Gene Expression of Nutrient Transporters and Digestive Enzymes in the Yolk Sac Membrane and Small Intestine of the Developing Embryonic Chick

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ABSTRACT

Chick embryos derive nutrients from the yolk during incubation and transition to intestinal absorption posthatch. Nutrient uptake is mediated by digestive enzymes and membrane bound transporter proteins. The objective of this study was to determine expression profiles of nutrient transporters and digestive enzymes during incubation in the yolk sac membrane (YSM) and small intestine of Leghorn and Cobb chickens derived from 22–30 wk (young) and 45–50 wk (old) breeder flocks. Genes examined included peptide transporter PepT1, amino acid transporters EAAT3, CAT1, and B^0AT, monosaccharide transporters SGLT1 and GLUT5, and digestive enzymes APN and SI. Expression of these genes was measured in YSM at embryonic day (e) 11, 13, 15, 17, 19, 20, and 21 and small intestine at e15, e17, e19, e20, and e21. Absolute quantification real-time PCR assessed gene expression. PepT1, APN, and B^0AT expression in YSM peaked between e15 and e17 and then decreased until e21, while expression increased over time in the small intestine. SGLT1 and EAAT3 expression increased over time in the small intestine and YSM. There was minimal gene expression of SI in the YSM, while the small intestine had high expression. GLUT5 and CAT1 expression decreased in the YSM, while peaking at e19 then decreasing in the small intestine. Breed and flock age affected expression levels in some genes. These results demonstrate that these genes show tissue- and development-specific patterns of expression and that the YSM expresses many digestive enzymes and nutrient transporters associated with the small intestine.

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CHAPTER I.

INTRODUCTION

As feed efficiency and meat yield have increased dramatically over the past several decades, the 21-day incubation period of the embryonic chick currently makes up approximately one-third of the growing period of the market weight broiler chicken. Consequently, factors affecting embryonic nutrition, growth, and development are of increasing importance over growth performance of the posthatch bird until market weight is reached.

The embryonic chick derives its nutrients from the yolk, which later shifts to the small intestine posthatch. During development, the yolk sac membrane (YSM) is continuous with the neonatal small intestine. Because the YSM has been linked to nutrient transport during embryonic growth, we hypothesized that the YSM would express digestive enzymes and nutrient transporters similar to the small intestine.

To initiate the process of nutrient assimilation, digestive enzymes must break down proteins and carbohydrates in the small intestine or yolk sac to yield oligopeptides, amino acids and monosaccharides. Transporters can then transfer these nutrients through the enterocyte to the blood. The objective of this study was to determine the developmental expression profiles of digestive enzymes (aminopeptidase N and sucrase-isomaltase), the peptide transporter PepT1, the amino acid transporters EAAT3, CAT1 and B^0AT, and the monosaccharide transporters SGLT1 and GLUT5 in YSM and small intestine of embryos from both Leghorn and broiler birds from different aged flocks.

CHAPTER II.

REVIEW OF LITERATURE

Development of the Embryonic Chicken Gastrointestinal Tract and Extra-embryonic Membranes

Development of the Chick Gastrointestinal Tract

Body functions and gene expression in a developing chick are highly different than that of a juvenile or adult bird. A closer look at the structural changes of the gastrointestinal tract during development is necessary in order to better understand these differences. The following section can be referenced to "The Atlas of Chick Development" (Bellairs and Osmond, 2005). By the time an egg is laid, the chick embryo is estimated to already consist of 32,000-42,000 cells. At this time, the chick blastoderm consists of several regions. One of these is the marginal zone, which lies between the area opaca, the peripheral, opaque region of the blastoderm that gives rise to extra-embryonic tissues, and area pellucida, the central, translucent region of the blastoderm that gives rise to embryonic tissues. Another region is Koller's sickle, which lies directly anterior to the posterior marginal zone and appears as two lateral horns. A third region is the endophyll area, which can be found in the central region of the area pellucida. Overall, there are two layers of the embryo, which consist of the upper layer, the epiblast, and the lower layer, the hypoblast. As the chick develops, the upper layer thins out to about 4 to 6 cells in depth and the lower layer cells actively differentiate.

In the first 2 days following laying, the group of undifferentiated cells making up the blastoderm undergo a major transformation that ultimately gives rise to the basic embryonic body. Initially, the lower layer of the blastoderm consists of clumps of cells

(deemed the endophyll) that are commonly known as polyinvaginated cells or the primary hypoblast. Many of these cells give rise to germ cells, and some are thought to contribute to the yolk sac stalk. The hypoblast arises shortly after and is then replaced by the endoblast. The endoblast, which is thought to be divided into the sickle endoblast and junctional endoblast subtypes, contributes to the development of the yolk sac endoderm and yolk sac blood vessels. The gut endoderm initially arises from the definitive endoderm, another contributing factor of the lower layer of the blastoderm.

The gut endoderm arises from the definitive endoderm as the body folds during the first 3 days of development. Continuing folding gives rise to the foregut, midgut and hindgut during this time. While the endoderm makes up the epithelial lining of the gut, it also creates the muscular exterior and other associated tissues. The movements that initially helped give rise to the foregut continue, causing the foregut to extend posteriorly. Consequently, cells migrate in the direction of the intestinal portal and stimulate the foregut to increase in length. From day 1 of development to around day 2, the anterior intestinal portal, which represents the posterior end of the foregut, slows displacement in the posterior direction from a rate of 0.1 mm/h to 0.02 mm/h, respectively. The hindgut forms comparatively like the foregut, and is originally present just after the beginning of embryonic day 2 as a simple tube. Through days 3 and 4 of development, it extends into the tail region of the developing chick, forming what is known as the tail gut. The midgut lacks a ventral wall, unlike the foregut and hindgut, and is thus in constant communication with the yolk sac (Figure 2-1). As the foregut and hindgut extend in opposite directions, the midgut narrows and around day 5 of development is left as a small region called the yolk sac stalk.

By day 6 of development, the future divisions of the small intestine can be discerned from the original gut formation. The underdeveloped duodenal loop is visible beneath the liver. Soon after, the pancreas will form within this loop and completes formation around day 9 of development. A duodenal-jejunal flexure follows the primitive duodenum, and is connected to the yolk sac stalk by an extended loop. The ileum herniates into the yolk stalk since the small intestine grows so extensively, and the apex of where the ileum and jejunum join runs continuously with the yolk sac. Two ceca are present as a small bulge where the small and large intestines join; these will further develop 30 millimeters in length at hatch.

After day 6 of development, the small intestine begins to rotate, with the anterior section moving in a direction opposite of the posterior section. This process is completed around day 11 of development. Further convolutions occur until day 17, after which the small intestine is gradually drawn back into the body cavity. At around day 19 the intestinal withdrawal is complete and the remainder of the yolk sac is also enclosed within the embryonic body cavity of the chick.

Development of the Extra-embryonic Membrane

Extra-embryonic membranes are essential for the proper growth and development of the embryonic chick. Inside the shell, the chorioallantoic, amniotic, and yolk sac membranes all work to ensure a healthy, fully-developed chick at hatch. The chorioallantoic membrane aids in respiration and the transport of calcium and nitrogenous wastes. The amniotic membrane forms a fluid-filled sac that surrounds the embryo, which serves to cushion the chick during development, along with maintaining hydration. The yolk sac membrane (YSM), which will be discussed in further detail

below, encloses and protects the yolk so that it can be used as a source of nutrition for the chick during development.

Originating from the area opaca of the blastoderm, the YSM initially consists of a thin epithelial ectoderm on top of a layer of yolk droplet-filled endodermal cells. These respective layers are continuous with the upper and lower layers of the area pellucida. The area opaca becomes encapsulated by the vitelline membrane, which is a transparent, elastic layer surrounding the yolk, by 12 hours after the onset of incubation. The membrane, also called the area vitellina, is tense and serves as a support system for cell migration and growth in the area opaca and its periphery. Mesoderm cells and blood islands congregate in the area opaca over the first 48 hours and eventually form a primitive vascular layer, called the area vasculosa, that coincides with that of the developing embryo. After 3 days of incubation, the vitelline membrane is broken down and lost.

The vascularization of the yolk sac is important for the nourishment and growth of the developing chick. Prior to its formation, a chick relies solely on intracellular yolk droplets found in its own cells. These stores are mostly depleted within 48 hours of embryonic development, and, in order to access the yolk stores in the area opaca, vascularization must occur. By day 4 of embryonic development, vascularization is highly increased because of ridges and folds that have formed on the inner surface of the endodermal layer of the yolk sac. This process continues and becomes more elaborate throughout incubation. Lipids in the yolk are partially absorbed by digestive enzymes that are secreted from the endodermal layer. These partially digested lipid particles can then

be absorbed into the endodermal cells via endocytosis and transported via the vascular system to the chick.

The embryo remains attached to the yolk by the yolk sac stalk during development, though the stalk narrows as the chick matures. Most yolk is absorbed by day 19, and the remainder of the sac is drawn into the body cavity by abdominal muscles prior to hatch. The remainder of the yolk acts as a form of nutritional reserves that nourish the chick during its first day of posthatch life.

Nutrient Digestion and Transportation from YSM to the Small Intestine

In the small intestine, proteins are broken down by peptidases to yield oligopeptides and free amino acids, and carbohydrates are broken down by saccharidases to produce monosaccharides. Products of digestion are then passed through the epithelial lining of the small intestine and into the blood stream via oligopeptide, amino acid, and monosaccharide transporters. The average chicken egg yolk contains approximately 50% water, 33% fat, 15% protein, and less than 1% carbohydrates (Yadgary et al., 2010). While it is already known that lipid droplets are transported by the vascular YSM via endocytosis, the process by which protein and carbohydrates are broken down and transported is yet unknown. In the small intestine, gene protein products of the digestive enzyme aminopeptidase N (APN), the peptide transporter peptide transporter 1 (PepT1), and amino acid transporters excitatory amino acid transporter 3 (EAAT3), cationic amino acid transporter 1 (CAT1) and the neutral amino acid transporter B^0AT aid in protein digestion and absorption, while protein products from genes of the digestive enzyme sucrase-isomaltase (SI) and monosaccharide transporters sodium-glucose cotransporter 1 (SGLT1) and facilitated glucose transporter 5 (GLUT5) take part in carbohydrate

digestion and absorption (Table 2-1). Expression levels of these genes in neonatal intestinal tissue compared to YSM tissue may help elucidate their role in embryonic chick nutrient assimilation and growth.

Protein Digestion and Amino Acid Uptake in the Chick Small Intestine

Introduction

Proteins are broken down into peptides, oligopeptides, and amino acids via proteases and peptidases present during digestion. Aminopeptidases further break down oligopeptides, while dipeptides and amino acids are absorbed via peptide transporters and amino acid transporters, respectively. As optimal growth during both embryonic development and post-hatch relies on a sufficient amino acid supply, determining the role different digestive enzymes and transporters play during development is essential (Gilbert et al., 2007).

Protein Digestion in the Chicken

Material presented in this section can be found in "Scott's Nutrition of the Chicken" (Leeson and Summers, 2001). The proventriculus, the glandular stomach of the chicken, is the first actual site of protein degradation in the chicken because it contains gastric juices that are secreted as a vagal reflex following the ingestion of food. These secretions include HCl and the enzyme precursor pepsinogen, which is converted to pepsin. The main goal of proteolysis by pepsin is to free peptide molecules that can be further broken down by digestive enzymes in the small intestine such as aminopeptidases.

The mixture of proteolytic enzymes and digesta forms chyme that is then ready to pass into the small intestine. Proteins, peptones, and peptides that were made available by pepsin digestion in the proventriculus can be further broken down into oligopeptides and amino acids by the pancreas-secreted digestive enzymes trypsin, chymotrypsin, elastase, and exopeptidases. Additionally, collagenases, also secreted by the pancreas, catalyze the hydrolysis of collagen into oligopeptides.

Hydrolysis of oligopeptides containing 2-6 amino acids must occur through peptidase action in the intestinal mucosa or on the surface of microvilli of the brush border membrane of the small intestine. A prominent oligopeptidase is aminopeptidase N (APN), which cleaves neutral and basic amino acids from N-terminal ends of peptides. Small peptides can be absorbed by the PepT1 transport system, while different types of amino acids are transported by their respective systems. For example, anionic and neutral amino acids can be transported through the brush border membrane by the excitatory amino acid transporter-3 (EAAT3) and B^oAT systems, respectively, while cationic amino acids are transported across the basolateral membrane of the enterocyte by the cationic amino acid transporter-1 (CAT1) system. These transporter systems will be described in greater depth later in this literature review.

The Digestive Enzyme Aminopeptidase N (APN)

Introduction. The aminopeptidase N (APN) digestive enzyme belongs to the M1 family, which is part of the MA clan of peptidases. APN is a metalloprotease and falls into the membrane-bound type II glycoproteins that also includes cystinyl aminopeptidase, glutamyl aminopeptidase, and pyroglutamyl-peptidase II (Look et al., 1989; Sjöström et al., 2002; Drag et al., 2010). The APN gene has been cloned in the human (Olsen et al., 1988), rat (Malfroy et al., 1989), rabbit (Yang et al., 1993), pig (Delmas et al., 1994), cat (Tresnan et al., 1996), mouse (Chen et al., 1996), and chicken (Midorikawa et al., 1998). Aminopeptidase N accounts for the majority of peptidase activity in the brush border membrane, making it essential for protein digestion and amino acid absorption (Maroux et al., 1973).

Tissue and Cellular Distribution of APN. Aminopeptidase N is expressed throughout the body, and is most concentrated in the brush border membranes of the kidney, mucosal cells of the small intestine and liver, and in cells and tissues of the nervous system (Luan and Xu, 2007). The intestinal aminopeptidase is highly abundant in the ileum and jejunum, constituting approximately 8% of the total protein amount within cellular vesicles (Maroux et al., 1973).

Substrates of APN. APN accepts many peptide substrates containing N-terminal neutral amino acids. Peptides with N-terminal amino acids containing small side chains, such as alanine derivatives, have been shown to elicit high APN activity. Peptides that contain bulky side-chains at the NH_2 terminus appear to have a weaker binding affinity but a faster turnover rate (Kania et al., 1977; Drag et al., 2010). Many bioactive peptides with hydrolysis-regulated activity, such as opioids, kinins, chemokines, and extracellular matrix proteins, are also substrates of APN (Gabrilovac et al., 2011).

Structure of APN. In humans, APN is located on chromosome 15 (Luan and Xu, 2007). The APN gene consists of 20 exons of 18-205 amino acids per exon (Sjöström et al., 2002). There are two separate promoters, which are separated by an 8 kb intron (Luan and Xu, 2007). Human and chicken APN share 66% identity. In chickens, APN consists of 3196 base pairs that code for a protein containing 967 amino acid residues (Midorikawa et al., 1998). The APN enzyme is incorporated into the lipid membrane and has a homodimeric, symmetrical structure (Midorikawa et al., 1998; Sjöström et al., 2002). The two components that compose APN are loosely connected through a noncovalent bond that can easily be disrupted (Luan and Xu, 2007). In total, APN consists of 7 domains, which either face the cytosol, span the membrane, support the

catalytic region, or act as an active site. The catalytic active site is outside of the liposome and the anchoring peptide on domains V and VI (Sjöström et al., 2002).

Digestive Mechanism of APN. The APN enzyme is zinc-dependent, and domains V and VI contain 3 zinc ligands for binding (Sjöström et al., 2002). Substrates of the APN enzyme are hydrolyzed by first binding to the active site and then having the neutral amino acid at the NH_2 terminus cleaved and released as a free amino acid. Remaining peptide fragments released from the reaction can be further hydrolyzed in secondary reactions (Kania et al., 1977).

Regulation of APN. Feeding and circulating protein changes can have regulatory effects on APN expression. In the Gila monster, a binge-feeding lizard, treatment groups containing lizards that were fed normally had a significantly higher level of APN expression than animals that were fasted (Christel et al., 2007). Similar results were obtained in studies involving blood pythons (Ott and Secor, 2007). Cytokines such as Interferon-γ (INF-γ) have been found to upregulate expression of APN, while anti-inflammatory drugs like dexamethasone downregulate the gene, showing that APN is sensitive to the inflammatory and immune responses of the body (Gabrilovac et al., 2011). Additionally, promotor-binding transcription factors can regulate the rate of APN transcription. For APN in myeloid cells, there is a distal promotor eight kilobases from the translation start codon, while the promotor for APN in endothelial and epithelial cells is located slightly upstream from the start codon (Mahoney et al., 2007). Tissue-specific transcription factors in their respective region can subsequently modulate APN transcription level (Inoue et al., 1998; Mahoney et al., 2007).

Summary. The aminopeptidase N digestive enzyme is the main source for oligopeptide hydrolysis in the small intestine. Seven domains make up the structure of APN and allow for the cleavage of neutral amino acids from the N-terminal domain of peptides, releasing oligopeptides and free amino acids. Expression levels of the APN protein are controlled by many factors including changes in substrate availability and circulating proteins. APN is necessary to provide substrates for amino acid transporters.

The Intestinal Oligopeptide Transporter, PepT1

Introduction. Studies showing oligopeptide disappearance from the small intestine and reappearance in the blood stream after hydrolysis disproved original conclusions that only amino acids were absorbed by the small intestine (Christensen, 1949; Peters and Macmahon, 1970; Adibi and Morse, 1971; Adibi and Mercer, 1973; Adibi et al., 1975). Oligopeptide absorption was confirmed after amino acid transport abnormality studies involving human patients were performed. Despite the inability to absorb specific luminal amino acids due to an autosomal recessive disorder called cystinuria, peptide administration was shown to elicit assimilation of these amino acids in the blood (Matthews, 1975; Leonard et al., 1976). Initially, the functionality of the oligopeptide transport system was characterized in the human (Adibi and Morse, 1971; Adibi and Soleimanpour, 1974; Adibi et al., 1975), and since has been found in every observed species. Since the confirmation of the peptide transport system, further research has supported that it is an extremely efficient route for amino acid absorption.

The PepT1 transporter belongs to the proton-dependent oligopeptide transporter (POT) and peptide transporter (PTR) families. The POT family consists of at least 4

genes including PepT1, PepT2, Proton-coupled histidine transporter (PHT) 1 and PHT2 (Botka et al., 2000). The PepT1 transporter was originally cloned in the rabbit (Fei et al., 1994) and has since been cloned and characterized in many other species including human (Liang et al., 1995), mouse (Fei et al., 2000), rat (Saito et al., 1995), cattle (Chen et al., 1999), sheep (Chen et al., 1999), chicken (Chen et al., 2002), turkey (Van et al., 2005), and pig (Klang et al., 2005). Chicken PepT1 shares approximately 60% amino acid identity with mammalian varieties (Chen et al., 2002). In avians and mammals, the PepT1 gene is used for peptide absorption along with maintaining adequate nitrogen supply (Yang et al., 1999; Botka et al., 2000; Buyse et al., 2001).

Tissue and Cellular Distribution of PepT1. In the adult rat, the cellular location of the PepT1 protein is exclusively in the brush border membrane of absorptive epithelial cells of the villus. The peptide transporter on the basolateral membrane is not associated with the PepT1 protein and thus requires a currently unknown peptide transporter (Hussain et al., 2002). PepT1 mRNA abundance in birds is localized primarily in the duodenum, with 33 and 25% of the duodenal mRNA amount distributed in the jejunum and ileum, respectively (Chen et al., 1999). Absorptive enterocytes that line the small intestinal tract in the human show strong peptide uptake activity. Overall, PepT1 transport occurs more at villi tips rather than in the crypts and do not occur in goblet cells, lamina propria cells, and vascular structures (Groneberg et al., 2001). Lesser amounts of PepT1 mRNA have also been found in the ceca, kidney and sertoli cells (Chen et al., 2002; Augustine et al., 2005). PepT1 mRNA is not found in preintestinal digestive tissue including the crop, proventriculus, gizzard and liver (Chen et al., 1999; Chen et al., 2002).

Both mammals and avian species do not demonstrate high levels of intestinal nutrient transporter expression prior to birth or hatch. This is because the fetus and embryonic chick derive much of their required nutrients from the placenta and yolk-sac membrane, respectively (Li et al., 2008). While PepT1 gene expression has previously been found to be low just prior to hatch (e18) and to increase dramatically following hatch (Gilbert et al., 2007), expression in the YSM increases early in incubation and decreases prior to hatch (Yadgary et al., 2011).

Substrates of PepT1. PepT1 as a nutrient transportation system is low affinity and high capacity (Vig et al., 2006). Chicken PepT1 has an affinity for many peptides 2-3 amino acids long and no affinity for single amino acids or peptides longer than 3 amino acids (Chen et al., 2002). Pharmacologically active peptidomimetics, such as β-lactam antibiotics, angiotensin-converting enzyme (ACE) inhibitors, rennin-inhibitors, and amino acid esters of nucleoside drugs are also transported by the PepT1 system (Groneberg et al., 2001; Vig et al., 2006). Some nonpeptides such as ω-amino fatty acids can act as PepT1 substrates as well when they contain at least 4 methylene groups between opposing carboxyl and amino groups (Döring et al., 1998). PepT1 has highest affinity for dipeptides, most likely because the dipeptide includes optimal properties for binding and transport (Vig et al., 2006). Additionally, PepT1 is stereospecific for oligopeptides containing L-amino acids over D-amino acids (Daniel el al., 1992; Amasheh et al., 1997; Brandsch, 2004).

Altering the N- and C-terminus construct of PepT1 substrates can change the affinity of the transporter. For example, in cases where the amino nitrogen is methylated or is part of an imino ring the substrate affinity is substantially decreased (Brandsch,

2004). Additionally, substrates with a greater number of hydrophobic and bulky side chains have a greater binding affinity (Vig et al., 2006). The combination of amino acid charge in the dipeptide also has an effect on PepT1 activation. Generally, dipeptides containing two neutral amino acids elicit the highest rate of PepT1 activation, those dipeptides with one charged and one neutral amino acid show moderate activation activity, dipeptides with acidic amino acids have low activation potential, and dipeptides with 2 basic amino acids never activate PepT1 at normal intestinal pH (Vig et al., 2006).

Although PepT1 only transports di- and tri- peptides and peptidomimetics, not all varieties of these molecules can be transported. In a study where 81 different oligopeptides were used as probes to test for PepT1 activation and binding affinity, 25 exhibited little to no ability to activate PepT1, though many of them still had a binding affinity to PepT1. At this point it is difficult to differentiate between inhibitor and substrate molecules, although this study showed that both kinds definitely exist (Vig et al., 2006).

Structure of PepT1. The chicken PepT1 gene is significantly smaller than the mammalian versions (Frazier et al., 2008). This size difference is due to larger introns present in mammalian versions of the gene. Both the chicken and mammalian PepT1 gene have 23 exons. The gene is located on chromosome 1 in the chicken (Frazier et al., 2008). The chicken PepT1 protein has 714 amino acids with a molecular mass of 79.3 kDa (Chen et al., 2002). Of the 23 exons, chicken and mammalian exons 3-23 share high sequence identity, while exons 1 and 2 have little homology (Frazier et al., 2008). Interspecies alignment results show that the first 15 amino acids of the chicken PepT1 protein on the N-terminus are different from mammalian species, but that turkeys share

14 of these 15 N-terminal amino acids (Chen et al., 2002; Frazier et al., 2008). In the promoter region of the chicken PepT1 gene, ranging from -60 to -1024 bp upstream of the transcription start site, numerous positive-acting and negative-acting elements have been mapped (Frazier et al., 2008).

Mammalian and chicken PepT1 have 12 putative transmembrane domains, with a large extracellular loop occurs between domains 9 and 10. The substrate binding domain within PepT1 consists of transmembrane domains 7, 8, 9, and their connecting loops (Chen et al., 2002). Several amino acids in PTR family member structure have been found to be important or essential for function of the transporters. (Rubio-Aliaga and Daniel, 2008). In PepT1, the residue His_{57}, which is located in the second transmembrane domain, has been shown to be of central importance for function (Fei et al., 1997; Chen et al., 2000; Terada et al., 2000)

Transport Mechanism of PepT1. As there is little dipeptide hydrolase activity in the intestinal lumen and most dipeptidase activity occurs intracellularly (Adibi and Morse, 1971), PepT1 is essential to transport small peptides from the lumen into the cell for further peptide digestion (Amasheh et al., 1997). Overall, the di- and tri-peptide transporter has been shown to be a prominent protein digesta transporter.

