Developmental Gene Expression of Nutrient Transporters in the Small Intestine of Chickens from Lines Divergently Selected for High or Low Juvenile Body Weight

by

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ABSTRACT

Nutrient transporters in the small intestine are responsible for dietary nutrient assimilation and therefore the expression of these transporters can influence the overall nutrient status as well as the growth and development of the animal. This thesis examined correlated responses to selection in the developmental gene expression of the peptide transporter PepT1, the glutamate/aspartate transporter EAAT3, the sodium-dependent glucose transporter SGLT1, and the fructose transporter GLUT5 in the small intestine of chickens from lines divergently selected for high (HH) or low (LL) eight-week body weight and their reciprocal crosses, (HL and LH). Chicks were weighed and killed on embryonic day 20 (E20), day of hatch (DOH with no access to feed), and days 3 (D3), 7(D7), and 14 (D14) post hatch. Duodenum, jejunum, ileum and liver were collected. DNA extracted from liver was used to sex birds by PCR. RNA was extracted from the intestinal segments of four males and four females from each mating combination (MC) and time point except E20 HL males (n = 3) and D7 LL females (n = 2). Expression of nutrient transporters was assayed by real-time PCR using the relative quantification method. In comparing HH and LL males and females there was a line by segment interaction in PepT1 gene expression, with no segment difference in HH and greatest expression in the ileum of the LL ($P < 0.05$). There was also a MC by age by sex interaction for PepT1 gene expression ($P < 0.0001$) with peak gene expression occurring on DOH for LL females, on D7 for HH females, on D7 for LL males and D14 for HH
males. Overall, females had greater EAAT3 expression \((P < 0.03)\). Gene expression of EAAT3 was greatest in the ileum, intermediate in the jejunum, and least in the duodenum \((P < 0.0007)\). There was an age by segment interaction for EAAT3 expression \((P = 0.0002)\) and a MC by segment interaction \((P < 0.02)\), with LL having greater expression than HH in the ileum. Females had greater SGLT1 expression than males \((P < 0.0001)\). There was a sex by age interaction for the expression of SGLT1 \((P < 0.0001)\). Females induced SGLT1 expression on DOH and maintained this level through D14, while males gradually increased expression through D7 and decreased expression by D14. These results indicate that expression of PepT1, EAAT3, SGLT1 are differentially expressed in male and female chickens regardless of selection for high or low juvenile body weight. These results also show a sexual dimorphism in the capacity to absorb peptides, anionic amino acids, and glucose from the intestine, which has implications for the poultry industry with regard to diet formulations for straight-run and sex-separate grow-out operations. In comparing male HH, HL, LH, and LL chicks, overall LL had the greatest level of expression \((P < 0.06)\), HH had the least level of expression \((P < 0.006)\) and HL and LH had intermediate levels of expression \((P < 0.06)\). Greatest PepT1 gene was expression in the ileum \((P < 0.0003)\) and there was a MC by segment interaction with expression increasing from duodenum to ileum in LL, but there was no segment difference in any other MC \((P < 0.08)\). Within each intestinal segment there was a MC difference \((P < 0.02)\). There was an effect of sire for PepT1 expression, with progeny from low weight selected sires (LWS) having greater expression than progeny from high weight selected (HWS) sires \((P = 0.0008)\). There was no difference between intestinal segments in progeny from HWS sires, however, greatest PepT1 gene expression was seen
in the ileum of progeny from LWS sires ($P < 0.0001$). Overall, expression of EAAT3 was greatest in the ileum, intermediate in the jejunum and least in the ileum ($P < 0.0001$) and there was a segment by age interaction for EAAT3 expression ($P < 0.0001$). In all MCs except HH, EAAT3 gene expression increased from duodenum to ileum ($P < 0.08$). Within the ileum, the LL had greatest EAAT3 gene expression, LH and HL had intermediate gene expression, and HH had least expression ($P < 0.08$). Expression of SGLT1 gradually increased through D7 and decreased by D14 ($P < 0.0001$) and overall, was greatest in the distal small intestine ($P < 0.0001$). There was a MC by segment interaction, with SGLT1 gene expression being greatest in the distal small intestine in LL, LH, and HL, but greatest in the jejunum of HH ($P < 0.04$). Within the ileum, LL had greater SGLT1 gene expression than HH ($P < 0.06$). Overall, greatest GLUT5 expression was in the distal small intestine ($P < 0.0001$) and there was a MC by segment interaction, with expression being greatest in the distal small intestine in LL and HL ($P < 0.02$), greatest in the ileum of LH ($P < 0.08$), and greatest in the jejunum of HH ($P < 0.09$). Within the ileum there was a MC difference ($P < 0.07$). These results indicate that selection for high or low juvenile body weight may have influenced the gene expression pattern of these nutrient transporters in the small intestine, which may contribute to the overall differences in the growth and development of these lines of chickens.
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CHAPTER I.
INTRODUCTION

In the broiler industry genetic selection has been practiced to produce a faster growing, larger, and leaner bird at market. To determine the correlated responses to selection for a single growth characteristic in chickens, a long term genetic selection project was begun in 1957. In this study White Plymouth Rock chickens were selected for high or low eight-week body weight. After 50 generations of selection, there is approximately a 10-fold difference between the lines in body weight at eight-weeks of age.

Although many studies have evaluated several correlated responses to selection in these lines at the animal, organ, and tissue level, little work has been done to examine the correlated responses to selection at the molecular level. In this thesis, the correlated response to selection in the developmental gene expression of the brush border membrane nutrient transporters was evaluated in the 48th generation of the selected lines. The transporters examined were the peptide transporter, PepT1, the glutamate/aspartate transporter, EAAT3, the glucose transporter, SGLT1, and the fructose transporter GLUT5.

Regulation and expression of these specific nutrient transport systems in the brush border membrane therefore impact the nutrient and energy availability to the animal for growth and development. This study was designed to provide insight into possible molecular mechanisms governing the differential growth characteristics of these selected lines.
CHAPTER II.

REVIEW OF LITERATURE

WHITE PLYMOUTH ROCK CHICKENS DIVERGENTLY SELECTED FOR HIGH OR LOW EIGHT-WEEK BODY WEIGHT

Introduction

The animal model for this research was White Plymouth Rock chickens divergently selected for high or low juvenile body weight. This section discusses the development of the selected lines as well as the correlated responses to long-term divergent selection for eight-week body weight that pertain to growth, development, metabolism, and appetite.

Development of the Selected Lines and Response in Eight-Week Body Weight

The selection experiment began in 1957 with the foundation stock coming from seven inbred lines of White Plymouth Rock chickens (Siegel, 1962). Parents for the high weight selected line (HWS) and low weight selected line (LWS) came from the chickens in this gene pool with the heaviest and lightest body weight, respectively, at eight weeks of age. In subsequent generations, individual single trait phenotypic selection for eight week body weight was conducted within each separate line.

The selected lines are currently in their 50th generation and show about a 10-fold difference in body weight at eight weeks of age. This difference should be interpreted with caution as feed restriction was implemented in the HWS, after the selection age (8 weeks) to reduce reproductive complications (Liu et al., 1995). Since the initiation of the selection trial, sublines of the initial HWS and LWS lines have been produced. Beginning in generations 6, 13, 19, and 26, within each selected line, males and females were randomly chosen as parents to establish sublines in which the selection for eight
week body weight was relaxed (Dunnington and Siegel, 1996). The result was lines, which had body weights that were regressed towards the mean, compared to the HWS and LWS of the respective generation. In the 13th generation, females of the HWS and LWS were crossed with males of a meat-type chicken which carried the sex-linked dwarfing gene (Reddy and Siegel, 1977). Backcrossing heterozygote males to females from the HWS and LWS was implemented for four generations to create the two dwarf populations (HWD and LWD). The HWD and LWD populations were closed and maintained by random breeding within the lines. There was no selection for body weight in the dwarf sublines. The result of the addition of the dwarfing gene was a depression on growth, which was greater in the LWS than the HWS genetic background.

Periodically throughout this selection study, reciprocal crosses of the HWS and LWS have been produced providing opportunities to study heterosis, recombination and other genetic effects. The result of this breeding scheme, with respect to the selected trait of eight week body weight, were chickens with an eight week body weight that is intermediate to that of the HWS and LWS.

**Correlated Responses to Selection in Growth and Development Related Traits**

Selection for eight-week body weight led to a correlated response of feed intake in the HWS and LWS. The HWS exhibits hyperphagia while the LWS demonstrates hypophagia with a certain percent of the population exhibiting anorexia (Dunnington and Siegel, 1996). The differences in eating behavior were apparent by Generation 5 (Siegel and Wisman, 1966) and were more evident for meal number than meal size. During a 24 hour period the HWS consumed a greater number of meals that were of a similar size to the meals consumed by the LWS (Barbato et al., 1980). The physiological mechanisms
causing the changes in eating behavior remain to be fully elucidated. However, results from electrolytic lesioning of the ventro-medial hypothalamus in hens from the selected lines suggest that the hyperphagia seen in the HWS may be partly due to a decrease of hypothalamic satiety mechanisms (Burkhart et al., 1983). Furthermore, studies using intracerebroventricular injections of the biogenic amines methoxamine and hydroxytryptamine in fully fed and fasted birds from HWS and LWS suggest that the selection for eight week body weight altered the brain response to these biogenic amines which may impact the appetite of HWS and LWS chickens (Denbow et al., 1986). There is also evidence that some component of plasma from feed deprived HWS impacts feed intake, causing stimulation of feed intake in LWS but not in similarly treated HWS (Lacy et al., 1987).

Anorexia was first noticed in Generation 25-26 of LWS when it was observed that a considerable proportion of the LWS pullets failed to reach sexual maturity (indicated by egg production). From that generation on it was common for 25 to 50 percent of LWS pullets to fail to reach sexual maturity by 275 days of age (Zelenka et al., 1988; Liu et al., 1995). In later generations it was observed that 5 to 20 percent of newly hatched LWS chicks do not survive after one week post-hatch because they never learn to eat (Noble et al., 1993; Liu et al., 1995). The percent of the LWS population that demonstrates anorexia is cyclical and varies with generation (Siegel, unpublished data).

There are differences in feed efficiency between the HWS and LWS. Chemical analyses of freshly laid and incubated Generation 5 eggs from the HWS and LWS revealed that the HWS embryos were more efficient at utilizing energy and certain amino acids, particularly the sulfur-containing amino acids, compared to the LWS embryos.
The embryonic differences in feed utilization did not appear to translate to post-hatch in the early generations of selection, with differences in feed consumption overriding differences in feed efficiency (Siegel and Wisman, 1966). In Generation 5, the HWS chicks consumed more feed *ad libitum* and grew faster than the LWS chicks but there was no difference in feed efficiency to a fixed age. However, when feed intake of the HWS was limited to that of the LWS in pair-fed feeding trials, the HWS utilized feed more efficiently than the LWS. Further generations of selection for eight week body weight resulted in differences in feed efficiencies that could be detected independent of feed consumption (Owens et al., 1971; Barbato et al., 1983a, b). Increased oxygen consumption (Owens et al., 1971), increased rate of feed passage (Cherry and Siegel, 1978), improved temperature regulation (Dunnington and Siegel, 1984), and superior intestinal glucose absorption capabilities (Walker et al., 1981) in the HWS are linked to the better feed efficiency observed in the HWS.

Body composition is also altered in the lines due to selection for eight week body weight. The HWS chickens have a higher percent of body fat compared to the LWS (Burgener et al., 1981; Dunnington et al., 1986). However, lipogenesis and lipolysis occurs at a faster rate in the LWS chickens (Calabotta et al., 1983, 1985). Therefore, the increased body fat in HWS chickens is probably due to decreased lipolysis rather than increased lipogenesis. There is also a size difference in organs that are important to the digestion and assimilation of nutrients in these selected lines of chickens. Of particular interest, the small intestine of the HWS line is larger per gram of body weight compared to the LWS (Katanbaf et al., 1988). This indicates that the HWS chickens may have a larger capacity to absorb nutrients.
Embryonic growth, yolk sac weight, and incubation times have been influenced by selection for eight week body weight in these lines of chickens. The HWS embryos are larger yet the relative weight of the residual yolk sac (g/100g BW) is greater in the HWS at hatch indicating that the HWS embryos are more efficient (Nitsan et al., 1991). The difference in relative residual yolk sac weight between the lines is gone by day 3 but may be due to the LWS using the yolk sac as a primary source of energy and nutrient intake to compensate for their hypophagia. The LWS hatches approximately 5 hours earlier than HWS chicks (Dunnington et al., 1992).

**Correlated Responses to Selection in Metabolic Related Traits**

The correlated changes in the growth characteristics listed above are associated with and in some cases inseparable from changes in the metabolic characteristics altered by the single trait genetic selection employed in these lines. Differences in phenomena such as thermoregulation, glucose tolerance, growth hormone (GH), thyroid hormones, and digestive enzymes exist in these selected lines of chickens.

The HWS and LWS differ in their ability to maintain core body temperatures. Surface and cloacal temperatures have been measured periodically throughout this long-term selection experiment. Chicks of the LWS have lower surface temperatures than the HWS chicks (Dunnington and Siegel, 1984). The difference in surface temperature between the lines disappears as the chicks mature. In addition to lower surface temperatures, the LWS chicks have lower core body temperatures than the HWS through 23 days of age (Dunnington and Siegel, 1984).

There are differences between the HWS and LWS in their ability to tolerate glucose. At all ages, LWS chicks are better able to clear glucose from their blood than
the HWS chicks (Sinsigalli et al., 1987). The HWS X LWS (HL) cross chicks have an intermediate glucose tolerance response. The lower glucose tolerance in the HWS and HL chicks is not associated with an insulin insufficiency. Therefore, it is believed that the excessive fat deposits in the HWS chickens is associated with elevated concentrations of glucagon and insulin in the blood plasma, which may lead to the observed insulin resistance (Sinsigalli et al., 1987).

Levels of GH and the thyroid hormones triiodothyronine (T₃) and thyroxine (T₄) differ between the LWS and HWS. The differences are more apparent in older chicks (61 days of age) than younger chicks. Plasma levels of GH are higher in the LWS compared to the HWS (Scanes et al., 1989). In all lines except the LWS, GH and T₄ increase with age (Nir et al., 1987a).

