ONTOGENESIS OF PEPTIDE TRANSPORT AND MORPHOLOGICAL CHANGES IN THE OVINE GASTROINTESTINAL TRACT

by

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ABSTRACT

Nutrient absorption is important in all stages of life. As the diet of an animal changes from birth on, morphological and biochemical adaptation can be anticipated in order to accommodate changing demands. The main focus of the present study was to examine the relationship between age and diet on the potential for peptide transport via PepT1 in the gastrointestinal tract of lambs and to relate changes of peptide transport capability to morphological changes. A 2x4 factorial arrangement of treatments was used with 32 crossbred lambs. Four blocks were created based upon gender, birth type (single or twin), birth weight, and birth date. Lambs were randomly allotted at birth to receive or not to receive a creep diet. All lambs were allowed to nurse. Sampling times of 2, 4, 6, or 8 wk were randomly allotted to lambs. Samples for RNA extraction and histological evaluation were taken from the dorsal rumen, ventral rumen, omasum, duodenum, jejunum, and ileum. Villi were about 7% shorter ($P < 0.09$) in lambs receiving creep feed. Papillary height and width increased linearly ($P < 0.001$ and $P < 0.0001$, respectively) with age. Total and keratinized epithelial cells in the stomach decreased ($P < 0.03$ and $P < 0.004$, respectively) with age and were fewer ($P < 0.0002$ and $P < 0.0001$, respectively) in lambs receiving creep feed. Creep feeding appears to have slightly altered the mucosal structure of the small intestine and it was advantageous in that it

stimulated papillary growth and thus predisposed the rumen for the introduction of feed into the diet. A 2.8 kb oPepT1 mRNA was present in all tissues studied by 2 wk, and age did not significantly influence the abundance of oPepT1 mRNA in the small intestine or stomach. In the small intestine, abundance of oPepT1 mRNA was greatest ($P < 0.0007$) in the jejunum. In the stomach, abundance of oPepT1 mRNA was greatest ($P < 0.01$) in the dorsal rumen. In the stomach, particularly in the rumen, a greater abundance of oPepT1 mRNA was observed in lambs not receiving the creep diet. It seems likely that a stimulus for development is coming from the non-luminal direction, possibly blood-borne, and may be involved in the ontogenesis of oPepT1. Peptide transport appears to be a physiologically important process in the young lamb and the rumen appears to be involved in the transport of peptides, particularly in nursing lambs.

Key Words: Ovine, Peptide, Transport, Ontogenesis, mRNA, PepT1

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"Celebrate we will because life is short but sweet for certain." –Dave Matthews Band

TABLE OF CONTENTS

Abstract ii

Acknowledgments iv

List of Tables viii

List of Appendix Tables ix

List of Figures x

Chapter I. Introduction 1

Chapter II. Literature Review 4
Cloning of Mammalian Peptide Transporters 4
Tissue Distribution of PepT1 6
Ruminal Histology and Papillary Development 8
Omasal Histology and Laminae Development 9
Small Intestinal Histology and Mucosal Development 10
Ontogenesis of Nutrient Transport 12
Dietary Regulation 16

Chapter III. Ontogenesis of Peptide Transport and Morphological Changes in the Ovine Gastrointestinal Tract 20
Abstract 20
Introduction 22
Materials and Methods 23
Materials 23
Experimental Design 23
Animal Management and Diets 24
Tissue Sampling 24
Morphometric Measurements 25
Preparation of Total RNA 26
Abundance of oPepT1 mRNA 27
Statistical Analysis 28
Results 28
Lamb Growth 28
Morphometric Measurements 28
Abundance of oPepT1 mRNA 31
Discussion 32
Lamb Growth 32
Morphometric Measurements 33
Abundance of oPepT1 mRNA 36
Implications 38

Chapter IV. Epilogue.....58

Literature Cited.....61

Appendix.....72

Vita.....81

LIST OF TABLES

TABLE

1 Ingredient composition of diets 40
2 Chemical composition of diets 41
3 Dry matter intake of ewes and lambs.....42

LIST OF APPENDIX TABLES

Appendix

1 Morphometric measurements of duodenal tissue from individual sheep 73
2 Morphometric measurements of jejunal tissue from individual sheep 74
3 Morphometric measurements of dorsal rumen tissue from individual sheep 75
4 Morphometric measurements of ventral rumen tissue from individual sheep 76
5 Morphometric measurements of omasum tissue from individual sheep 77
6 Example of analysis of variance for effect of age, diet, and block on total gain 78
7 Example of analysis of variance for effect of age, diet, and block on villus height in tissues of the small intestine 79
8 Example of analysis of variance for effect of age, diet, and block on abundance of oPepT1 in tissues of the small intestine 80