PepT1 action is driven by H^+, rendering the PepT1 transport system electrogenic and pH dependent (Ganapathy, 1987). In order for the optimal luminal pH to be maintained, a Na^+/H^+ exchanger (NHE) exists on the apical membrane of the enterocyte. This exchanger functions by transporting 1 H^+ from the cytosol into the lumen in exchange for 1 Na^+, which is transported from the lumen into the cytosol. The cytosol is maintained at a negative potential by a basolateral Na^+/K^+-ATPase pump, which pumps

out 3 Na^+ in exchange for 2 K^+ (Adibi, 1997). Substrates of PepT1 are coupled with either 1 or 2 protons when they are cotransported across the brush border membrane. The proton-substrate coupling ratios for the PepT1 transporter are 1:1, 2:1, and 1:1 for neutral, basic and acidic substrates, respectively. At physiological pH 5.5-6.0 PepT1 transportation of neutral and acidic dipeptides is preferred (Steel et al., 1997). As pH levels increase the transport maximum of PepT1 shifts to basic amino acids which suggests that titrating amino acid side-chains of the transporter such as histidine may aid in the transportation of substrates (Chen et al., 2000).

Regulation of PepT1. Diet, feeding and outside factors such as temperature can ultimately regulate levels of PepT1 gene expression. PepT1 expression can be induced by ingested dietary protein. Once ingested, hydrolytic enzymes break the protein down into free amino acids and small peptides that are absorbed by enterocytes. These nutrients then go on to activate transcriptional activity of PepT1 and other nutrient transporter genes. Specifically, Lys, Arg, and Phe, along with several dipeptides, have been shown to induce PepT1 gene expression. In rats that were fed a protein-free diet, dipeptide transport in the ileum significantly decreased after 4 days, whereas rats fed a 50% casein diet showed an increase in dipeptide uptake. Between the two extreme diets there was a 2.4-fold difference in PepT1 mRNA level (Shiraga et al., 1999). These findings explain why rats have highest PepT1 expression levels in the first week after birth since they have a high-peptide milk diet (Shen et al., 2001). Fasting also impacts the level of PepT1 expression in the rat. In two studies, rats with free access to water were starved for either 1 or 4 days. In both studies PepT1 expression was upregulated in the jejunum and

downregulated by the dietary administration of amino acids (Ogihara et al., 1999; Thamotharan et al., 1999a).

In chickens, genetic predispositions and altered incubation temperature during embryonic development can ultimately impact expression levels of PepT1 post-hatch. Broiler chicks that were incubated at 39.6°C instead of the normal 37.4°C showed a decline in PepT1 expression on day 6 after hatch compared to day 10 for chicks incubated at 37.4°. This change in expression pattern indicates that changes in incubation temperature might alter energy expenditure and thus the expression profile of PepT1 (Barri et al., 2011). Developmental changes in PepT1 expression is predicted to be a bodily response to outside challenges in order to optimize chances of survival through development (Shen et al., 2001). Genetic predispositions regulate the amount of PepT1 expression, which was seen in different lines of birds within the same species. In White Plymouth Rock chicken lines divergently selected for high and low body weight a natural change in PepT1 expression levels was observed. In the low weight birds PepT1 gene expression was induced prior to eating, while in the high weight line this induction was absent, showing its altered role in birds with different genetically-induced metabolic needs (Mott et al., 2008).

Hormones and neurotransmitters are also known to alter PepT1 function. Protein kinase C activation or increased intracellular calcium levels inhibits peptide transport and increases gastrointestinal secretion. Therefore, protein kinase C activators such as cholera toxin or forskolin treatment upregulate peptide transport (Buyse et al., 2001; Thwaites et al., 2002). Additionally, hyperthyroidism and hypothyroidism downregulate and upregulate PepT1 expression, respectively, due to either a direct or indirect effect of the

thyroid hormone (Ashida et al., 2002; Ashida et al., 2004). The hormone leptin also increases transport activity of PepT1, while expression downregulation is observed in its absence (Buyse et al., 2001; Hindlet et al., 2007). Insulin also increases PepT1 activity by gathering intestinal protein stores into the enterocyte membrane (Thamotharan et al., 1999b). These and other such hormones help determine the time and intensity of PepT1 activation and expression.

Summary. The oligopeptide transporter PepT1 provides a method of transportation of di- and tri- peptides across the brush border membrane in both mammalian and avian species. Although present in many tissues, PepT1 expression levels are highest in the small intestine, allowing for transport of small peptides from the small intestine into the intestinal enterocyte. Gene expression and transport activity depends upon the nutritional and developmental state of the animal, along with its blood hormone concentrations and genetic makeup. Environmental factors such as diet and temperature can also influence final intestinal PepT1 activity.

The Intestinal Glutamate/Aspartate Transporter, EAAT3

Introduction. Ingested proteins and peptides are broken down into small peptides or free amino acids to be transported into the cell and utilized by the body. The excitatory amino acid transporter 3 (EAAT3) is a Na^+-dependent, brush-border membrane-bound amino acid transporter with a high affinity for anionic amino acids, including glutamate and aspartate (Kanai and Hediger, 2004; Gilbert et al., 2007). EAAT3 belongs to the solute carrier family 1 (SLC1), which consists of 5 high-affinity glutamate transporters,

including EAAT3, and two neutral amino acid transporters (Kanai and Hediger, 2004). All glutamate transporters share 44-55% sequence identity (Kanai and Hediger, 2004).

In the small intestine, glutamate is ingested and metabolized to provide the main form of energy and nitrogen to enterocytes (Iwanaga et al., 2005). The EAAT3 transporter has been found in the intestine of animals such as rabbits (Kanai and Hediger, 1992), humans (Nakayama et al., 1996), sheep (Howell et al., 2001), cattle (Howell et al., 2001), and chickens (Gilbert et al., 2007).

Tissue and Cellular Distribution of EAAT3. The amino acid transporter EAAT3, also known as EAAC1, is a neuronal and epithelial transporter. Gene expression of EAAT3 is found primarily in neurons of the hippocampus, cerebral cortex, olfactory bulb, striatum, superior colliculus and thalamus brain tissue, along with the kidney (Kanai and Hediger, 2004), heart, lung, liver, placenta, and the brush border of the small intestine (Iwanaga et al., 2005).

Distinct differences in gut EAAT3 spatial expression has been seen among species (Howell et al., 2001). For example, in rats there is a low to high mRNA expression gradient along the intestinal axis running from the proximal to distal regions, respectively (Erickson et al., 1995; Rome et al., 2002). In rabbits, strong expression levels were detected in the ileum and duodenum, but not the colon (Kanai and Hediger, 1992). Contrastingly, humans exhibit EAAT3 expression in both the small intestine and the colon (Nakayama et al., 1996), while ruminants have additional expression detected in the forestomach (Howell et al., 2001). In the chicken, mRNA levels are highest in the ileum, lowest in the duodenum, and intermediate in the jejunum (Gilbert et al., 2007).

Substrates of EAAT3. The EAAT3 transporter has a stereospecific affinity for L-glutamate, while having a nonselective affinity for D- and L- forms of aspartate (Nicholson and McGivan, 1996). In addition to glutamate and aspartate, excitatory amino acid transporters have been shown to transport cysteine (Chen and Swanson, 2003; Lee et al., 2009). The role of cysteine as a substrate has been confirmed and appears to play a role in cellular oxidative stress (Lee et al., 2009).

Structure of EAAT3. Members of the SLC1 family have a putative structure consisting of 8 α-helical transmembrane domains. Within the structure there is a predicted large, extracellular glycosylated loop between domains 3 and 4, a reentrant loop between domains 7 and 8, and a third loop that is predicted to extend into the reentrant loop (Zerangue and Kavanaugh, 1996). Additionally, 2 amino acid residues important for binding with K^+, Tyr 403 and Glu 404, have been found in transmembrane domain 7 (Kavanaugh et al., 1997; Zhang and Kanner, 1999).

Transport Mechanism of EAAT3. All glutamate transporters have similar transport mechanisms that entail the coupling of glutamate uptake with inorganic ions. An electrochemical potential gradient develops from the free energy and is used to power the uphill transport of glutamate (Kanai and Hediger, 2004). In glutamate transport, the current belief is that 3 Na^+ and 1 H^+ ions are coupled with glutamate during forward transport, while 1 K^+ ion is counter-transported (Zerangue and Kavanaugh, 1996).

Regulation of EAAT3. Expression level of EAAT3 is downregulated by activation of PKC in *Xenopus* oocytes. Rather than directly phosphorylating EAAT3, PKC indirectly alters its expression by intracellular trafficking. Although affinity for glutamate does not change, upon activation of PKC, the maximal transport rate of

EAAT3 decreases as the protein is redistributed in the plasma membrane and intracellular vesicles (Trotti et al., 2001). Specific proteins also interact with EAAT3 to regulate its activity. A protein called GTRAP3-18 associates with the carboxyl-terminal domain of EAAT3, decreasing its affinity for glutamate, causing transport rates to decrease (Lin et al., 2001; Butchbach et al., 2002).

Summary. The neuronal and epithelial EAAT3 transporter provides the primary source of energy for enterocytes by aiding in the absorption of glutamate and aspartate. Eight transmembrane domains make up the structure of EAAT3 and allow for the cotransport of Na^+, H^+ and cationic amino acids, while countertransporting K^+. Expression levels of the glutamate transporter can be controlled by protein interaction and oxidative stress.

The Intestinal Cationic Amino Acid Transporter, CAT1

Introduction. Cationic amino acid transporters (CATs) have their own subfamily within the solute carrier family, called SLC7. Within the subcategory resides four proteins including CAT1 (SLC7A1), CAT2A, CAT2B, and CAT3 (Deves and Boyd, 1998; Closs et al., 2006). Cationic amino acids are necessary to feed reactions such as nitric oxide, urea, creatine, and agmatine synthesis from the cationic amino acid arginine; polyamines, proline, and glutamine from cationic amino acid ornithine; and protein synthesis from combining these cationic and other amino acids (Mann et al., 2003; Closs et al., 2006; San MartÌn and Sobrevia, 2006)

Tissue and Cellular Distribution of CAT1. Aside from the liver, the CAT1 transporter is expressed at varying levels in most tissues throughout the body (Closs et

al., 2006). In the small intestines, CAT1 proteins are found on the basolateral membrane of the enterocyte. In the chicken, CAT1 gene expression also occurs in the bursa and thymus 1 and 7 days after hatch, respectively, and is expressed at very low levels in the spleen immediately following hatch (Humphrey et al., 2004). In the chick, CAT1 expression is relatively high in the small intestine at day 18 of incubation, and decreases linearly through hatch and early post-hatch (Gilbert et al., 2007), while expression is moderate and variable in the YSM during prenatal development (Yadgary et al., 2011).

Substrates of CAT1. The cationic amino acid transporter CAT1 has a high affinity for L-cationic amino acids including L-arginine, L-lysine, and L-ornithine. Activity of the transporter is stimulated by substrate appearance at the *trans* side of the membrane (Kim et al., 1991; Closs et al., 1997). An additional substrate that binds to CAT1 includes the murine leukemia virus. As briefly discussed below, the virus can bind to the extracellular third loop of the CAT1 structure (Albritton et al., 1993; Wang et al., 1996).

Structure and Transport Mechanism of CAT1. All members of the CAT family have 14 putative transmembrane domains (Deves and Boyd, 1998). Both the C- and N-termini are thought to be located intracellularly due to a lack of glycosylation (Albritton et al., 1989; Closs et al., 2006). A recognized amino acid site essential for transport in mice is Glu_{107}. When mutated from Glu to the amino acid Asp, CAT1 loses its ability to transport amino acids, while still being able to target and bind with viruses. This mutation analysis shows that Glu_{107} may be necessary for the binding of cationic amino acids (Wang et al., 1994).

The third extracellular loop of mouse CAT1 is known to be located extracellularly since it is known to contain a receptor for virus particles and some glycosylated amino acids (Albritton et al., 1993; Kim and Cunningham, 1993). A 42 amino acid stretch around the fourth intracellular loop of the CAT1 protein sequence has been shown to be important in the function of the protein (Closs et al., 1993). This region is highly conserved among CAT1, CAT2B, and CAT3, and substitution of different amino acids in this region highly affect transport ability. Altogether, not many polymorphic varieties of CAT1 have been found, suggesting that the protein is probably not tolerable of mutations (Closs et al., 2006)

The transportation pattern of CAT1 is widely expressed and is Na^+- and pH-independent (White and Christensen, 1982; White et al., 1982; Deves and Boyd, 1998). Hyperpolarization of the enterocyte membrane elicits activation of cationic amino acid transport (Bussolati et al., 1989; Kavanaugh, 1993).

Regulation of CAT1. Intestinal amino acid availability maintains the expression level of the CAT1 gene. During amino acid starvation, gene transcription and mRNA stability and translation increases (Hatzoglou et al., 2004). Conversely, feeding chickens a diet composed mostly of amino acids leads to an increase in CAT1 expression (Gilbert et al., 2010). The amount of cationic amino acids present within the intracellular compartment of the enterocyte and the polarization of the cell regulates uptake through the CAT1 transport system. An increase in intracellular concentration of substrate causes an increase in uptake via CAT1, while hyperpolarization of the cell increases influx while decreasing efflux of substrate (Kavanaugh, 1993; Rotmann et al., 2004). Hyperpolarization of the cell, and thus increase in substrate transport, can be caused by

administration of bradykinin (Bogle et al., 1996), adenosine (Sobrevia et al., 1997), insulin (González et al., 2004), or glucose (Flores et al., 2003). The CAT1 transporter is essential in post-natal life. Although the CAT3 nutrient transporter may compensate for CAT1 during embryonic development, mice with homozygous knockouts of these genes are 25% smaller and die within the first day after birth (Ito and Groudine, 1997; Perkins et al., 1997; Nicholson et al., 1998).

Developmental age also has a regulatory effect on CAT1 expression in chickens. From embryonic day 20 of development to 14 days post-hatch, expression levels of CAT1 decrease linearly (Gilbert et al., 2007). This post-hatch decrease suggests that CAT1 may play an important role during embryonic development and a lesser role as the bird ages.

Summary. The cationic amino acid transporter CAT1 transports cationic amino acids and provides substrates for many biological reactions. Fourteen transmembrane domains comprise the CAT1 structure and have binding sites for both cationic amino acid and viral substrates. Expression of the CAT1 protein is modulated by amino acid availability, cell polarization, and the developmental state of the organism.

The Intestinal Sodium-dependent Neutral Amino Acid Transporter, B^0AT

Introduction. The broad neutral amino acid transporter (B^0AT), denoted SLC6A19, belongs to the SLC6 subfamily of solute carriers that contains transporters for neurotransmitters, osmolytes, and amino acids (Bröer et al., 2004). As an amino acid transporter, B^0AT transports all neutral amino acids at varying levels on the brush-border membrane of the intestinal enterocyte. B^0AT has been characterized mainly in the mouse

since heterologous transport systems in the human have exhibited little activity (Bröer, 2008).

***Tissue and Cellular Distribution of* B^0AT.** *In situ* hybridization studies show high B^0AT expression in the kidney and intestine, and expressed sequence tags have also found existing expression in the thymus, spleen, uterus, blood stem cells, diencephalon, inner ear and blood vessels. In the kidney, highest expression levels were found in the proximal convoluted tubule, where most amino acid reabsorption occurs. In the intestine, expression occurs all through the small intestinal tract, with highest concentrations found in the jejunum and ileum (Terada et al., 2005). There has not been any gene expression found in the colon (Bröer, 2008).

At the molecular level, expression occurs in a gradient that is highest toward the tips of the villi (Bröer et al., 2004). Gene expression of B^0AT has also been found in the skin. As tyrosine is a precursor for melanin (Hearing and Tsukamoto, 1991), expression of this transporter in the skin suggests a possible role in UV light protection (Bröer et al., 2004).

***Substrates of* B^0AT.** Aside from transporting osmolytes, such as Na^+, and neurotransmitters, the B^0 transporter is low affinity for most neutral amino acids and carries them across the apical brush-border membrane (Bröer et al., 2004). Of the neutral amino acids, the substrate most preferred for uptake is leucine (Bröer et al., 2004). Overall, B^0AT has a larger affinity for large aliphatic amino acids such as phenylalanine, methionine and valine (Bröer, 2008; Ducroc et al., 2010). Additionally, B^0AT has shown some affinity for the anionic amino acid lysine. B^0AT may therefore transport negatively charged amino acids at a low pH, but this remains to be shown (Bröer, 2008).

***Structure and Transport Mechanism of* B^0AT.** The cDNA sequence of B^oAT consists of 1904 base pairs, which encodes a protein of 634 amino acids. The protein consists of 12 transmembrane domains, with a predicted larger loop between domains 7 and 8 (Bröer et al., 2004). The transport system of B°AT is Na^+-dependent, Cl^- -independent, and is powered by an electrogenic field. For every amino acid carried through the apical membrane, 1 Na^+ is cotransported (Bröer et al., 2004; O'Mara et al., 2006). The Na^+ ions generate the inward electrical current, and are necessary for proper transporter function (Bröer et al., 2004). Additionally, there is a strong pH dependence (Bröer et al., 2004).

***Regulation of* B^0AT.** Changing the concentration of one of the two cosubstrates, either the neutral amino acid or Na^+, has a definite impact on the affinity of substrates for binding and a possible impact on transport velocity. An increased concentration of Na^+ leads to a decrease in neutral amino acid affinity. Increasing neutral amino acid concentration affects Na^+ binding similarly, decreasing its affinity for binding. These results demonstrate that there is some competition for binding between cosubstrates, which may mean there is an overlap of binding sites (Bohmer et al., 2005; Camargo et al., 2005). Conversely, removing Na^+ entirely causes neutral amino acid transport to cease (O'Mara et al., 2006). Coexpression of the gene collectrin, whose role in amino acid uptake is still uncertain, with B^0AT is a regulatory mechanism that has started to be examined in the past 5 years. In collectrin-deficient mice B^0AT gene expression is downregulated and amino acid uptake decreases. It is hypothesized that collectrin must be coexpressed with B^0AT for sufficient neutral amino acid transport by B^0AT to occur (Danilczyk et al., 2006; Malakauskas et al., 2007)

Summary. The neutral amino acid transporter, B^0AT, is a neutral amino acid transporter that supplies nutrients such as amino acids and osmolytes to the body by aiding in intestinal absorption. Twelve transmembrane domains make up B^0AT construct and requires specific pH levels and a Na^+ to power transport. Amino acid and Na^+ concentrations, cell polarization, and coexpression of other genes such as collectrin regulate B^0AT gene expression.

Carbohydrate Digestion and Monosaccharide Uptake in the Chick Small Intestine

Introduction

Carbohydrates are broken down into monosaccharides via intestinal hydrolases (Van Beers et al., 1995). Glucose and other monosaccharides are mediated through the lipid bilayer of the intestinal enterocyte by two different types of transporters belonging to the solute carrier gene series (SLC): the Na^+-coupled carrier system (SGLT) and the facilitative glucose transporters (GLUT) (Scheepers et al., 2004). The monosaccharide transporters GLUT2, GLUT5, and SGLT1 are highly expressed in the small intestine, while other hexose transporters have negligible expression levels (Yoshikawa et al., 2011). Absorptive ability is displayed throughout the small intestine, with the duodenum acting as the primary site of monosaccharide absorption (Yoshikawa et al., 2011). To assure proper intestinal sugar delivery, both monosaccharide transporter and nutrient hydrolase gene expression must occur (Le Gall et al., 2007).

Carbohydrate Digestion in the Chicken

Information in this section can be referenced to "Scott's Nutrition of the Chicken" (Leeson and Summers, 2001). Most ingested carbohydrates are in the form of digestion-resistant, insoluble granules that need additional preparation before digestion begins. By physical wetting, grinding and amylase activation, which in wild birds and some domestic poultry occurs in the crop, these feed particles can be prepared for further digestion in the jejunum, while monosaccharides ingested directly from feed are absorbed in the duodenum.

Once passing through the proventriculus, gizzard, and duodenum with little active carbohydrate digestion occurring, feed arrives at the jejunum where the highest

concentration of carbohydrate digestive enzymes exist. The pancreas initially secretes α-amylase into the duodenum. After mixing with feed and passing into the jejunum 1,4 α-linkages on either side of the 1,6 attachment sites are hydrolyzed. The resulting molecules consist of maltose along with oligosaccharides, which are further cleaved by pancreatic enzymes maltase and isomaltase, respectively, to yield glucose. Isomaltase is derived from the protein sucrase-isomaltase (SI), which is cleaved by pancreatic enzymes into sucrase and isomaltase. Sucrase acts to hydrolyze sucrose in order to yield glucose and fructose. Glucose is readily transported into mucosal cells by the intestinal transporter Na^+-coupled glucose transporter-1 (SGLT1), which creates an electrochemical Na^+ gradient to couple glucose transport. Fructose is transported by facilitated glucose transporter-5 (GLUT5).

The Digestive Enzyme Sucrase Isomaltase (SI)

Introduction. The intestinal brush-border membrane contains 4 main glycohydrolases, which include trehalase, maltase-glucoamylase, lactase, and sucrase-isomaltase (SI) (Van Beers et al., 1995). Sucrase-isomaltase is much more abundant than maltase-glucoamylase in humans, and thus conducts approximately 80% of the maltase and maltotriase activity in the small intestine (Semenza, 1986). Additionally, all sucrose breakdown in the small intestine relies on the sucrase from SI (Van Beers et al., 1995).

Tissue and Cellular Distribution of SI. About 10% of proteins in the intestinal membrane are sucrase-isomaltase complexes (Van Beers et al., 1995). In transgenic mouse experiments, reporter genes revealed SI gene expression in the jejunum, ileum and minimally in the enterocytes of the colon (Markowitz et al., 1993). Expression of SI in

the small intestine is limited to the villi and does not appear until the crypt-villus junction, where activity commences. Similar to the glucose transporter SGLT1, which will be discussed later, expression is maximal at the lower half of the villi and decreases towards the tip (Van Beers et al., 1995). In embryonic and early post-hatch chickens SI expression is also found in the small intestine, showing expression early in development and increasing dramatically at day 19 of incubation (Sklan, 2003). SI has also been found in the embryonic YSM of the chick, although in minimal amounts (Yadgary et al., 2011)

Substrates of SI. The two active sites within the SI complex have both overlapping and unique substrate specificities. Although both sites can degrade maltose and maltitriose and are unable to hydrolyze larger glucans, only the sucrase subunit is responsible for sucrose degradation while the isomaltase subunit displays unique isomaltase activity (Semenza, 1986; Quezada-Calvillo et al., 1993; Van Beers et al., 1995). Additionally, the sucrase subunit cannot hydrolyze α(1-6) glucosidic bonds, while the isomaltase subunit has the ability (Van Beers et al., 1995). The ultimate product of all sucrase and isomaltase activity that can be absorbed is glucose.

Structure of SI. The nucleotide sequence of sucrase-isomaltase cDNA has been determined for several animals including the rabbit, human, and rat, and a partial sequence has been generated from the chicken (Hunziker et al., 1986; Chantret, 1992; Chandrasena et al., 1994; Uni, 1998). For the rabbit, human and rat the full peptide sequence was found to be 1827, 1827, and 1841 amino acids in length, respectively (Hunziker et al., 1986; Chantret, 1992; Chandrasena et al., 1994). The partial sequence of the chicken is 262 amino acids long (Uni, 1998). The enzyme complex, SI, contains two polypeptides, or domains, sucrase and isomaltase that interact noncovalently with one

another (Skovbjerg et al., 1979; Cowell, 1986). The sizes of the polypeptides varies among species but has been found to be 120-140 kDa for the sucrase component, and 140-151 kDa for the isomaltase component (Van Beers et al., 1995). A unique feature of chickens appears to be that sucrase, which is usually the smaller of the two enzyme components, is actually the larger of the two (Hu et al., 1987).

SI begins as a precursor, pro-sucrase-isomaltase, which, in the rabbit, is about 270 kDa (Wacker et al., 1981; Van Beers et al., 1995). The pro-enzyme of SI is cleaved proteolytically in the gut lumen via proteases provided by the pancreas (Montgomery et al., 1981). Of the two polypeptide components, only isomaltase interacts directly with the brush-border membrane of the intestinal wall (Cowell, 1986). Mammalian and chicken SI contain a hydrophobic sequence at the N-terminus of the isomaltase subunit that is responsible for enzyme-membrane interaction (Van Beers et al., 1995). The 3-dimensional structure of the SI complex is currently unavailable, but algorithmic predictions propose that the two protein domains are folded similarly and can be referred to as a pseudo-dimer (Jacob et al., 2002).

