Splicing. Solid boxes indicate constitutive exons, open boxes indicate alternative sequences, single dashed lines indicate different pathways. Solid lines indicate introns. Double arrow is cryptic splice acceptor site.

MATERIALS AND METHODS

Birds and Tissue Collection

Tissue samples used in this study were those collected by Zwarycz and Wong (2013). Fertile eggs were obtained from the S37 generation of Dr. Paul Siegel's high antibody selected line of Leghorn chickens (Zhao et al., 2012). Birds were sacrificed by cervical dislocation and brain, kidney, liver and ileum samples (n = 5) on E18, E20, D1, D7, and D14 were collected. On E18, whole intestine rather than duodenum jejunum, and ileum was collected. All samples were stored at -80°C.

Generation of Variant Schematic

Each of the variants were separately compared to the chicken genome published on the National Center for Biotechnology Information (NCBI, Bethesda, MD) website using the BLAST tool. Further analysis of the PepT2 variants was performed using Biology Workbench 3.2 (SDSC, San Diego, CA) using Nucleic Tool CLUSTALW for all 4 variants. The open reading frame for each variant was determined using the Biology Workbench 3.2 (SDSC) nucleic tool SIXFRAME and then the open reading frames of each of the 4 variants were compared to each other using CLUSTALW. The official numeral nomenclature for these variants start with variant 5 and continue to variant 6, 7, and 8.

Total RNA Extraction and PCR

Total RNA was extracted on E18, E20, D1, D7 and D14 ileum, brain and kidney samples and E18 and E20 liver samples using Direct-zol™ RNA Miniprep (Zymo Research, Irvine, CA). The RNA samples were quantified using a Nanodrop®-1000 (Thermo Scientific, Wilmington, DE) and then diluted to 200 ng/μL. Total RNA (2 μg) was used to synthesize cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a 20

μ L reaction. Using 5 μ L of the cDNA reaction, a 25 μ L end point PCR reaction was performed for 30 cycles on a PTC-2000 Peltier Thermocycler (MJ Research, Watertown, MA) using 12.5 μ L of AccuStart™ II PCR SuperMix (2X) (Quanta Biosciences, Gaithersburg, MD). Primers specific to four PepT2 variants and β -actin as the endogenous control are shown in Table 4.1. The initial denaturation step was 94 °C for 2 minutes, followed by 30 cycles at 94 °C for 20 seconds; 60 °C for 20 seconds; 72 °C for 30 seconds. After the cycles were completed the samples were held at 4 °C. A total of 5 μ L of PCR product was separated on a 3% agarose TBE gel stained with ethidium bromide. The DNA ladder Hi-Lo™ DNA Marker (Minnesota Molecular, Inc., Minneapolis, MN) was used to compare the PCR products.

Table 4.1. Chicken PepT2 variant specific primers for PCR.

Gene	Accession #	PCR primers, forward/reverse	Product
PepT2 Variant 5	XM_422093	CGAGAGGGGAGATGAACACT/ GCAGAACTCGTTCACCACAA	176 bp
PepT2 Variant 6	XM_004942922	AAAGCAAGAAGGAATGGCAGA/ GCAGAACTCGTTCACCACAA	168 bp
PepT2 Variant 7	XM_004942923	ATGATCCAACCCCAGGAGAT/ GCAGAACTCGTTCACCACAA	122 bp
PepT2 Variant 8	XM_004942924	GCTCGTGAATCCAGGCTTAG/ GCAGAACTCGTTCACCACAA	207 bp
β -actin	NM_205518	GTCCACCGCAAATGCTTCTAA/ TGCGCATTTATGGGTTTTGTT	76 bp

Primers designed by Primer3Plus software (Untergasser et al., 2012)

RESULTS

The organization of the chicken PepT2 gene is shown in Figure 4.2. The PepT2 gene consists of 23 exons (1-23), which are common to mammalian PepT2 genes. Exons 1A, 1B, and 1C are unique to chicken PepT2 and are involved in alternative splicing. All chicken PepT2 variants contain exons 2-23. Chicken PepT2 variant 5 includes exon 1. Chicken PepT2 variants 6 and 7 include exons 1A and 1B spliced to a cryptic splice acceptor site in exon 1. Variant 8 includes exon 1C, which is located between exon 1 and exons 2. The amino acid sequences of the four chicken PepT2 variants are shown in Figure 4.3. All four chicken PepT2 variants share the same amino acid sequence starting from exon 2, but vary at their N-termini.