Alternate day feeding decreased pancreatic digestive enzyme (trypsin, chymotrypsin, and amylase) activities at 25 days of age in HWS, HXL, and LWS chickens (Nir et al., 1987b). In selected lines from day of hatch to 10 or 15 days of age levels of amylase, trypsin, and chymotrypsin located in the pancreas and gut content varied with age (Nitsan et al., 1991; Dunnington and Siegel, 1995). There was no difference between lines when comparisons of amylase, trypsin, chymotrypsin, and lipase levels in the pancreas and gut contents were made at a common body weight in females from the LWS, HWS, HWR, and LWR (Dunnington and Siegel, 1995). These results suggest that the correlated response to selection in feed intake were overriding the regulation of the expression and activity of these digestive enzymes in the pancreas and gut content.
Summary

Divergent selection for high or low juvenile body weight in White Plymouth Rock chickens has led to the development of a model system for the study of correlated responses of single trait selection to a variety of traits, particularly of growth characteristics and mechanisms. Understanding of mechanisms related to growth and development can lead to improved animal production in agriculture.

CHICKEN GASTROINTESTINAL ANATOMY AND SMALL INTESTINE MICROANATOMY

Upper Gastrointestinal Anatomy

The information provided in the following two sections can be referenced to “Scott’s Nutrition of the Chicken” (Leeson and Summers, 2001). The process of feed digestion begins with the beak which is used to gather feed as well as begin to mechanically break down the feed. The efficiency of the beak to break down feed is low. Feed enters the esophagus and then moves into the crop. This structure, which is unique to avians, functions to store and moisten feed. However, the crop serves little function in the domesticated chicken, except for young broiler breeders or where there are severe food availability limitations. Feed enters the proventriculus, which is analogous to the stomach of other monogastrics. The normal pH here is about 2.5 due to HCl secretion. From the proventriculus, feed and secretions are moved into the gizzard, another structure unique to the avian gastrointestinal tract. The gizzard is a strong, muscular organ that functions to mechanically break down feed into particles fit for enzymatic digestion and to mix the feed with secretions from the proventriculus. In the domesticated chicken the
gizzard is rudimentary compared to the gizzard of wild birds as a consequence of the feed milling processes used to increase digestibility of feed.

**Lower Gastrointestinal Anatomy**

Contents of the gizzard are then passed into the small intestine, which is divided into three defined sections: duodenum, jejunum, and ileum. The duodenum starts at the gizzard, forms a loop surrounding the pancreas and ends at the top of the loop. The jejunum begins at the end of the duodenal loop and is separated from the ileum by Meckel’s diverticulum. Meckel’s diverticulum is the remnant of the yolk stalk. The ileum starts at Meckel’s diverticulum and extends to the ileal-cecal junction. The last part of the gastrointestinal tract is a pair of ceca and the large intestine. Fecal material is then excreted through the cloaca.

*Figure 1. Chicken Digestive Tract*
Small Intestine Microanatomy

The information in the following section can be referenced to “Molecular and Cellular Basis of Digestion” (Desnuelle et al., 1986). The microanatomy of the small intestine is very important to the overall function of this absorptive organ. The primary objective of the microanatomical features of the small intestine is to maximize absorptive area. Three structures accomplish this goal: the mucosal folds, the villi, and the microvilli that constitute the brush border. Goblet cells are also present in the intestinal epithelium and play an indirect role in nutrient absorption.

The mucosal folds are the circular folds on the luminal side of the intestine which give it the rough appearance. Not only do these folds increase absorptive surface area they also aid in mixing of luminal contents. On the surface of these mucosal folds are many finger-like projections called villi. The villus is divided into two anatomical parts: the villus and the crypt. The crypt is an invagination of the epithelial surface and the villus is the part that projects into the lumen of the small intestine.

On the surface of the villus are the enterocytes or absorptive epithelial cells of the small intestine. Enterocytes originate from stem cells in the crypts. As the enterocyte migrates towards the villus tip it gains its absorptive function. After reaching the tip it is sloughed off into the lumen of the small intestine. The life span of an enterocyte is short, lasting only about 2-5 days.

Enterocytes are polarized cells that have a basolateral membrane that faces the blood stream, and an apical membrane that faces the lumen of the small intestine. The apical membrane is comprised of more microscopic finger-like projections called microvilli. These microvilli form what is called the brush border membrane, which is the
absorptive interface of the small intestine. This is where the transport systems and enzymes reside, which facilitate nutrient assimilation.

Arising from stem cells in the villi crypts, goblet cells are part of the villus epithelium along with enterocytes. Goblet cells secret mucus, which forms the glycocaylx of the small intestine. This mucus layer of the small intestine plays an important role in nutrient digestion and absorption. It creates a microenviroment, sometimes referred to as the unstirred water layer, surrounding the brush border membrane. This covering serves to protect the fragile brush border from the movement of gut contents in the mainstream of the lumen, which may cause premature sloughing of the enterocytes or damage to the microvilli. It also functions as a pathogen barrier and maintains an environment that has a consistent pH and levels of ions and other molecules to optimize digestive enzyme and nutrient transporter function.

*Figure 2. Small Intestine Microanatomy.*
**Summary**

The chicken is a monogastric with the addition of several unique structures including the crop, gizzard, proventriculus and Meckel’s diverticulum. The small intestine of the chicken is the absorptive organ of the digestive tract and the enterocyte is the absorptive cell of the intestinal epithelium. The apical surface of the enterocyte is the brush border membrane, where nutrient transporters and enzymes are located to facilitate nutrient assimilation.

**CARBOHYDRATE DIGESTION IN THE CHICKEN AND THE BRUSH BORDER MEMBRANE GLUCOSE AND FRUCTOSE TRANSPORTERS IN THE SMALL INTESTINE**

**Introduction**

In the chicken most digestive enzyme secretion occurs in the proventriculus, pancreas, and duodenum, while most absorption of nutrients occurs in the jejunum and ileum (Leeson and Summers, 2001). Dietary carbohydrates are broken down into a variety of end products including glucose, galactose, and fructose. Transport of glucose across the brush border membrane by the sodium-dependent glucose transporter 1 (SGLT1) is the primary route for glucose assimilation. Fructose is transported across the brush border membrane by the facilitative fructose transporter (GLUT5). In this section chicken carbohydrate digestion and the sugar transporters SGLT1 and GLUT5 will be discussed.

**Carbohydrate Digestion in the Chicken**

The information provided in the following section can be referenced to “Scott’s Nutrition of the Chicken” (Leeson and Summers, 2001). The majority of the carbohydrates in the grains, which make up poultry feed, are in the form of starch and
most of this starch is in the form of digestion resistant granules. This starch can be made available by physical breakdown of the granules and wetting with saliva which contains the enzyme amylase that can initiate starch digestion.

The feed passes through the proventriculus, gizzard, and duodenum with little additional digestion of carbohydrates. Most carbohydrate digestion occurs in the jejunum. Overall, the highest concentrations of carbohydrate digestive enzymes are found in jejunum followed by ileum and duodenum. The pancreas produces and secretes α-amylase (pancreatic amylase) into the duodenum, which is then passed with feed into the jejunum. The α-amylase hydrolyzes α 1,4 links of starch generating maltose and maltase. The intestinal mucosa produces and secretes the enzyme complex sucrase-isomaltase, which is cleaved into the disacharidases sucrase and isomaltase. Isomaltase acts on maltose and isomaltose to yield glucose. Sucrase acts on sucrose to generate glucose and fructose. The glucose and fructose are assimilated into the enterocyte by the brush border membrane nutrient transporters SGLT1 and GLUT5, respectively.

**The Intestinal Sodium-Dependent Glucose Transporter, SGLT1**

*Introduction.* Glucose is the primary energy source for an animal and is provided in the diet in the form of carbohydrates. Complex carbohydrates are broken down during digestion to free glucose, galactose, and fructose which are then available for absorption by the brush border membrane of the small intestine. The major route for glucose assimilation in enterocytes is by the SGLT1 transporter (Hediger and Rhoads, 1994; Wright and Turk, 2004). Re-uptake of glucose from the glomerular filtrate is also
facilitated by SGLT1 located in the proximal tubule of the kidney (Hediger and Rhoads, 1994; Wright and Turk, 2004).

The SGLT1 is a member of the SLC5A gene family, which is a part of the larger family of glucose transporters including all the SGLT proteins as well as the GLUT proteins (Wood and Trayhurn, 2003). The SGLT1 protein was first cloned in rabbit intestine (Hediger et al., 1987) and has been identified in several other animals including humans (Hediger et al., 1989), rat (Aoshima et al., 1997), pig (GenBank Accession # AAW69922), horse (Dyer et al., 2002), cattle (Zhao et al., 2005), sheep (Wood et al., 1994), and chicken (Gal-Garber et al., 2000). The SGLT1 transporter is the major, if not only, route of glucose uptake by the intestine as absence of functional SGLT1 leads to the development of glucose/galactose malabsorption disease (Turk et al., 1991). Therefore, the SGLT1 protein has a major influence on the glucose availability to the animal.

**Tissue and Cellular Distribution of SGLT1.** The SGLT1 protein and transcript are expressed highest in the small intestine with lower levels in the renal proximal tubules (Hediger and Rhoads, 1994; Wright and Turk, 2004). Expression of SGLT1 has also been observed in rumen and omasal tissue (Zhao et al., 1998; Aschenbach et al., 2002), as well as mammary tissue from lactating cattle (Zhao et al., 1999) and lung from rat (Basset et al., 1987, 1988) and sheep (Basset et al., 1987, 1988; Barker et al., 1989; Zhao et al., 2005).

The cellular localization of SGLT1 is controversial and may be influenced by many factors. In rats under normal physiological and environmental conditions, experiments utilizing immunofluorescence showed that SGLT1 protein location is specific to differentiated mature enterocytes (Hwang et al., 1991; Takata et al., 1992;
Yoshida et al., 1995). Immunogold (Takata et al., 1992) and immunohistochemical (Hwang et al., 1991; Yoshida et al., 1995) staining in rat intestine demonstrated that the SGLT1 protein is primarily localized to the brush border membrane of these mature enterocytes. Some SGLT1 may be found in the Golgi apparatus (Takata et al., 1992) as well as the cytoplasm (Takata et al., 1992; Kipp et al., 2003).

In rats, while the SGLT1 protein appears to be found only in the mature enterocyte, in situ hybridization studies show that SGLT1 mRNA is specific to the brush border membrane of enterocytes in the lower two thirds of the villus (Lee et al., 1994; Dong et al., 1997). There is also limited SGLT1 mRNA expression in the crypts (Lee et al., 1994). This indicates that as the enterocyte begins to differentiate from the stem cells found in the crypts, they begin to express SGLT1 mRNA. As the enterocytes migrate up the villus and mature they begin to synthesize and accumulate SGLT1 protein, which becomes incorporated into the brush border membrane as early as the crypt-villus junction.

Although the cellular expression of SGLT1 at the gene and protein level is fairly well characterized in the rat, other species demonstrate a slightly different picture. In rabbits, while phlorizin (a high affinity, non transported, competitive inhibitor of SGLT1) sensitive glucose uptake occurs in the upper two thirds of the villus, indicating that the SGLT1 protein is localized to mature enterocytes (as seen in rats); the SGLT1 mRNA expression is different. In rabbits, SGLT1 mRNA is expressed in enterocytes along the entire length of the villus (Hwang et al., 1991; Smith et al., 1992) with SGLT1 mRNA abundance increasing 6-fold from villus base to tip (Hwang et al., 1991). In chickens,
immunohistochemical analysis revealed that SGLT1 protein is expressed throughout the entire length of the villus but not in the crypts (Barfull et al., 2002a).

**Substrates of SGLT1.** The primary substrate for the SGLT1 transporter is glucose (Hediger and Rhoads, 1994; Wright and Turk, 2004). The SGLT1 transporter, when expressed in *Xenopus* oocytes, transported a range of other sugars and sugar analogs including D-galactose, α-methyl-D-glucopyranoside and 3-O-methylglucopyranoside, although with a lower affinity than glucose (Ikeda et al., 1989).

Along with sugars, the SGLT1 transporter cotransports Na⁺ (Hediger and Rhoads, 1994; Wright and Turk, 2004) with a stochiometry of glucose: Na⁺ of 1:2 (Wright and Turk, 2004). Transport of Na⁺ into the blood by a basolateral Na⁺ / K⁺ ATPase creates a transepithelial osmotic gradient that increases fluid absorption and through this mechanism, SGLT1 may also play a role in water absorption across the brush border membrane. The SGLT1 may also directly cotransport water (Wright and Turk, 2004), which would be facilitated by osmosis due to the high concentration of glucose next to the plasma membrane generated by SGLT1 (Lapointe et al., 2003; Gagnon et al., 2004). It is also possible that a combination of the two mechanisms may contribute to water absorption by SGLT1 (Wright and Turk, 2004).

**Structure of SGLT1.** The primary structure of SGLT1 varies in number of amino acids depending on species, but most average about 663 amino acids. The predicted weight of the SGLT1 protein is approximately 73 kD (Wright and Turk, 2004). Evaluation of consensus sequences determined that SGLT1 contains a number of potential cyclic-AMP (PKA) and protein kinase C (PKC) dependent phosphorylation
sites, although the exact number of each appears to be species dependent (Kennelly and Krebs, 1991).

The current model for the membrane topology of SGLT1 shows that the protein contains 14 α-helical trans-membrane domains (Turk et al., 1996; Wright and Turk, 2004). In this model the N-terminus is extracellular, while the C-terminus is the trans-membrane domain 14 (Wright and Turk, 2004). The regions between trans-membrane domains 10-13 appear to be important for extracellular glucose binding and the regions between trans-membrane domains 10-14 appear to be important for intracellular glucose binding (Wright and Turk, 2004). It is thought that transmembrane domains 2-8 are important for Na⁺ binding because the homology in this area is high between SGLT1, SGLT2, and a sodium dependent E. coli transporter (Hediger and Rhoads, 1994).