Digestive Mechanism of SI. The mechanism by which poly- and disaccharides are cleaved are similar between SI and the related enzymes lactase and maltase-glucoamylase. The process begins with the substrate binding to the active site of the enzyme, followed by the protonation of the substrate glucosyl oxygen. The now positively charged glucosyl-oxygen is transferred to the C-1 carbon of the glucosyl, causing the bond between the C-1 atom and attached oxygen to split. This cleavage causes the first product to be released, an α-glycan. The remaining glucose oxycarbium ion is initially stabilized by a negative charge, until the oxycarbium ion is substituted by

an –OH, rendering an α-glucose as the enzyme's second product (Van Beers et al., 1995). The glucose produced can then be absorbed by the small intestine with the help of glucose transporters such as SGLT1.

Regulation of SI. Feeding and starving patterns regulate SI expression levels. Administration of sucrose to the apical membrane of enterocytes stimulates human SI promoter activity (Le Gall et al., 2007). Starving rats causes precocious, enhanced expression of SI, which ceases to occur at the crypt-villus junction once feeding is reinstated. However, enterocytes that are already exhibiting SI expression at an enhanced level are unable to downregulate their expression even when feeding begins again. Since it takes several days for enterocytes to migrate to the villus tip, effects from starvation are seen temporarily after the condition has ended (Nsi-Emvo et al., 1994).

Protein and gene stability play regulatory roles in determining SI expression levels. Studies in various healthy animals have shown that the amount of SI mRNA directly correlates with protein amount. However, as animals age the half-lives of the enzymes decrease as degradation rate increases, causing SI protein concentration to decrease while mRNA levels stay the same (Van Beers et al., 1995). If the SI gene itself contains mutations, proper transportation to the apical membrane, and hydolysis of sucrose and maltose, cannot occur (Naim et al., 1988).

Outside factors such as sugar transporter and circulating hormone levels also impact SI expression. Sugar transporter gene expression is necessary in order to regulate SI mRNA levels. In mice with the GLUT2 glucose transporter, inactivated, SI expression did not occur or was dramatically reduced. Thus, it has been inferred that a GLUT2-dependent signaling pathway is required for normal SI expression (Le Gall et al., 2007).

Several hormones, such as glucocorticoids and insulin, can impact expression levels of the SI enzyme. In suckling rats different types of glucocorticoids have been found to either stimulate or inhibit gene expression (Van Beers et al., 1995). Diabetic mice show an increase in SI gene activity, showing a possible role of insulin in upregulating SI expression (Hoffman and Chang, 1992; Van Beers et al., 1995).

Summary. The SI enzyme is found mainly in the small intestine, and in smaller amounts in the large intestine. In order to obtain monosaccharides for absorption via sugar transporters, SI must be present to digest sucrose, maltitriose, maltose and isomaltose. Sucrase-isomaltase consists of two domains, sucrase and isomaltase, that fold to form a pseudo-dimer that contains two binding regions for substrates. Feeding, starvation, and hormonal activity all have a role in regulating SI expression.

The Intestinal Sodium-Dependent Glucose Transporter, SGLT1

Introduction. In order to supply an adequate amount of energy to cells and tissues, blood glucose level must be maintained between 80-110 mg/dL (Scheepers et al., 2004). Once dietary carbohydrates are broken down into monosaccharides, the D-glucose products can be absorbed by mature enterocytes by the coexpression of Na^+-glucose cotransporters SGLT1 and SGLT2 (Hediger and Rhoads, 1994). These transporters belong to the sodium/glucose cotransporter family (SLC5), which contains 220 or more members in human and bacterial cells (Wright and Turk, 2004). The SGLT1 gene has been cloned in many animals including the rabbit (Hediger et al., 1987), human (Hediger et al., 1989), pig (Ohta et al., 1990), and sheep (Wood et al., 1994).

Six different members of the SGLT family have been identified including high-affinity SGLT1, low-affinity SGLT2, Na^+-dependent neutral amino acid transporter (SAAT1), Na^+-dependent *myo*-inositol transporter (SMIT), proline transporter from *E. coli* (*put*P), and the pantothenic acid transporter from *E. coli* (*put*F). The mammalian sequences of SGLT1, SGLT2, SAAT, and SMIT share 50-60% sequence identity and are predominantly Na^+ coupled transporters (Hediger et al., 1989).

SGLT1 may be the most prominent if not only Na^+-glucose cotransporter in the intestine, as demonstrated by glucose-galactose malabsorption arising from a single point mutation in SGLT1 (Turk et al., 1991). Therefore, the SGLT1 transporter is important for the absorption of glucose into the blood.

Tissue and Cellular Distribution of SGLT1. The SGLT1 transporter is expressed primarily within the intestine, heart, and kidney (Zhou et al., 2003), with strong expression in the small intestine. In the intestinal and renal brush-border membranes SGLTs have a relatively low abundance, constituting approximately 0.05-0.7% of brush-border membrane protein. Transcription level and location varies among species, and is higher in the lower two-thirds of the small intestinal villi in rats beginning at the crypt-villus junction, and decreases towards the tip of the villi (Hediger and Rhoads, 1994; Lee et al., 1994; Dong et al., 1997; Yoshikawa et al., 2011). In addition to high mRNA abundance in the basal portion of the villi, there are small amounts of SGLT1 expression in the crypts (Lee et al., 1994; Dong et al., 1997). Protein incorporation into the membrane occurs as mRNA is translated, which begins as early as the crypt-villus junction and ceases slightly before the villus tip.

In situ hybridization of tissue from rabbits shows how spatial localization of gene expression can differ among species. While SGLT1 mRNA expression is spread throughout the length of the villus, the cotransporter protein is found primarily on the villus tip. Additionally, no significant amount of SGLT1 protein has been found in the crypts, cytoplasm, or basolateral membrane of enterocytes. In rabbits, mRNA abundance increases 6-fold from the base to the tip, and protein localization to the tip occurs as these mRNAs are produced and translated in the mature enterocyte (Hwang et al., 1991).

In addition to the small intestine, glucose transporters including SGLT1 are also localized in the large intestine of humans, pigs, mice and possibly many other species. Glucose is needed in the large intestine to supply energy to enterocytes. Germ-free mice that are unable to produce short-chain fatty acids from energy express SGLT1 at a tenfold higher level (Yoshikawa et al., 2011). In chick embryos SGLT1 gene expression has been found in the small intestine and YSM, as well (Barfull et al., 2002; Yadgary et al., 2011). During the last days of development, gene expression begins to occur within the developing intestine of the chicken and by maturity SGLT1 is uniformly expressed throughout the length of the small intestine, excluding the crypts (Barfull et al., 2002). From embryonic day 15 to day 20 gene expression in the YSM increases and then decreases as yolk contents diminish before hatch, showing its importance in glucose assimilation during prenatal development (Yadgary et al., 2011)

Substrates of SGLT1. SGLT1 has an affinity for monosaccharide substrates such as D-glucose, with the highest affinity, followed by D-galactose, α-MeGlc, 3-O-MeGlc, and L-glucose in order of descending affinity strength (Hediger and Rhoads, 1994). Monosaccharide recognition and selection is hypothesized to occur based on a series of

docking sites where hydroxyl groups on the monosaccharide molecules are evaluated. Carbons 2 and 3 of sugars are the most important in SGLT1 substrate specificity (Puntheeranurak et al., 2007). Phlorizin (phloretin-2'-β-glucoside), a plant product from apple tree bark, is also a substrate of SGLT1. Unlike glucose and galactose, phlorizin acts competitively as an inhibitor of SGLT1 function (Gerardi-Laffin et al., 1993).

Many cotransporters in the SLC5 family have others functions besides sugar transport. Specifically, SGLT1 also behaves as a Na^+ uniporter, water channel, urea channel and a cotransporter of water and urea (Wright and Turk, 2004).

Structure of SGLT1. The large SGLT1 gene is about 112 kb in length with 15 exons distributed throughout (Turk et al., 1994). Rabbit SGLT1 has been sequenced and found to be a 662 amino acid, hydrophobic protein and approximately 74 kDa in weight. Similar to the water transporter aquaporin, SGLT1 functions as a homotetramer (Beliveau, 1988; Hediger and Rhoads, 1994).

The monomeric unit of SGLT1 has 14 α-helical transmembrane domains, with both the N- and C- termini facing the extracellular area (Puntheeranurak et al., 2007; Liu et al., 2009). While the N- terminal portion of SGLT1 provides for Na^+ binding, the C-terminal half of the SGLT1 transmembrane domains (10-13) retains sufficient tertiary structure, allowing it to bind and transport sugar molecules down a concentration gradient in a stereospecific manner (Hirayama et al., 2007; Puntheeranurak et al., 2007). Amino acids 457 and 460 of SGLT1 interact with substrates and determine specificity (Diez-Sampedro et al., 2001).

Transport Mechanism of SGLT1. Each glucose molecule transported via SGLT1 is coupled with 2 Na^+. In order to maintain an electrochemical gradient of inwardly

directed Na^+ that allows for the uphill transport of glucose, a Na^+-K^+-ATPase pump located on the basolateral membrane of the enterocyte pumps Na^+ out of the cell and into the blood. Along with allowing glucose transport, the transepithelial flux of Na^+ creates an osmotic gradient that stimulates salt and water absorption, allowing for proper bodily hydration (Hediger and Rhoads, 1994). In a constructed 8-order reaction model, a Na^+ must first bind to SGLT1, inducing a conformational change. This structural change allows glucose to bind, along with a second Na^+ that causes additional conformational changes that proceeds to translocate the glucose and Na^+ into the cytosol of the enterocyte (Bennett and Kimmich, 1992).

Regulation of SGLT1. Diet has an impact on the regulation of SGLT1 expression. An increase in dietary carbohydrates increases SGLT1 expression and sugar transport activity (Sharp et al., 1996). Influences of diet on SGLT1 protein concentration must occur within the enterocyte life cycle, which is 2-5 days due to a fast turnover rate. Since crypt cells are the only cells competent to alter cotransporter density, upregulation of transporter expression in response to diet is much quicker (<1 day) in comparison to downregulation (>2 days) (Diamond and Karasov, 1988). For example, infusion of glucose in rat jejunum elicited an increase in SGLT1 expression within 30 minutes (Sharp et al., 1996). In chicks, feeding, starvation, and refeeding all have an impact on expression level of SGLT1. Both starved birds and birds that were starved and refed after 4 days of starvation experienced higher levels of SGLT1 expression than those birds given free access to feed (control). Simultaneously, control birds and refed birds exhibited higher net glucose uptake than starved birds. Therefore, when high levels of

substrate are present, a lower amount of transporters are necessary to transport a sufficient amount of substrate to meet nutritional needs (Gal-Garber et al., 2000).

Circadian rhythms also contribute to the regulation of SGLT1 gene expression. In trials observing SGLT1 expression over a 24-hour period, peak gene expression in rats, which are nocturnal creatures, consistently occurred right before dark, whereas in diurnal mammals this was achieved 12 hours later (Rhoads et al., 1998). Metabolic conditions such as diabetes mellitus can alter the regulation of SGLT1 expression. Hyperglycemia and/or reduced insulin levels, which can be imparted by either type I or II diabetes, can impact expression level or onset time of expression (Hediger and Rhoads, 1994). In other medical conditions involving glucose-galactose malabsorption (GGM) the SGLT1 gene is defective (Hediger and Rhoads, 1994). This condition arises as a rare autosomal recessive genetic disease due to missense, nonsense, frame-shift, or splice-site mutations in the SGLT1 reading frame (Wright and Turk, 2004). Protein kinases also play a role in regulating transport activity of SGLT1. For example, protein kinases such as PKA and PKC can regulate transport by rapidly inserting or retrieving the transporter into or out of the plasma membrane (Wright and Turk, 2004).

Summary. Sodium-glucose cotransporters are found mainly in tissues related to glucose recovery such as the small intestine (Hediger and Rhoads, 1994).The Na^+-glucose cotransporter SGLT1 is essential for glucose absorption and, thus, nutritional homeostasis. The SGLT1 functions as a homotetramer, each component of which is comprised of 14 transmembrane domains having specific, separate regions for the binding of Na^+ and sugar. Diet composition, meal timing, molecular components and health conditions regulate gene activity.

The Facilitated Fructose Transporter, GLUT5

Introduction. Saccharidases and other enzymes break down carbohydrates and disaccharides into monosaccharides that can be transported across enterocytes by their respective nutrient transporter (Le Gall et al., 2007). While SGLT1 carries glucose across the apical membrane, GLUT5 transports fructose (Le Gall et al., 2007). The GLUT family (SLC2A) consists of 14 members that can be subdivided into 3 classes designated by their sequence similarities and characteristic elements. Fructose transporter GLUT5 belongs to class II of the sugar transport facilitators (Scheepers et al., 2004).

The GLUT5 gene has been cloned in many species including the human (Kayano et al., 1990), rat (Inukai et al., 1993), rabbit (Miyamoto et al., 1996), mouse (Corpe et al., 2002), and horse (Merediz et al., 2004). Expression of GLUT5 has also been found in the chicken (Garriga et al., 2004; Mott et al., 2008), and expression levels have been found to increase linearly with age, even in the absence of fructose (Mott et al., 2008).

Tissue and Cellular Distribution of GLUT5. The fructose transporter GLUT5 is expressed primarily in intestinal tissue, kidney, and spermatozoa, and is also found at lower levels in adipose and muscle tissue (Yang et al., 2002). In the small intestine of mice, the GLUT5 transporter is primarily expressed in the first half of the small intestine, peaks in the jejunum, and decreases at the end of the ileum. Like SGLT1, intestinal GLUT5 mRNA is expressed most abundantly from the crypt-villus junction to the middle of the villi and decrease in expression towards the tip (Yoshikawa et al., 2011). Additionally, the glucose transporter GLUT5 has an elevated level of expression in follicle associated epithelium in lymphoid follicles of the large intestine (Yoshikawa et al., 2011).

Substrates of GLUT5. GLUT5 is low affinity-high capacity and has a specificity for D-fructose (TatibouÎt et al., 2000; Jones et al., 2011). Along with the standard D-fructose form, the furanose and pyranose ring forms of D-fructose also bind with GLUT5. Epimers of D-fructose do not combine with GLUT5, suggesting an important role in the positioning of hydroxyls on the sugar (TatibouÎt et al., 2000). The affinity of GLUT5 for 2,5-anhydro-D-mannitol is comparable to D-fructose (TatibouÎt et al., 2000), and has a 10-fold higher affinity than D-fructose when the C-6 or C-1 hydroxyl group is replaced with a secondary amine group (-NH) (Yang et al., 2002).

Structure of GLUT5. Genes in the GLUT family range from 8 to 35 kb in length (Turk et al., 1994). Although GLUT5 varies among species, in the mouse it has been characterized as a 7.7 kb-genomic fragment, a 2069 bp cDNA clone, and a protein consisting of 501 amino acids. The molecular weight of the mouse protein is approximately 55 kDa and has between 69% and 88% amino acid homology with human, rat, and rabbit GLUT5 (Corpe et al., 2002).

The fructose tranpsorter GLUT5 has a putative structure consisting of 12 transmembrane domains, with a large intracellular loop occurring between transmembrane domains 6 and 7 (Bell et al., 1993; Corpe et al., 2002). This intracellular loop was found to be important for sorting along the apical or basolateral membrane in a study with constructed chimeras of GLUT1 and GLUT5 (Inukai et al., 1997). Two protein domains in the GLUT5 structure have been found to be associated with fructose uptake. Chimeras constructed between GLUT5 and GLUT3, revealed that the sections between the amino terminus and intracellular loop, and the 5th and 11th transmembrane domains are essential for fructose binding and transport (Buchs et al., 1998).

Transport Mechanism of GLUT5. Overall, fructose is transported across the apical membrane of the enterocyte by the facilitative transporter, GLUT5 (Jones et al., 2011). After the sugar has bound, fructose is transported across the lipid bilayer by alternating the conformational state of GLUT5 from facing the extracellular fluid to facing the opposing, cytoplasmic side (Bell et al., 1993). Once fructose binds to either an inward or outward binding site, depending on the direction of the concentration gradient, a conformational change occurs, causing the binding site to be displaced (Walmsley, 1988; Carruthers, 1990).

Similar to glucose conditions with SGLT1, when fructose malabsorption occurs, symptoms such as chronic diarrhea and abdominal pain occur and cease when fructose is removed from the diet (Choi et al., 2003). Malabsorption occurrences increase with the increase of concentration of available fructose (Jones et al., 2011). When a large amount of fructose is available in the basolateral membrane, the sugar transporter, GLUT2, is recruited to the apical membrane to assist in fructose transport (Jones et al., 2011).

Regulation of GLUT5. Sugar ingestion regulates GLUT5 gene expression. In one study where mice were fed fructose, an increase in GLUT5 expression resulted. This increase in expression did not occur when glucose was administered, showing the affinity of GLUT5 for fructose (Le Gall et al., 2007; Jones et al., 2011). Increasing sugar levels also increases cAMP levels, showing that adenylate cyclase/PKA pathways may be necessary to regulate GLUT5 expression (Le Gall et al., 2007). Blood sugar levels and metabolic syndromes also affect the expression level of sugar transporters. In experimentally-induced diabetic rats SGLT1, GLUT2, and GLUT5 expression levels and

sugar absorption amount increased. Similar results for GLUT5 expression were seen in humans with type 2 diabetes (Yang et al., 2002; Le Gall et al., 2007).

Two promoters have been identified upstream of exon 2 to exhibit transcriptional and translational control over the expression of the GLUT5 gene. Additionally, there is a transcription factor, caudal homeobox gene (CdxA), and an upstream stimulatory factor (USF) present on the promotor of the GLUT5 gene that are essential for intestinal development and carbohydrate response, respectively. These transcription factors could elicit variations in GLUT5 expression variation and responses to changes in fructose levels (Corpe et al., 2002).

Age of individual and taste receptor functionality also have important regulatory effects on the expression and function of the GLUT5 transporter. Toddlers and infants have a significantly lower expression level of GLUT5 as much of their carbohydrate ingestion comes in the form of glucose and galactose (Jones et al., 2011). Taste receptors on the basolateral membrane combined with fructose exposure initiates GLUT5 expression. When sweet taste receptors are blocked by inhibitors, the stimulation of GLUT5 gene expression by fructose is decreased (Le Gall et al., 2007).

Summary. Fructose transport across the apical membrane of enterocytes in the small intestine is carried out primarily by the transporter GLUT5. The protein has 12 transmembrane domains with a large intracellular loop important for localization and other specific regions for fructose binding. Gene expression is controlled by fructose availability and abundance, transcription factors that bind to the promoter region, age of the individual, and functionality of taste receptors. In order to maintain fructose

circulation the GLUT5 transporter is necessary to transport luminal fructose into the enterocyte.

Conclusion

The digestive enzymes APN and SI, the peptide transporter PepT1, the amino acid transporters EAAT3, CAT1, and B^0AT, and the monosaccharide transporters SGLT1 and GLUT5 provide a means by which nutrient assimilation can take place in the chicken. Previous studies have evaluated expression of these genes in the late-term embryo and post-hatch bird small intestine, but have not yet looked at expression in embryonic small intestine and YSM simultaneously. Therefore, in this thesis the correlated responses of age and breed of the laying flock of the embryo with the embryonic expression of these genes over the last half of the 21-day incubation period was evaluated in the neonatal small intestine and YSM.

Table 2-1. Gene Description and Function.

Gene	Transporter Family	Description	Function
APN		Aminopeptidase N	Peptide digestive enzyme
SI		Sucrase-isomaltase	Carbohydrate digestive enzyme
B^0AT	**SLC6**	Neutral amino acid transporter	Na^+ -dependent neutral amino acid transporter
CAT1	**SLC7**	Cationic amino acid transporter 1	Na^+ -independent cationic amino acid transport
EAAT3	**SLC1**	Excitatory amino acid transporter 3	Na^+, H^+, K^+ -dependent, anionic amino acid transport
GLUT5	**SLC2A**	Glucose transporter 5	Na^+ -independent fructose transporter
PepT1	**SLC15**	Peptide transporter 1	Oligopeptide transporter
SGLT1	**SLC5**	Sodium/glucose cotransporter 1	Na^+ -dependent glucose and galactose transporter

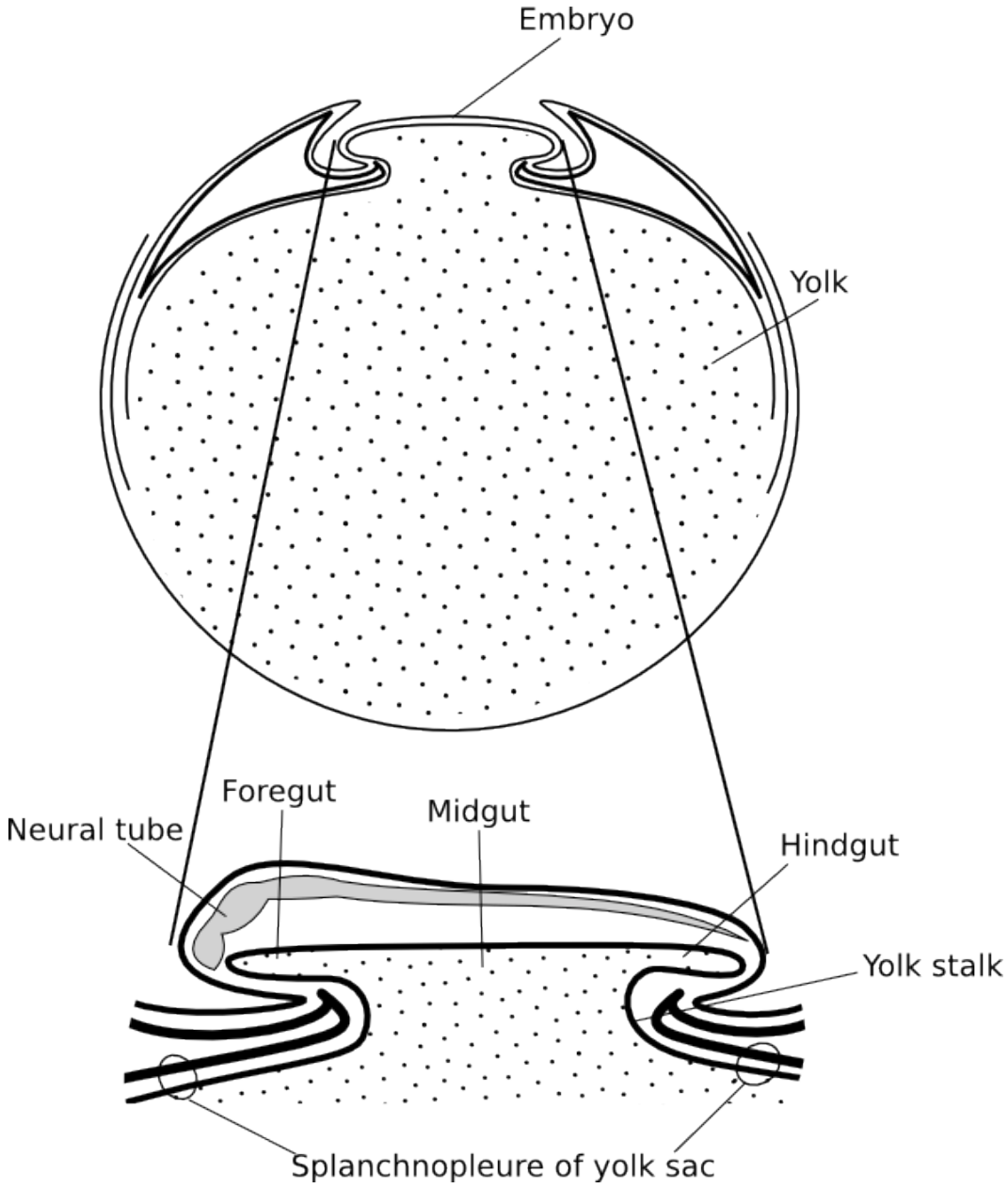

Figure 2-1. Development of the Embryonic Chick YSM and Small Intestine (e3).