LIST OF FIGURES

FIGURE

1 Weight gain of lambs sampled during the first 8 wk postnatal.....43
2 Measurements of villus height in the small intestine of lambs 44
sampled during the first 8-wk postnatal
3 Measurements of villus width in the small intestine of lambs 45
sampled during the first 8-wk postnatal
4 Measurements of crypt depth in the small intestine of lambs 46
sampled during the first 8-wk postnatal
5 Measurements of villus height:villus width in the small intestine 47
of lambs sampled during the first 8-wk postnatal
6 Measurements of villus height:crypt depth in the small intestine 48
of lambs sampled during the first 8-wk postnatal
7 Measurements of papillae height in the rumen of lambs sampled 49
during the first 8-wk postnatal
8 Measurements of papillae width in the rumen of lambs sampled 50
during the first 8-wk postnatal
9 Measurements of papillae height:papillae width in the rumen of 51
lambs sampled during the first 8-wk postnatal
10 Total epithelial cell counts from ruminal and omasal papillae of 52
lambs sampled during the first 8-wk postnatal
11 Keratinized epithelial cell counts from ruminal and omasal papillae 53
of lambs sampled during the first 8-wk postnatal
12 Nonkeratinized epithelial cell counts from ruminal and omasal 54
papillae of lambs sampled during the first 8-wk postnatal
13 Keratinized:nonkeratinized cell ratios from ruminal and omasal 55
papillae of lambs sampled during the first 8-wk postnatal
14 Abundance of oPepT1 mRNA in small intestinal epithelium of.....56
lambs sampled during the first 8-wk postnatal
15 Abundance of oPepT1 mRNA in forestomach epithelium of 57
lambs sampled during the first 8-wk postnatal.

Chapter I

INTRODUCTION

Although the abomasum of ruminants is both well developed and highly functional at birth, the forestomach (rumen, reticulum, and omasum) remains rudimentary. Yet, by approximately 56 d of age in lambs, the four compartments of the ruminant stomach have reached adult proportions (Wardrop and Coombe, 1960b). During this time, development of the forestomach can be divided into three phases: preruminant (0 to 3 wk), transition (3 to 6 wk), and ruminant (8 wk; Wardrop, 1960a). During the preruminant phase, the reticulorumen is small and flaccid with rudimentary papillae. Diet heavily influences the transitional phase, during which the reticulorumen greatly increases in size and papillary development.

It is important to remember that, in order for the newborn to survive, the gastrointestinal tract must be sufficiently differentiated to accommodate the postnatal changes that occur in diet after birth. There are two dramatic developmental transitions that are associated with the changing functional demands on the gastrointestinal tract. The first occurs at birth when placental nutrition is abruptly terminated and the gastrointestinal tract must assume sole responsibility for extracting nutrients from the lumen of the gut. The second transition occurs with weaning, when solid food becomes the primary nutrient source. The mucosal structure of the small intestine is characterized by marked changes at these times that appear to differ among species.

Absorption of small peptides is known to contribute significantly to meeting the protein requirements of animals (Matthews, 1991). Traditionally, it was believed that the only way for protein to enter portal circulation was in the form of amino acids. Today we

realize that amino acids are not the only absorbable form of protein. The current model for protein absorption begins with the digestion of dietary protein by gastric and pancreatic proteases and peptidases yielding primarily large peptides (Ganapathy et al., 1994). These large peptides are then further hydrolyzed by pancreatic and brush border peptidases to yield free amino acids and small peptides, both of which are absorbed by separate transport systems into the enterocyte. Once inside the enterocyte, peptides are subjected to hydrolysis by cytoplasmic peptidases yielding free amino acids. Free amino acids then exit the enterocyte and enter portal circulation. Even though free amino acids are the predominant protein form exiting the enterocyte, some peptides will escape hydrolysis and subsequently enter the portal vein (Ganapathy et al., 1994).

Di- and tripeptides as well as some peptidomimetics such as β-lactam antibiotics and angiotensin converting enzyme (ACE) inhibitors are transported by the peptide transporter, PepT1 (Daniel, 1996). This transporter has 12 transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10 (Fei et al., 1994). Overall, amino acid similarity is highly conserved with the exception of the large extracellular loop where the amino acid sequence similarity is least conserved (Daniel, 1996).