There are few studies focusing on the tertiary or quaternary structure of SGLT1. The molecular size of the functional unit of SGLT1 was investigated using radiation inactivation studies, which yielded a functional unit size of 290 kD suggesting that SGLT1 can exist as a homo tetramer in the brush border membrane (Beliveau et al., 1988; Stevens et al., 1990). The SGLT1 is still functional as a monomer (Wright and Turk, 2004) but oligomerization may be a mechanism of altering the overall transport velocity and capacity of SGLT1. Studies removing N-linked glycosylation at Asn 248 in rabbit SGLT1 (Hediger and Rhoads, 1994) and site-directed mutagenesis (Lee et al., 1994) demonstrated a reduction in molecular weight of SGLT1 to approximately 52 kD without N-glycosylation, suggesting that SGLT1 is N-glycosylated. The implications of this glycosylation remain uncharacterized.
**Transport Mechanism of SGLT1.** Overall, glucose is transported from the lumen of the small intestine, across the brush border membrane by SGLT1 into the enterocyte. According to the 8-state ordered reaction model one external Na\(^+\) ion binds initiating a conformational change to allow glucose binding, then the second Na\(^+\) ion binds causing a series of conformational changes in SGLT1 to allow translocation of Na\(^+\) and glucose into the enterocyte (Bennett and Kimmich, 1992).

The intracellular concentration of glucose is maintained by transport of glucose across the basolateral membrane out of the enterocyte by the GLUT2 transporter or exocytosis (Wright and Turk, 2004). The intracellular concentration of Na\(^+\) is maintained lower than the Na\(^+\) concentration in the lumen of the small intestine by the basolateral Na\(^+\)/K\(^+\) ATPase to facilitate the cotransport of glucose (Wright and Turk, 2004).

**Regulation of SGLT1.** Expression of SGLT1 is regulated by diet. There is an acute increase in SGLT1-mediated glucose transport in response to luminal infusion of glucose in rat jejunum (Sharp et al., 1996). High carbohydrate diets and both metabolizable and nonmetabolizable SGLT1 substrates can cause an increase in glucose transport in rats, sheep, and mice (Diamond et al., 1984; Ferraris and Diamond, 1992; Reimer et al., 1997; Solberg and Diamond, 1987; Weiss et al., 1998). The increase in glucose transport is correlated with an increase in the amount of SGLT1 protein in the brush border membrane (Ferraris and Diamond, 1992; Ferraris et al., 1992; Dyer et al., 1997). There is also an increase in SGLT1 mRNA abundance; however the magnitude of change is less than what is seen with SGLT1 protein levels in response to a high carbohydrate diet (Lescale-Matys et al., 1993; Miyamoto et al., 1993; Shirazi-Beechey et
indicating that transcription may not be the main site of SGLT1 regulation induced by dietary carbohydrate.

The mechanisms by which dietary carbohydrates induce SGLT1 protein expression and SGLT1 mediated glucose transport remain to be fully characterized. There is evidence that there is a SGLT1 substrate sensing mechanism in the intestine. This mechanism may involve a single receptor with a broad specificity for sugars or many receptors for various SGLT1 substrates that communicate via second messengers, transcription factors, etc. to stimulate the increase in SGLT1 expression in response to the presence of luminal SGLT1 substrates (Ferraris, 2001).

Changes in Na⁺ levels in the diet may also influence SGLT1 expression and glucose transport. Studies in chickens demonstrated that low salt diets decrease the rate of SGLT1-mediated glucose transport (Jaso et al., 1995). Phlorozin binding studies (Garriga et al., 1999) and western blot analysis (Donowitz et al., 1998) showed that the decrease in glucose transport was due to a decrease in the number of SGLT1 transporters. The decrease in SGLT1 expression and glucose transport could be seen within one day of switching to a low salt diet and peaked in two days (De La Horra et al., 2001). In contrast to low Na⁺ levels, high Na⁺ levels did not affect SGLT1 mediated glucose transport or protein level in chickens (Bindslev et al., 1997). However, increasing the Na⁺ levels in chickens adapted to a low Na⁺ diet caused an increase in glucose uptake, which was similar to chickens receiving a high Na⁺ diet (Garriga et al., 2000). Similarly, a decrease in Na⁺ intake led to a decrease in transport of a non metabolizable glucose analogue in the ileum and colon but not in the jejunum of chickens (Barfull et al., 2002b). The decrease in the glucose analogue transport was correlated with a decrease in SGLT1
mRNA abundance and number of SGLT1 transporters in the ileum and colon but not the jejenum indicating that SGLT1 in different regions of the small intestine may be regulated differently.

There is a distinct circadian rhythm to glucose assimilation in the intestine (Stevenson et al., 1980). In rats, there is a doubling of glucose transport in rats in the mid afternoon which then decreases at night (Ferraris et al., 1990; Tavakkolizadeh et al., 2001). This increase in glucose transport was correlated to an increase in SGLT1 mRNA and protein expression (Corpe and Burant, 1996; Rhoads et al., 1998). This increase in expression was seen even when animals are food deprived (Stevenson et al., 1980).

Expression and function of SGLT1 are developmentally regulated. The intestine of fetal mammals expressed SGLT1 mRNA (Wang et al., 1994) and demonstrated active glucose transport (Matsumoto et al., 1993). In rabbits, active glucose and galactose transport increased 3-fold during the last week of gestation (Phillips et al., 1990). In mink (Buddington et al., 2000), rat (Vazquez et al., 1997; Khan et al., 2000), and chick (Moreno et al., 1996), glucose transport rate was highest right after birth or hatch but decreased thereafter and was lowest in adults. However, in rats SGLT1 mRNA abundance increased proportional to pup age (Kojima et al., 1999). In rats there was also a brief increase in glucose transport and SGLT1 expression at weaning, which was thought to be correlated to the change in diet at this time (Khan et al., 2000).

Changes in the environment can impact the expression and function of SGLT1. Food deprivation can affect SGLT1 mediated glucose uptake, but the effect may be species dependent. Brush border membrane vesicles isolated from chicks that had undergone a 4 day fast had lower net glucose uptake than brush border membrane
vesicles isolated from chicks fed *ad libitum* or chicks that had been refed for 2 days post fast (Gal-Garber et al., 2000). In contrast, higher glucose transport that correlated with an increased number of SGLT1 transporters was observed in malnourished neonatal rabbits (Butzner et al., 1990).

Other environmental stressors such as heat can impact SGLT1 expression. Heat stress in chickens caused a decrease in food intake. In chickens exposed to chronic high environmental temperatures the intestinal mucosa has an increased sugar uptake capacity (Mitchell and Carlisle, 1992). The increased SGLT1 activity and expression seen in heat stressed chickens is an adaptation due to heat stress and not reduced food intake (Garriga et al., 2006).

There has been some investigation into the underlying molecular mechanisms for changes in SGLT1 expression and function. Although, cellular location studies suggest that trafficking of SGLT1 from the cytoplasmic pool may be an important step for regulation of SGLT1 expression (Takata et al., 1992; Kipp et al., 2003), however the exact pathways involved are not known. There is evidence that second messengers may act to regulate SGLT1. Using a reporter gene assay in an intestinal cell line (STC-1) with a section of the sheep SGLT1 promoter, it was found that a PKA antagonist decreased glucose-induced SGLT1 promoter activity (Dyer et al., 2003). This group also observed that when glucose concentrations in the culture medium were increased, there was an increase in cAMP levels. In addition when a cAMP analogue was added to SCT-1 cells transfected with a reporter gene driven by a fragment of the sheep SGLT1 promoter in a low glucose medium, SGLT1 promoter activity increased. Further, the addition of a cAMP analogue to these cells in a high glucose medium led to an augmentation of
glucose-induced SGLT1 promoter activity. In support of these results the addition of forskolin (an inducer of intracellular cAMP) caused an increase in intestinal glucose transport (Grubb, 1995). These results suggest that PKA, and therefore PKA dependent phosphorylation, as well as cAMP play roles in the regulation of SGLT1.

Analysis of the SGLT1 promoter sequence has led to the identification of potential binding sites for the nuclear factors hepatocyte nuclear factor 1 (HNF-1) and major late transcription factor/upstream stimulatory factor (MLTF/USF) (Rhoads et al., 1998). This group observed a diurnal expression pattern with HNF-1 and proposed that this nuclear factor was involved in the circadian periodicity of SGLT1 transcription.

**Summary.** The SGLT1 is the primary glucose transporter found in the intestine and SGLT1 utilizes secondary active transport to cotransport Na⁺. The SGLT1 is a 14 transmembrane domain protein that is regulated by many mechanisms including diet, environment, developmental stage, second messengers and transcription factors. The expression and function of intestinal SGLT1 is important to study and understand due to its importance in maintaining overall energy homeostasis in the animal.

**The Intestinal Facilitated Fructose Transporter, GLUT5**

**Introduction.** Dietary carbohydrates can be digested into sucrose, which can then be further broken into free fructose and glucose by the digestive enzyme sucrase. Fructose is then assimilated into the enterocyte by GLUT5 (Kayano et al., 1990; Gould et al., 1991; Burant et al., 1992).

The GLUT5 is a facilitated fructose transporter and is part of the SLC2A5 gene family, which includes all of the facilitated glucose/fructose transporters (GLUTs)
(Mueckler, 1994; Joost and Thorens, 2001). All GLUTs are included in the larger sugar transporter family of proteins which includes SGLT1 (Wood and Trayhurn, 2003). The GLUT5 has been cloned in several species including human (Kayano et al., 1990), rat (Inukai et al., 1993; Rand et al., 1993), rabbit (Miyamoto et al., 1994), mouse (Corpe et al., 2002), and horse (Merediz et al., 2004). Fructose transport studies and mRNA detection indicate that GLUT5 is present in chicken (Garriga et al., 2004) and cattle (Zhao et al., 1993).

**Tissue and Cellular Distribution of GLUT5.** The GLUT5 transporter is found primarily in the small intestine and proximal tubule of the kidney (Kayano et al., 1990; Rand et al., 1993; Sugawara-Yokoo et al., 1999; Corpe et al., 2002; Merediz et al., 2004). It is also expressed in low levels in skeletal muscle (Kayano et al., 1990; Hundal et al., 1998; Stuart et al., 2006), brain (Shepherd et al., 1992; Rand et al., 1993), testis (Kayano et al., 1990), adipose tissue (Kayano et al., 1990; Litherland et al., 2004), mammary tissue (Zhao et al., 1993), and erythrocytes (Concha et al., 1997). This review will focus on expression and function of GLUT5 in the small intestine.

Studies utilizing immunohistochemical analysis (Davidson et al., 1992; Inukai et al., 1997), western blot analysis (Miyamoto et al., 1994), immunofluorescence (Harris et al., 1992; Inukai et al., 1995) and immunoelectron microscopy (Harris et al., 1992) demonstrate that the GLUT5 transporter is primarily localized to the brush border membrane of the enterocyte. However, expression of GLUT5 has also been observed in the basolateral membrane of enterocytes (Harris et al., 1992; Blakemore et al., 1995) and intracellular membranes of enterocytes (Harris et al., 1992). The cause and implications for this differential localization has yet to be determined. In another study with human
fetal intestine, GLUT5 was localized along the intracellular junctions of the developing villus (Davidson et al., 1992). In situ hybridization analysis using rat tissue showed that GLUT5 mRNA was differentially expressed along the crypt-villus axis, with highest GLUT5 mRNA expression in the mid villus region (Rand et al., 1993).

**Substrates of GLUT5.** The GLUT5 is a low affinity-high capacity and highly stereospecific transporter of fructose (Kayano et al., 1990; Gould et al., 1991; Burant et al., 1992; Miyamoto et al., 1994; Corpe et al., 2002; Merediz et al., 2004). Fructose appears to be the only significant substrate of GLUT5 as most studies indicate that GLUT5 does not transport glucose. However, there are studies that indicate that GLUT5 may be capable of transport of glucose and galactose in minute amounts. Rat GLUT5 expressed in *Xenopus* oocytes transported glucose (Rand et al., 1993) and fructose transport by rabbit GLUT5 was inhibited by D-glucose and D-galactose (Miyamoto et al., 1994).

**Structure of GLUT5.** Expression cloning of GLUT5 revealed that the primary protein structure varies between species. Rat has 502 amino acids (Rand et al., 1993), mouse, human, and horse have 501 amino acids (Kayano et al., 1992; Corpe et al., 2002; Merediz et al., 2004), and rabbit has 457 amino acids (Miyamoto et al., 1994). The GLUT5 protein isolated in rabbits has a molecular weight of 49 kD (Miyamoto et al., 1994).

The putative membrane topology of GLUT5 has 12 transmembrane spanning domains with intracellular N- and C-termini (Bell et al., 1993). According to this model there is also a large intracellular loop between transmembrane domains 6 and 7 (Bell et al., 1993). A study using chimeric GLUT-1 and GLUT-5 proteins have implicated this loop in regulating apical membrane localization of GLUT5 (Inukai et al., 1997).
Mutagenesis and chimeric protein studies have indicated that a large portion of the C-terminus is important for fructose transport (Bell et al., 1993; Inukai et al., 1995). The GLUT5, like the other GLUTs, is predicted to be glycosylated at the large extracellular loop (Bell et al., 1993). The function of this glycosylation is not yet characterized.

There is little information in the literature on the tertiary or quaternary structure of GLUT5, but it is known that GLUT5 facilitates the transport of fructose as a monomer (Burant and Bell, 1992).

**Transport Mechanism of GLUT5.** The mechanism of GLUT5-mediated fructose transport is one of classical facilitated diffusion. The lipid bilayer of the brush border membrane is impermeable to hydrophilic molecules such as fructose. Therefore, GLUT5 provides transport of fructose across the membrane down its concentration gradient (Bell et al., 1993). The kinetic mechanism of GLUT5-mediated fructose transport is thought to be a mechanism where GLUT5 alternates between two conformational states (Walmsley, 1988; Carruthers, 1990). Fructose binds to either the extracellular or intracellular side of the protein which initiates a conformational change that causes fructose to be translocated across the plasma membrane and released into the cytoplasm of the enterocyte or lumen of the small intestine. Direction of fructose transport depends on its concentration gradient.

**Regulation of GLUT5.** Expression of GLUT5 is regulated by dietary fructose but not by other sugars such as glucose and galactose (Inukai et al., 1993; Miyamoto et al., 1993; Miyamoto et al., 1994). Changes in fructose transport are typically correlated with similar changes in GLUT5 protein and mRNA abundance. Fructose transport increased 2-fold within 3 days of rats consuming a high fructose diet (Crouzoulon and Korieh,
Similarly, Burant and Saxena (1994) observed an increase in fructose transport, which correlated with a similar increase in GLUT5 mRNA and protein abundance within one day of rats consuming a high fructose diet. The increase in protein abundance was maintained as long as fructose consumption continued, which suggests that the increase in fructose transport capacity may be due to de novo synthesis of GLUT5.