J. S. Speier, 2011

CHAPTER III

OBJECTIVES

Gene expression of the digestive enzymes and nutrient transporters APN, SI, PepT1, EAAT3, CAT1, B^0AT, SGLT1, and GLUT5 in the YSM and small intestine of embryonic chicks derived from young and old flocks of Cobb and Leghorn breeds of chickens were examined in this thesis. The gene expression was determined using real-time PCR using the absolute quantification method. The objective of the first study was to examine the effect of chicken breed and line on the expression of PepT1 in the post-hatch small intestine segments. The objective of the second experiment was to determine correlated responses to flock age and bird breed in the gene expression of the 8 digestive enzyme and nutrient transporter genes in the small intestine and YSM of the developing neonatal chick.

CHAPTER IV.

MATERIALS AND METHODS

Animals and Tissue Collection

Developmental Trial. From commercial broilers (Cobb) and commercial layers (Leghorn), 224 fertile eggs were gathered and incubated simultaneously at the hatchery located in the Faculty of Agriculture, Hebrew University, Israel. Eggs were from young 22 wk (young) and 45 wk (old) Leghorn flocks or 30 wk (young) and 50 wk (old) Cobb flocks. Each age subcategory within each flock species had 5-8 embryos collected for each sampled tissue at embryonic d (e) 11, 13, 15, 17, 19, 20, and 21 (day of hatch, DOH). Yolk sac membrane was sampled from all collected embryos, and small intestinal tissue was only sampled from embryos at e15, e17, e19, e20, and e21 since the small intestine is underdeveloped until e15. From e15 and e17 the whole small intestine was sampled, while only the duodenum of the small intestine was sampled from e19, e20, and e21. The tissues were rinsed in PBS, flash frozen within cryogenic tubes in liquid nitrogen and stored at -80 °C until being shipped to Virginia Tech.

Across Breed and Line Trial. Chicks from 7 different chicken breeds or lines (Aviagen A, Aviagen B, Cobb, High Weight Select (HWS), Low Weight Select (LWS), Leghorn, and Virginia Tech Barred Plymouth Rock (VT BPR) were sampled for intestinal tissue 7 d posthatch. The small intestine was rinsed in PBS and separated into duodenum, jejunum, and ileum segments. The whole segments were then flash frozen individually within cryogenic tubes in liquid nitrogen and stored at -80 °C.

Total RNA extraction

For both experiments, RNA was extracted from tissue samples using the RNeasy Kit according to the animal tissue protocol (Qiagen, Valencia, CA). All excised tissues were stored at -80 °C prior to extraction. For extraction the tissues were ground with a mortar and pestle for the developmental trial and by homogenization for the across breeds and lines trial. In the developmental trial, tissues were retrieved from the -80 °C freezer and placed in liquid nitrogen until needed. Metal bowls for holding tissue and pestles were autoclaved prior to grinding. The mortar was filled halfway with liquid nitrogen, the bowl was placed on top, and the tissue was placed within the bowl. All of the samples were ground to a fine powder, placed in new cryogenic tubes, and placed back in the -80 °C freezer for later use.

Before RNA extraction in both trials, lysis buffer RLT solution (2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) diluted 1:100 in Buffer RLT (Qiagen, Valencia, CA)) was prepared and 600 µL aliquots were made in 2 mL tubes. After all samples were ground in the developmental trial, 20-30 mg of tissue were added to the 2 mL tubes for most RNA extraction procedures. For older YSM tissues, 40-50 mg were used since RNA yield was low. Once ground tissue was added to the RLT solution, the tube was vortexed for 30 s for proper mixing. For both experiments, the manufacturer's protocol was followed after the tissue was in solution. In the last step, RNA was eluted by rinsing the column membrane with 30 µL of RNase-free water two times. Concentration was determined using the NanoDrop, while purity was verified using Agilent RNA 6000 Nano chips on the Agilent 2100 Bioanalyzer (Agilent, Foster City, CA). In each chip 12 samples were run in parallel following the manufacturer's protocol. All samples that had

an RNA integrity number (RIN) below 7.0 were extracted again from their respective tissues. All extracted RNA samples were stored at -80 °C.

Standard Curve Generation

In both trials, fragments of genes were cloned into a pGEM®-T Easy Vector (Promega) by following the manufacturer's protocol. Genes were inserted into the vector either in a sense (T7) or antisense (SP6) orientation. The plasmids were linearized with the enzymes PstI or SacII for transcription from the T7 and Sp6 promoters, respectively. Five micrograms of plasmid, 1 µL of BSA (10 mg/ml), 4 µL of the appropriate restriction enzyme (Pst1 (10u/µL) or SacII (20,000 U/mL)), 10 µL of the 10X restriction enzyme buffer, and DEPC water to bring the total volume to 100 µL. The Pst1 restriction enzyme with NE Buffer 3 and the SacII restriction enzyme with NE Buffer 4 (New England Biolabs, Ipswich, MA) were used for T7 and SP6 genes, respectively. The 100 µL reactions were incubated at 37°C for 4 h followed by either 80°C (PST1) or 65 °C (SacII) for 20 min. The cut plasmid was purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA) and the purity of the product was verified using the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). All plasmid stock and cut plasmid were stored at 4 °C.

RNA was transcribed from the cut DNA plasmid by using the respective MEGAscript® T7 or SP6 in vitro transcription kit (Ambion, Austin, TX). For each gene, a 20 µL reaction was created using 2 µL each of ATP, CTP, GTP, and UTP (stored at 75 mM for T7 kits 50 mM for SP6 kits), along with 2 µL of 10X buffer, 1 µg of template DNA, 2 µL of 10X enzyme and DEPC water. After mixing, the reaction was incubated in

a 37°C water bath for 16 h, followed by DNase I treatment. Following incubation, RNA was eluted by adding 30 µL LiCl and 15 µL DEPC water, and incubating for 2 h at -20 °C. RNA was collected by centrifugation and washed with 70% ethanol. Pelleted RNA was resuspended in 20 µL of DEPC water. The concentration of RNA was then determined using a ribogreen assay (Molecular Probes, Eugene, OR) and a FLUOstar OPTIMA microplate reader (BMG LABTECH, Germany).

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

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

Each gene cRNA was diluted in a series of 10^{10} to 10^{4} molecules per microliter containing 10 µg/mL tRNA.

Reverse Transcription

Both experiments followed the same reverse transcription protocol to prepare complementary DNA (cDNA) for Real-Time PCR. Using the cDNA Archive Kit (Applied Biosystems, Foster City, CA) cDNA was generated from the RNA samples and the standard curve dilution series. For each reaction, a 2X master mix containing 2 µL 10X reverse transcription buffer, 0.8 µL 25X dNTPs, 2 µL 10X random primers, 1 µL Multiscribe reverse transcriptase (50 U/µL), and 4.2 µL DEPC water were added to 10 µL of 0.2 µg/µL diluted sample RNA. The RNA and master mix were combined in a thin-walled PCR tube, which was then run in a PCR thermocycler for 10 min at 25 °C followed by 120 min at 37 °C. The generated cDNA was stored at -20 °C.

Absolute Quantitative Real-Time PCR

The Applied Biosystems 7300 real-time PCR machine was used along with 96-well plates to perform quantitative real-time PCR. All cDNA samples and standards were diluted 1:30 using DEPC water prior to real-time PCR analysis. While the plate was set on ice, 2 μL of diluted sample or standard RNA were added to each well, and 23 uL of real-time PCR master mix (per reaction: 12.5 μL 2X SYBR Green Master Mix (Applied Biosystems, Foster City, CA); 0.5 μL of forward primer (5 μM) and 0.5 μL of reverse primer (5 μM); 9.5 μL DEPC water) were added on top and gently mixed. The plate containing samples and master mix was loaded into the 7300 machine and run under the following settings: 95 °C hold for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The genes analyzed included APN, B^0AT, CAT1, EAAT3, GLUT5, PepT1, SGLT1, and SI. Their respective primer sequences can be found in Table 4-1. Two housekeeping genes, Beta-actin and GAPDH, were also quantified, but not included in the data since they were not found to be constantly expressed over time (see Appendix A). All primers were created using the Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by MWG-BIOTECH, Inc. (Huntsville, AL). All samples were run in duplicate along with each standard curve. The mRNA molecules/ng of total RNA was determined from each sample by dividing the Mean Quantity generated from the sample duplicates by 2000.

Statistical Analysis

All data were analyzed using JMP® Statistical Discovery Software from SAS (SAS Institute, Cary, NC). For the developmental trial, the model included all main effects of flock age, breed, embryonic age, tissue type and all 2- and 3-way interactions. For the

across breeds trial, the model included the main effects of bird breed and tissue segment and the 2-way interaction between them. Significant effects and interactions ($P < 0.05$) were further evaluated with Tukey's test for pairwise comparisons.

Table 4-1. Forward and Reverse Primers of Genes.

Gene	Forward Primer	Reverse Primer
APN	AATACGCGCTCGAGAAAACC	AGCGGGTACGCCGTGTT
B^0AT	GGGTTTTGTGTTGGCTTAGGAA	TCCATGGCTCTGGCAGAGAT
CAT1	CAAGAGGAAAACTCCAGTAATTGCA	AAGTCGAAGAGGAAGGCCATAA
EAAT3	TGCTGCTTTGGATTCCAGTGT	AGCATGACTGTAGTGCAGAAGTAATATAT
GLUT5	TTGCTGGCTTTGGGTTGTG	GGAGGTTGAGGGCCAAAGTC
PepT1	CCCCTGAGGAGGATCACTGTTGGCAGTT	CAAAAGAGCAGCAGCAACGA
SGLT1	GCCATGGCCAGGGCTTA	CAATAACCTGATCTGTGCACCAGTA
SI	CGCAAAAGCACAGGGACAGT	TCGATACGTGGTGTGTGCTCAGTT

CHAPTER V.

PEPT1 GENE EXPRESSION IN INTESTINAL SEGMENTS ACROSS CHICKEN BREEDS AND LINES

Introduction

The 7 chicken breeds and lines involved in this study included Aviagen A, Aviagen B, Cobb 500, High Weight Select (HWS), Low Weight Select (LWS), Leghorn, and Virginia Tech Barred Plymouth Rock (VT BPR). Aviagen A and B lines are 2 commercial broiler lines genetically selected under different nutritional environments. These included a corn- and soy-based diet for Aviagen A and a wheat-based diet with higher amino acid concentrations compared to Aviagen A for Aviagen B (Gilbert et al. 2007). The HWS and LWS birds are pedigree White Plymouth Rock that were selected over 50 generations for either high weight (HWS) or low weight (LWS) (Dunnington and Siegel, 1996). The White Plymouth Rock along with the VT BPR chickens are dual purpose, laying large eggs and producing meat. Finally white Leghorn layers, and Cobb 500 broilers, were evaluated in this study as well.

Results

Gene Expression of PepT1. Across breeds and lines of chickens (Aviagen A, Aviagen B, Cobb, HWS, Leghorn, LWS, and VT BPR) there was a 2-way interaction with intestinal segment ($P < 0.001$). The duodenum had significantly higher PepT1 gene expression than jejunum or ileum in VT BPR and LWS, and Cobb 500 had significantly higher PepT1 gene expression in the duodenum compared to the jejunum (Figure 5-1).

Other breeds and lines showed no differences in PepT1 expression between intestinal segments.

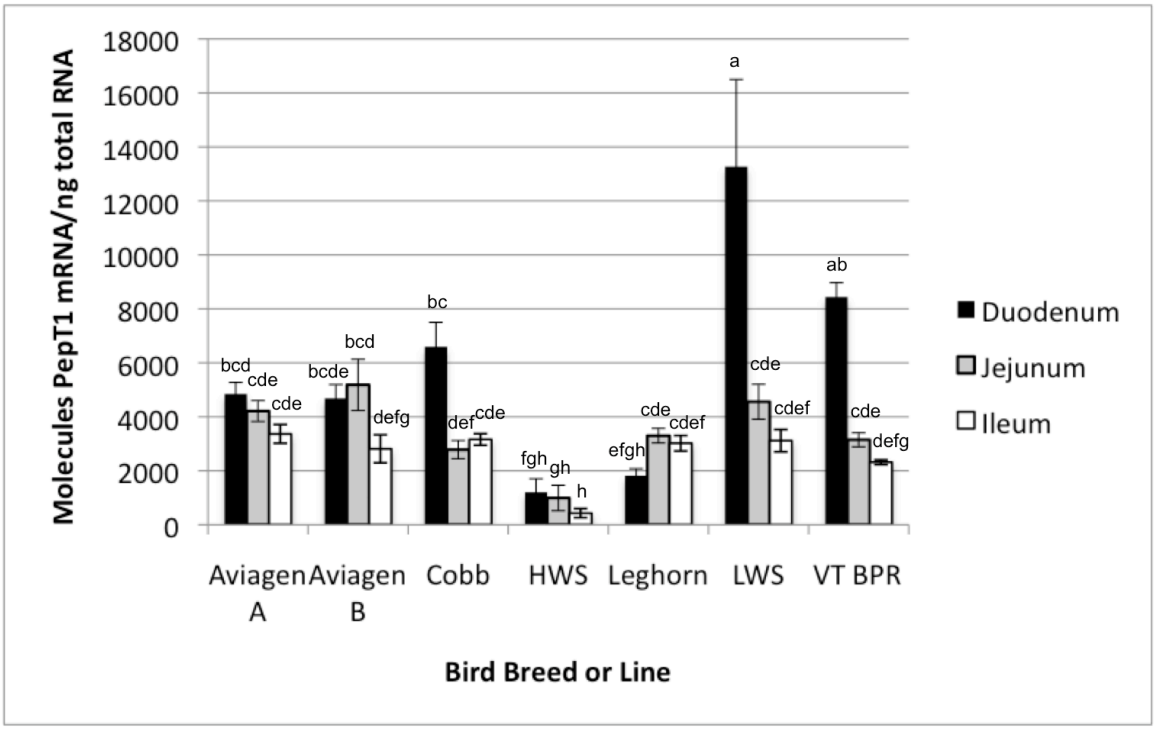

Figure 5-1. Small Intestine Segment by Bird Breed/Line Interaction PepT1 Gene Expression. The PepT1 gene expression of the sampled intestinal segments of Aviagen A, Aviagen B, Cobb 500, High Weight Select (HWS), Leghorn, Low Weight Select (LWS), and VT BPR is shown for d 7 post hatch (n = 8). There was a small intestine segment by bird line effect on PepT1 gene expression in d 7 post hatch chicks ($P < 0.001$). Bars within bird breed or line type without common letters (a - h) differ significantly by $P < 0.05$.

Discussion

Chen et al. (1999) found that in both broiler and Leghorn birds PepT1 expression was highest in the duodenum, with 33% and 25% as much expression in the jejunum and ileum, respectively. In this study, the breeds with significant differences showed this same pattern. In the study performed by Chen et al. (1999), experimental birds were 14 weeks of age. In the current study, all birds were 7 days old, which could mean that adult PepT1 distribution in the small intestine may not yet be reached. In this study, the HWS birds had the lowest PepT1 expression levels while LWS birds had the highest expression levels. Similarly, in the study performed by Mott et al. (2008), LWS birds had 6-fold higher expression levels of PepT1 than HWS birds. All of the Plymouth Rock lines, Cobb and Leghorn fell in between these two extremes. As the HWS and LWS line demonstrate the extremes of growth in the broiler chicken, low expression in the high weight line may be linked to the high feed efficiency of the bird, while the low weight line may need more gene activity in order to grow minimally enough to survive (Dunnington and Siegel, 1996). Previous studies have linked starvation/feed restriction with PepT1 activation in the rat (Ogihara et al., 1999) and in the chicken (Madsen, 2009). The LWS line of birds are anorexic and nutritionally starved, and thus the PepT1 upregulation found here is logical (Mott et al., 2008).

CHAPTER VI.

GENE EXPRESSION OF NUTRIENT TRANSPORTERS AND DIGESTIVE ENZYMES IN THE YSM AND SMALL INTESTINE OF THE DEVELOPING CHICK.

Introduction

In the chicken, digestive enzymes break down ingested feed and yolk in the intestine and yolk sac, respectively. The nutrient products are then absorbed by the small intestine and, in the yolk sac, by the YSM in order to provide nutrition to the embryonic and post-hatch bird. In this study, eggs from 30 wk (young) or 50 wk (old) commercial broilers (Cobb) and 22 wk (young) and 45 wk (old) commercial layers (Leghorn) were incubated simultaneously. At embryonic day 11 (e11), e13, e15, e17, e19, e20, and e21. YSM was sampled from all collected embryos, and intestinal tissue was sampled from embryos at e15, e17, e19, e20, and e21. Absolute quantification of mRNA molecules per ng total RNA was measured for all genes of interest, and analyzed for interactions with the tissue, breed, flock age, and embryonic age variables. Additionally, expression profiles for each gene were observed for YSM from young and old flock embryos (YY and YO) and small intestine from young and old flock embryos (IY and IO) within breeds. Expression of the housekeeping genes Beta-actin and GAPDH was also measured, but these data were not included in the data analysis since expression was not constant over time (see Appendix A).

Results

Introduction. All main effects and interaction effects can be found in Table 6-1. Breed had a main effect on the CAT1, EAAT3, and GLUT5 genes where gene expression of CAT1 and GLUT5 was higher in Cobb embryos than in Leghorn embryos and gene expression of EAAT3 was higher in Leghorn embryos than in Cobb embryos. Flock age had a main effect on the APN, B^0AT, and CAT1 genes where all 3 genes had higher expression in embryos derived from young flock than in embryos derived from old flocks. Tissue had a main effect on all genes except for GLUT5, and embryonic age had a main effect on all genes (Table 6-1).

Gene Expression of APN. Aminopeptidase N is a digestive enzyme that cleaves N-terminal amino acids from proteins. In this experiment, APN gene expression in the embryonic YSM and small intestine ranged from 60,000 to 500,000 mRNA molecules per ng total RNA (Figure 6-1). For both the Cobb and Leghorn breeds, APN gene expression in the YSM increased initially, peaked between e13 and e15, and then decreased to low levels toward the end of incubation, while the small intestine, which started with low expression levels, increased in expression toward the end of incubation (Figure 6-1). There was a 4-way interaction of embryonic age by tissue by flock age by breed ($P = 0.023$) for APN gene expression (Figure 6-2). In this interaction, on e15 YSM tissue of embryos from young flocks of Cobb birds (YYC) had greater APN gene expression than YSM tissue of embryos from old flocks of Cobb birds (YOC), while Leghorn embryos from either flock age (YYL and YOL) did not have significantly different APN expression levels. On e19, YSM tissue of embryos from young flocks of

Leghorn birds (YYL) had significantly higher APN expression than YSM from Leghorn embryos from old flocks (YOL) and all embryonic Cobb YSM (YOC and YYC).

Gene Expression of PepT1. The nutrient transporter PepT1 is a brush-border membrane-bound protein that transports luminal di- and tri- peptides into the intestinal enterocyte. In this experiment, PepT1 gene expression in the embryonic YSM and small intestine ranged from 50 to 2,500 mRNA molecules per ng total RNA (Figure 6-3). For both the Cobb and Leghorn breeds, PepT1 gene expression in the YSM increased initially, peaked between e13 and 15, and then decreased to low levels toward the end of incubation, while the small intestine, which started with low expression levels, increased in expression toward the end of incubation (Figure 6-3). These expression patterns were reflected in the embryonic age by tissue interaction ($P < 0.001$) seen from e15 to e21 (Figure 6-4) where intestinal mRNA levels increased throughout incubation while YSM expression levels decreased. From e15 to e17 YSM had higher PepT1 expression than small intestine, while from e19 to e21 small intestine had higher PepT1 expression than YSM. There was also a breed by tissue interaction ($P = 0.018$) (Figure 6-5), where PepT1 expression in the YSM was higher in the Leghorn breed than in Cobb, while expression was similar in the small intestine for Cobb and Leghorn embryos.

Gene Expression of EAAT3. The nutrient transporter EAAT3 is a brush-border membrane-bound protein that transports anionic amino acids, such as glutamate and aspartate, into the intestinal enterocyte. In this experiment, EAAT3 gene expression in the embryonic YSM and small intestine ranged from 175 to 700 mRNA molecules per ng total RNA (Figure 6-6). For both the Cobb and Leghorn breeds, EAAT3 gene expression in the YSM increased gradually throughout the incubation period examined, while gene

expression in the small intestine increased to a lesser degree (Figure 6-6). There was a 4-way interaction of embryonic age by tissue by flock age by breed (P = 0.002) for EAAT3 gene expression (Figure 6-7). On e19, YSM tissue of embryos from young flocks of Leghorn birds (YYL) had higher EAAT3 expression than YSM from Leghorn embryos from old flocks (YOL) and all Cobb embryos (YYC and YOC), and higher expression than the small intestine. On e20, YSM expression of EAAT3 from Leghorn embryos (YOL and YYL) was higher than expression within the small intestine, while YSM expression in Cobb embryos (YOC and YYC) was not significantly higher than expression in the small intestine. On e21, EAAT3 gene expression in YSM of embryos derived from old flocks of Leghorn birds (YOL) was higher than all small intestine gene expression, while YSM expression from embryos derived from old and young flocks of Cobb and Leghorn birds (YOC and YYL), respectively, was higher than all small intestine expression except that of small intestine from embryos derived from young flocks of Leghorn birds (IYL). Expression in YSM of embryos derived from young flocks of Cobb birds (YYC) was not significantly different from any other tissue samples.

Gene Expression of CAT1. The nutrient transporter CAT1 is a basolateral membrane-bound protein that transports cationic amino acids out of the intestinal enterocyte. In this experiment, CAT1 gene expression in the embryonic YSM and small intestine ranged from 30 to 1,275 mRNA molecules per ng total RNA (Figure 6-8). For both the Cobb and Leghorn breeds, CAT1 gene expression in the YSM decreased gradually to e17 and had very low expression levels thereafter, while gene expression in the small intestine was much higher than in the YSM and increased initially after e15, peaked between e17 and e19, and then decreased towards the end of incubation (Figure

6-8). The embryonic age by tissue interaction observed for CAT1 demonstrated this overall profile ($P < 0.001$) (Figure 6-9) where intestinal mRNA expression, which was higher than YSM expression throughout incubation, initially increased, peaked at e17 then decreased, while YSM gene expression continually decreased. There was a breed by flock age interaction for CAT1 gene expression ($P = 0.042$) (Figure 6-10). Within the Cobb breed, gene expression for embryos derived from young flocks was higher than embryos derived from old flocks, while in the Leghorn gene expression was similar between the young and old flocks.

Gene Expression of B^0AT. The nutrient transporter B^0AT is a brush-border membrane-bound protein that transports neutral amino acids into the intestinal enterocyte. In this experiment, B^0AT gene expression in the embryonic YSM and small intestine ranged from 35 to 2,575 mRNA molecules per ng total RNA (Figure 6-11). For both the Cobb and Leghorn breeds, B^0AT gene expression in the YSM began at low levels and increased gradually at varying rates, peaked in expression between e17 to e20, and then decreased to low expression levels around e21, while gene expression in the small intestine increased through the examined incubation period (Figure 6-11). There was a 4-way interaction of embryonic age by tissue by flock age by breed ($P < 0.001$) for B^0AT gene expression (Figure 6-12). In this interaction, on e15 and e20 YSM tissue of embryos from young flocks of Leghorn birds (YYL) had greater B^0AT gene expression than YSM tissue of all embryos derived from old flocks (YOC and YOL), while Cobb embryos from young flocks (YOC and YYC) did not differ significantly in B^0AT expression levels from any Leghorn embryos or Cobb embryos from old flocks (YOL and YYL). On e19, YSM tissue of embryos from old flocks of Cobb birds (YOC) had

higher B^0AT expression than YSM tissue from Leghorn embryos from old flocks (YOL), while B^0AT expression in YSM tissue from Cobb embryos did not differ significantly within flock ages (YYC compared to YOC) or from YSM tissue from Leghorn embryos derived from young flocks (YYL). B^0AT expression within YSM did not differ greatly from the small intestine until e21 when intestinal gene expression was significantly higher than YSM gene expression.