Early research in our laboratory indicated that peptides might be an important form of absorbed amino acids from the ruminant gastrointestinal tract (Koeln et al., 1993). This led to further research, which indicated the presence of a peptide transport protein(s) in the forestomach of sheep (Matthews et al., 1996; Pan et al., 1997). Additionally, our laboratory reported the tissue distribution of PepT1 in sheep, cows, pigs, and chickens (Chen et al., 1999). The PepT1 mRNA was present in the small

intestine of all animals studied as well as in the rumen and omasum of the sheep and cows.

Ontogenic development has been studied extensively for many gut functions, but until recently few data have been available on the ontogenesis of nutrient transporters. Studying the ontogenesis of peptide transporters will provide us with information that will contribute to understanding the regulation of peptide transport. The purpose of this review is to describe information related to ontogenesis of the ovine gastrointestinal tract and peptide transport.

Chapter II

Literature Review

Although the existence of a transport process for intact peptides has been acknowledged for several years (Matthews, 1991; Leibach and Ganapathy, 1996), it was only recently that peptide transporters have been identified (Boll et al., 1994, 1996; Dantzig et al., 1994; Fei et al., 1994, 2000; Liang et al., 1995; Liu et al., 1995; Saito et al., 1995, 1996; Yamashita et al., 1997; and Pan et al., 2001). To gain a full understanding of these transporters, it is important to study their development and regulation during the postnatal period.

Cloning of Mammalian Peptide Transporters. Rabbit PepT1 was the first mammalian peptide transporter cloned (Boll et al., 1994; Fei et al., 1994). In both cases, expression cloning was the technique used. Poly(A)$^+$ mRNA from small intestinal epithelium was size fractionated, and an RNA-pool containing the message was used in the construction of a cDNA library. The cDNA were then transcribed into corresponding cRNA and injected into *Xenopus laevis* oocytes. Oocytes were monitored for transport activity by their ability to uptake radiolabelled peptides. The clones eliciting a positive response in terms of peptide uptake were then sequenced. The rabbit PepT1 cDNA encodes a protein 707 amino acids in length (Fei et al., 1994). The amino acid sequence predicted 12 transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10 (Fei et al., 1994).

Human PepT1 was identified by screening a human intestinal cDNA library with a probe derived from rabbit PepT1 cDNA (Fei et al., 1994; Liang et al., 1995). The

predicted protein contained 708 amino acids with 12 transmembrane domains (Liang et al., 1995).

Saito et al. (1995) also took advantage of the published rabbit PepT1 sequence. Primers for reverse transcription coupled polymerase chain reaction (RT-PCR) were designed based upon the rabbit PepT1 sequence. A 380-bp PCR product was then used as a probe to screen a rat kidney cDNA library from which rat PepT1 was isolated. The cDNA for rat PepT1 encoded a protein that contained 710 amino acids.

Recently, mouse PepT1 was cloned (Fei et al., 2000). Rat PepT1 cDNA was used as a probe to screen a mouse kidney cDNA library. The isolated clone was sequenced and mouse PepT1 cDNA encoded a protein 709 amino acids in length.

The first reported ruminant peptide transporter, ovine PepT1, was recently cloned in sheep (Pan et al., 2001). The predicted protein contained 707 amino acids with a molecular weight of 78 kDa. Additionally, ovine PepT1 was predicted to contain 12 transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10.

Liu et al. (1995) discovered human PepT2 while screening a human kidney cDNA library with a probe derived from rabbit PepT1 cDNA (Fei et al., 1994). This was followed by the cloning of rabbit PepT2 (Boll et al., 1996; Saito et al., 1996). PepT2 cDNA encoded a 729 amino acid protein and, like PepT1, PepT2 had 12 transmembrane domains with a large extracelluar loop between transmembrane domains 9 and 10 (Liu et al., 1995).

The HPT-1 peptide transporter was discovered in human intestinal cells using Caco-2 cells (Dantzig et al., 1994). It is a unique peptide transporter that shows no amino

acid sequence similarity with other known peptide transporters. However, it did have 20% to 30% identity with the cadherin superfamily of calcium-dependent cell–cell adhesion proteins. Predicted to have only one transmembrane domain, HPT-1 could be a member of a new class of a peptide transporter or it may be a protein associated with a transporter.