Rats demonstrate a proximal to distal gradient of GLUT5 mRNA abundance with highest levels in the proximal small intestine (Castello et al., 1995). The expression of GLUT5 mRNA expression pattern parallels luminal sugar concentrations (Ferraris et al., 1990), which suggests that GLUT5 expression is correlated with availability of its substrate.

Developmental age can also influence GLUT5 expression. In rabbits fructose transport increases during the last week of gestation (Phillips et al., 1990). After birth, in rabbits and rats, there is a gradual decrease in fructose transport (Toloza and Diamond, 1992; Goda, 2000) until weaning, when there is a dramatic increase in fructose transport that correlates with high levels of GLUT5 mRNA abundance (Toloza and Diamond, 1992; Rand et al., 1993; Castello et al., 1995; Shu et al., 1997). Interestingly, this increase in GLUT5-mediated fructose transport appears to be “hard wired” as it occurs independent of dietary fructose. However, dietary fructose can increase GLUT5 expression ahead of the “hard wired” induction of GLUT5 expression (David et al., 1995; Shu et al., 1997).

Expression of GLUT5 is also subject to circadian rhythm. Similar to the diurnal rhythm of SGLT1, highest expression of GLUT5 mRNA and protein are at the start of the
dark phase in rats (Castello et al., 1995). Dietary fructose can augment the diurnal expression of GLUT5 (Corpe et al., 1996).

Hormones may also influence the expression of GLUT5. In Caco 2 cells, thyroid hormone, in the presence of glucose, stimulated GLUT5 gene transcription and promoter activation (Matosin-Matekalo et al., 1999). This glucose dependent-thyroid hormone activation has yet to be observed in vivo.

The main underlying molecular mechanism regarding GLUT5 expression appears to be changes in transcription, as changes in fructose transport and GLUT5 protein expression correlate with similar changes in GLUT5 mRNA abundance. Several transcription factor binding sites have been identified in the mouse GLUT5 5’ flanking region including sites for CdxA, USF and sex determining region Y (Corpe et al., 2002). The transcription factors c-fos and c-jun are upregulated preceding the increase in GLUT5 mRNA following intestinal fructose infusion suggesting that these transcription factors may be involved in the fructose-mediated upregulation of GLUT5 expression (Jiang et al., 2001). The exact roles of these transcription factors in GLUT5 expression have yet to be characterized but they may be involved in the regulation of GLUT5.

There are also other molecular mechanisms, such as second messengers, which may influence GLUT5 expression, albeit indirectly. Sequence analysis of the human GLUT5 promoter revealed cAMP response elements (Mahraoui et al., 1994). Treatment of Caco2 cells with forskolin, an inducer of cAMP, caused a 2-fold increase in fructose transport which correlated with a 5-fold and 7-fold increase in GLUT5 protein and mRNA levels, respectively (Mahraoui et al., 1994). This suggests that cAMP plays a role in the regulation of GLUT5 expression and fructose transport.
**Summary.** The GLUT5 is a facilitated fructose transporter, which is primarily expressed in the brush border membrane of the small intestine. The GLUT5 protein has 12 transmembrane spanning domains and is regulated by dietary fructose, developmental stage, hormones, second messengers and possibly transcription factors. Understanding of the expression and regulation of GLUT5 are important to understanding fructose availability to the animal.

**PROTEIN DIGESTION IN THE CHICKEN AND THE BRUSH BORDER MEMBRANE PEPTIDE AND GLUTAMATE/ASPARTATE TRANSPORTERS, PEPT1 AND EAAT3**

**Introduction**

In the chicken, most digestive enzyme secretion occurs in the proventriculus, pancreas, and duodenum, while most absorption of nutrients occurs in the small intestine, mainly the jejunum and ileum (Leeson and Summers, 2001). Dietary protein is hydrolyzed into free amino acids and peptides, which are assimilated into the enterocyte by specific brush border membrane transporter systems. The anionic amino acids glutamate and aspartate are transported by the excitatory amino acid transporter 3 (EAAT3), while di- and tri- peptides are transported by the proton-coupled oligopeptide transporter (PepT1). This section discusses general protein digestion in the chicken, and properties of the peptide transporter PepT1 and the amino acid transporter EAAT3.

**Protein Digestion in the Chicken**

Even though there is no meaningful digestion of dietary protein in the mouth or crop, the physical process of ingesting feed stimulates the vagus nerve which initiates the secretion of gastric juices into the proventriculus in preparation for feed arrival. The main components of this gastric juice are HCl and the zymogen pepsinogen, which are
transformed into its active form, pepsin, by acid hydrolysis. Pepsin cleaves proteins between amino acids Leu-Val, Leu-Tyr, or Leu-Leu, as well as between aromatic amino acids Phe-Phe, or Phe-Tyr. Thus, the proventriculus is the first site of protein digestion in the chicken. The mix of feed and secretions, now called chyme, passes into the gizzard where it is mixed and undergoes further proteolysis by pepsin. The resultant peptides can then be further broken down by other proteolytic enzymes produced by the small intestine.

As the acidic chyme enters the duodenum it lowers the luminal pH. This decrease in pH and other mechanisms stimulate gastrin secretion, which stimulates increased HCl secretion into the proventriculus, and thus further conversion of pepsinogen to pepsin. The pancreas produces and releases elastase and the zymogens trypsinogen and chymotrypsinogen into the duodenum. Elastase hydrolyzes collagen proteins. Enterokinases convert trypsinogen into its active form called trypsin, which is an important proteolytic enzyme as it activates many other zymogens including chymotrypsinogen. Trypsin and chymotrypsin further hydrolyze polypeptides which were produced from protein breakdown by pepsin. The result of hydrolysis by these enzymes is the release of many terminal peptide bonds. The newly formed terminal peptide bonds are further broken down by aminopeptidases and carboxypeptidases A and B and the numerous other specific peptidases produced by the mucosa of the duodenum. Peptides are also produced from the breakdown of collagen by collagenase in the duodenum. In the jejunum, the proteolytic enzyme erepsin is produced, which breaks down polypeptides into amino acids.
The end result of hydrolysis by these enzymes in the duodenum and jejunum are small oligopeptides and free amino acids. Interestingly, the majority of the hydrolysis of peptides by the proteolytic enzymes occurs in the unstirred water layer surrounding the microvilli rather than in the intestinal lumen. There are more digestive and other enzymes near the tip of the microvilli, incidently where the majority of the transport systems are located. The free amino acids and oligopeptides produced by hydrolysis in the unstirred water layer are assimilated by the enterocytes by specific transport systems such as oligopeptide transporter 1 (PepT1), which transports di- and tri-peptides and the excitatory amino acid transporter 3 (EAAT3), which transports the anionic amino acids glutamate and aspartate. In general the PepT1 transporter is a low affinity, high capacity transporter, while the free amino acid transporters, like EAAT3, are high affinity, low capacity transporters. These transporters are discussed in greater detail elsewhere in this literature review. The assimilation of peptides and amino acids occurs along the length of the small intestine although certain transport systems are differentially expressed in specific segments.

The Intestinal Oligopeptide Transporter, PepT1

Introduction. Until the 1970’s it was generally thought that all amino acids derived from dietary protein were absorbed by the small intestine in the form of free amino acids and that oligopeptides did not cross the brush border membrane. The physiological importance of oligopeptides became apparent in studies, which documented their hydrolysis and disappearance from the lumen of the small intestine as well as their appearance in the blood after a meal (Adibi, 1971; Adibi and Mercer, 1973; Adibi et al., 1975). Although these findings were met with skepticism, studies using patients with
impaired transport of certain single amino acids supported the new hypothesis. These studies showed normal assimilation of these amino acids when they were supplied to these patients in peptide form (Hellier et al., 1972; Silk et al., 1975; Leonard et al., 1976). The peptide transport system, first functionally characterized in humans (Adibi, 1971; Adibi and Soleimanpour, 1974; Adibi et al., 1975), has been found to exist in every animal species studied. Today, assimilation of amino acids in the form of peptides by an intestinal peptide transport system is recognized as an energy efficient pathway for the uptake of amino acids.

The peptide transporter (PepT1) facilitates the assimilation of di- and tripeptides in the small intestine and was first cloned in rabbit (Fei et al., 1994). Since this time, PepT1 has been cloned and characterized from several other animal species including human (Liang et al., 1995), mouse (Fei et al., 2000), rat (Saito et al., 1995), chicken (Chen et al., 2002), turkey (Van et al., 2005), cattle (Chen et al., 1999), sheep (Chen et al., 1999), pig (Klang et al., 2005), and dog (GenBank Accession # AF461733.2). The PepT1 is a member of the Peptide Oligo Transporter (POT) superfamily, which includes all peptide transporters in all species including the peptide transporters found in bacteria, plants, and fungi (Botka et al., 2000). The variety of organisms that have members of the POT family indicate that the peptide transport function has been evolutionarily conserved. In humans the POT family has at least four known members all displaying tissue specific expression. These transporters are PepT1, PepT2, Proton-coupled histidine transporter (PHT) 1 and PHT 2 (Botka et al., 2000).

*Tissue and Cellular Distribution of PepT1.* The PepT1 protein is expressed mainly in the small intestine with lesser expression in the kidney (Meredith and Boyd,
2000), although there are exceptions to this. For example, PepT1 is expressed in the omasum and rumen of dairy cattle and sheep (Chen et al., 1999). The abundance of PepT1 mRNA and protein in the small intestine in animals under normal dietary and metabolic conditions is varied. In rabbits (Fei et al., 1994), and pigs (Chen et al., 1999; Klang et al., 2005) maximal PepT1 mRNA abundance and/or protein is in the proximal (duodenum and jejunum) small intestine. In sheep (Chen et al., 1999), dairy cattle (Chen et al., 1999), and black bear (Gilbert et al., 2007a) maximal PepT1 mRNA abundance is in the distal small intestine. Tissue mRNA expression of chicken PepT1 (cPepT1) is highest in the small intestine with very low expression in the kidney and ceca (Chen et al., 1999, 2002). In the small intestine highest cPepT1 mRNA levels are seen in the duodenum followed by jejunum and ileum (Chen et al., 1999, 2002; Gilbert et al., 2007b). The physiological importance governing these species differences is not fully understood.

The PepT1 has a specific cellular distribution that is independent of intestinal region (Groneberg et al., 2001; Hussain et al., 2002). In the small intestine of mice (Groneberg et al., 2001) and rats (Ogihara et al., 1999; Groneberg et al., 2001; Hussain et al., 2002), PepT1 protein expression is limited to the brush border membrane of mature enterocytes. The PepT1 protein expression level may be related to the maturity of the enterocytes as the density of the PepT1 transporter decreases from the apical tip to the base of the villus in rats (Ogihara et al., 1999) and there is no PepT1 expression in the crypts of the villus in mice and rats (Ogihara et al., 1999; Hussain et al., 2002). The cellular location of PepT1 may be influenced by developmental stage. Immediately after birth in rats, PepT1 is localized in the subapical cytoplasm and basolateral membrane of
the enterocyte (Hussain et al., 2002). This basolateral localization of PepT1 in newborn rats may serve an important physiological function as the newborn rat switches from assimilation of nutrients from the bloodstream to the lumen of the small intestine.

**Substrates of PepT1.** The PepT1 has a broad substrate range that includes di- and tri- peptides, and amino acid derivatives (Brandsch et al., 2004; Vig et al., 2006) as well as pharmacologically important compounds such as the β-lactam antibiotics and several prodrugs (Brodin et al., 2002). Substrates for PepT1 do not possess a common structural feature. However, there are structural characteristics of PepT1 substrates that dictate the binding affinity as well as transport efficiency (Amasheh et al., 1997; Brandsch et al., 2004; Vig et al., 2006). Substrate size, charge, amino and carboxy termini modifications, side chain modifications, presence of proline, stereospecificity all affect the ability of a substrate to bind to and be transported by PepT1. Although a compound may bind the PepT1 protein, this does not necessarily mean that the compound will be transported by PepT1. Vig et al. (2006) showed that transport by PepT1 is influenced by substrate charge, hydrophobicity, size, and side chain flexibility. Di-peptides with two positively charged amino acids or with extreme physical bulk are not substrates of PepT1 (Vig et al., 2006). In studies with cPepT1, transport of di-and tri-peptides is saturable and requires an acidic environment to facilitate optimal peptide transport (Chen et al., 2002). Of the 16 di- and tri-peptides tested (all contained the essential amino acids Met, Lys, or Trp) most had substrate affinities in the 20 to 100 micromolar range and were transported by cPepT1 in Chinese hamster ovary cells. However, the dipeptide Lys-Lys, the tripeptide Lys-Trp-Lys, and tetrapeptides tested had substrate affinities in the 4 to 27
millimolar range. Further, free amino acids were not transported by cPepT1 in Chinese hamster ovary cells.

**Structure of PepT1.** The PepT1 protein is an integral membrane protein with 12 trans-membrane domains with a large extracellular loop between domains 9 and 10 (Fei et al., 1994; Meredith and Boyd, 2000; Chen et al., 2002). Evidence from studies using PepT1 and PepT2 chimeras indicate that the putative peptide binding site in PepT1 consists of transmembrane domains 7, 8, 9 and the loops in between (Chen et al., 2000).

The PepT1 protein has several potential N-linked glycosylation sites (Fei et al., 1994; Saito et al., 1995; Chen et al., 2002; Van et al., 2005). The number and location of these putative N-linked glycosylation sites are species dependent. The PepT1 protein also has been shown in some species to contain potential phosphokinase C (PKC) and cyclic AMP (PKA) dependent phosphorylation sites (Fei et al., 1994; Saito et al., 1995; Chen et al., 2002; Meredith and Boyd, 2000; Van et al., 2005). While rabbit PepT1 has both the putative PKA and PKC dependent phosphorylation sites (Fei et al., 1994), most other species have only the putative PKC dependent phosphorylation site (Saito et al., 1995; Meredith and Boyd, 2000; Chen et al., 2002; Van et al., 2005). The implications of the modifications by glycosylation or phosphorylation have yet to be characterized but are speculated to play a role in regulating PepT1 expression.

The cPepT1 has 714 amino acids with a molecular weight of 79.3 kDa and an isoelectric point of 7.48. The predicted cPepT1 is 62.4, 62.5, 63.8, 64.8, 65.1 percent homologous to rabbit, human, mouse, rat, and sheep PepT1 respectively, but has no sequence identity in the first 15 amino acids (Chen et al., 2002). Two cPepT1 cDNAs
were cloned which encode two naturally occurring cPepT1 protein variants (Chen et al., 2002). The point mutation in the cDNA results in a Leu 703 Ser change.