Gene Expression of SI. Like proteins, carbohydrates must be broken down by digestive enzymes, such as sucrase-isomaltase, to yield monosaccharides that can be transported through the enterocyte via sugar transporters, like SGLT1 and GLUT5. Sucrase-isomaltase is a digestive enzyme that degrades sucrose and isomaltose to yield glucose. In this experiment, SI gene expression in the embryonic YSM and small intestine ranged from 2 to 6,000 mRNA molecules per ng total RNA (Figure 6-13). For both the Cobb and Leghorn breeds, SI gene expression in the YSM was very low throughout the observed incubation except for some increases right before hatch, while SI expression in the small intestine was on average 20-fold higher than YSM expression and increased at the onset of the observed period and changed little thereafter (Figure 6-13) (Table 6-1). A 3-way interaction between embryonic age, tissue, and flock age for SI gene expression occurred ($P < 0.001$) (Figure 6-14). On e19 and e21 of development, YSM from embryos derived from young flocks (YY) had higher expression than those from embryos derived from old flocks (YO).

Gene Expression of SGLT1. The nutrient transporter SGLT1 is a brush-border membrane-bound protein that transports glucose into the intestinal enterocyte. In this experiment, SGLT1 gene expression in the embryonic YSM and small intestine ranged

from 270 to 6,000 mRNA molecules per ng total RNA (Figure 6-15). For both the Cobb and Leghorn breeds, SGLT1 gene expression in the YSM was low until the end of incubation where it increased slightly, while SGLT1 expression in the small intestine was low initially then increased to a larger degree than YSM (Figure 6-15). There was a 4-way interaction of embryonic age by tissue by flock age by breed ($P < 0.001$) for SGLT1 gene expression (Figure 6-16). On e20, YSM from embryos derived from old flocks of Leghorn birds (YOL) had higher expression than YSM from all Cobb embryos (YOC and YYC), while expression in YSM from embryos derived from young flocks of Leghorn birds (YYL) was not significantly different from YSM from Cobb embryos (YOC and YYC) or other Leghorn embryos (YOL). Additionally, on e21 within the small intestine SGLT1 expression was higher in Cobb embryos derived from young flocks (IYC) than all Leghorn embryos, while Cobb embryos derived from old flocks (IOC) and Leghorn embryos derived from young flocks (IYL) had higher expression than Leghorn embryos derived from old flocks (IOL).

Gene Expression of GLUT5. The nutrient transporter GLUT5 is a brush-border membrane-bound protein that transports fructose into the intestinal enterocyte. In this experiment, GLUT5 gene expression in the embryonic YSM and small intestine ranged from 25 to 500 mRNA molecules per ng total RNA (Figure 6-17). For both the Cobb and Leghorn breeds, GLUT5 gene expression in the YSM decreased to low levels throughout the incubation period and ended at very low levels, while the small intestine increased slightly in expression, peaked around e19, and then decreased at the end of incubation (Figure 6-17). A 3-way interaction between tissue, flock age and breed occurred for GLUT5 gene expression ($P < 0.001$) (Figure 6-18). GLUT5 gene expression in YSM and

small intestine from embryos derived from old flocks was higher within the Cobb breed (OC) than in the Leghorn (OL). GLUT5 gene expression of small intestine from embryos derived from young flocks was also higher in the Cobb breed (OC) than the Leghorn breed (OL). A second 3-way interaction was observed between embryonic age, flock age, and breed ($P = 0.008$) (Figure 6-19). On e15 and e19 embryonic tissue derived from young flocks was higher in the Cobb breed (YC) than in the Leghorn embryo (YL). Additionally, on e17, e19, e20, and e21, GLUT5 expression in embryos from old flocks was higher in the Cobb breed (OC) than in the Leghorn (OL). There was also an embryonic age by tissue interaction observed for SI ($P < 0.001$) (Figure 6-20) where GLUT5 gene expression in the YSM decreased over time, while expression in the small intestine increased, peaked at e19, then decreased. From e15 to e17 GLUT5 expression in the YSM was higher than expression in the small intestine, while from e19 to e21 intestinal expression was higher than YSM expression.

Table 6-1. Main Effects and Interactions of Variables within Nutrient Transporter and Digestive Enzyme Gene Expression.

	Gene							
Item	**APN**	**B^0AT**	**CAT1**	**EAAT3**	**GLUT5**	**PepT1**	**SGLT1**	**SI**
	Molecules of mRNA/ng of total RNA							
Breed								
Cobb	241,042	627	352	357	178	736	1,205	1,700
Leghorn	252,943	439	220	440	97	812	1,245	2,535
SEM	10,227	37	20	13	6	43	87	137
P-value	0.2521	0.0575	0.0086	<.0001	<.0001	0.0753	0.0002	0.0632
Flock Age								
Old	228,286	505	257	385	144	719	1,233	1,846
Young	265,665	564	319	411	132	828	1,217	2,379
SEM	10,143	39	21	13	7	43	88	139
P-value	0.0257	0.0135	0.0029	0.1724	0.9188	0.0985	0.1641	0.0697
Tissue								
Intestine	306,983	680	502	328	122	727	1,742	4,020
YSM	186,037	386	71	469	154	821	699	183
SEM	9,540	36	14	12	6	43	72	84
P-value	<.0001	<.0001	<.0001	<.0001	0.2331	<.0001	<.0001	<.0001
Embryonic Age								
E15	306,444	223	317	288	183	950	496	1,519
E17	238,526	418	414	386	157	458	639	2,387
E19	220,074	517	365	370	179	623	852	2,206
E20	246,745	533	196	484	92	594	1,256	2,106
E21	221,520	968	136	479	73	1,183	2,837	2,375
SEM	15,427	47	30	19	9	55	79	219
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	2-Way Interaction *P*-value							
Interaction								
B X F	0.9823	0.0419	0.0025	0.7244	0.0028	0.9106	0.7646	0.9493
B X T	0.0267	0.0137	0.1691	0.0001	0.4307	0.0180	<.0001	0.2860
B X E	0.4094	0.0835	0.0631	0.6468	0.6520	0.2915	0.0614	0.8869
F X T	0.7623	0.0889	0.7218	0.2229	0.3252	0.4923	0.0181	0.0084
F X E	0.8054	0.0681	0.7218	0.2575	0.0466	0.2150	0.3659	0.6857
T X E	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	3-Way Interaction *P*-value							
B X F X T	0.5702	0.3297	0.9292	0.3554	0.0148	0.6720	0.6964	0.6752
B X F X E	0.3103	0.2781	0.4147	0.0003	0.0080	0.3205	0.0520	0.9145
B X T X E	0.0057	0.0237	0.9977	0.2319	0.2656	0.3412	0.0010	0.3574
F X T X E	0.0104	0.0037	0.7082	0.0646	0.0726	0.2059	0.0053	<.0001
	4-Way Interaction *P*-value							
B X F X T X E	0.0203	<.0001	0.4301	0.0020	0.2548	0.2507	<.0001	0.1072

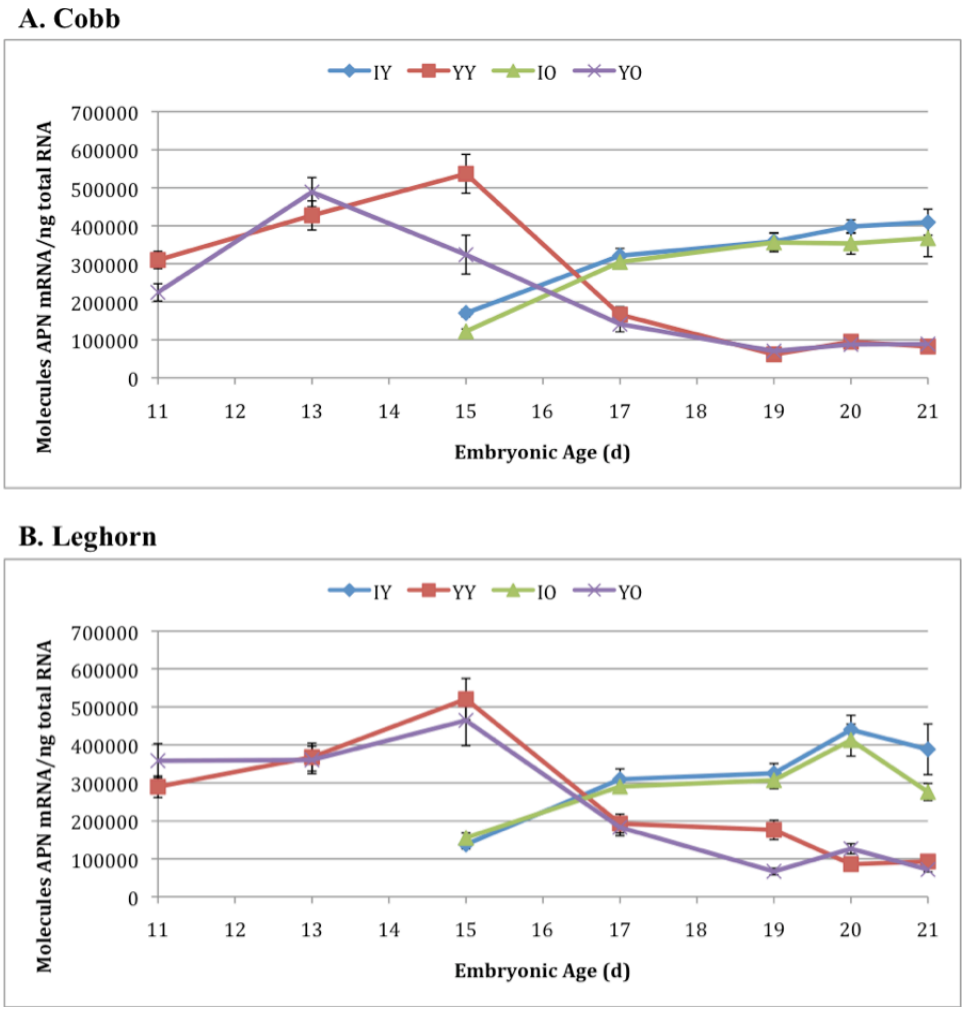

Figure 6-1. APN Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. APN gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), YO tissue increased in APN expression from e11 to e13 ($P = 0.001$), and decreased gradually from e13 to e17 ($P < 0.001$), while YY tissue increased gradually from e11 to e15 ($P = 0.002$), and decreased from e15 to e17 ($P < 0.001$). From e17 to e20 gene expression for both flock ages fell slightly, then rose slightly ($P< 0.001$) and had minimal expression changes at e21. Expression in both IY and IO tissue increased gradually from e15 to e21 ($P < 0.001$). In the Leghorn breed (B), gene expression in YY tissue increased gradually from e11 to e15 ($P = 0.007$), while expression in YO did not change significantly. After peaking at e15, both flock types decreased in gene expression until e21 ($P < 0.001$). Gene expression in both IY and IO increased minimally from e15 to e20 and appeared to decrease thereafter. [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

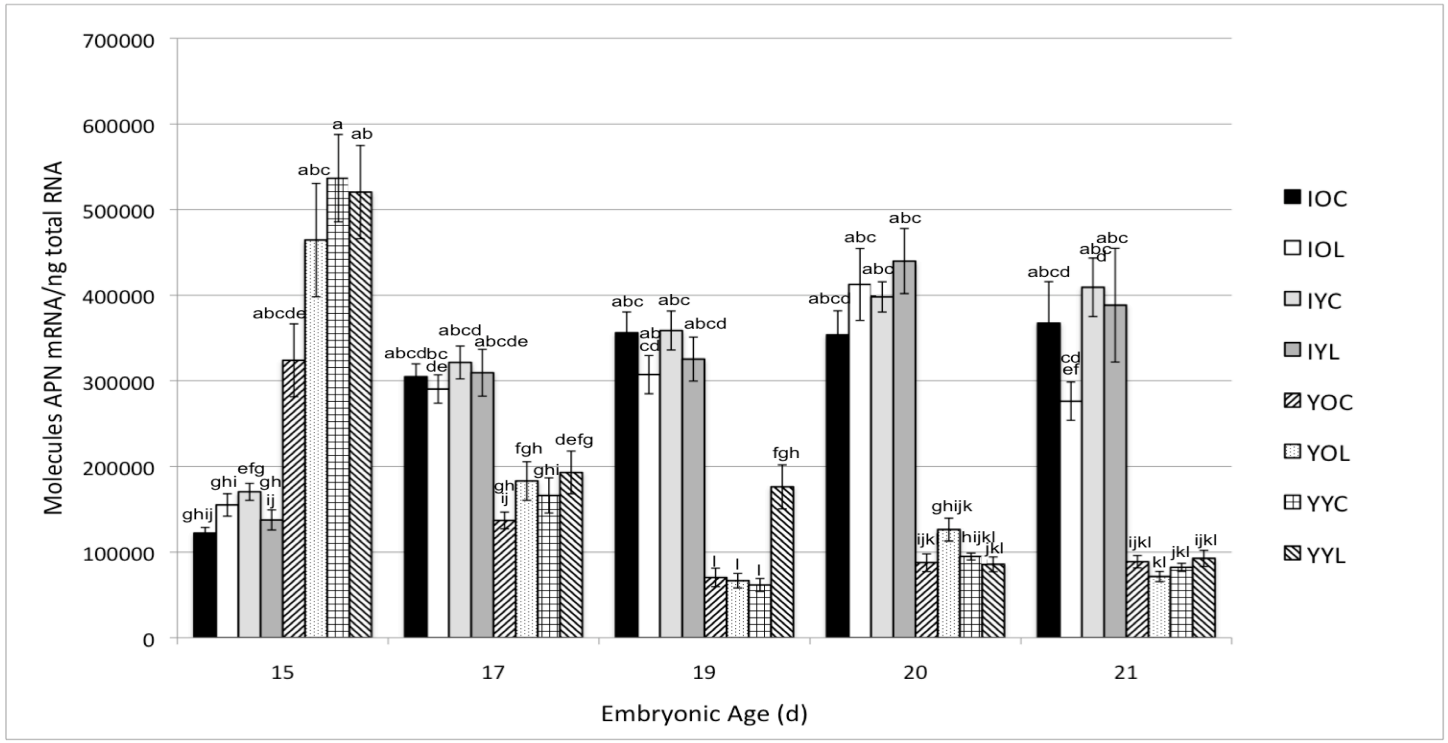

Figure 6-2. Embryonic Age by Tissue by Flock Age by Breed Interaction for APN Gene Expression in Small Intestine and Yolk Sac Membrane in Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks. APN gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). There was an embryonic age by tissue by flock age by breed interaction for APN gene expression in small intestine and YSM within chick embryos from young and old laying flocks of Cobb and Leghorn birds at e15, e17, e19, e20, and e21 ($P = 0.023$). Bars without common letters (a - l) differ significantly by $P < 0.05$. [IOC = Small Intestine/Old Flock/Cobb, IOL = Small Intestine/Old Flock/Leghorn, IYC = Small Intestine/Young Flock/Cobb, IYL = Small Intestine/Young Flock/Leghorn, YOC = YSM/Old Flock/Cobb, YOL = YSM/Old Flock/Leghorn, YYC = YSM/Young Flock/Cobb, YYL = YSM/ Young Flock/Leghorn (embryonic tissue/embryo laying flock age/breed).]

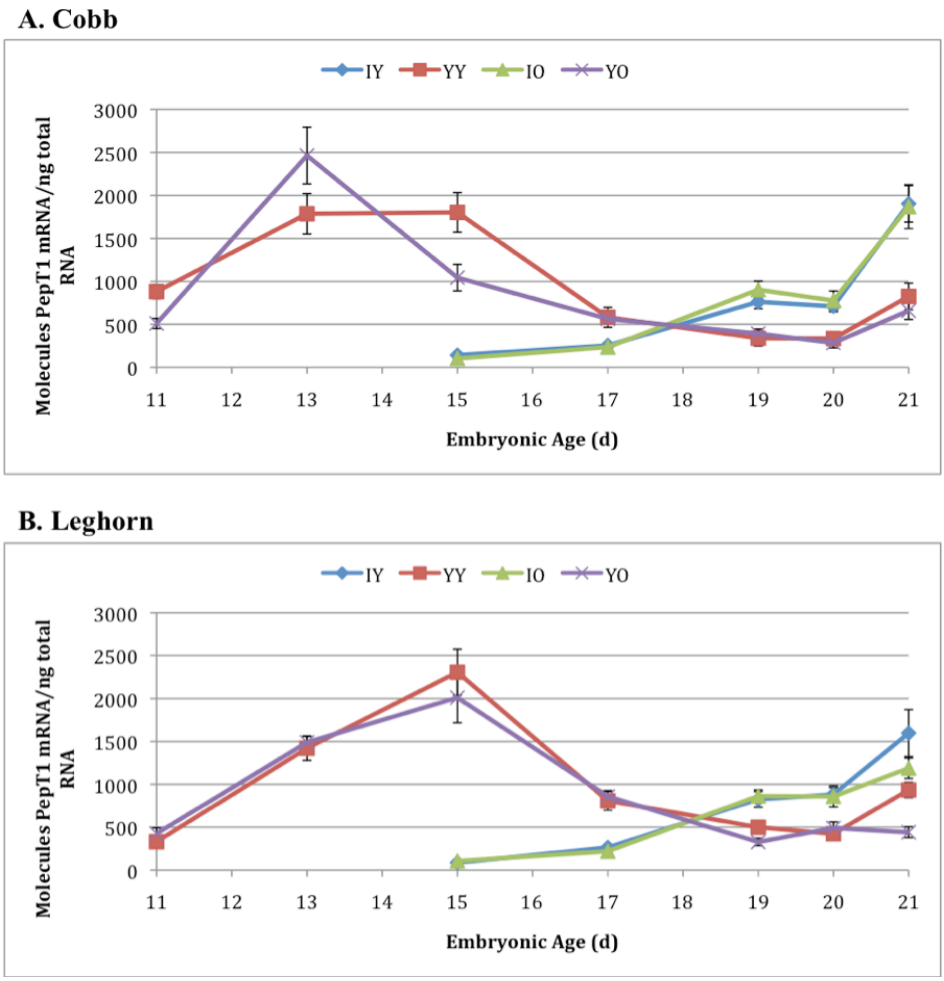

Figure 6-3. PepT1 Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. PepT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), PepT1 expression in YO tissue increased from e11 to e13 ($P < 0.001$) and decreased from e13 to e17 ($P < 0.001$). Expression in YY tissue increased slightly, then, from e15 to e17 decreased in expression ($P < 0.001$). Expression in both YY and YO tissues decreased from e17 to e20 and increased from e20 to e21 ($P < 0.001$). Both IY and IO tissues had an increase in PepT1 expression from e15 to e19 ($P < 0.001$) and a second expression increase from e20 to e21 ($P < 0.001$). In the Leghorn breed (B), PepT1 expression in both YY and YO tissues increased from e11 to e15 ($P < 0.001$) and decreased from e15 to e19. While YO tissue showed minimal expression change thereafter, YY tissue expression increased from e20 to e21 ($P < 0.001$). Both IY and IO tissues showed an increase in PepT1 expression from e15 to e19 ($P < 0.001$). While IO tissue showed minimal expression change thereafter, IY tissue expression increased from e20 to e21 ($P = 0.031$). [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

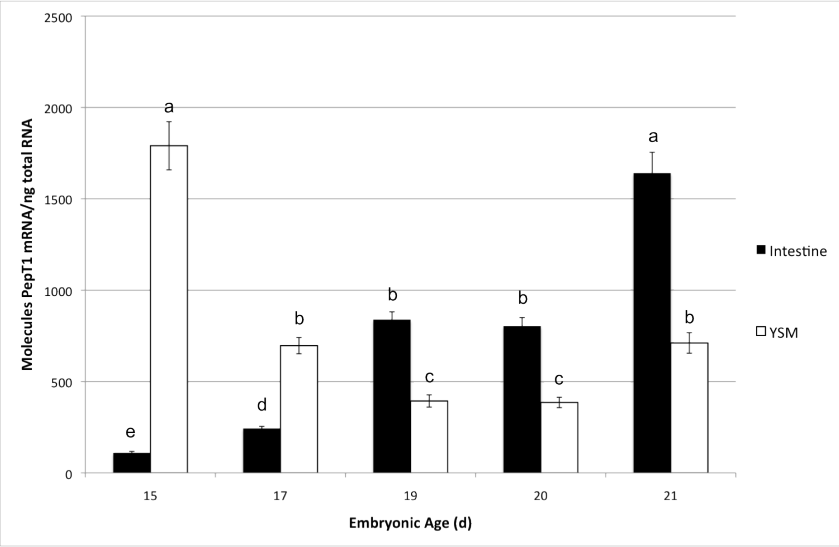

Figure 6-4. Embryonic Age by Tissue Interaction for PepT1 Gene Expression.
PepT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from embryonic chick yolk sac membrane (YSM) and small intestine tissues at e15 to e21 (n = 26 – 32). There was an embryonic age by tissue interaction for APN gene expression in Cobb and Leghorn embryonic chicks at e15, e17, e19, e20, and e21 ($P < 0.001$). Bars without common letters (a - e) differ significantly by $P < 0.05$.

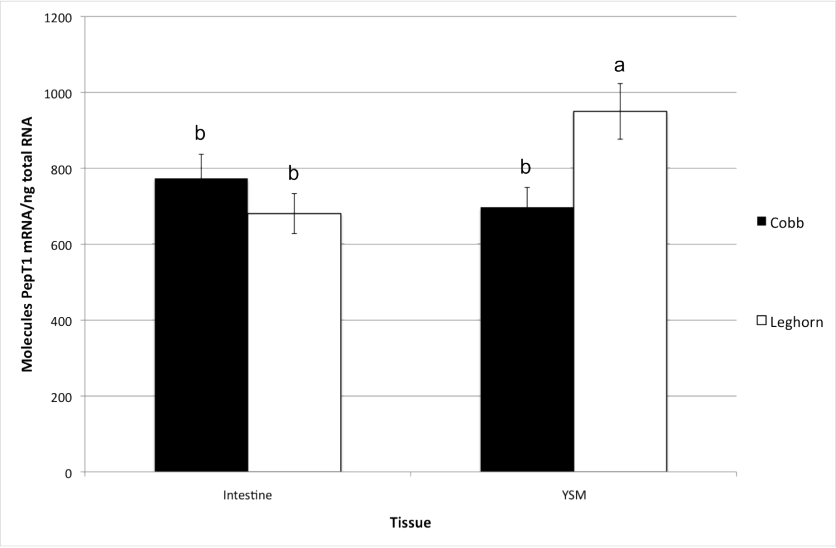

Figure 6-5. Tissue by Breed Interaction for PepT1 Gene Expression.
PepT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues of Cobb and Leghorn chicks (n = 72 – 77). There was a tissue by flock age interaction for PepT1 gene expression in Cobb and Leghorn developing embryonic chicks ($P = 0.018$). Bars without common letters (a or b) differ significantly by $P < 0.05$.