More recently, Yamashita et al. (1997) reported the cloning of a peptide/histidine transporter (PHT1) from rat brain. The predicted protein contained 572 amino acids and was predicted to have 12 transmembrane domains, but did not have the large extracellular loop between transmembrane domains 9 and 10. The authors reported that PHT1 had 17% and 12% sequence similarity with PepT1 and PepT2, respectively. They did not indicate the species to which the comparison was made, but presumably they were referring to the rat.

The amino acid sequence of the 12 transmembrane domains of PepT1 is highly conserved, yet the amino acid sequence of the large extracellular loop between domains 9 and 10 is less conserved (Daniel, 1996). Overall, amino acid sequence similarities among PepT1 are quite homologous. Ovine PepT1 is 83%, 78%, and 81% homologous with human, rabbit, and rat, respectively (Pan et al., 2001). Rat PepT1 is 77% homologous with rabbit PepT1 and 83% homologous with human PepT1 (Saito et al., 1995). Rabbit and human PepT1 are 81% homologous (Liang et al., 1995). Rat PepT2 is 83% and 48% homologous to human PepT2 and rat PepT1, respectively (Saito et al., 1996).

Tissue Distribution of PepT1. Previous studies in our laboratory indicate that a peptide transport protein(s) is present in the forestomach of sheep (Matthews et al., 1996; Pan et al., 1997). Northern blot analysis has revealed that PepT1 is primarily expressed

in the small intestine while PepT2 is primarily expressed in the kidney (Fei et al., 1994; Liang et al., 1995; Miyamoto et al., 1996; Saito et al., 1996). More recently, Chen et al. (1999) reported the tissue distribution of PepT1 in sheep, dairy cows, pigs, and chickens. A cloned 446-bp cDNA fragment of PepT1 from sheep omasum was used as a probe for northern blot analysis. Positive hybridization was seen in the small intestine of all animals as well as the rumen and omasum of sheep and dairy cows. For sheep and dairy cows, the size of the hybridized mRNA was 2.8 kb. The greatest abundance of PepT1 was observed in the jejunum followed by the ileum. The duodenum and omasum had lesser abundance with the rumen having the least abundance. In chickens, the size of the hybridized mRNA was 2.9 kb (Chen, 2001) with greatest abundance occurring in the jejunum. Unlike the other species studied, two RNA transcripts, 2.9 kb and 3.5 kb, were observed in the small intestine of pigs. Greatest abundance of hybridized mRNA was present in the jejunum of pigs. This provided evidence for the presence of peptide transport proteins in farm animals.

Although there has been extensive advancement into the research of peptide transporters, few studies have focused on the distribution and localization of PepT1 at the level of protein expression. Saito et al. (1995) conducted western blot analysis of brush border membranes from rat small intestine and kidney cortex using rabbit antiserum raised against a synthetic peptide corresponding to the carboxy-terminal amino acids of rat PepT1. Rat PepT1 had an apparent molecular mass of 75 kDa. Ogihara et al. (1996) examined the localization of PepT1 protein along the rat digestive tract. Immunohistochemical analysis revealed PepT1 protein in the duodenum, jejunum, and

ileum. The PepT1 protein was localized in the brush border membrane of the enterocytes.

The progress that has been made in peptide transport research over the past several years is impressive. Yet, few studies have focused on the ontogenesis of these transporters. As this area becomes elucidated, it will become more important to consider the morphological changes that are simultaneously occurring in the gut.

Ruminal Histology and Papillary Development. The mucosal lineing of the rumen is composed of a keratinized stratified squamous epithelium (Wardrop, 1961a). Within this, four distinct cell layers, or stratas, can be differentiated. The outermost layer, or stratum corneum, serves as a protective barrier against digesta. It consists of cornified epithelial cells and varies in thickness from one to ten to twenty cells (Frappier, 1998). The stratum granulosum, typically one to three cells in thickness, consists of flattened cells with keratohyalin granules present in the cytoplasm. Below the stratum granulosum is the stratum spinosum in which the cells are polyhedral and slightly larger than those found in the stratum basale. The stratum basalae, or the innermost layer, is composed of columnar cells. There is no distinct boundary between the lamina propria and submucosa, thus it is sometimes referred to as the propria-submucosa. The tunica muscularis is composed of an inner circular layer of smooth muscle and an outer longitudinal layer of smooth muscle. Finally, the serosa is the outer layer of connective tissue.