The model of cPepT1 indicates that it has several features that are similar to mammalian PepT1 as well as many signatures of the POT superfamily, including 12 transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10 and cytoplasmic amino- and carboxy-termini (Chen et al., 2002). The cPepT1 contains a putative PKC dependent phosphorylation site.

**Transport Mechanism of PepT1.** The PepT1 is a proton-coupled transport protein (Fei et al., 1994) that is dependent on a pH gradient as well as a negative intracellular membrane potential (Adibi, 1997) to drive the transport of its substrates. The pH of the unstirred water layer that surrounds the tip of the villi is approximately 6.0 (Shimada, 1987). Transport of neutral and cationic substrates by PepT1 is most efficient at this slightly acidic physiologic pH (Steel et al., 1997).

Di- and tri peptides as well as other substrates of PepT1 are co-transported along with one or two protons by the PepT1 transporter across the brush border membrane of enterocytes (Steel et al., 1997). Neutral and cationic peptides are transported with a proton:peptide stoichiometry of 1:1, while anionic peptides are transported with a proton:peptide stoichiometry of 2:1 (Steel et al., 1997). It is thought that one proton transported with the acidic peptides neutralizes the peptide, while the second proton binds the PepT1 transporter (Steel et al., 1997). The proton first binds to the binding site on PepT1 followed by substrate binding and translocation (Mackenzie et al., 1996).

Intracellular pH is maintained by a Na\(^+\) / H\(^+\) exchanger protein (NHE) located on the brush border membrane so that the intracellular proton concentration is less than the
extracellular concentration (Adibi, 1997). The NHE transports a proton from the cytosol of the enterocyte to the lumen of the small intestine, while it transports one Na\(^+\) from the lumen of the small intestine into the enterocyte (Adibi, 1997). A basolateral Na\(^+\)/K\(^+\) ATPase pumps out the cytosolic Na\(^+\) in exchange for a K\(^+\), thus maintaining the negative intracellular membrane potential (Adibi, 1997).

The intracellular peptide concentration is regulated by two mechanisms. Peptides can be broken down by intracellular peptidases into free amino acids (Adibi, 1997). These free amino acids can be used by the enterocyte or they can be transported out of the enterocyte and into the blood by free amino acid transporters located on the basolateral membrane (Adibi, 1997). There is also evidence that peptides not broken down by intracellular peptidases are transported across the basolateral membrane as peptides by a currently uncharacterized basolateral peptide transporter which is distinct from PepT1 (Adibi, 1977; Adibi and Krzysik, 1977; Saito and Inui, 1993; Thwaites et al., 1993).

**Regulation of PepT1.** Substrates of PepT1 can influence expression and activity of this transporter. In human intestinal cells (Caco-2), dipeptides were shown to increase the transport of substrate by PepT1 which correlated with an increased amount of PepT1 protein in the membrane, resulting from an increase in PepT1 mRNA stability coupled with an increase in gene expression (Walker et al., 1998; Shiraga et al., 1999). In Caco-2 cells, certain amino acids could up-regulate PepT1 transport activity and amount of PepT1 protein in the membrane (Shiraga et al., 1999). In transient expression studies using luciferase reporter vectors and Caco-2 cells, selected peptides as well as the amino acids Phe, Arg, and Lys stimulated the rat PepT1 promoter (Shiraga et al., 1999).
Analysis of the rat PepT1 promoter revealed the presence of an AP-1 binding site as well as an amino acid response element, which may mediate the effect of the amino acids (Shiraga et al., 1999).

Hormones also play a role in the regulation of PepT1. In Caco-2 cells, insulin increased the abundance of PepT1 in the membrane by increasing PepT1 trafficking from the cytoplasmic pool to the membrane (Thamotharan et al., 1999). Leptin can also increase the amount of PepT1 protein present in the membrane (Buyse et al., 2001). Like insulin, leptin increases PepT1 trafficking from the cytoplasmic pool to the membrane (Buyse et al., 2001). In Caco-2 cells, epidermal growth factor (Nielsen et al., 2001) and thyroid hormone (Ashida et al., 2004) decreased the expression of PepT1 protein in the membrane. Thyroid hormone also decreased PepT1 expression in vivo in rats (Ashida et al., 2004). The decrease in PepT1 protein was caused by a decrease in PepT1 gene expression and/or PepT1 mRNA stability (Ashida et al., 2002).

The diurnal rhythm in rats influences both PepT1 mRNA and protein expression and transport (Pan et al., 2003). Transport of Gly-Sar and levels of PepT1 mRNA and protein were low during the light phase and high during the dark phase (Pan et al., 2003).

PepT1 gene expression changes during development. The PepT1 gene expression was highest in 4-day old rats and decreased to adult levels by day 28 of age (Miyamoto et al., 1996). Similarly, Shen et al. (2001) found that PepT1 gene and protein expression was highest in rats at 3-5 days of age and then decreased rapidly thereafter. They also observed an increase of PepT1 expression at day 24 of age which was attributed to the stresses of weaning. Adult PepT1 protein levels in rats were 70 percent that of day 3-5 levels. In chickens and turkeys, PepT1 is developmentally regulated. In turkeys there is a
3.2-fold increase in PepT1 mRNA abundance from embryonic day 25 to day of hatch (Van et al., 2005). In chickens there is a 14- to 50-fold increase in PepT1 mRNA abundance from embryonic day 16 to day of hatch (Chen et al., 2005).

Diet also influences PepT1 expression. When rats were fed a high protein diet of 50% gelatin, PepT1 mRNA abundance increased (Erickson et al., 1995). The greatest increase in PepT1 mRNA abundance occurred in the distal regions of the small intestine and therefore, Erickson et al. (1995) concluded that these regions were more responsive to dietary induced changes in peptide transport. There was a decrease in PepT1 mRNA abundance in intestinal tissue as well as transport of Gly-Sar in brush border membrane vesicles (BBMV) isolated from rats that were on a protein free diet (Shiraga et al., 1999). This was in contrast to the increase in Gly-Sar transport in BBMV isolated from rats that were on a high protein, 50% casein, diet (Shiraga et al., 1999). The increase in functionality of the PepT1 protein in the presence of a high protein diet was due to enhanced PepT1 protein expression as a result of increased gene transcription (Shiraga et al., 1999).

Chicken PepT1 is regulated by dietary protein level. Chicks that were fed a 12% crude protein diet had a decrease in PepT1 mRNA abundance from day of hatch to 35 days of age (Chen et al., 2005). This was in contrast to an increase in PepT1 mRNA abundance in chicks that had been fed an 18% or 24% crude protein diet (Chen et al., 2005). In this study, the feed intake of chicks fed the higher protein diets were restricted to the amount of feed consumed by the 12% group and therefore the results may have been confounded because of effects of restricted food intake. To separate out the effects of food restriction and protein level on PepT1 gene expression, Chen et al. (2005)
measured PepT1 mRNA abundance in chicks that were fed a 24% crude protein *ad libitum* diet and observed a decrease of PepT1 mRNA abundance through day 14 of age followed by an increase in PepT1 mRNA abundance so that by day 35 of age PepT1 mRNA abundance was higher than chicks on the 12% crude protein diet but lower than chicks on the 18% crude protein diet and the chicks on restricted 24% crude protein diet. These results indicate that dietary protein levels affect gene expression of PepT1, however amount of food intake can influence these effects.

Metabolic conditions such as starvation affect PepT1 expression. Vazquez et al. (1985) noted that starvation altered peptide transport in the human jejunum. Thamotharan et al. (1999) observed that transport by PepT1 increased 2-fold in rats that had undergone a 24 hour fast. This increase in transport was coupled with a 3-fold increase in PepT1 protein and mRNA abundance. These results indicate that acute food withdrawal induces an up-regulation in PepT1 gene expression leading to an increase in membrane PepT1 protein levels and an overall increased capacity to transport substrates.

Shimakura et al. (2006) focused their investigations on the transcription factor PPARα, which plays a role in the adaptive response to fasting in the liver and other tissues. In rats that had been fasted for 48 hours, PPARα mRNA in the small intestine increased and was accompanied by an elevation of serum levels of free fatty acids, which are endogenous PPARα ligands. Oral administration of a PPARα agonist to rats also increased intestinal PepT1 mRNA levels. Further, treatment of Caco-2 cells with the same PPARα agonist increased PepT1 mRNA and transport of Gly-Sar. This group concluded that PPARα plays a role in mediating the increase in fasting levels of PepT1 mRNA in the small intestine. Naruhashi et al. (2002) observed that in fed rats, PepT1
mRNA abundance as well as transport activity was highest in the distal small intestine. In starved rats, PepT1 mRNA abundance and transport activity increased in all segments of the small intestine but the greatest increase was seen in the proximal small intestine.

**Summary.** Transport of di- and tri-peptides across the brush border membrane is facilitated by the proton-dependent oligopeptide transporter, which is localized to the brush border membrane of mature enterocytes. The intestinal expression and functionality of PepT1 is dependent on species, nutritional or metabolic status, diet, hormones, age, time of day, functionality of proteins that maintain chemical, electrical or pH gradients, as well as other factors such as second messengers, PepT1 substrates and amino acids. In the chicken, PepT1 is proximally expressed in the small intestine and varies with diet and developmental age.

**The Intestinal Glutamate/Aspartate Transporter, EAAT3**

**Introduction.** The end products of protein hydrolysis in the lumen of the small intestine are peptides and free amino acids, which are transported across the brush border membrane by specific transport systems. The EAAT3 transporter is a high-affinity low-capacity transporter of free aspartate and glutamate in the small intestine and was first cloned in rabbit (Kanai and Hediger, 1992). This transporter is important to the enterocyte because glutamate is the primary fuel source for the enterocyte (Newsholme et al., 2003). The EAAT3 is a member of the amino acid transporter system X_{AG} which includes the other aspartate/glutamate amino acid transporters.

**Tissue and Cellular Distribution of EAAT3.** Expression of EAAT3 has been observed in a variety of tissues including intestine (Kanai and Hediger, 1992; Erickson et
al., 1995; Howell et al., 2001; Fan et al., 2004), kidney (Kanai and Hediger, 1992; Howell et al., 2001), brain, neurons (Rothstein et al., 1994; Lehre et al., 1995), liver (Kanai and Hediger, 1992; Howell et al., 2001), pancreas (Howell et al., 2001), rumen (Howell et al., 2001), omasum (Howell et al., 2001), and heart (Kanai and Hediger, 1992).

The EAAT3 mRNA is differentially expressed along the longitudinal axis of the small intestine with highest quantities in the distal portions of the small intestine (Erickson et al., 1995; Rome et al., 2002; Iwanaga et al., 2005). In situ hybridization revealed that EAAT3 mRNA is also differentially expressed along the crypt-villus axis with highest quantities seen in the crypts and basal half of the intestinal villi (Iwanaga et al., 2005). The EAAT3 protein is localized to the brush-border membrane of the enterocyte (Iwanaga et al., 2005) and only found in differentiating enterocytes in the crypt of the intestinal villus (Rome et al., 2002). This villus location is independent of intestinal region. The localization of the EAAT3 transcript and protein suggests that the EAAT3 transporter and its substrates play a role in the growth and differentiation of the premature enterocyte.

**Substrates of EAAT3.** The intestinal EAAT3 transporter is highly specific and stereospecific for the anionic amino acids L-glutamate and D- or L-aspartate (Nicholson and McGivan, 1996; Castagna et al., 1997). Studies with neuronal and brain EAAT3 indicate that the transporter may also have a low affinity for cysteine (Zerangue and Kavanaugh, 1996; Chen and Swanson, 2003). Studies confirming cysteine transport by intestinal EAAT3 have not been conducted.
**Structure of EAAT3.** Sequence analysis indicates that EAAT3 has a primary structure of 524 amino acids in rabbit (Kanai and Hediger, 1992), human (GenBank Accession # P43005), and dog (Sato et al., 2001). Rat (Kanai et al., 1995a) and mouse (Tanaka, 1993) had primary EAAT3 sequences of 523 amino acids. There is some controversy over the membrane topology of EAAT3. The first model proposed that EAAT3 had 10 transmembrane domains with intracellular N- and C-termini (Kanai and Hediger, 1992). However, the large hydrophobic stretch of amino acids near the C-terminus makes other models plausible, such as a 12 transmembrane domain model.

The tertiary and quaternary structures of EAAT3 are not well understood. However, freeze fracture electron microscopy did reveal a pentameric 3-D structure of neuronal EAAT3 expressed in the plasma membrane of *Xenopus* oocytes (Eskandari et al., 2000). It remains unclear if the pentameric structure is made of subunits of a single EAAT3 protein or individual EAAT3 proteins forming a homopentamer.

**Transport Mechanism of EAAT3.** The EAAT3 mediated transport of glutamate and aspartate is coupled to the transport of 2 Na⁺ ions, either cotransport of 1 H⁺ ion or countertransport of 1 OH⁻ ion, and possibly the countertransport of 1 K⁺ ion (Kanai et al., 1995b). Kinetic studies indicate that transport of glutamate and aspartate by EAAT3 follows a 10 step ordered kinetic mechanism (Kanai et al., 1995b). First one Na⁺ ion binds to the extracellular face of EAAT3, which increases the affinity for glutamate. Glutamate (or aspartate) then binds followed by the binding of the second Na⁺ ion and translocation to the intracellular side of the membrane. The bound Na⁺ and glutamate (or aspartate) are released into the cytoplasm, then an OH⁻ ion binds followed by binding of K⁺. Relocation of the transporter to the extracellular side of the membrane occurs
followed by release of K<sup>+</sup> and OH<sup>-</sup>. The low intracellular Na<sup>+</sup> concentration and the high intracellular K<sup>+</sup> concentration is maintained by the basolateral Na<sup>+</sup> / K<sup>+</sup> ATPase (Hyde et al., 2003).