Expression of PepT2 variants on embryonic day 18 is shown in Figure 4.4. On E18, PepT2 variant 5 was expressed in brain, with lower expression in liver, kidney, and ileum. Variant 6 was expressed greater in liver and kidney than brain and ileum. Variant 7 was not expressed in liver, brain, kidney or ileum. Variant 8 was expressed in liver, brain, and kidney and expressed lower in the ileum.

Expression of PepT2 variants on day 7 after hatch is shown in Figure 4.5. On D7, expression of PepT2 variants in brain, kidney, and ileum were also analyzed. Liver was not included because Zwarycz and Wong(2013) reported that PepT2 was only expressed in fetal liver. Variant 5 was expressed in the brain and kidney. Variant 6 was only expressed in the kidney. Variant 7 was not expressed in any tissue. Variant 8 was expressed in brain and kidney and at a lower level in the ileum.

Variant 7 was not expressed in any tissue or time point shown in Figures 4.4 or 4.5. Variant 7, was however expressed at different time points and tissues of different birds, but the expression pattern was not consistent. Some samples did not express Variant 7 at any time point

or in any tissue, while other samples expressed small amounts of Variant 7 in all tissues and time-points except embryonic liver (data not shown).

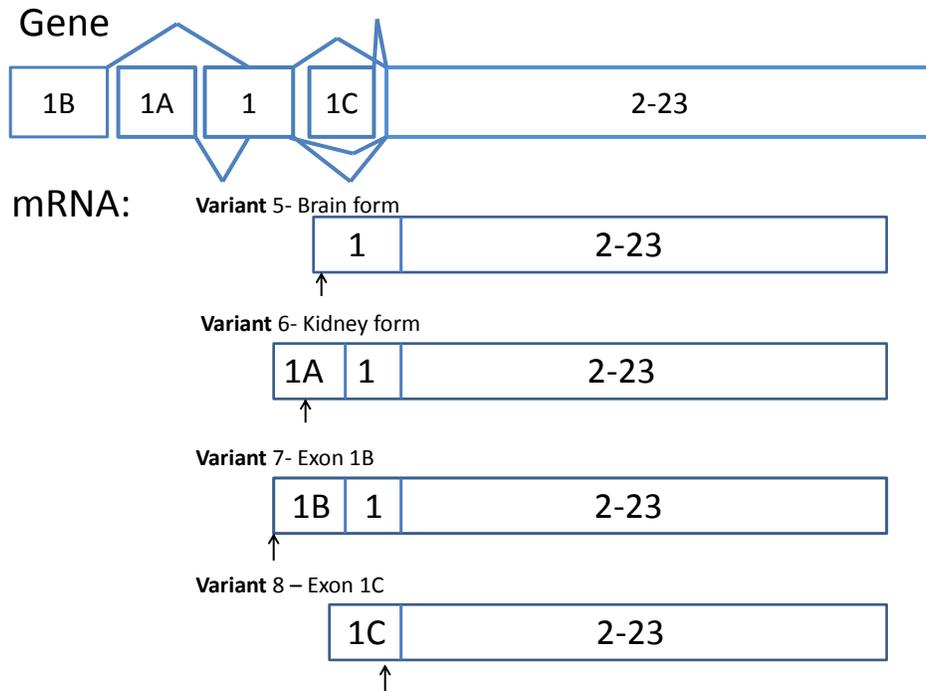


Figure 4.2. Chicken PepT2 gene and its four splice variants.

Boxes indicate genomic order of exons. Arrows indicate location of methionine codon (AUG).

Variant 5:

MVGDEARGEMNTFQRNESKESLFTFISTGDDPPKGDHFVQKKSPKLCGSNPLSIAFIVVNEFC...
Exon 1 Exon 2

Variant 6:

MAEKDSELQTAKETQAGDDPPKGDHFVQKKSPKLCGSNPLSIAFIVVNEFC...
Exon 1A 3' end of Exon 1 Exon 2

Variant 7:

MKGMDDPTPGDDPPKGDHFVQKKSPKLCGSNPLSIAFIVVNEFC...
Exon 1B 3' end of Exon 1 Exon 2

Variant 8:

MDEGCPLLDVQAGPMQKLCGSNPLSIAFIVVNEFC...
Exon 1C Exon 2

Figure 4.3. Amino acid sequences of the four chicken PepT2 variants.

The amino acid sequence was determined using the nucleic tool SIXFRAME on Biology Workbench 3.2 software (SDSC, San Diego, CA) and the reading frames were compared using nucleic tool CLUSTALW.