The mucosal surface of the rumen is covered by many tongue shaped projections, known as papillae. Ruminal papillae begin to develop at 100 d of fetal life and by birth the mucus membrane has folded to form numerous papillae (Wardrop, 1961a). By 56 d,

there has been extensive development of the papillae in height, width, and surface area (Wardrop, 1961a; Zitnan et al., 1999).

The stimulation of papillary development occurs in parallel with the establishment of ruminal fermentation and its resulting end products, known as volatile fatty acids (VFA). Butyrate and propionate have been found effective in stimulating papillary development (Sander et al., 1959; Omar et al., 1964). Butyrate was not present in the rumen until after solid feed became a part of the diet (Omar et al., 1964). Results indicate that maintaining young on a liquid diet delays development of the forestomach. Under these conditions, the reticulorumen was smaller than normal for the age with thinner walls, less capacity, and lacked normal coloration and development of the papillae (Warner et al., 1956; Smith, 1961; Tamate et al., 1962; Stobo et al., 1966; Swan and Groenewald, 2000). By contrast, animals maintained on hay, grain, or hay and grain had extensive forestomach and papillary development (Brownlee, 1956; Warner et al., 1956). Exposure of lambs to a whole barley and protein mineral pellet diet (32 % CP, 1.5 to 2.5% Ca, 1 % P, and 34, 000 IU vitamin A/kg) while nursing, resulted in longer papillae and 38 % more surface area than lambs that were not exposed to the whole barley and protein mineral pellet diet (Ortega-Reyes et al., 1992). In calves, feeding increasing amounts of concentrate resulted in longer papillae and a more dense covering over both the dorsal and ventral rumen in comparison to calves fed lesser amounts of the same concentrate (Stobo et al., 1966).

Omasal Histology and Laminae Development. The omasum is lined by keratinized stratified squamous epithelium and possesses the same four cell layers as the rumen: stratum basalae, stratum spinosum, stratum granulosum, and stratum corneum

(Wardrop, 1961a; Lubis and O'Shea, 1978). There is a thick layer of muscularis mucosae beneath the laminae propria followed by a thin layer of connective tissue and serosa. Like in the rumen, the tunica muscalaris is composed of an outer layer of longitudinal smooth muscle and an inner layer of circular smooth muscle.

The mucosal surface of the omasum contains numerous leaves or laminae that extend longitudinally from the reticulo-omasal orifice to the omaso-abomasal orifice (Yamamoto et al., 1994). These laminae begin to develop at 46 d of fetal life and are visible by 70 d of fetal life (Wardrop, 1961a). By 20 d postnatal, all orders of laminae are greatly increased in length (Wardrop, 1961a).

Omasal papillae have been described as highly varied in shape with small conical papillae being visible on all orders of laminae by 56 d postnatal (Wardrop, 1961a Yamamoto et al., 1994). Papillae decrease gradually in size from the reticulo-omasal orifice to the omaso-abomasal orifice (Yamamoto et al., 1994). In addition, it was noted that, in a 72-d-old milk-fed calf, the omasal laminae were small and the papillae were poorly developed (Yamamoto et al., 1994).

Small Intestinal Histology and Mucosal Development. Histologically, the small intestine is comprised of the mucosa and submucosa (Frappier, 1998). The epithelial lining (simple columnar epithelium), lamina propia, and muscularis mucosae comprise the mucosa of the small intestine. The submucosa is a layer of connective tissue that is denser than the lamina propria. Within the submucosa are Brunner's glands that function to secrete mucus to protect the epithelial surface. Also present in the submucosa are isolated lymphoid nodules. Aggregations of 10 or more lymphoid nodules, or Peyer's patches, are most common in the ileum, but can occur in all segments of the small