**Regulation of EAAT3.** The expression of EAAT3 is modulated by levels of dietary protein. A change from a low (4% casein) to high protein (50% gelatin) diet fed to rats caused a 2- to 3-fold increase in EAAT3 mRNA in the mid small intestine, while there was little change in the proximal and distal regions of the small intestine (Erickson et al., 1995). Further, in the absence of luminal nutrients, induced by total parenteral nutrition feeding, in rats caused an increase in ileal EAAT3 mRNA (Howard et al., 2004). These results suggest that the enterocytes have adaptation mechanisms to reduced luminal nutrients. The expression of EAAT3 mRNA is influenced by growth stage of the animal. A comparison between growing and non-growing lambs that were fed diets that had similar levels of metabolizable protein, found that the ileal epithelium of growing lambs contained approximately 313% more EAAT3 mRNA than non-growing lambs (Howell et al., 2003). The expression of EAAT3 mRNA was increased throughout the small intestine of rats from days 4 through 21 of age, indicating that the gene expression of this transporter is developmentally regulated (Rome et al., 2002). The second messenger phosphokinase C (PKC) is also involved in the regulation of EAAT3. In neural cell lines, phosphorylation of EAAT3 at serine residues by PKC caused a 2-fold increase in L-glutamate transport *in vivo* (Casado et al., 1993).

**Summary.** The intestinal EAAT3 transporter facilitates the assimilation of glutamate and aspartate, thus providing the enterocyte with its primary fuel. Expression of this transporter is highest in the rapidly growing and differentiating premature
enterocytes in the crypts of the intestinal villi indicating the importance of EAAT3 to these cells. Diet, growth, and second messengers may influence EAAT3 gene expression.
CHAPTER III.

OBJECTIVES

The gene expression of the nutrient transporters PepT1, EAAT3, SGLT1 and GLUT5 in the small intestine of lines of chickens divergently selected for high or low juvenile bodyweight was examined in this thesis. Gene expression was assayed using real-time PCR using the relative quantification method. The objective of the first experiment was to determine the effect of sex on the expression of these nutrient transporters in the small intestine of these divergently selected lines of chickens. The objective of the second experiment was to examine the correlated response to selection in the gene expression of these nutrient transporters in the small intestine of the high and low weight selected chicken lines and their reciprocal crosses.
CHAPTER IV.
MATERIALS AND METHODS

*Animals*

Pooled semen was used to inseminate females from the 48th generation of LWS and HWS to obtain HH (HWS X HWS) and LL (LWS X LWS) and their reciprocal crosses, LH (LWS males crossed with HWS females) and HL (HWS males crossed with LWS females). Eggs from the four mating combinations (MC) were incubated for 21 days. On day of hatch (DOH, without access to feed and water) chicks were wing banded for identification and placed in battery cages with 10-15 chicks per cage. Chicks had *ad libitum* access to a corn/soy mash diet that contained 20% crude protein and 2,685 kcal ME/kg. For complete diet formulation see Tables 1, 2, and 3. The LL and HL chicks were also offered feed on paper in addition to feeders for 7 days to enhance chick survival.

*Tissue Sampling*

All chicks were sampled on embryonic day 20 (E20), DOH, and days 3 (D3), 7 (D7), and 14 (D14) post hatch. On E20 12-16 eggs per MC were weighed and broken open. A portion of the liver was rinsed in ice cold phosphate buffered saline (1.47 mM NaH2PO4, 8.09 mM Na2HPO4, 145 mM NaCl) and frozen on dry ice. The small intestine was separated into duodenum (portion extending from the gizzard to end of duodenal loop), jejunum (portion from the end of the duodenal loop to Meckel’s diverticulum), and ileum (portion from Meckel’s diverticulum to the ileal-cecal junction). All segments were then rinsed in ice cold PBS and minced. One 20-30 mg tissue aliquot was placed in a 2-mL microfuge tube and frozen on dry ice, while the remaining tissue
was divided into \( \leq 1 \) g aliquots and flash frozen in liquid nitrogen. All samples were stored at -80°C.

On post hatch sampling days all chicks were weighed. Chicks sampled were killed by cervical dislocation and the liver and small intestine were subsequently collected using the same method employed for E20.

**Chick Sexing by PCR**

DNA was extracted from liver samples using the DNeasy kit according to the animal tissue protocol (Qiagen, Valencia, CA). DNA was quantified spectrophotometrically at OD 260 (U-2000, Hitachi North America). DNA was diluted to 0.05 µg/µL in TE buffer (10 mM Tris HCl, pH 7.5; 1 mM EDTA). The following 20 µL PCR reaction was set up: 12.7 µL sterile ultra-pure water, 2.0 µL 10X PCR Buffer B (Fisher Scientific, Pittsburgh, PA), 1.2 µL 25 mM MgCl₂ (Fisher Scientific), 0.4 µL 10 mM each dNTP’s, 0.4 µL each of 5 uM primers for a “W” chromosome specific gene (forward: 5’ CTGTGATAGAGACCGCTGTGC 3’, reverse: 5’ CAACGCTGACACTTCCGATGT 3’) (MWG, Charlotte, NC), 0.4 µL each of 5 µM primers for a 376 bp amplicon of the PepT1 gene (forward: 5’ TTGTCTCCCTGTCCATTGTCTATACT 3’, reverse: 5’ GTTCTTTCAAAACTGATCCCCACCCAAA 3’) (MWG), 0.1 µL of Taq DNA polymerase, 5 units/µL (Fisher Scientific), and 2.0 microliters of 0.05 µg/µL DNA. A PTC-200 Peltier thermocycler (MJ Research, Waltham, MA) was used to carry out the PCR reaction: 2 min at 94 °C followed by 35 cycles of denaturing (30 s at 94 °C), annealing (1 min at 55 °C), extension (1 min at 72 °C), and ending with a 7 min extension at 72 °C.
Following PCR, 10 µL of the PCR product and 10 µL of loading dye (0.2% bromophenol blue, (Sigma-Aldrich, St. Louis, MO), 0.2% xylene cyanole (Sigma-Aldrich) 80% glycerol (Sigma-Aldrich)) were mixed and run along with DNA standards (DNA Hi-Lo ladder; Fisher Scientific) on a 1% (w/v) agarose gel. Females were identified by the presence of two bands: a 376 bp amplicon of PepT1 and a 1200bp amplicon of the “W” chromosome, while males were identified by the presence of one band corresponding to the 376 bp amplicon of PepT1.

**Total RNA Extraction**

Total RNA from four males from all MC at all time points, except E20 HL (n = 3), and 4 females from HH and LL at all time points, except D7 LL (n = 2), was extracted using the RNeasy kit according to the animal tissue protocol (Qiagen). Briefly, the 20-30 mg aliquots of tissue were removed from storage at -80 °C and kept on dry ice until homogenization. Immediately before homogenization, 600 µL of the working RLT solution (2-mercaptoethanol (Sigma-Aldrich) diluted 1:100 in buffer RLT (Qiagen)) was added to the tissue aliquot. The tissue was homogenized using a 7 mm tip on a homogenizer (Ultra-Turrax T-25 Basic, Ika, Wilmington, NC) at a speed of 13,500 rpm for 20-30 s. The manufacturer’s protocol was then followed and the RNA was eluted by rinsing the column membrane twice with 30-40 uL of RNAse free water. The RNA purity was determined by evaluating the ratio of OD 260 to OD 280 in a spectrophotometer. The RNA concentration was calculated based on the OD 260 value.

The RNA quality was examined by gel electrophoresis. Two micrograms of total RNA were added to 20-25 µL of RNA sample buffer (0.75 mL deionized formamide (Sigma-Aldrich) 0.15 mL 10X MOPS (200mM MOPS, 50mM NaAc, 10mM EDTA, pH
7.0) 0.24 mL 37% formaldehyde (Sigma-Aldrich), 0.17 mL DEPC water (1 mL Diethylpyrocarbonate (Sigma-Aldrich) per liter ultra-pure water, autoclaved); 0.1 mL glycerol; 8 uL 10% (w/v) bromophenol blue for a final volume of 30 uL. Samples were incubated at 65 °C for 15 min then run out on a denaturing 1% agarose-formaldehyde gel (1X MOPS; 1% (w/v) agarose; 2.2 mol/L formaldehyde). The RNA was stored at -80 °C.

**Reverse Transcription**

Total RNA was diluted to 0.2 µg/µL in DEPC water. The reverse transcription was performed using the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). A 2X reverse transcription master mix was prepared containing, per reaction, 2 µL 10X reverse transcription buffer, 0.8 µL 25X dNTP’s, 2 uL 10X random primers, 1 µL Multiscribe reverse transcriptase (50U/µL), and 4.2 µL nuclease free ultra-pure water. The final reverse transcription reaction contained 10 µL of 0.2 µg/µL RNA and 10 µL of the 2X reverse transcription master mix. A thermocycler was used to perform the reverse transcription reaction for 10 min at 25 °C followed by extension (120 min at 37 °C). The cDNA was stored at -20 °C.

**Quantitative Real-Time PCR**

Quantitative real-time PCR (qPCR) was conducted using a 7300 Real-time PCR System (Applied Biosystems) in a 96-well plate format. Two microliters of cDNA diluted 1:30 in sterile ultra-pure water were added to each well. Then, 23 µL of a real time PCR master mix (Per reaction: 12.5 µL 2X SYBR Green Master Mix (Applied Biosystems); 0.5 µL each of a 5 µM forward primer and reverse primer; 9.5 µL sterile ultra-pure water) were added to each well. Then the following real time PCR reaction was run: 95 °C hold for 10 min followed by 40 cycles of 95 °C for 15s and 60 °C for 1
min. Genes analyzed were PepT1, EAAT3, GLUT5, and SGLT1. The endogenous control was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences were:

PepT1 forward (5’ CCCCTGAGGAGGATCACTGTT 3’)
PepT1 reverse (5’ CAAAAGAGCAGCAGCAACGA 3’)
EAAT 3 (forward 5’ TGCTGCTTTGGATTCCAGTG 3’)
EAAT 3 reverse (5’ AGCAATGACTGTAGTGCAGAAGTAATATATG 3’)
GLUT 5 forward (5’ TTGCTGGCTTTGGGTTGTG 3’)
GLUT 5 reverse (5’ GGAGGTTGAGGGCCAAAGTC 3’)
SGLT1 forward (5’ TGTCTCTCTGGCAAGAACATGTC 3’)
SGLT1 reverse (5’ GGGCAAGAGCTCAGGTATCC 3’)
GAPDH forward (5’ GCCGTCCTCTCTGGCAAAG 3’)
GAPDH reverse (5’ TGTAACCAGTCAGTCAGATCGATA 3’).

Primers were designed using the Primer Express software (Applied Biosystems) and synthesized by MWG. Each reaction was run in duplicate.

Two independent real-time PCR experiments were conducted using the same cDNA samples. The cDNA from the male and female HH and LL chicks at all ages were used for the first experiment (results described in Chapter V). The cDNA from the male LL, LH, HL, and HH were used for the second experiment (results described in Chapter VI).

**Quantitative Real-Time PCR Analysis**

All plates were analyzed individually using the software provided with the 7300 Real-Time PCR machine using the Auto function. Average gene expression relative to
the endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The calibrator for each gene in both experiments was the average ΔCt value of male E20 LL duodenum.

**Statistical Analysis.**

Data were analyzed using the PROC MIXED procedure of SAS (1996, Cary, NC) including the main effects of body weight, MC, age, sex, intestinal segment and all appropriate two and three way interactions. Segment, line, and sex differences, as well as two way interactions were further evaluated using linear contrasts. The main effects of age were tested for linear, quadratic, and cubic responses using orthogonal contrasts in the MIXED procedure.
Table 1. Minimum and Maximum Nutrient and Ingredient Content of the Chicken Starter Diet for the LWS and HWS.

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<thead>
<tr>
<th>Nutrients</th>
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<tr>
<td>Metabolizable energy</td>
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</tr>
<tr>
<td>Crude protein</td>
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</tr>
<tr>
<td>Crude fat</td>
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</tr>
<tr>
<td>Crude fiber (Max.)</td>
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</tr>
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<tr>
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<tr>
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<tr>
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CHAPTER V.
SEXUAL DIMORPHISM IN THE DEVELOPMENTAL GENE EXPRESSION OF NUTRIENT TRANSPORTERS IN THE SMALL INTESTINE OF CHICKENS FROM LINES DIVERGENTLY SELECTED FOR HIGH OR LOW BODY WEIGHT.

RESULTS

Egg and Body Weight

Eggs from HH were heavier than eggs from LL ($P = 0.0004$), with no difference between males and females in either mating combination, with HH eggs averaging $34.1 \pm 1.0$ g and LL eggs averaging $27.0 \pm 1.0$ g. Chicks from HH were heavier throughout the experiment and grew faster than chicks from LL ($P < 0.0001$), with no difference between males and females in either mating combination (Figure 3). By D14 there is about a 4.5-fold difference in body weight between the mating combinations.

Gene Expression of PepT1

Overall, the LL had about 6-fold greater PepT1 gene expression than the HH ($P < 0.0001$). Although there was no difference in PepT1 gene expression between the segments in HH, expression in LL was greatest in the ileum, intermediate in the jejunum, and least in the duodenum ($P < 0.05$) (Figure 4). There was a MC by age by sex interaction for PepT1 gene expression ($P < 0.0001$) (Figure 5). Females had peak PepT1 gene expression 7 d earlier than males for both LL and HH. For LL females peak expression occurred on DOH, while in HH females the peak expression occurred on D7. For LL males peak expression occurred on D7, while in HH males the peak expression occurred on D14. This line difference was similar in males with LL males peaking on D7 and HH males peaking on D14.
**Gene Expression of EAAT3**

Overall, females had approximately 2-fold greater EAAT3 gene expression than the males ($P < 0.03$) (Figure 6). Expression of EAAT3 was greatest in the ileum, intermediate in jejunum, and least in the duodenum regardless of sex or MC ($P < 0.0007$) (Figure 7). There was an age by segment interaction ($P = 0.0002$) (Figure 8). Expression of EAAT3 in the duodenum increased slightly on or before DOH and remained at this level through D14. In the jejunum and ileum, gene expression of EAAT3 was induced on DOH and decreased by D14. There was a MC by intestinal segment interaction in which there was no difference between MCs in the duodenum or jejunum, but in the ileum LL had greatest EAAT3 gene expression ($P < 0.02$) (Figure 9).