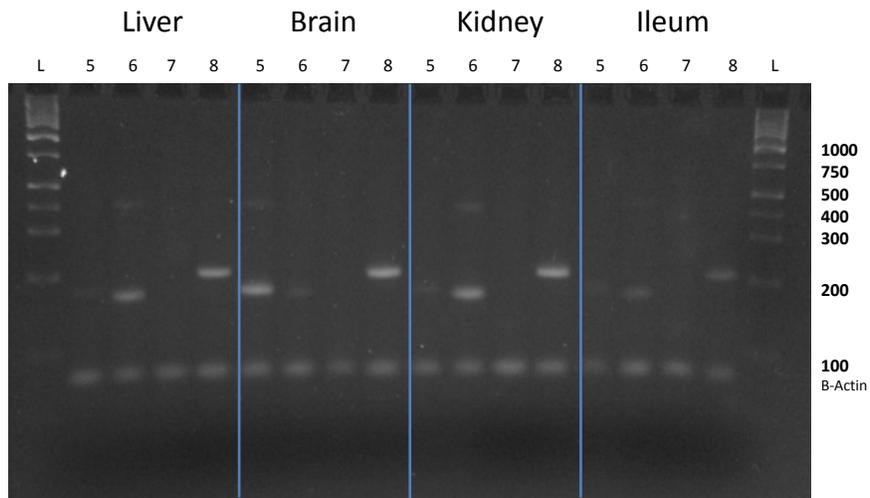


Figure 4.4. Expression of PepT2 variants on embryonic day 18.

3% TBE gel of each variant after 30 cycles of end-point PCR using variant specific primers on tissues from a single representative chicken. The lane designated with an L indicates the DNA ladder. Lanes labeled 5, 6, 7, and 8 correspond to PepT2 variants 5, 6, 7, and 8 respectively. β -actin was the reference gene.

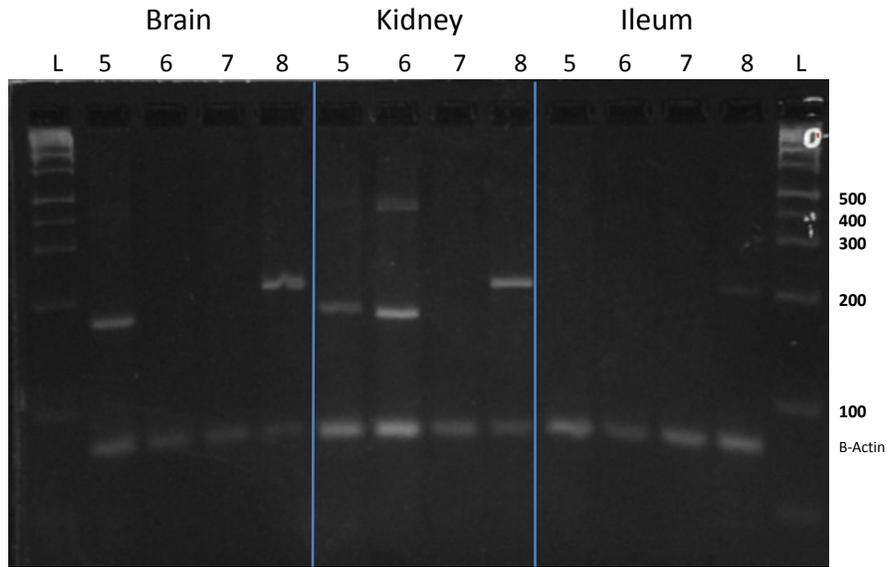


Figure 4.5. Expression of PepT2 variants on D7 post-hatch.

3% TBE gel of each variant after 30 cycles of end-point PCR using variant specific primers on tissue from a single representative chicken. The lane designated with an L indicates the DNA ladder. Lanes labeled 5, 6, 7, and 8 correspond to PepT2 variants 5, 6, 7, and 8 respectively. β -actin was the reference gene.

DISCUSSION

In chicken during late stage embryonic (E18) and post-hatch (D7) development, PepT2 variants are expressed with tissue specificity. For both time points, PepT2 variant 5 is brain specific. PepT2 variant 6 is expressed in the liver, kidney and ileum pre-hatch, but only the kidney post-hatch. Variant 7 is not expressed in any tissue on E18 and D7. PepT2 variant 8 is expressed in all tissues and all time points indicating that it could be the ubiquitous form.