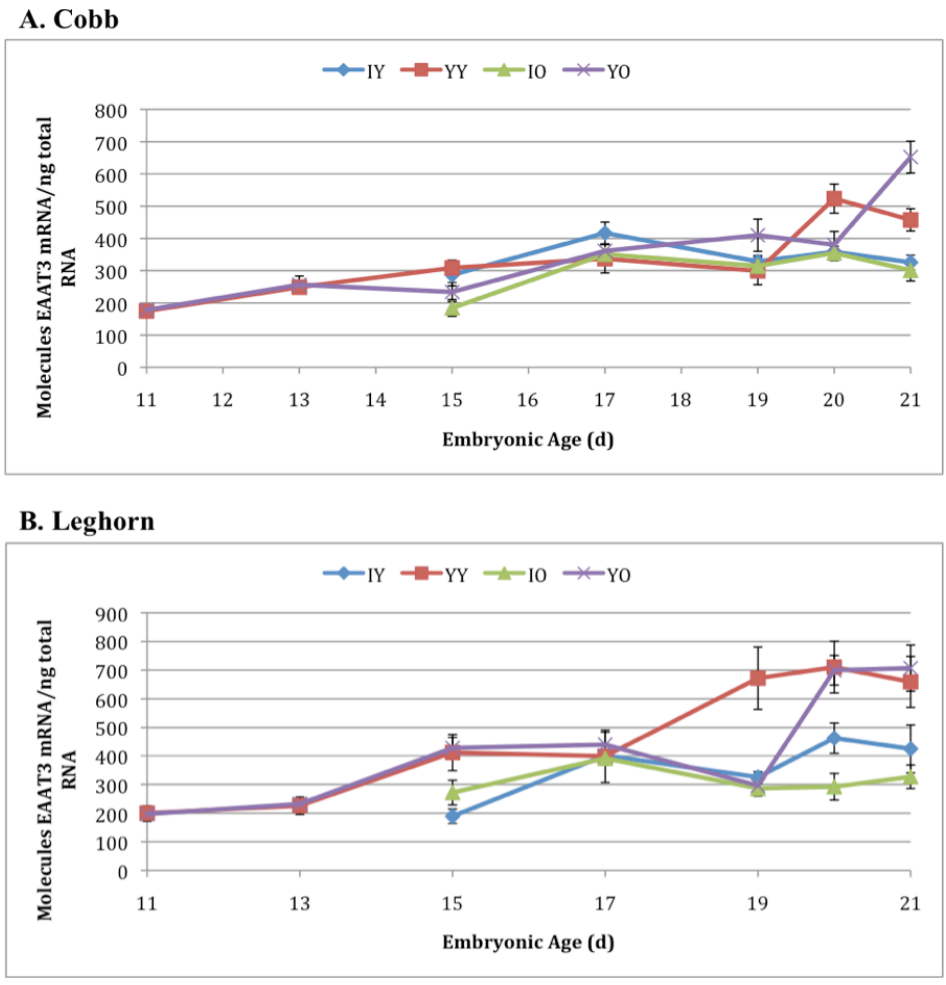

Figure 6-6. EAAT3 Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. EAAT3 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), EAAT3 expression in YO tissue is consistent until e15 when it increased gradually until e19 (P = 0.003) and then from e20 to e21 it increased (P = 0.0086). Gene expression in YY tissue increased gradually from e11 to e15 (P = 0.026), maintained expression until e19 where it increased to e20 (P = 0.001) and maintained expression levels thereafter. Expression of EAAT3 in both IY and IO tissues increased from e15 to e17 (P < 0.001) and had minimal expression changes thereafter. In the Leghorn breed (B), both YY and YO gene expression increased between e11 and 15 (P < 0.001). Expression increased in YO tissue from e19 to e20 while YY tissue expression increased from e17 to e20 (P = 0.036). Both YY and YO tissues had minimal expression changes thereafter. EAAT3 expression in the IY tissue increased from e15 to e17 (P < 0.001). No significant changes occurred thereafter or in the small intestine of the old flock subcategory. [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

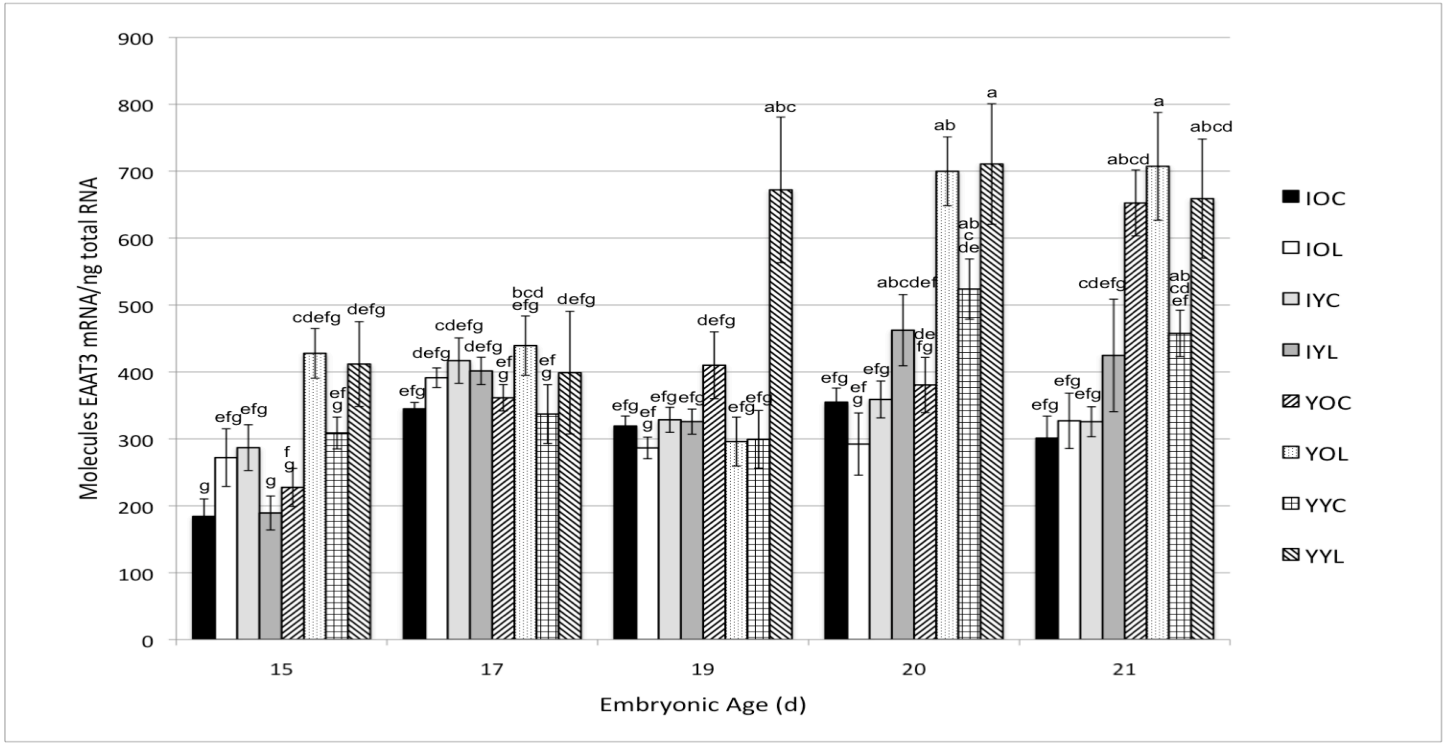

Figure 6-7. Embryonic Age by Tissue by Flock Age by Breed Interaction for EAAT3 Gene Expression in Small Intestine and Yolk Sac Membrane in Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks. EAAT3 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). There was an embryonic age by tissue by flock age by breed interaction for APN gene expression in intestine and YSM within chick embryos from young and old laying flocks of Cobb and Leghorn birds at e15, e17, e19, e20, and e21 ($P = 0.002$). Bars without common letters (a - g) differ significantly by $P < 0.05$. [IOC = Small Intestine/Old Flock/Cobb, IOL = Small Intestine/Old Flock/Leghorn, IYC = Small Intestine/Young Flock/Cobb, IYL = Small Intestine/Young Flock/Leghorn, YOC = YSM/Old Flock/Cobb, YOL = YSM/Old Flock/Leghorn, YYC = YSM/Young Flock/Cobb, YYL = YSM/ Young Flock/Leghorn (embryonic tissue/embryo laying flock age/breed).]

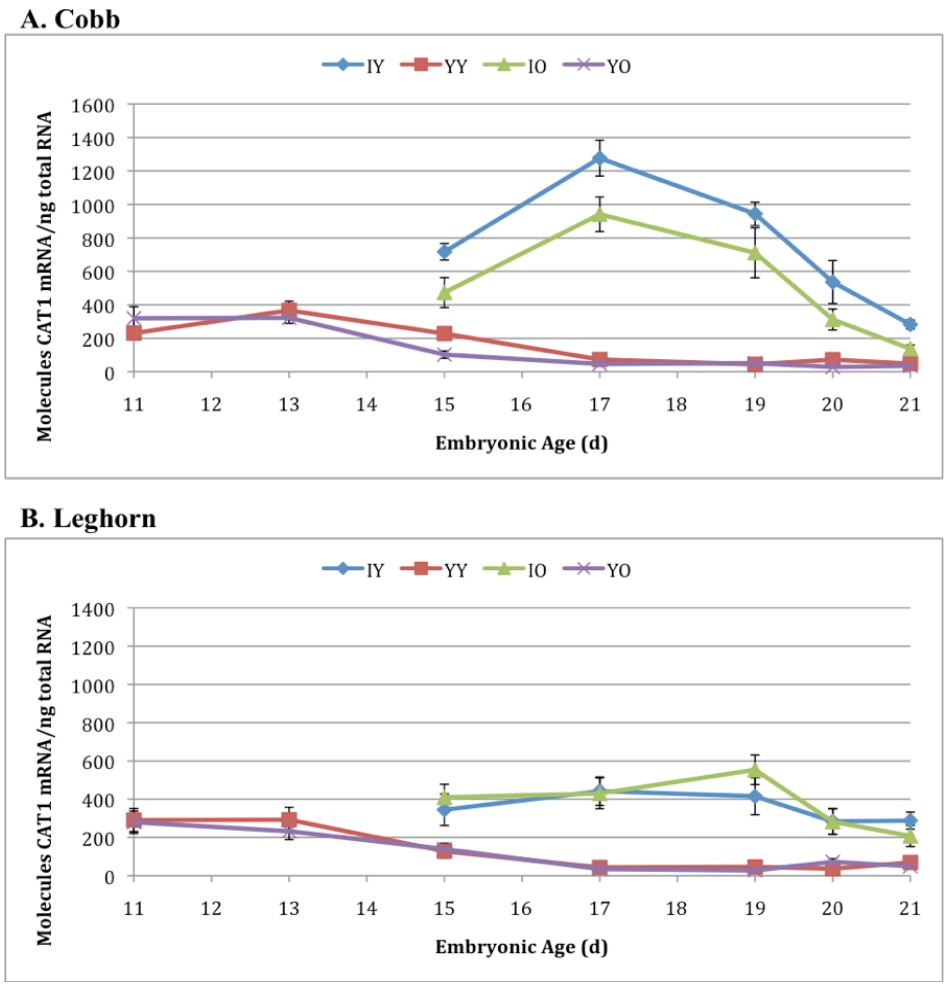

Figure 6-8. CAT1 Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. CAT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), CAT1 expression YO tissue decreased from e13 to e17 ($P < 0.001$) and maintained levels thereafter. In YY tissue expression decreased gradually from e11 to e17 ($P < 0.001$) and maintained expression levels thereafter. Expression in IO tissue increased slightly from e15 to e17. Small Intestine expression in IY tissue increased from e15 to e17 ($P = 0.047$). Both IY and IO tissues decreased in expression from e17 to e21 ($P < 0.001$). In the Leghorn breed (B) both YY and YO had decreasing amounts of expression from e11 to e17 ($P < 0.001$) and minimal expression changes thereafter. The IO tissue maintained expression levels until e19 where it decreased until e21 ($P = 0.005$). No significant variation during embryonic development for IY tissue. [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

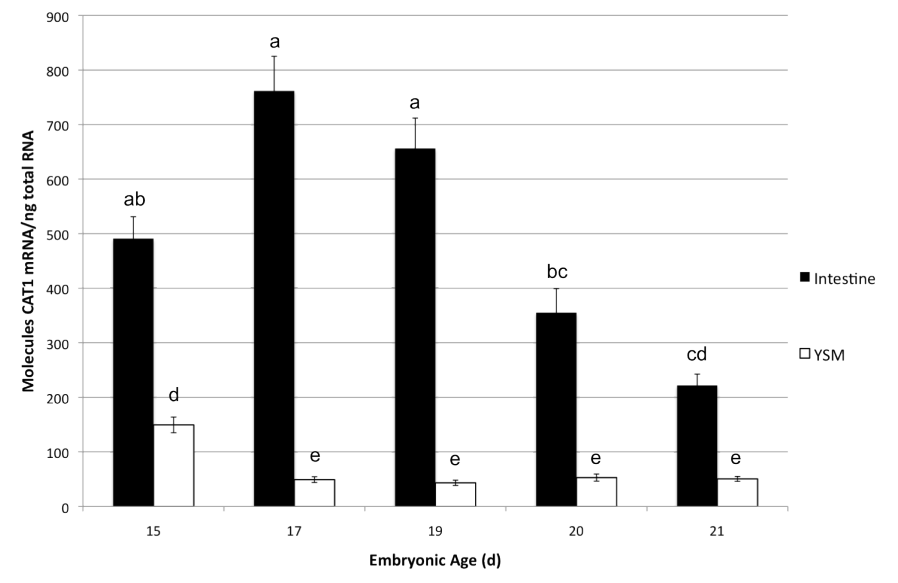

Figure 6-9. Embryonic Age by Tissue Interaction for CAT1 Gene Expression. CAT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from embryonic chick yolk sac membrane (YSM) and small intestine tissues at e15 to e21 (n = 24 – 32). There was an embryonic age by tissue interaction for CAT1 gene expression in Cobb and Leghorn embryonic chicks at e15, e17, e19, e20, and e21 ($P < 0.001$). Bars without common letters (a - e) differ significantly by $P < 0.05$.

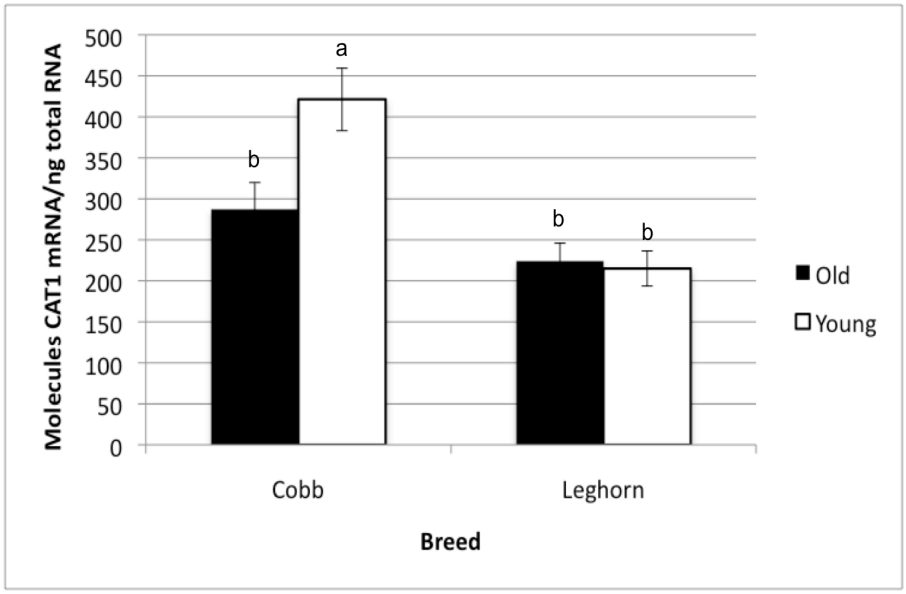

Figure 6-10. Breed by Flock Age Interaction for CAT1 Gene Expression. CAT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from chick embryos from 22-30 wk (young) and 45-50 wk (old) flocks of Cobb and Leghorn birds (n = 73 – 78). There was a tissue by flock age interaction for CAT1 gene expression in Cobb and Leghorn developing embryonic chicks ($P = 0.042$). Bars without common letters (a or b) differ significantly by $P < 0.05$.

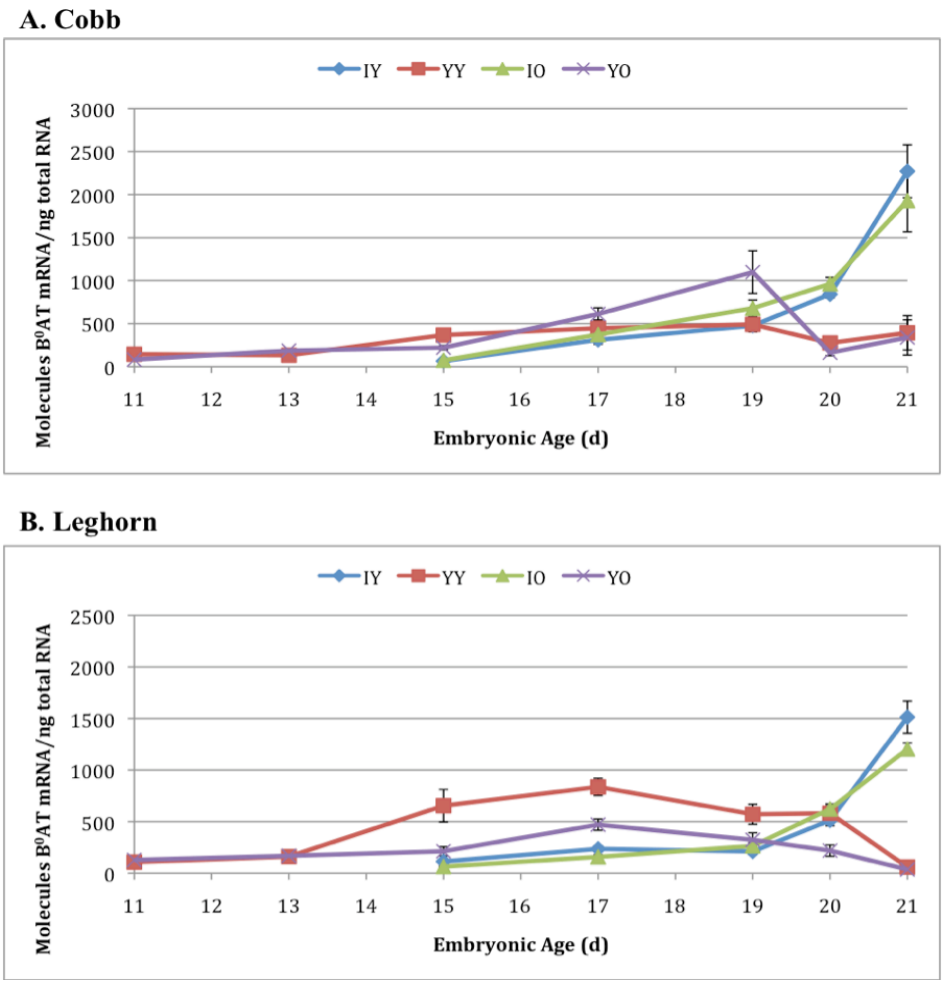

Figure 6-11. B^0AT Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. B^0AT gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), B^0AT expression in YO tissue increased from e11 to e19 ($P < 0.001$), decreased from e19 to e20 ($P < 0.001$), and increased from e20 to e21 ($P < 0.001$). Expression in YY tissue increased from e11 to e15 ($P = 0.048$), maintained expression levels from e15 to e20, and decreased in expression from e20 to e21 ($P = 0.003$). Both IY and IO tissues gene expression increased continually from e15 to e21 ($P < 0.001$). In the Leghorn breed (B) B^0AT expression YO increased from e13 to e17 ($P < 0.001$), and decreased from e17 to e21 ($P < 0.001$). Expression in YY tissue increased from e13 to e17 ($P < 0.001$), maintained expression levels from e19 to e20, and decreased expression from e20 to e21 ($P < 0.001$). Both IY and IO gene expression increased continually from e15 to e21 ($P < 0.001$). [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

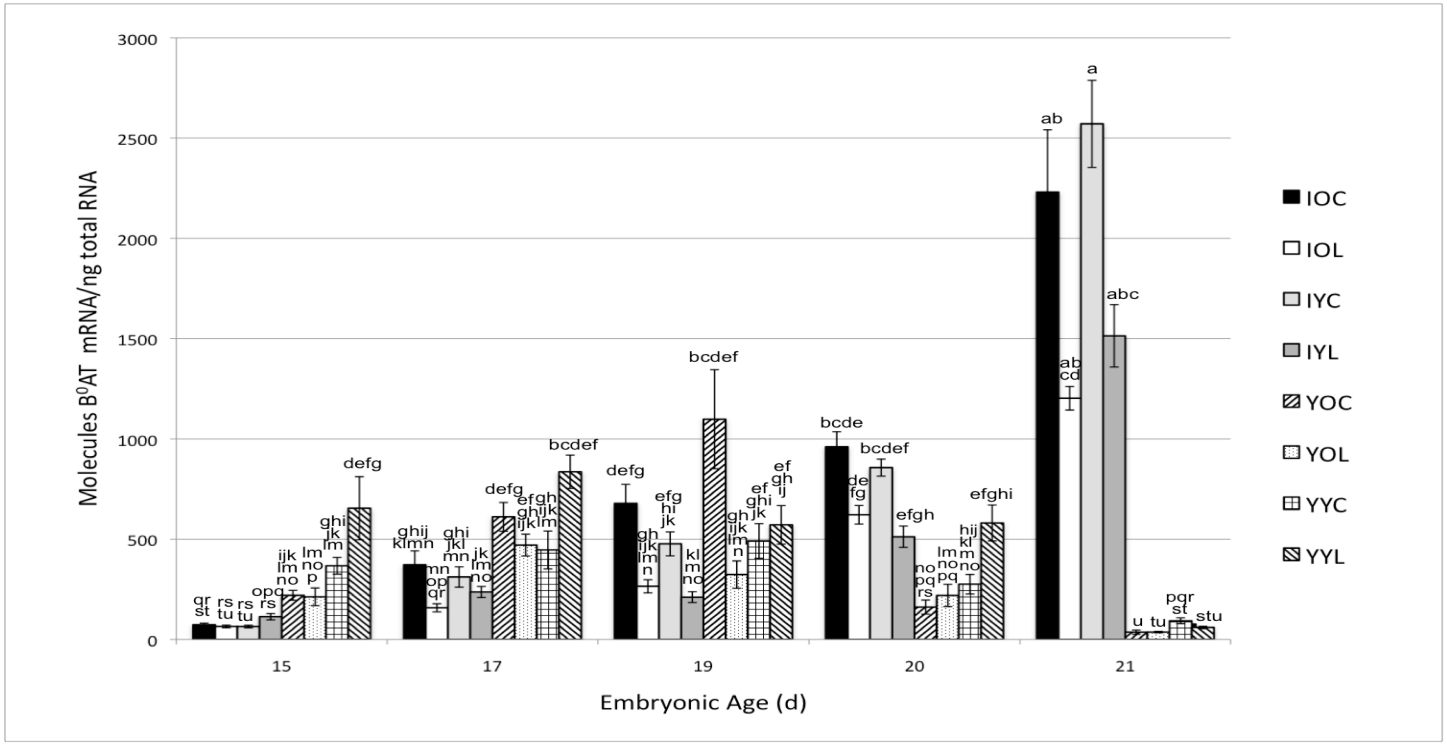

Figure 6-12. Embryonic Age by Tissue by Flock Age by Breed Interaction for B^0AT Gene Expression in Small Intestine and Yolk Sac Membrane in Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks. B^0AT gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). There was an embryonic age by tissue by flock age by breed interaction for APN gene expression in small intestine and YSM within chick embryos from young and old laying flocks of Cobb and Leghorn birds at e15, e17, e19, e20, and e21 ($P < 0.001$). Bars without common letters (a - u) differ significantly by $P < 0.05$. [IOC = Small Intestine/Old Flock/Cobb, IOL = Small Intestine/Old Flock/Leghorn, IYC = Small Intestine/Young Flock/Cobb, IYL = Small Intestine/Young Flock/Leghorn, YOC = YSM/Old Flock/Cobb, YOL = YSM/Old Flock/Leghorn, YYC = YSM/Young Flock/Cobb, YYL = YSM/ Young Flock/Leghorn (embryonic tissue/embryo laying flock age/breed).]