intestine (Frappier, 1998). They can cause alterations of the mucosal surface and thus alter villi height (Frappier, 1998). Peyer's patches, the largest of the mucosal lymphoid tissue, belong to a group of lymphoid tissues referred to as GALT (gut associated lymphoid tissue; Tizzard, 1996). GALT is a collective term for all lymphoid nodules, Peyer's patches, and individual lymphocytes present in the intestine. The covering epithelium of Peyer's patches is composed of epithelial cells known as M cells. M cells, or microfold cells, endocytose antigens and present them to the lymphoid system to initiate an immune response. Therefore, Peyer's patches play a critical role in the intestinal immunological system. Intestinal villi, or fingerlike mucosal projections, are present on the epithelial surface. Simple tubular glands, known as crypts of Leiberkuhn, are also present in the mucosa. The stratum compactum separates the laminae propria from the muscularis mucosae, which is composed of an inner layer of circular smooth muscle and an outer layer of longitudinal smooth muscle. Regions of the small intestine include the duodenum, jejunum, and ileum. A gradient of maturation exists along the length of the small intestine whereby the proximal regions mature earlier than the distal regions (Trahair and Robinson, 1986). Villi began to form in the proximal intestine at 50 d gestation, whereas crypts were not observed, in all segments of the small intestine, until 90 to 125 d gestation (Trahair and Robinson, 1986). From 75 d gestation until term, enterocytes present at the tip of the villus were more developed than enterocytes present at the villus base. This is similar to the enterocyte migration pattern, from the crypt to the tip of the villus, seen in the small intestine of adults (Klein and McKenzie, 1983).

The early postnatal period is characterized by cell proliferation in the small intestine (Klein and McKenzie, 1983). Increases are observed in villus height, crypt

depth, numbers of villi/cross sectional area, cells/crypt, cells/villi, and crypts/villi. Crypt depth in calves and lambs (Moon and Joel, 1975), pigs (Moon, 1971), rats (Koldovsky et al., 1966), and chickens (Moon and Skartvedt, 1975) was observed to increase during the first few weeks after birth. Although cellular proliferation, and thus resulting growth, is an important feature of the small intestine at this time, there are also marked changes in mucosal structure occurring that appear to differ among species. For example, in rodents, villus height and crypt depth increased postnatally beginning at approximately d 18 (Klein, 1989). Whereas, villus height was reported to gradually decrease in pigs during the first 2 to 3 wk of life (Hampson, 1986; Cera et al., 1988; Kelly et al., 1992). Small increases in crypt depth accompanied these changes in villus height. Additionally, changes in mucosal structure were more abrupt immediately after weaning. Moon and Joel (1975) reported the villi of newborn lambs and calves to be longer in comparison to older animals. In weaned pigs, villus height decreased and crypt depth increased in comparison to suckling pigs, but this was uneffected by prior exposure to creep feed (Kelly, 1990). Therefore, creep feeding did not affect the mucosal structure and function of the small intestine.

As morphological structure of the gut undergoes changes during the postnatal period, nutrient transporters localized in the epithelium of the gut may also be effected. It is therefore necessary to understand the development and regulation of nutrient transporters during the postnatal period.

Ontogenesis of Nutrient Transport. There are two major developmental transitions that are associated with the changing functional demands on the gastrointestinal tract. The first occurs at birth when placental nutrition is abruptly

terminated and the gastrointestinal tract must assume sole responsibility for extracting nutrients from the lumen of the gut. At this time, milk serves as the primary nutrient source for the animal. The second transition coincides with weaning, in mammals, when solid food becomes the primary nutrient source. Ontogenic development has been studied extensively for many gut functions, but few data are available on the ontogenesis of nutrient transporters.

It was shown by Fitzgerald et al. (1971) that intestinal transport of sugars (α-methyl-D-glucoside and D-galactose) and L-valine, L-lysine, and glycine were maximal in the proximal and distal intestine of 2- to 5-d-old rats in comparison to older rats. More recently, the ontogenesis of the Na^+/glucose cotransporter (SGLT1) during the suckling period in rats was investigated (Kojima et al., 1999). Northern blot analyses were performed to measure the change in SGLT1 mRNA abundance in the various segments of the small intestine at ages 5, 7, 10, 13, 15, and 18 d. The proximal intestine of 5-d-old rats had higher SGLT1 mRNA abundance than did the distal segments of the small intestine. The abundance of SGLT1 mRNA increased in the mid-intestine with respect to postnatal age. In the distal intestine, SGLT1 mRNA abundance was low, but gradually increased to adult levels by 21 d of age.