A PepT2 variant has been identified in humans. The human PepT2 (hPepT2) variant includes three non-synonymous amino acid substitutions; F350L, S409P, and K509R (Pinsonneault et al., 2004). A major difference between the hPepT2 variant and the chicken PepT2 (cPepT2) variants is that for hPepT2 there are single amino acid changes and for cPepT2 there are different N-termini sequences. In both cases, changes in amino acid sequence might change the structure and function of the protein. The hPepT2 variant only demonstrated functional differences in K_m and differences in uptake at extracellular pH of 6.0, but no differences were observed at other extracellular pH or in drug uptake.

The four cPepT2 variants each start with a different exon, which suggests that there may be a different promoter for each first exon. This would allow more precise control of PepT2 gene expression in different tissues. In the kidney, PDZ (PSD-95, DglA, and ZO-1)-domain protein PDZK1 couples with PepT2 allowing it to remain anchored to the plasma membrane and continue transporting oligo-peptides (and possibly pharmaceuticals) inside the cell (Noshiro et al., 2006). An *in vitro* study of PepT2 in kidney showed that serum and glucocorticoid inducible kinase SGK1 and Na^+/H^+ exchanger regulatory factor 2 (NHERF2) recognize the PDZ1 domain and PepT2 expression and transport increase in the presence of both regulatory proteins (Boehmer et al., 2008). Regulation of PepT2 is characterized in kidney; however, little is known

about the regulation of PepT2 in other tissues. If the kidney has a specific pathway to regulate PepT2, it is probable that other tissues have their own regulatory proteins, which recognize their own specific binding sites. Multiple regulatory pathways might explain why chicken PepT2 variants differ only before exon 2 because of the need to accommodate a tissue-specific promoter. The presence of PepT2 variant mRNA in different tissues does not imply differences in functionality. Further studies are required to determine functional differences between the four chicken PepT2 variants.

Chapter 5

EPILOGUE

Commercial avian species spend a large portion of their life in the egg, and therefore embryonic development has been studied closely. As a whole, there is greater interest in chicken broiler morphological and developmental changes in regards to nutrient transporters for feed formulations because the chicken broiler industry is larger than the turkey industry within the United States. However, turkey exports still represent a US market worth more than half a billion dollars. More time, man power, research, and money should be spent studying the assimilation of nutrients in the turkey diet during development to increase production profits in an already booming market.

Young turkey poults are more difficult to raise than their chicken broiler counterparts. Feed conversion rates for young turkeys are very high, relative to chicken and their grow-out times are quadrupled compared to chickens. A better understanding of the intestinal morphology from late embryogenesis to hatch would allow for optimization of feed formulations to better accommodate the challenges that occur in young turkeys. Nutrient transporters overall are more highly expressed in females than in males and it is possible that females have an increased need for nutrients because they are less efficient at gaining weight, or it could be that their intestines are even less developed than males at hatch, as research is only available for male poults.

EAAT3 is expressed more in turkey in the ileum on D14 than chicken. This increase continues to D28. In the wild, turkeys are omnivores whose diet ranged from insects to nuts and berries. Commercial birds are granivores and the industry has selected birds, which perform best on such a diet. It is unclear at this point whether wild turkeys have an increased need for glutamate on D14, however it is possible that this need is necessary for the enterocyte to reach

maturity. Thus histological data from both wild turkeys and commercial turkeys would be necessary to determine if the increased need for glutamate is a product of commercial selection or an inherent trait.

The oligo-peptide transporter PepT2 is closely related to PepT1 in both amino acid sequence and structure. The chicken PepT2 gene has four tissue- and development- specific variants. If functional differences can be detected between the variants, it may be helpful to not only continue the study of PepT1, but also PepT2 expression in the avian small intestine. Furthermore, if PepT2 has two variants located in the small intestine, which may be the case because this research indicates that variant 8 is an ubiquitous form, it may be useful to take differing Km and di- and tri- protein affinities into account when formulating the protein content in the diet.

Drawbacks to previous research in poultry include the limited knowledge of female intestinal transporters. Also, many studies use a limited number of time points. For many of the brush border membrane proteins, they may not be functioning in the gut to their full potential until D14, which is when many of these studies end. Therefore it might be useful for future studies to collect data for longer periods of time, for example to grow out age.

Nutrient absorption is critical to increased body weight; correct feed formulation will allow for improved feed conversion and increased feed efficiency. It is expensive and time consuming to obtain exact nutrient profiles on all the components of poultry feed. Instead, studies on intestinal development and absorption capacity in conjunction with applied nutrition are likely the future of the poultry industry. While studying male broiler chickens may be adequate in order to get a clear picture of changes in the small intestine, more research should be

dedicated to female turkeys as there appears to be a difference between male and female expression of most nutrient transporters.

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