**Gene Expression of SGLT1**

Females had approximately 2-fold greater SGLT1 gene expression than the males ($P < 0.0001$) (Figure 10). There was a sex by age interaction in gene expression of SGLT1 ($P < 0.0001$) (Figure 11). Regardless of MC, females induced SGLT1 gene expression on DOH and maintained this level through D14. In contrast, males gradually increased SGLT1 gene expression through D7 and then decreased expression by D14.
Figure 3. Body Weights of Sampled Male and Female HH and LL Chicks. The average body weight ± SEM (g) of the sampled male and female HH (HWS X HWS) and LL (LWS X LWS) chicks is shown for day of hatch (no access to feed) and days 3 (D3), 7 (D7), and 14 (D14) post hatch (n = 4, except female LL D7, n = 2). Chicks from HH were heavier throughout the experiment and grew faster than chicks from LL ($P < 0.0001$), with no difference between males and females in either mating combination. By D14 there is about a 4.5-fold difference in body weight between the mating combinations.
Figure 4. Mating Combination by Intestinal Segment Interaction for PepT1 Gene Expression in LL and HH Male and Female Chicks. There was a mating combination by segment interaction for PepT1 gene expression in male and female HH (HWS X HWS) and LL (LWS X LWS) chicks (n = 40 except for LL, n = 38). Relative gene expression (2^{−\Delta\Delta Ct}) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. Expression in LL was greatest in the ileum, intermediate in the jejunum, and least in the duodenum (P < 0.05).
Figure 5. Mating Combination by Age by Sex Interaction for PepT1 Gene Expression in LL and HH Male and Female Chicks. There was a line by age by sex interaction for PepT1 gene expression in male and female LL (LWS X LWS) (A) and HH (HWS X HWS) (B) chicks ($P < 0.0001$) ($n = 4$, except for D7 LL F, $n = 2$). Relative gene expression ($2^{\Delta\Delta Ct}$) ± SEM was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. Overall, the LL had about 6-fold greater PepT1 gene expression than the HH ($P < 0.0001$).
Figure 6. Effect of Sex on EAAT3 Gene Expression in LL and HH Male and Female Chicks. There was an overall effect of sex for EAAT3 gene expression in male and female HH (HWS X HWS) and LL (LWS X LWS) chicks ($P < 0.03$) ($n = 60$ except female, $n = 54$). Relative gene expression ($2^{-\Delta\Delta Ct} \pm$ SEM) was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator.
**Figure 7. Effect of Intestinal Segment on EAAT3 Gene Expression in LL and HH Male and Female Chicks.** There was a main effect of intestinal segment for EAAT3 gene expression in male and female HH (HWS X HWS) and LL (LWS X LWS) chicks (*n* = 80). Relative gene expression ($2^{\Delta\Delta Ct}$) ± SEM was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. Expression of EAAT3 was greatest in the ileum, intermediate in jejunum, and least in the duodenum regardless of sex or mating combination (*P* < 0.0007).
There was an age by segment interaction for EAAT3 gene expression in male and female HH (HWS X HWS) and LL (LWS X LWS) chicks ($P = 0.0002$) (n = 16 except for D7, n = 14). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator.
Figure 9. Mating combination by Intestinal Segment Interaction for EAAT3 Gene Expression in LL and HH Male and Female Chicks. There was a mating combination by intestinal segment interaction for EAAT3 gene expression in male and female HH (HWS X HWS) and LL (LWS X LWS) chicks (n = 40 except for LL, n = 38). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. There was no difference between the mating combinations in the duodenum or jejunum, but in the ileum LL had the greatest EAAT3 gene expression ($P < 0.02$).
Figure 10. Effect of Sex on SGLT1 Gene Expression in LL and HH Male and Female Chicks. There was an overall effect of sex for SGLT1 gene expression in male and female HH (HWS X HWS) and LL (LWS X LWS) chicks ($P < 0.0001$) (n = 120 except female, n = 114). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator.
Figure 11. Age by Sex Interaction for SGLT1 Gene Expression in LL and HH Male and Female Chicks. There was an age by sex interaction for SGLT1 gene expression in male and female HH (HWS X HWS) and LL (LWS X LWS) chicks ($P < 0.0001$) ($n = 24$ except for female D7, $n = 18$). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator.
DISCUSSION

There was a difference in the time of peak expression between LL and HH birds. In the LL, PepT1 gene expression was induced on DOH in both sexes, with DOH being the peak expression for the females. This peak in PepT1 gene expression is similar to previous studies in chickens (Chen et al., 2005; Gilbert et al., 2007b). However, no increase in PepT1 gene expression was seen in HH until D7 in females and D14 in males. One possible reason for this difference is that selection for a high juvenile body weight has delayed induction of PepT1 because it is not needed by HH for maximal nitrogen absorption in early life due to the overall greater feed intake and feed efficiency (O’Sullivan et al., 1992) of the line.

There was a difference in nutrient transporter expression between males and females in chickens. Currently there has been no published data suggesting there is a difference between males and females of any species in the developmental gene expression of PepT1, EAAT3 and SGLT1 in the small intestine. Females have an earlier peak in PepT1 gene expression. In this study we showed a 7 day difference in peak expression between males and females in each line. Females are known to be more metabolically efficient (Frisch, 2002) in an effort to prepare for reproduction. Earlier expression of PepT1 may be reflective of this improved metabolic efficiency. Lu and Klassen (2006) reported that there was no difference in the level of PepT1 gene expression between males and females in the small intestine of rats and mice. This study used 8-week-old rats and analyzed PepT1 expression at a single time point. Because of the experimental design, this study may have not detected a difference in PepT1 gene expression between males and females simply because the rodents were too old.
Females of both lines had a higher overall SGLT1 gene expression and an earlier induction of SGLT1 gene expression compared to males. It has been observed that female turkeys and broiler type chickens have an average of a 7.7% less energy requirement than male turkeys or broiler type chickens (Shalev and Pasternak, 1998). If greater SGLT1 gene expression in females ultimately correlates with greater glucose absorption, then the earlier and greater SGLT1 gene expression in female chickens seen in this thesis may begin to explain, at the molecular level, a reason for the difference in energy requirements between male and female chickens and turkeys.

The up-regulation and earlier expression of SGLT1 in females may also be a reflection of the need for females to be more metabolically efficient in order to reach the minimum body weight and fat percent needed to achieve sexual maturity (Frisch, 2002) at the same time that the males become sexually mature. A positive energy balance is needed to shift energy expenditures from growth to reproduction (i.e., increased fat deposition). Therefore having a greater capacity to assimilate glucose, the body’s primary fuel, early would facilitate the generation of energy stores and thus a positive energy balance earlier in life.

It has been reported that renal SGLT1 gene expression, protein expression and transport capacity is greater in both intact and ovarectomized female rats than intact or castrated male rats (Sabolic et al., 2006). Further, castrated male rats had greater renal SGLT1 gene expression, protein expression and transport capacity than intact male rats, indicating a negative effect of androgens. There was no difference in renal SGLT1 gene expression between the intact and ovarectomized females, indicating that progesterone and estrogen did not affect renal SGLT1 expression. The rat SGLT1 gene sequence has
two androgen response elements (Sabolic et al., 2006). If the chicken also has an
androgen response element in the SGLT1 gene, it is possible that androgens in the
chicken could cause a down-regulation of the SGLT1 gene in all tissues where it is
expressed, in particular the small intestine.

Females had greater expression of EAAT3. Glutamate is the primary fuel source
for enterocytes. In order to facilitate greater gene expression of nutrient transporters,
such as SGLT1 and PepT1 and possibly growth and development of the small intestine,
females may upregulate the gene expression of EAAT3, which may lead to increased
protein expression and increased glutamate/aspartate assimilation.

This study indicates that expression of nutrient transporters in males and females
is not the same during the first 2 weeks post hatch and might have implications for the
poultry industry. Altering the nutritional guidelines to compensate for these differences
may allow for a larger or faster maturing female bird which would allow for an increase
in straight run broiler flock size at market and sooner onset to lay in the breeders and
layers in the egg industry.
CHAPTER VI.
DEVELOPMENTAL GENE EXPRESSION OF NUTRIENT TRANSPORTERS IN THE SMALL INTESTINE OF MALE CHICKENS FROM LINES DIVERGENTLY SELECTED FOR HIGH OR LOW JUVENILE BODY WEIGHT AND THEIR RECIPROCAL CROSSES.

RESULTS

Egg and Body Weight

Sampled eggs with male embryos out of LWS dams (HL and LL eggs) at E20 weighed less than sampled eggs with male embryos out of HWS dams (HL and LL eggs) ($P < 0.0001$). Sampled eggs with male embryos out of LWS dams at E20 weighed 27.65 ± 1.6 g, while sampled eggs with male embryos out of HWS dams (LH and HH eggs) at E20 weighed 36.0 ± 1.5 g. The average body weights of the male sampled chicks of the HH, LL, HL, and LH are shown in Figure 12. There was an age by mating combination interaction ($P < 0.0001$). Chicks from HH were heaviest and grew fastest, while LL chicks were lightest and grew the slowest. The body weight on DOH of reciprocal crosses reflected the body weight of the dam line, however by D14 the HL and LH had body weights that were intermediate of the HH and LL ($P < 0.0001$). On D14 there was a 5.4 fold difference in body weight between the HH and LL mating combinations.

Gene Expression of PepT1

Overall, LL had greatest level ($P < 0.06$) of PepT1 gene expression while HH had the least level ($P < 0.006$) of PepT1 gene expression (Figure 13). The reciprocal crosses had intermediate levels of gene expression ($P < 0.06$) and were not different from each other (Figure 13). Greatest gene expression of PepT1 was seen in the ileum ($P < 0.0003$) (Figure 14). Gene expression of PepT1 increased from duodenum to ileum in LL, with no segment difference in any other MC ($P < 0.08$). Within each segment there
was a MC difference ($P < 0.02$). In the duodenum and jejunum, HH had the least PepT1 gene expression, while the LH, HL, and LL had the greatest gene expression ($P < 0.02$). In the ileum, LL had greatest gene expression, LH and HL had an intermediate level of gene expression, and the HH had the least gene expression ($P < 0.003$). There was also an effect of sire on PepT1 gene expression ($P = 0.0008$), with chicks from LWS sires (LL and LH) having greater expression than chicks from HWS (HH and HL) sires (Figure 16). There was no difference between intestinal segments in progeny from HWS sires, however, greatest PepT1 gene expression was seen in the ileum of progeny from LWS sires ($P < 0.0001$) (Figure 17).

**Gene Expression of EAAT3**

In this study expression of EAAT3 was greatest in the ileum, intermediate in the jejunum, and least in the duodenum ($P < 0.0001$), which was similar to that seen in the male and female LL and HH chicks (Figure 7). There was an intestinal segment by age interaction ($P < 0.0001$), which was similar to the pattern of expression seen in the male and female LL and HH chicks (Figure 8). In all MCs except HH, EAAT3 gene expression increased from duodenum to ileum ($P < 0.08$) (Figure 18). In the HH greatest gene expression was in the ileum ($P < 0.08$) with no difference between the duodenum and jejunum (Figure 18). Within the ileum, LL had greatest EAAT3 gene expression, LH and HL had intermediate gene expression, and HH had least expression ($P < 0.08$) (Figure 18).

**Gene Expression of SGLT1**

The expression of SGLT1 in this study was developmentally regulated with an increase in expression through D7 and then a decrease by D14 ($P < 0.0001$) (Figure 19).
Overall, greatest expression of SGLT1 was in the distal (jejenum, ileum) small intestine ($P < 0.0001$) (Figure 20). Gene expression of SGLT1 was greatest in the distal small intestine in LL, LH, and HL, but greatest in the jejunum of HH ($P < 0.04$) (Figure 21). Within the ileum, LL had greater SGLT1 gene expression than HH ($P < 0.06$) (Figure 21).

**Gene Expression of GLUT5**

Greatest gene expression of GLUT5 was in the distal small intestine ($P < 0.0001$) (Figure 22). In the jejunum, LL had greater gene expression than LH and HL ($P < 0.07$) (Figure 23). In the ileum, LL had greater gene expression than HH and HL ($P < 0.03$) and LH had greater gene expression than HH ($P < 0.07$) (Figure 23). Expression of GLUT5 was greatest in the distal small intestine in LL and HL ($P < 0.02$), greatest in the ileum of LH ($P < 0.08$), and greatest in the jejunum of HH ($P < 0.09$) (Figure 23).