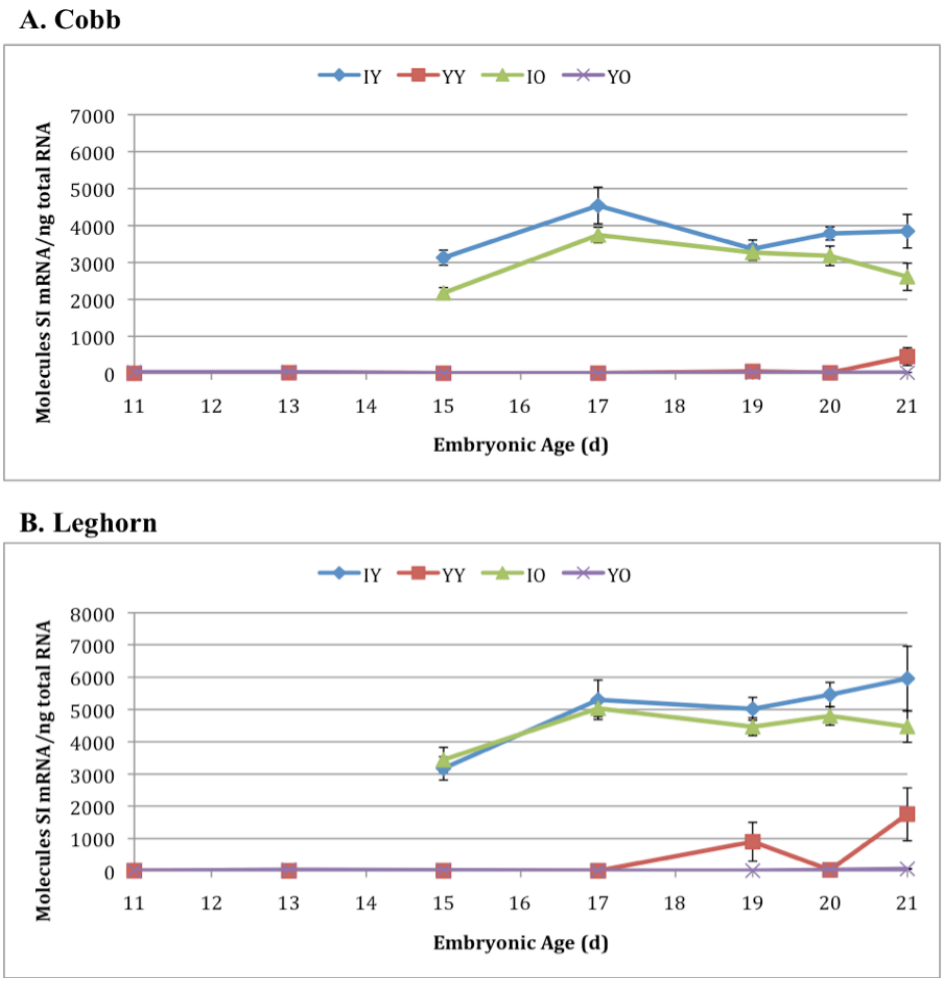

Figure 6-13. SI Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. SI gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), SI expression in YO tissue was consistently low and increased between e17 and 19 ($P = 0.013$) and maintained expression levels until e21. Expression of YY tissue was consistently low and increased from e17 to e19 ($P < 0.001$) and again from e20 to e21 ($P < 0.001$). Expression in IO tissue increased from e15 to e17 ($P = 0.001$), then decreased gradually from e17 to e21 ($P = 0.011$). In IY tissue expression increased from e15 to e17 ($P = 0.045$) and had no significant change in expression thereafter. In the Leghorn breed (B), SI expression in YO tissue maintained a low level of expression and increased between e19 to e20 ($P = 0.019$), and maintained expression thereafter. Expression in YY tissue was consistently low until increasing from e17 to e19 ($P < 0.001$) and increasing again from e20 to e21 ($P < 0.001$). From e15 to e17 both IO ($P = 0.005$) and IY ($P = 0.037$) tissue gene expression increased and minimal expression changes occurred thereafter. [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

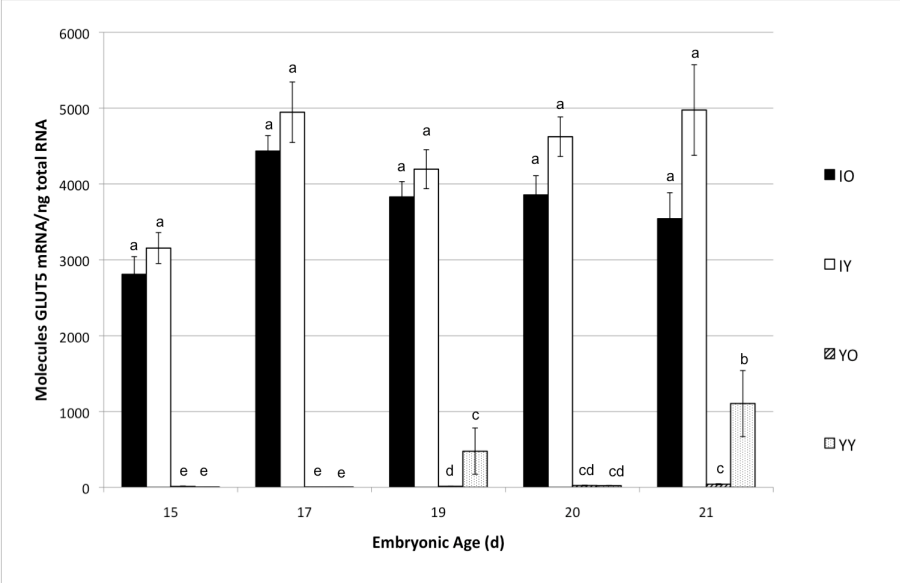

Figure 6-14. Embryonic Age by Tissue by Flock Age Interaction for SI Gene Expression in Small Intestine and Yolk Sac Membrane in Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks. SI gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks at e15 to e21 (n = 13 - 16). There was an embryonic age by tissue by breed interaction for SI gene expression in small intestine and YSM within chick embryos from young and old laying flocks at e15, e17, e19, e20, and e21 ($P < 0.001$). Bars without common letters (a - e) differ significantly by $P < 0.05$. [IO = Small Intestine/Old Flock, IY = Small Intestine/Young Flock, YO = YSM/Old Flock, YY = YSM/Young Flock (embryonic tissue/embryo laying flock age).]

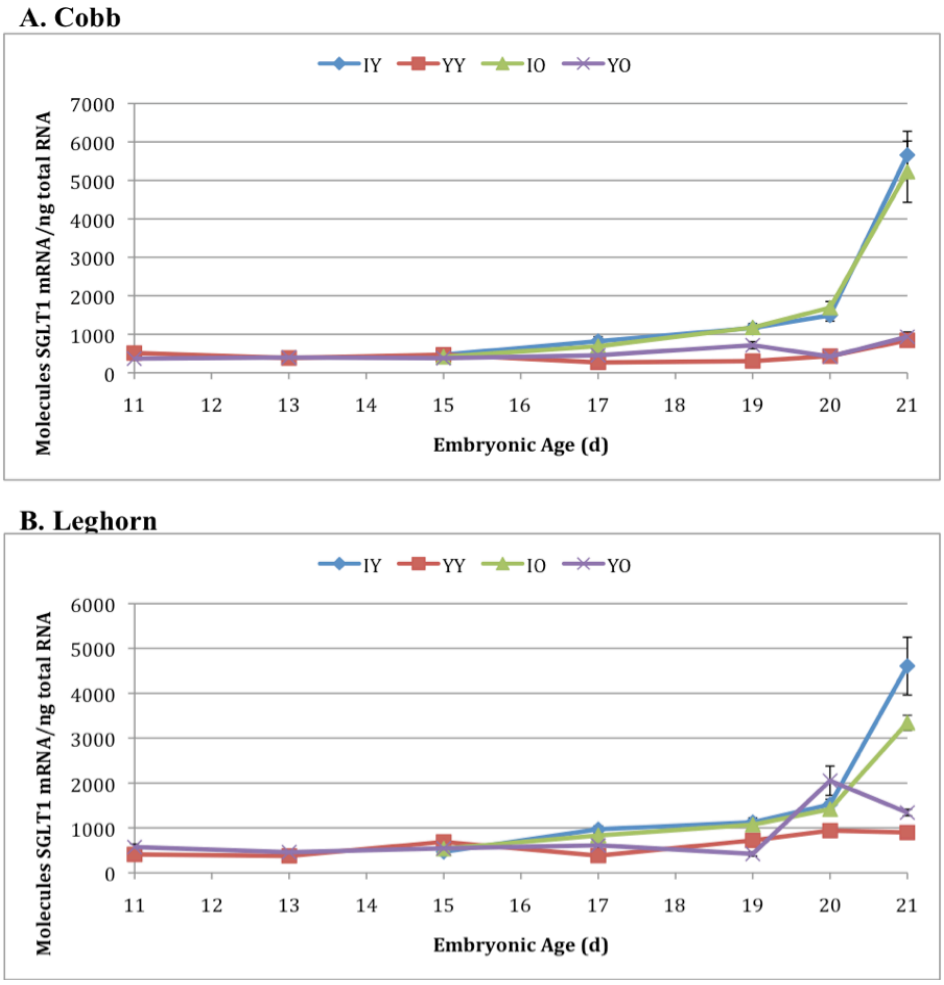

Figure 6-15. SGLT1 Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. SGLT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), SGLT1 expression in YO tissue was maintained at low levels until increasing from e20 to e21 ($P = 0.006$). Gene expression in YY tissue was maintained at low levels until e15 to e17 where it increased ($P = 0.004$) and increased again from e20 to e21 ($P = 0.021$). Both IY and IO tissue gene expression increased from e15 to e21 ($P < 0.001$). In the Leghorn breed (B), SGLT1 expression in YO maintained low levels of expression until increasing from e19 to e20 ($P < 0.001$) and maintaining expression thereafter. Gene expression in YY increased from e13 to e15 ($P = 0.042$), decreased from e15 to e17 ($P = 0.018$), increased from e17 to e19 ($P = 0.018$), and maintained expression levels thereafter. Both IY and IO tissue gene expression increased from e15 to e21 ($P < 0.001$). [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

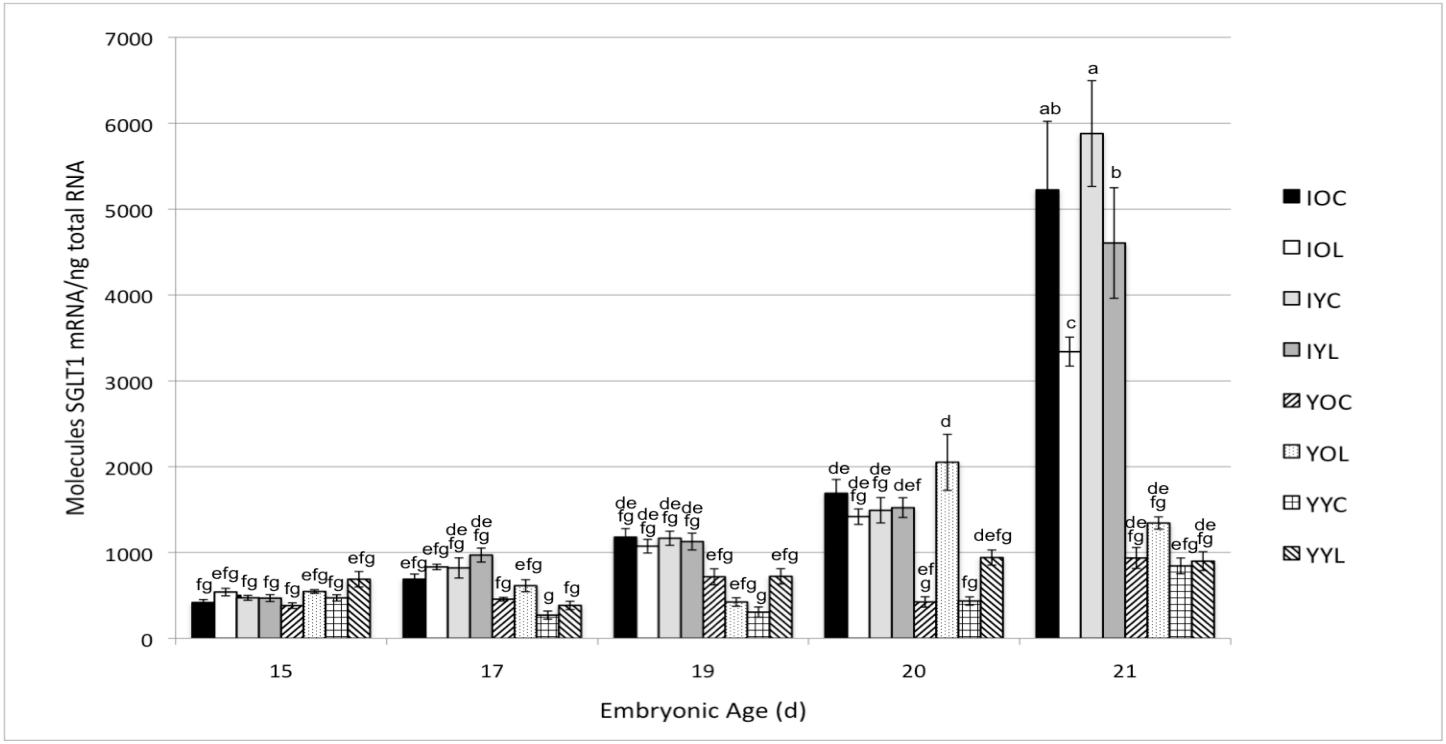

Figure 6-16. Embryonic Age by Tissue by Flock Age by Breed Interaction for SGLT1 Gene Expression in Small Intestine and Yolk Sac Membrane in Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks. SGLT1 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). There was an embryonic age by tissue by flock age by breed interaction for APN gene expression in small intestine and YSM within chick embryos from young and old laying flocks of Cobb and Leghorn birds at e15, e17, e19, e20, and e21 ($P < 0.001$). Bars without common letters (a - g) differ significantly by $P < 0.05$. [IOC = Small Intestine/Old Flock/Cobb, IOL = Small Intestine/Old Flock/Leghorn, IYC = Small Intestine/Young Flock/Cobb, IYL = Small Intestine/Young Flock/Leghorn, YOC = YSM/Old Flock/Cobb, YOL = YSM/Old Flock/Leghorn, YYC = YSM/Young Flock/Cobb, YYL = YSM/ Young Flock/Leghorn (embryonic tissue/embryo laying flock age/breed).]

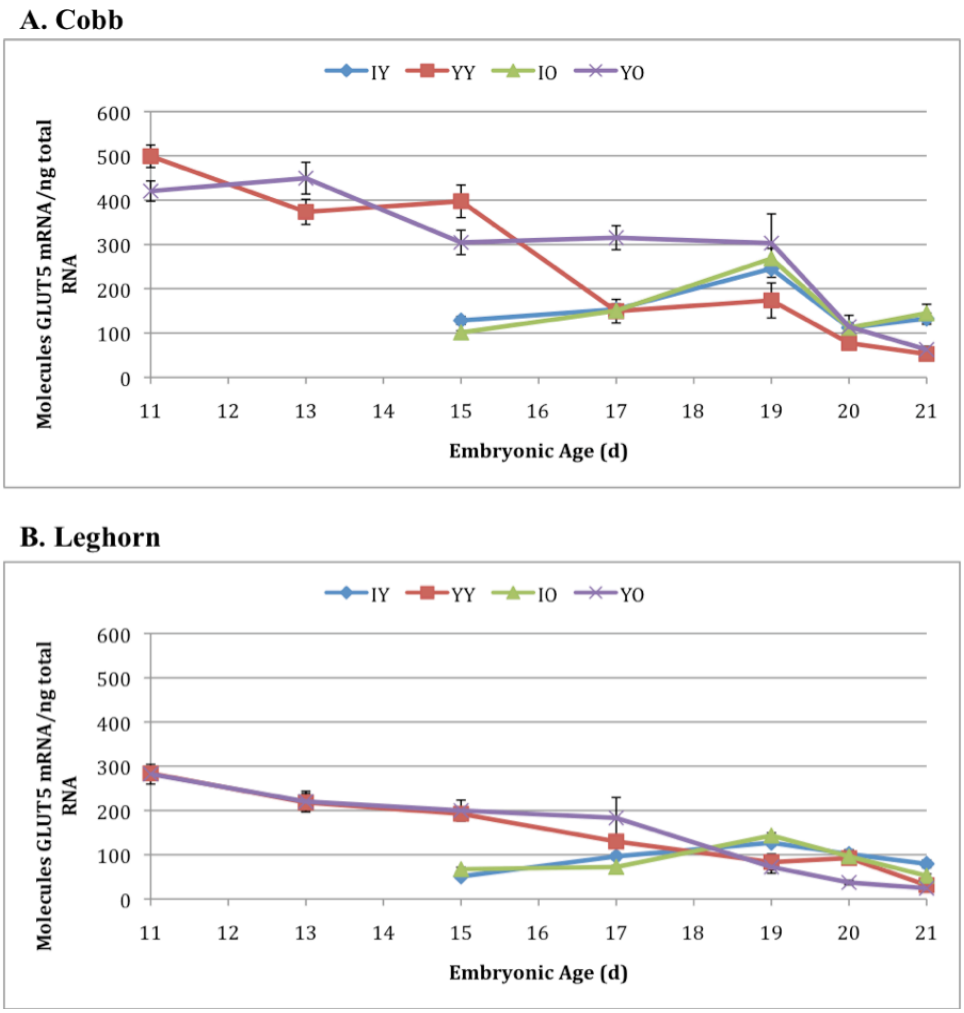

Figure 6-17. GLUT5 Gene Expression in the Yolk Sac Membrane and Small Intestine of Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks within Cobb and Leghorn Breeds. GLUT5 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), GLUT5 expression in YO tissue maintained levels of expression from e11 to e13, decreased gradually from e13 to e19 ($P = 0.005$), and decreased from e19 to e21 ($P < 0.001$). Gene expression in YY tissue decreased significantly from e15 to e17, and again between e19 and e21 ($P < 0.001$). GLUT5 expression in both IY and IO tissues increased from e15 to e19 ($P < 0.001$), then decreased from e19 to e20 ($P < 0.001$) and did not change significantly thereafter. In the Leghorn breed (B) GLUT5 expression in both YY and YO decreased gradually throughout incubation ($P < 0.001$). GLUT5 expression in both IY and IO tissues increased from e15 to e19 ($P < 0.001$), and decreased from e19 to e21 ($P < 0.001$). [IY = Small Intestine/Young Flock, YY = YSM/Young Flock, IO = Small Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).]

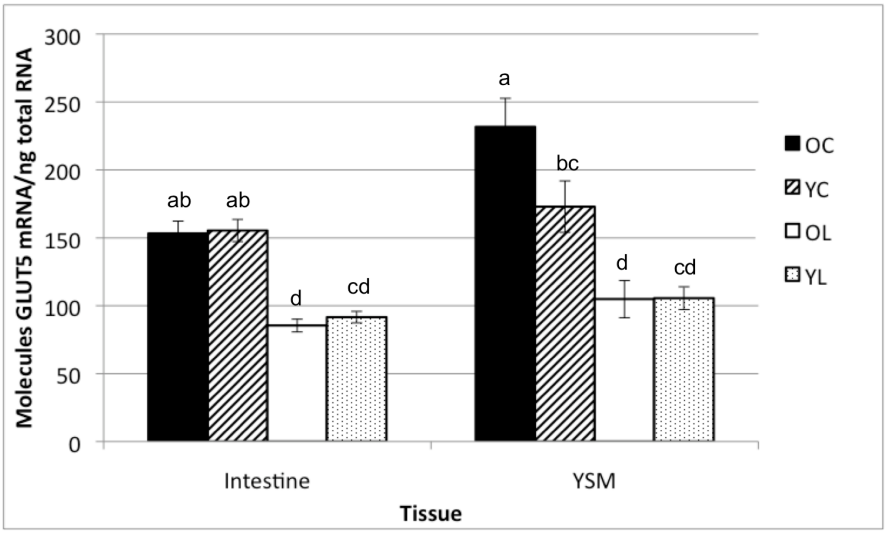

Figure 6-18. Tissue by Flock Age by Breed Interaction for GLUT5 Gene Expression in Small Intestine and Yolk Sac Membrane in Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks of Cobb and Leghorn Birds. GLUT5 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and small intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds (n = 36 - 39). There was a tissue by flock age by breed interaction for GLUT5 gene expression in small intestine and YSM embryonic chick tissue from Cobb and Leghorn birds from young and old flocks ($P = 0.015$). Bars without common letters (a - d) differ significantly by $P < 0.05$. [OC = Old Flock/Cobb, YC = Young Flock/Cobb, OL = Old Flock/Leghorn, YL = Young Flock/Leghorn (embryo laying flock age/breed).]

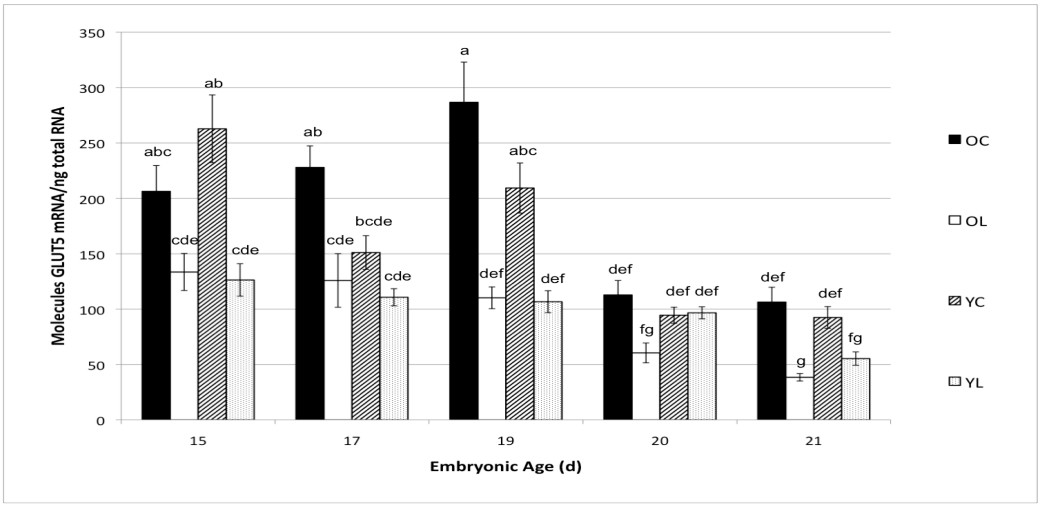

Figure 6-19. Embryonic Age by Flock Age by Breed Interaction for GLUT5 Gene Expression in Embryonic Chicks from 22-30 wk (Young) and 45-50 wk (Old) Flocks of Cobb and Leghorn Birds. GLUT5 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from embryonic tissues derived from young and old laying flocks of Cobb and Leghorn birds at e15 to e21 (n = 12 - 17). There was an embryonic age by flock age by breed interaction for GLUT5 gene expression in chick embryos from young and old flocks of Cobb and Leghorn birds at e15, e17, e19, e20, and e21 ($P = 0.008$). Bars without common letters (a - g) differ significantly by $P < 0.05$. [OC = Old Flock/Cobb, YC = Young Flock/Cobb, OL = Old Flock/Leghorn, YL = Young Flock/Leghorn (embryo laying flock age/breed).]

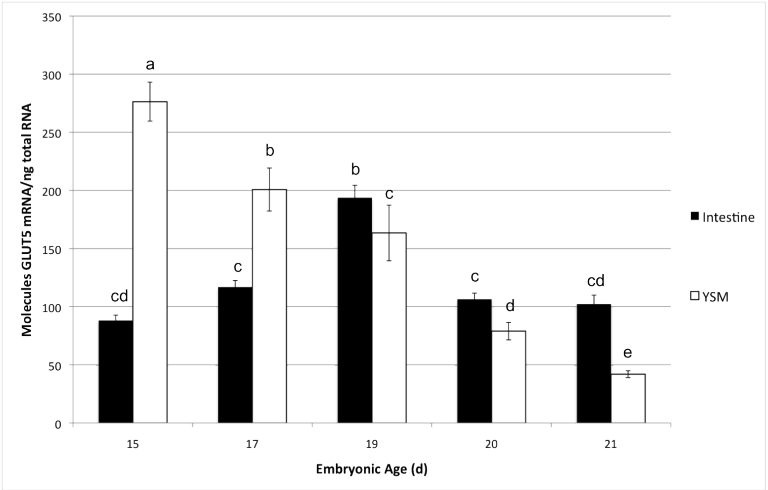

Figure 6-20. Embryonic Age by Tissue Interaction for* GLUT5 *Gene Expression. GLUT5 gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from embryonic chick yolk sac membrane (YSM) and small intestine tissues at e15 to e21 (n = 25 – 32). There was an embryonic age by tissue interaction for GLUT5 gene expression in Cobb and Leghorn embryonic chicks at e15, e17, e19, e20, and e21 ($P < 0.001$). Bars without common letters (a - e) differ significantly by $P < 0.05$.

Discussion

In this study, the observed nutrient transporters and digestive enzymes showed a diverse array of developmental gene expression patterns in the YSM and neonatal small intestine. In the small intestine, the genes APN, PepT1, EAAT3, B^0AT and SGLT1 showed varying degrees of expression increases, the genes SI and GLUT5 had minimal expression changes, and the amino acid transporter CAT1 showed decreases in expression throughout prehatch development. In the YSM, the genes APN, PepT1, and B^0AT increased in expression during development and decreased to much lower levels prior to hatch, the genes CAT1 and GLUT5 decreased in expression throughout development, and the genes EAAT3, SI, and SGLT1 had slight increases in expression during incubation.