**Mating Combination Effect in the Ileum**

In all genes, LL had greater expression than HH ($P < 0.0001$ for PepT1, $P < 0.0001$ for EAAT3, $P < 0.06$ for SGLT1, and $P = 0.001$ for GLUT5) (Figure 24). The LH and HL had intermediate levels of gene expression for PepT1 ($P < 0.003$) and EAAT3 ($P < 0.07$). For SGLT1 and GLUT5 the LH and HL had numerically intermediate levels of gene expression.
Figure 12. Body Weights of the Sampled Male LL, LH, HL, and HH Chicks.
The average body weight ± SEM (g) of the sampled male HH (HWS X HWS) LH (LWS X HWS), HL (HWS X LWS) and LL (LWS X LWS) chicks is shown for day of hatch (no access to feed) and days 3 (D3), 7 (D7), and 14 (D14) post hatch (n = 4). There was an age by mating combination interaction ($P < 0.0001$). The body weight on DOH of reciprocal crosses reflected the body weight of the dam line, however, by D14 the HL and LH had body weights that were intermediate of the HH an LL ($P < 0.0001$).
Figure 13. Effect of Mating Combination on PepT1 Gene Expression in Male LL, LH, HL, and HH Chicks. There was a main effect of mating combination for PepT1 gene expression of PepT1 in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 60 except HL, n = 57). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. The LL had the greatest gene expression ($P < 0.06$), LH and HL had intermediate gene expression ($P < 0.06$), and HH had the least gene expression ($P < 0.006$).
Figure 14. Effect of Intestinal Segment on PepT1 Gene Expression in Male LL, LH, HL, and HH Chicks. There was a main effect of intestinal segment on PepT1 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 79). Relative gene expression (2^{-\Delta\Delta Ct}) \pm SEM was calculated using the \Delta\Delta Ct method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. Highest expression is in the ileum (P < 0.0003).
Figure 15. Intestinal Segment by Mating Combination Interaction for PepT1 Gene Expression in Male LL, LH, HL, and HH Chicks. There was a mating combination (MC) by intestinal segment interaction for PepT1 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 20 except HL, n = 19). Relative gene expression ($2^{\Delta\Delta C_t}$ ± SEM) was calculated using the $\Delta\Delta C_t$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. Gene expression increased from duodenum to ileum in LL but there was no segment difference in any other MC ($P < 0.08$). Within each segment there was a MC difference ($P < 0.02$). In the duodenum and jejunum, HH had the least PepT1 gene expression, while the LH, HL, and LL had the greatest gene expression ($P < 0.02$). In the ileum, LL had greatest gene expression, LH and HL had an intermediate level of gene expression, and the HH had the least gene expression ($P < 0.003$).
Figure 16. Effect of Sire on PepT1 Gene Expression in Male Progeny from LWS and HWS Sires. There was an effect of sire \((P = 0.0008)\) on PepT1 gene expression in male progeny from low weight selected (LWS) sires (LWS x LWS (LL) and LWS x HWS (LH)) and male progeny from high weight selected (HWS) sires (HWS x HWS (HH) and HWS x LWS (HL)) \((n = 120\) except for “HWS” sire, \(n = 117\)). Relative gene expression \((2^{\Delta\Delta Ct}) \pm SEM\) was calculated using the \(\Delta\Delta Ct\) method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator.
Figure 17. Sire Line by Intestinal Segment Interaction for PepT1 Gene Expression in Male Progeny from LWS and HWS Sires. There was a sire by intestinal segment interaction for PepT1 gene expression in male progeny from low weight selected (LWS) sires (LWS x LWS (LL) and LWS x HWS (LH)) and male progeny from high weight selected (HWS) sires (HWS x HWS (HH) and HWS x LWS (HL)) (n = 40 except for progeny from HWS sires, n = 39). Relative gene expression (2^{-\Delta\Delta Ct}) \pm SEM was calculated using the \Delta\Delta Ct method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. There was no difference between intestinal segments in progeny from HWS sires, however greatest PepT1 gene expression was seen in the ileum of progeny from LWS sires (P < 0.0001).
Figure 18. Mating Combination by Intestinal Segment Interaction of EAAT3 Gene Expression in Male LL, LH, HL, and HH Chicks. There was a mating combination by intestinal segment interaction for EAAT3 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 20 except for HL n = 19). Relative gene expression (2^{-\Delta\Delta Ct}) ± SEM was calculated using the \Delta\Delta Ct method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. In all mating combinations except HH, EAAT3 gene expression increased from duodenum to ileum (P < 0.08). In the HH greatest gene expression was in the ileum (P < 0.08) with no difference between the duodenum and jejunum. Within the ileum, LL had greatest EAAT3 gene expression, LH and HL had intermediate gene expression, and HH had least expression (P < 0.08).
Figure 19. Developmental Gene Expression of SGLT1 in Male LL, LH, HL, and HH Chicks. Expression of SGLT1 was developmentally regulated ($P < 0.0001$) in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks on embryonic day 20 (E20) day of hatch (DOH), and days 3 (D3), 7 (D7), and 14 (D14) post hatch (n = 48 except E20, n = 45). Relative gene expression ($2^{-\Delta\Delta\text{Ct}}$ ± SEM of SGLT1 was calculated using the $\Delta\Delta\text{Ct}$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator.
Figure 20. Main Effect of Intestinal Segment on SGLT1 Gene Expression in Male LL, LH, HL, and HH Chicks. There was a main effect of segment on SGLT1 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 79). Relative gene expression ($2^{-\Delta \Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. Overall, highest expression of SGLT1 was in the distal (jejunum, ileum) small intestine ($P < 0.0001$).
There was a mating combination by segment interaction for SGLT1 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 20 except for HL, n = 19). Relative gene expression ($2^{\Delta\Delta C_t}$) ± SEM was calculated using the $\Delta\Delta C_t$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. The gene expression of SGLT1 was greatest in the distal small intestine in LL, LH, and HL, but greatest in the jejunum of HH ($P < 0.04$). Within the ileum, LL had greater SGLT1 gene expression than HH ($P < 0.06$).
Figure 22. Effect of Intestinal Segment on GLUT5 Gene Expression in Male LL, LH, HL, and HH Chicks. There was an effect of segment on GLUT5 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 79). Relative gene expression (2^{-ΔΔCt}) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. Gene expression was greatest in the distal small intestine (P <0.0001).
Figure 23. Mating Combination by Intestinal Segment Interaction of GLUT5 Gene Expression in Male LL, LH, HL, HH Chicks. There was a mating combination by intestinal segment interaction for GLUT5 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 20 except for HL, n = 19). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the $\Delta\Delta Ct$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. In the jejunum LL had greater gene expression than LH and HL ($P < 0.07$). In the ileum, LL had greater gene expression than HH and HL ($P < 0.03$) and LH had greater gene expression than HH ($P < 0.07$). Expression of GLUT5 was greatest in the distal small intestine in LL and HL ($P < 0.02$), greatest in the ileum of LH ($P < 0.08$), and greatest in the jejunum of HH ($P < 0.09$).
Figure 24. Mating Combination Effect in the Ileum in the Expression of PepT1, EAAT3, SGLT1, and GLUT5 in Male LL, LH, HL, and HH Chicks. There was a mating combination effect in the ileum for PepT1, EAAT3, SGLT1, and GLUT5 gene expression in male LL (LWS X LWS), LH (LWS X LWS), HL (HWS X LWS), and HH (HWS X HWS) chicks (n = 20 except for HL, n = 19). Relative gene expression ($2^{\Delta\Delta C_t}$) ± SEM was calculated using the $\Delta\Delta C_t$ method with GAPDH as the endogenous control and the average Ct value for E20 LL male duodenum as the calibrator. In all genes, LL had greater expression than HH ($P < 0.0001$ for PepT1, $P < 0.0001$ for EAAT3, $P < 0.06$ for SGLT1, and $P = 0.001$ for GLUT5). The LH and HL had intermediate levels of gene expression for PepT1 ($P < 0.003$) and EAAT3 ($P < 0.07$).
DISCUSSION

In this study there was a difference in the expression of the PepT1 transporter gene between LL and HH, with LL having overall greater gene expression with greatest levels in the distal small intestine. In interpreting these results, differences in food intake between the mating combinations must be considered. The LL chicks exhibit hypophagia whereas HH chicks exhibit hyperphagia. This hypophagia in LL leads to a suboptimal nutrient intake, which can affect the growth of these chicks. The pattern of increased PepT1 gene expression seen in LL is comparable to PepT1 expression observed in starvation and reduced feed intake studies conducted in rats (Ihara et al., 2000; Naruhasi, et al., 2002; Howard et al., 2004). Ihara et al. (2000) reported an increase in both mRNA and protein expression of PepT1 in response to starvation, reduced feed intake and total parenteral nutrition treatment. Naruhasi et al. (2002) and Howard et al. (2004) observed in starved rats that PepT1 mRNA expression increased, with highest levels in the distal small intestine. Naruhasi et al. (2002) demonstrated that activity levels, as measured by cefadroxil transport, were highest in the proximal intestine and, despite higher PepT1 gene expression levels, lowest in the mid and distal intestine. They suggested that the increase in distal PepT1 gene expression was a compensatory mechanism used to counteract the increased pH of the distal small intestine, which hinders PepT1 activity by affecting the proton gradient. They concluded that the increased distal PepT1 gene expression would allow the nitrogen starved animal to maximize its nitrogen absorption.

It is important to realize, however, that there exists an unstirred water layer surrounding the luminal side of the brush border membrane that has many functions including pH maintenance (Shiau et al., 1985). Therefore, the pH of the luminal content
does not necessarily reflect the pH of the microenvironment surrounding the transporters. Mucins make up part of the unstirred water layer (Smithson et al. 1981). In the chicken, the mucin barrier is uniform throughout the length of the small intestine and starvation has been shown to change the composition of the barrier and decrease its thickness, with the greatest depletion seen in the ileum (Smirnov et al., 2004). If this barrier is responsible in part for maintaining the unstirred water layer and thus pH of the environment surrounding PepT1, mucin barrier depletion may alter the activity of the transporter through disruption of the environment that normally allows maximal activity. Thus, an increase in gene expression of PepT1 in the distal portion of the small intestine may be a mechanism to allow maximal nitrogen absorption by compensating for the reduced transport activity due to the increase in pH.

Recently, Shimakura et al. (2006) concluded that the increase in PepT1 gene expression and protein levels in response to fasting was mediated by the peroxisome proliferator-activated receptor α (PPARα). The PPARα is a nuclear hormone receptor that plays a vital role in the adaptive response to starvation in the liver as well as other tissues. Its function is to aid in regulating fatty acid metabolism, which helps the body switch from metabolizing carbohydrates and fats in the fed state to only fat in the starved state (Kersten et al., 1999). The finding that regulation of PepT1 expression, in particular in the fasted state, by PPARα provides more evidence that upregulation of PepT1 gene expression is an adaptive response to suboptimal feed intake.

The induction of PepT1 gene expression in LL, which was absent in the HH, prior to feed intake suggests that genetic selection has altered the regulatory factors for PepT1 in response to the correlated responses in feed intake in these lines. The induction in LL
as well as overall greater PepT1 gene expression may be necessary to maximize nitrogen assimilation for survival of these chicks.

Expression of the EAAT 3 gene was highest in the ileum, which supports the findings of Iwanaga et al. (2005) and Gilbert et al. (2007b). They also found greatest levels of EAAT 3 gene expression as well as protein in the ileum and concluded that most glutamate transport occurs in the ileum. That EAAT 3 gene expression was greater in the LL ileum than the HH ileum is consistent with the findings of Howard et al. (2004) of an increase in ileal gene expression of EAAT 3 in starved rats. Therefore, the mating combination by segment interaction is biologically significant with respect to adaptations to reduced feed intake. Like the mating combination difference seen in PepT1, this difference in EAAT 3 gene expression may be an adaptive response to a reduced feed intake in LL, thus maximizing assimilation of glutamate, which is the primary fuel for the enterocytes (Matthews, 2000).

There was a linear increase in GLUT5 gene expression despite a lack of fructose in the feed. The increase in GLUT5 expression may indicate that this expression is a genetically “hard wired” event that would maximize nutrient absorption in wild birds, which would have access to fructose containing foods such as fruit. This demonstrates that although the domesticated chicken has undergone intense selection pressures, there still remains genetically hard wired events which are a throw back to its ancestor, the red jungle fowl. As GLUT5 has a low efficiency of glucose transport (Matthews, 2000), increasing gene expression of the transporter may ultimately allow for more GLUT5 protein to be made and thus maximize total glucose transport. It is also possible that sugars, such as sucrose, in the soybean and corn as well as other ingredients may be
broken down into glucose as well as fructose by enzymes, such as sucrase. Fructose would then cause the up-regulation of GLUT 5 similar to what is seen in mice and rats (Ferraris, 2001).

These results reveal differences in expression of nutrient transporters between chickens that have been selected for high or low juvenile body weight. It is important to note, however, that only mRNA levels were examined and not protein levels. Further investigation into the transporter proteins and their functionality would be valuable to understand the nutrient absorption capacity of these selected lines.
CHAPTER VI. EPILOGUE

My results indicate that differences exist between males and females in the gene expression of the nutrient transporters PepT1, EAAT3 and SGLT1 in the small intestine of chickens. These differences observed may be a reflection of a need for females to be more metabolically efficient to help facilitate reproduction later in life. The results from this thesis may also begin to help understand the reason for difference between male and female broiler chickens and turkeys with respect to energy requirement, at the molecular level.

Further investigation into the sexual dimorphism in the gene expression, protein expression, and transport capacities during development and beyond of nutrient transporters in chickens and other species is warranted. It is beginning to be understood that nutrient requirements are different between males and females, although most of these observations have been done in humans. A better understanding of differences that may exist in poultry and other livestock species may ultimately allow for more efficient feeding of these animals and thus a cheaper food for human consumption.

In these mating combinations of chickens it has been well documented that LL chickens are less efficient in feed and energy utilization than HH chickens with the differences associated with several factors including changes in oxygen consumption, feed passage rate, and intestinal glucose absorption capacity and temperature regulation (Dunnington and Siegel, 1996). The increase in PepT1 gene expression may be an adaptation of the LL line to compensate for inefficient nitrogen absorption. There are many possible causes for inefficient peptide absorption. There may be a reduction in PepT1 transporter numbers due to post translational modifications or the transporters
present have a reduced activity. Also, the enzymes responsible for the formation of di- and tri-peptides may not be functioning properly and therefore cause a reduction in PepT1 substrate, which in turn leads to an increase in PepT1 transporter expression in order to maximize nitrogen absorption.

Although there is a maternal effect, as demonstrated by egg size, between LL and HH chickens, there was a sire effect in the gene expression of PepT1. Although the reason for the up regulation in the progeny from “L” sires is not known, it may be an adaptive response to maximize the survival of both sexes as both males and females inherit a copy of the “Z” chromosome from the sire. The PepT1 gene is located on chicken chromosome 1 and it is possible that LL males contain a modified gene on the Z chromosome that is involved in upregulating PepT1 gene expression.

There was an interesting trend for the expression of all genes examined to be influenced by mating combination in the ileum. The LL had highest gene expression followed by LH, then HL, and HH. This additive genetic effect may point to a correlated response to selection in these mating combinations with respect to the ability of the small intestine, in particular, the ileum to upregulate the gene expression of nutrient transporters. In particular there may be a gene in the LL that causes up-regulation of these genes or conversely a gene in HH that causes down-regulation of these genes. If the former is true, greatest gene expression of these nutrient transporters is seen in LL chicks because they have two copies of this “up-regulating” gene, intermediate expression is seen in LH and HL chicks because they have one copy of this “up-regulating” gene and HH chicks would have the least expression because they do not
have any copies of this “up-regulating” gene. The reverse would hold true of the HH had a gene that caused a down-regulation of these nutrient transporter genes.

It is also possible that the MC effect seen in the ileum is an adaptation to food intake and thus nutrient availability. The ileum is the last segment of the small intestine in chickens, therefore it is the last place where any appreciable nutrient absorption can take place, chickens do not possess much of a large intestine. Up-regulation of nutrient transporters gene expression in the ileum may be a mechanism to maximize nutrient absorption in situations of reduced nutrient availability (i.e. the LL chicks).

These results suggest that long term divergent selection for high or low juvenile body weight has impacted, either directly or indirectly, the gene expression of PepT1, EAAT3, SGLT1, and GLUT5. Further investigation to separate out the effect of food intake and the effect of long term genetic selection for high or low juvenile body weight on gene expression of these nutrient transporters should be considered. Protein expression and transport capacity of these nutrient transporters in these selected lines of chickens should also be examined to evaluate the importance of these changes in gene expression of these transporters with respect to overall nutrient absorption capacity of the selected lines.
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