The digestive enzyme APN cleaves neutral amino acids from the N-terminus of proteins and thus provides substrate for neutral amino acid transporters, such as B^0AT (Kania et al., 1977; Drag et al., 2010). Gilbert et al. (2007) observed developmental regulation of nutrient transporters and APN in broiler small intestine and found APN mRNA abundance to be the highest of all the studied genes. Similarly, of all of the observed genes in the present study, APN exhibited the highest expression level in both the developing small intestine and YSM. High expression of APN in these tissues provides a constant generation of amino acids available for transport. Overall, APN expression in the small intestine increased 2-fold from e15 to e21, which could be in response to amnion consumption or a genetic predisposition to prepare the chick for outside feed. In the YSM, highest levels of APN occurred at e13 or e15 and decreased thereafter, suggesting that gene activity diminishes as yolk contents are used up. Yadgary

et al. (2011) also found peak YSM expression levels of APN to occur at e17, supporting this theory. At e19 of development embryos derived from young flocks of Leghorn birds had significantly higher APN gene expression in the YSM than YSM from any other breed and flock combination. Yadgary et al. (2010) found that at e19 and e21 eggs produced by older flocks of birds have more protein in the YSM. Since there is less protein available for degradation in the YSM of eggs produced by younger flocks, upregulation of APN gene expression may occur for an extended period in Leghorn birds to maximize protein utilization. Cobb birds may be more efficient with protein degradation at this stage in incubation.

In this study, intestinal PepT1 mRNA levels increased from e15 to e21. Gilbert et al. (2007) and Chen et al. (2005) found a similar linear increase from e18 to d14 post hatch and e16 to e21, respectively. This increase in expression throughout development and early post hatch suggests that peptide transport is an important form of nutrient assimilation in both the neonatal and early posthatch chick. In the YSM, expression of PepT1 followed a similar pattern to APN, though with lower mRNA levels. Results from Yadgary et al. (2011) confirm this pattern in chick YSM, with PepT1 expression levels increasing about 3-fold in the middle of incubation, then decreasing towards hatch. Similar to what APN displayed on e19, overall PepT1 expression in the YSM was higher in Leghorn embryos than Cobb embryos, which could be linked to protein degradation and absorption efficiency differences between breeds during embryonic development.

The 3 amino acid transporters, EAAT3, CAT1 and B^0AT transport anionic, cationic and neutral amino acids, respectively. The transporter EAAT3 provides intestinal enterocytes with glutamate, the anionic amino acid that fuels cellular processes (Iwanaga

et al., 2005). In this study, expression levels of EAAT3 increased by about 50% from e15 to e21, and Gilbert et al. (2007) found that EAAT3 expression increases by 3-fold from e20 to d14 posthatch. As a great amount of intestinal change and rapid growth occurs during late embryonic development (Bellairs and Osmond, 2005), increases in EAAT3 gene expression logically is necessary to fuel many of the intestinal cells. In the yolk sac, protein composition does not change much throughout embryonic development (Yadgary et al., 2010). EAAT3 gene expression in the YSM increases from e11 to e21. Since EAAT3 transport is essential to transport glutamate, the main form of energy for enterocytes, upregulation may help ensure that the glutamate made available by digestive enzymes is transported from the yolk to the growing chick. Similar to APN, embryos derived from young flocks of Leghorn birds had significantly higher YSM gene expression than YSM from embryos of other breed and flock age combinations. Additionally, from e19 to e21 expression of EAAT3 in the YSM varied among breed and flock combinations, showing that divergences in amino acid assimilation of chicks from different genetic and metabolic backgrounds may begin as early as embryonic development.

The basolaterally-expressed cationic amino acid transporter CAT1 peaked in expression levels around e17 and e19 for the Cobb and Leghorn breeds, respectively. Gilbert et al. (2007) had similar findings with expression levels peaking at e18 and decreasing thereafter. Vascularization of the yolk sac increases throughout development until the yolk stores are mostly absorbed around e19 (Bellairs and Osmond, 2005). Since CAT1 is a basolateral transporter, it may be more active earlier than brush-border transporters such as EAAT3 and B^0AT as it is essential to transport amino acids from the

blood into the developing small intestine. Once the yolk stores are depleted, expression levels of CAT1 will understandably drop. Expression levels of CAT1 in the YSM were significantly lower than the small intestine and diminished late in incubation. Yadgary et al. (2011) found a similar decrease in CAT1 in the middle of incubation. As the YSM degrades, the need for basolateral nutrient transporters may become less, leading to downregulation of associated genes.

The neutral amino acid transporter B^0AT had a similar expression pattern to that of PepT1 in the small intestine. B^0AT has been characterized within the past 10 years and has been found to transport many different neutral amino acids in many different tissues. Gilbert et al. (2007) found B^0AT gene expression to increase with age in chicken small intestine, with greatest mRNA quantity located in the ileum. Similarly, this study showed that B^0AT expression increased from e15 to e21 in the small intestine, perhaps preparing for the approaching increase in amino acid availability or in response to amnion consumption at the end of incubation. In the YSM, B^0AT gene expression increases in late incubation and decreases just prior to hatch. The pattern is similar to the expression pattern of APN in the YSM, but begins and ends several days later. Like EAAT3, B^0AT may be responding to the increase in aminopeptidase activity and amino acid availability. As neutral amino acids are depleted, which may occur sooner than anionic depletion, B^0AT expressional activity may diminish in response. Similar to APN and EAAT3 embryos derived from young flocks of Leghorn birds had significantly higher B^0AT gene expression during incubation, except on e15 and e20 instead of e19. Embryos derived from old flocks of Cobb birds showed significantly higher expression than Leghorn embryos on e19. Initial levels of neutral amino acids in the yolk may differ from peptide

and anionic amino acid levels, causing B^0AT expression patterns in the YSM to differ from APN and EAAT3 even though they are all affiliated with protein digestion and absorption.

The enzyme sucrase-isomaltase was expressed at much greater levels in the small intestine than the YSM throughout development. In the small intestine, there was a 50% increase in expression level from e15 to e17 and high levels of expression were maintained thereafter. In a study performed by Sklan et al. (2003), an increase was found similarly at e19 of development of neonatal chicks. In order to prepare the chick for a high-starch diet post-hatch, genetically pre-programmed activation of SI early in development may be necessary. Expression at the villus tips allows for the degradation of carbohydrates into sugars that can then be transported into the cell and blood stream via sugar transporters such as SGLT1 and GLUT5 (Van Beers et al., 1995). In the current study and in the YSM study performed by Yadgary et al. (2011), SI expression in the YSM was minimal early in incubation and spiked several times towards the end of incubation. In an earlier experiment, Yadgary et al. (2010) determined that carbohydrate levels are low in the yolk but increase 4-fold between e13 and e19. As carbohydrate levels increase, activation of the SI gene may be necessary to initiate digestion of the molecules so their respective sugar transporters can transport them. Interestingly, increases in YSM gene expression of SI were more dramatic in YSM from embryos derived from young flocks. Yassin et al. (2008) found that the hatchability of chicks from 25 wk old flocks was 22% lower than birds at prime egg-laying age (between 31 and 35 wks). Since the young flocks in this study were only 22 to 30 wk of age, it is possible that

the chick is less efficient at digesting yolk carbohydrates, causing upregulation of SI and overall lower hatchability.

As intestinal SI expression must increase to prepare for digestion of carbohydrates into monosaccharides, monosaccharide transporters must be activated to prepare for sugar absorption. In the current study, the sodium-dependent glucose cotransporter, SGLT1, small intestinal expression increased 10-fold from e15 to e21. Gilbert et al. (2005) found a similar 9-fold increase from e18 to e21. As Na^+-glucose cotransport is already detectable towards the end of incubation (Barfull et al., 2002), increases in expression of SGLT1 and similar transporters must occur earlier in order to prepare for the absorption process. Similar to the increase in carbohydrate abundance in the yolk toward the end of incubation (Yadgary et al., 2010), SGLT1 gene expression in the YSM increased at the end of incubation in this study. Yadgary et al. (2011) had similar findings, though SGLT1 expression dropped just prior to hatch in their respective study. As carbohydrate levels increase, their respective sugar transporters must become more active to ensure delivery to the developing chick. At e21 expression in Cobb embryos was higher than Leghorn embryos and embryos derived from old flocks had significantly higher expression than embryos derived from young flocks. A greater rate of glucose uptake after the increase in carbohydrates in the yolk could show where Cobb birds begin their pattern of increased muscle deposition compared to Leghorn birds.

The GLUT5 fructose transporter was expressed at much lower quantities than SGLT1 ranging from 4-fold lower at the onset of expression measurement to 40-fold lower right before hatch. Although Gilbert et al. (2005) found that GLUT5 begins to rise post-hatch, the low levels during incubation indicate that GLUT5 is less active as a sugar

transporter during neonatal development than other sugar transporters, such as SGLT1. In the YSM, GLUT5 expression levels were at their highest at e11 and decreased throughout incubation. Although carbohydrate levels increase in the yolk until e19 (Yadgary et al., 2010), this study demonstrates that glucose transport predominates over fructose transport throughout incubation in the YSM, increasing towards the end.

All genes had a tissue by embryonic day interaction. Although the YSM and embryonic small intestine are a continuous entity (Figure 3-1) throughout development, their roles in nutritional assimilation differ significantly. Flock age appears to impact some genes in their expression during development. Some of the genes involved in protein digestion and amino acid absorption (e.g. APN, B^0AT, and CAT) all had significantly higher levels of gene expression in embryonic tissue of young flocks than embryonic tissue from old flocks. The carbohydrate digestive enzyme and transporters SI, SGLT1 and GLUT5 also exhibited some interactions with flock age that yielded significant changes in mRNA abundance. Interestingly, in an egg composition study, protein composition was found to be consistently higher in eggs derived from older flocks than those from younger flocks (Yadgary et al., 2010). However, Yadgary et al. (2010) also found that eggs from older flocks were 21.5% larger, which may make the volume of the yolk larger and accessibility to its contents lower for the YSM. Breed also had a varying impact on gene expressional activity. CAT1 and GLUT5 expression was significantly higher in the Cobb breed, while EAAT3 and SGLT1 were higher in the Leghorn breed. Various interactions involving breed occurred for all of the studied genes. The two different breeds used, Leghorn and Cobb, serve two different production

purposes, egg and meat production, respectively. Therefore, it is not surprising that the nutrient requirements and enzyme and transporter activity may vary among them.

These results reveal differences in expression of digestive enzymes and nutrient transporters in embryonic YSM and small intestine derived from old and young flocks of Cobb and Leghorn chickens. Although the YSM and neonatal small intestine are a contiguous entity during development, these results indicate that they have different roles in nutrient assimilation throughout embryonic development and that bird breed and age of the breeder flock may impact expression patterns at varying developmental stages. It is important to note, however, that only mRNA levels were examined in this study and not protein levels. Further research of the proteins derived from these genes along with their functionality would be valuable to understand their full role in digestion and nutrient assimilation in the developing chicken. Additionally, the sizes of the YSM and small intestine in proportion to one another change dramatically throughout development. Comparing tissue sizes (ng) to mRNA expression per ng could show the whole tissue capacity relative to one another.

CHAPTER VI.

EPILOGUE

My results indicate that there are nutrient transporter and digestive enzyme gene expression pattern differences that exist between the chick neonatal YSM and small intestine, and that laying flock age and breed impact some, but not all, of these gene expression patterns. Although the YSM and small intestine are a continuous entity during development (Bellairs and Osmond, 2005), nutrient availability in these regions differs and may cause different gene expression profiles. Additionally, the results from this thesis may begin to show the importance of specific nutrients during development through the expression of their respective enzyme or transporter, which may help in our understanding of what nutrients are important for embryonic growth.

Little knowledge is available on the protein and carbohydrate breakdown and transport mechanism in the embryonic YSM. Further research on this mechanism and how the chick metabolically transitions from pre- and post-hatch life may ultimately illuminate important aspects of chick development that help with pre- and post-hatch growth efficiency. If a specific nutrient is more active in facilitating chick growth, adding more of this nutrient to the yolk may provide embryonic chicks with more fuel for growth.

In this study, the expression pattern of digestive enzymes SI and APN, oligopeptide transporter PepT1, amino acid transporters EAAT3, CAT1, and B^0AT, and sugar transporters SGLT1 and GLUT5, were observed in the YSM and small intestine during embryonic chick growth. It has been found in this study and previously that the digestive enzyme APN is expressed at a much greater level than nutrient transporter

genes (Gilbert et al., 2007). In order to provide sufficient substrate for the oligopeptide and amino acid transporters, the APN digestive enzymes may need to be present at higher levels. As yolk contents consist of 15% protein and less than 1% carbohydrate (Yadgary et al., 2010), the lower expression of SI in the yolk is understandable. The low levels of carbohydrates in the yolk may also already be in the transportable monosaccharide form.

In the small intestine, all observed genes had increased expression through incubation except CAT1 and GLUT5. As the chick is preparing for post-hatch life most gene expression should increase so that the chick can continue gaining nutrients from feed instead of the yolk. As CAT1 is a basolateral transporter, expression may increase earlier in incubation as it picks up nutrients transported from the yolk through the blood stream. Low GLUT5 expression means that little fructose absorption occurs immediately post-hatch, although previous studies indicate that expression levels increase dramatically post-hatch, allowing the chick to absorb fructose more efficiently (Gilbert et al., 2007).

Overall, all genes except for EAAT3 either decreased in expression or maintained low levels of expression up until or through e20. SI, SGLT1, and PepT1 exhibited spikes in expression at the last day end of incubation, indicating that the chick may be obtaining a large amount of glucose just before hatch to provide it with the necessary energy to begin life. Since EAAT3 provides cells with their main form of energy, glutamate, expression in the YSM may need to increase in order to supply the organ with necessary glutamate, despite its depleting abundance. For the most part, nutrients are largely depleted by the end of incubation, which causes digestive enzyme and nutrient transporter genes to be downregulated.

Bird breed impacted expression levels of the CAT1, EAAT3, and GLUT5 nutrient transporters. The Cobb breed had higher expression of CAT1 and GLUT5, while Leghorns had higher EAAT3 expression. Since these bird breeds have different metabolic needs post-hatch, these observed gene expression differences may begin to show how these birds begin to diverge before hatch in order to fulfill their genetic path after hatch. APN, B^0AT, and CAT1 gene expression was influenced by flock age. Interestingly, all three genes were expressed higher in embryos obtained from young flocks. It has been documented that older birds produce larger eggs and larger chicks (Yadgary et al., 2010). Perhaps embryos from older hens are more nutritionally efficient, and do not need higher expression to obtain adequate nutrition.

These results suggest that the stage of embryonic development impacts the comparable level of digestive enzyme and nutrient transporter gene expression in the YSM and small intestine, and that expression levels of some genes are influenced by the age of the laying flock and breed of the bird. Future studies involving in-ovo feeding may further show the role that substrate has on gene activation, and may lead to new techniques toward creating a faster growing chick. Research on the process by which nutrients are transported from the yolk, through the vascularized YSM and into the chick, may help explain various gene expression patterns observed in the YSM. Additionally, further investigations about why flock age and breed influence some genes and not others are warranted. With further knowledge on nutrient assimilation processes in developing chicks with different metabolic genetic traits, advances in growth efficiency in the developing pre- and post-hatch chick can be made.

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APPENDIX A

Expression of housekeeping genes are often measured along with genes of interest in real-time PCR in order to "normalize" the data against experimental error such as concentration errors. In this study expression of the housekeeping genes Beta-actin and GAPDH were measured for all samples. For the housekeeping gene to work properly in this experiment, expression within a tissue would have to be constant over time. For both Beta-actin and GAPDH expression was variable throughout incubation in individual tissues, and therefore was not used to normalize sample data.

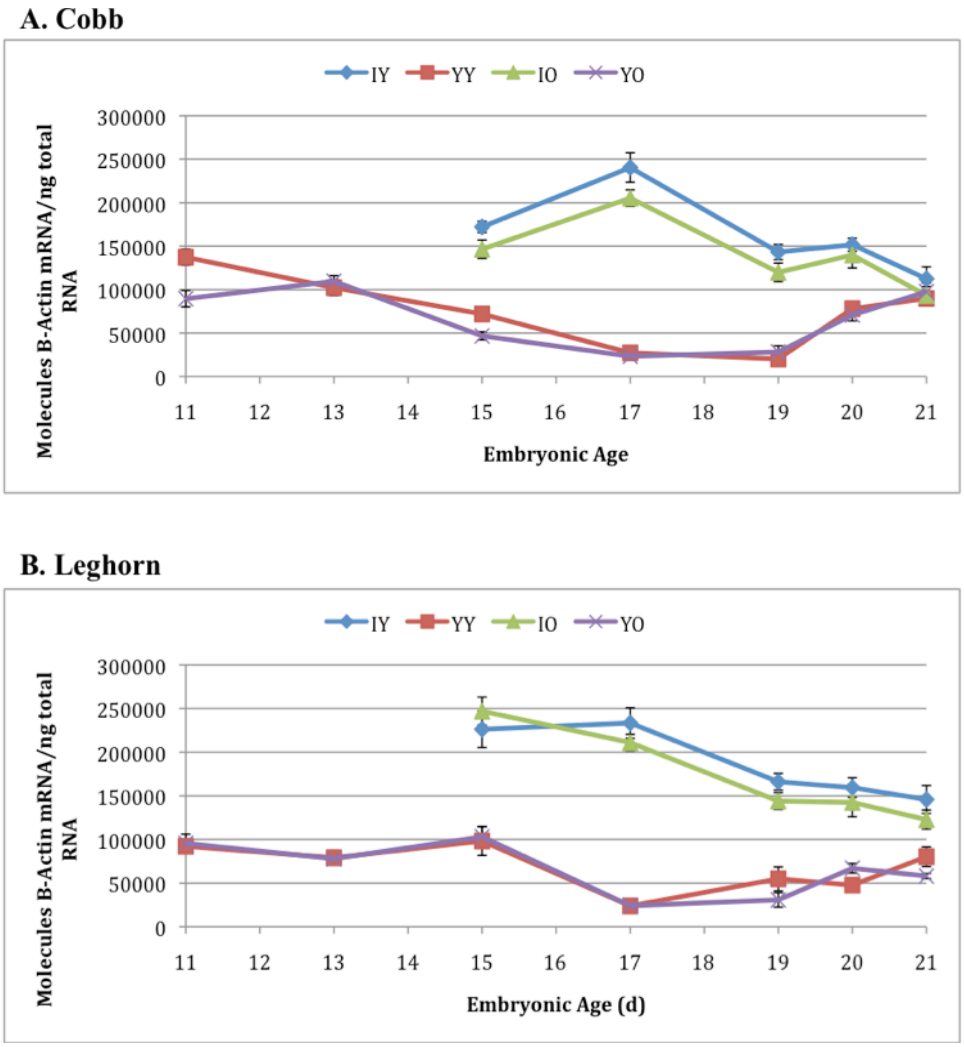

Figure A-1. Beta-actin Gene Expression in the Yolk Sac Membrane and Intestine of Embryonic Chicks from Young and Old Flocks within Cobb and Leghorn Breeds. Beta-actin gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), Beta-actin expression in both YY and YO tissue decreased from e11 to e17 ($P < 0.001$) and then increased from e19 to e21 ($P < 0.001$). Both IY ($P = 0.002$) and IO ($P = 0.008$) tissue gene expression increased from e15 to e17, decreased from e17 to e19 ($P < 0.001$) and had minimal changes thereafter. In the Leghorn breed (B), Beta-actin expression in both YY and YO tissues had minimal changes until decreasing from e15 to e17 ($P < 0.001$) and slightly increasing in expression from e17 to e21 ($P < 0.001$). Gene expression in YY increased from e13 to e15 ($P = 0.042$), decreased from e15 to e17 ($P = 0.018$), increased from e17 to e19 ($P = 0.018$), and maintained expression levels thereafter. Both IY ($P = 0.003$) and IO ($P < 0.001$) tissue gene expression decreased from e15 to e21 ($P < 0.001$). IY = Intestine/Young Flock, YY = YSM/Young Flock, IO = Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).

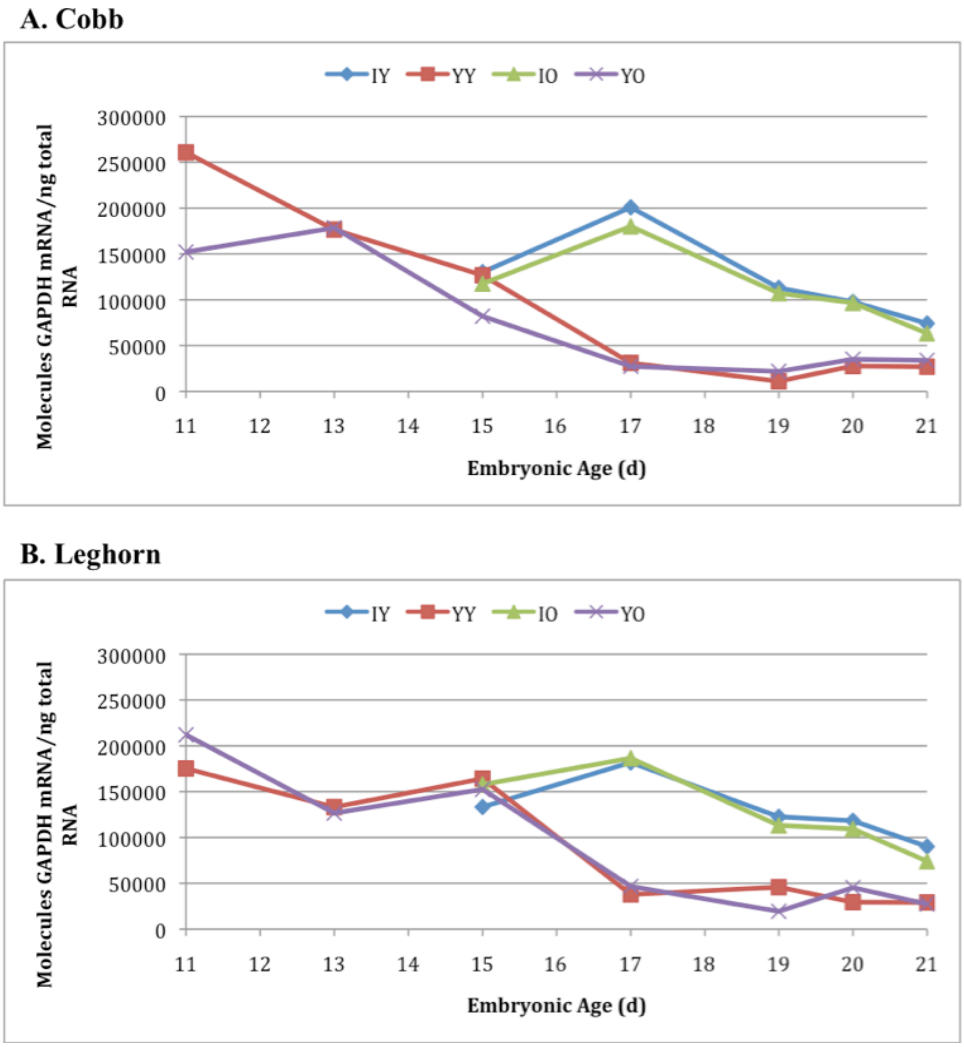

Figure A-2. GAPDH Gene Expression in the Yolk Sac Membrane and Intestine of Embryonic Chicks from Young and Old Flocks within Cobb and Leghorn Breeds. GAPDH gene expression (mean ± SE) was calculated from real-time PCR absolute quantification assays of cDNA from yolk sac membrane (YSM) and intestine tissues. Tissues were derived from embryos from young and old laying flocks of Cobb and Leghorn birds at e11 to e21 (n = 5 - 8). In the Cobb breed (A), GAPDH expression in both YY and YO tissue decreased from e11 to e15 ($P < 0.01$) and maintained expression thereafter. Both IY and IO tissue gene expression increased from e15 to e17 ($P < 0.001$) then decreased from e17 to e21 ($P = 0.001$). In the Leghorn breed (B), GAPDH expression in both the YY and YO tissues had minimal expression change until decreasing from e15 to 17 and had minimal expression changes thereafter. Gene expression in IY increased from e15 to e17 ($P = 0.025$), and both IY and IO tissue gene expression decreased from e17 to e21 ($P < 0.001$). IY = Intestine/Young Flock, YY = YSM/Young Flock, IO = Intestine/Old Flock, YO = YSM/Old Flock (embryonic tissue/embryo laying flock age).