Miyamoto et al. (1992) studied the ontogenesis of mRNA levels of glucose transporters in the jejunum of rats of various ages (5, 10, 15, 20, 25, and 90 d). In contrast to Kojima et al. (1999), no change was seen in SGLT1 mRNA abundance during development. Different timelines were used in the two studies for sampling the animals and it is possible that this may have had an affect on the contradictory results. In addition to SGLT1, GLUT2 and GLUT 5 were also analyzed. The GLUT2 is a basolaterally

located transporter that is primarily expressed in the liver and small intestine (Burant et al., 1991). Substrates for GLUT2 include glucose and fructose. The GLUT5 is located on the apical membrane and is expressed primarily in the small intestine (Burant et al., 1991). Fructose is the substrate for GLUT5. GLUT2 mRNA was low in 5- and 10-d-old rats, but beginning at 20 d GLUT2 mRNA abundance rapidly increased reaching adult levels by 25 d of age (Miyamoto et al., 1992). In contrast, GLUT5 mRNA levels were greatest in 5- and 10-d-old rats, yet the abundance of GLUT5 mRNA began to steeply decline at 15 d of age (Miyamoto et al., 1992). These data indicate marked changes in mRNA abundance in the third week for both GLUT2 and GLUT5. This corresponds with the time of weaning in rats, therefore, these changes may be the result of dietary regulation.

Recently, Buddington et al. (2001) studied the absorption of five amino acids during the development of the pig. The amino acids were aspartate (acidic), lysine (basic), leucine (neutral), methionine (neutral), and proline (imino). Lysine, leucine, and methionine represented essential amino acids, whereas aspartic acid and proline were representative of nonessential amino acids. Absorption rates were greatest for all amino acids at birth except proline, which was greatest at d 7. The first 24-h following birth were accompanied by an average decrease of 30 % in absorption rates for all five amino acids. Leucine, methionine, and proline continued to decline, whereas aspartate and lysine absorption rates began to rise at about 28 d. Because the proteolytic capabilities of newborn mammals are less developed than adults (Hamosh and Hamosh, 1999), the authors emphasized the need to quantify peptide absorption during the early postnatal period.

Rubino and Guandalini (1977) reported the first developmental data on intact-peptide transport. Influxes of radiolabelled glycyl-L-proline and glycyl-L-phenylalanine were measured in the jejunum of fetal, newborn, suckling, and adult rabbits. Influxes of glycyl-L-proline and glycyl-L-phenylalanine were greater in sucklings than in adults. This was particularly true for glycyl-L-proline where uptake gradually increased from 25 d gestation to reach a peak shortly after birth. Uptake of glycyl-L-proline then remained constant for 6 d postnatal, declined steadily, and reached adult levels by 50 d postnatal. Glycine influxes did not differ between fetal, newborn, or adult rabbits. These data indicate that the developmental pattern of dipeptide transport and amino acid transport may be quite different.

That younger animals have a greater ability to absorb dipeptides was supported by the report of Himukai et al. (1980). They used isolated, everted intestinal segments to study the age-dependent change in intestinal absorption of dipeptides in the guinea pig. Glycylglycine and glycyl-L-leucine influxes were greatest in sucklings, intermediate in weanlings and least in adults. The age-dependent difference was most evident in the jejunum. A decrease in maximal transport capacity in the suckling and weanlings (V_{max}; 50 and 20 nmoles·cm^{-2}·min^{-1}, respectively) was attributed to the age-dependent difference observed. In comparison to adults, younger animals may have a greater proportion of amino acid absorption occurring as peptides. It is also possible that the association with membrane digestion and free amino acid transport in the young may not be as functional as it is in adults.

The mRNA abundance of rat PepT1 was noted to markedly change in the jejunum during development (Miyamoto et al., 1996). A ^{32}P-labeled, randomly primed 1.9 kb rat

PepT1 cDNA probe was used to perform northern blot analyses on jejunal epithelium of rats at the following ages: 2, 4, 14, 21, 28, and 70 d. The PepT1 mRNA abundance was greatest in 4-d-old rats and then decreased, reaching adult levels by 28 d of age.

Shen et al. (2001) recently reported the developmental expression of PepT1 and PepT2 in the small intestine, colon, and kidney of the rat. The PepT1 mRNA abundance was maximal in all segments of the small intestine 3 to 5 d after birth and then declined. Particularly in the ileum, PepT1 mRNA abundance temporarily increased at 24 d. Rats were weaned at 21 d of age, therefore the temporary increase in PepT1 mRNA abundance may be a result of dietary regulation. PepT1 mRNA was present in the colon, but decreased to undetectable levels by 2 wk of age. In the kidney, PepT1 and PepT2 mRNA abundance were maximal at 24 d. Overall, changes in mRNA abundance and protein expression in the small intestine paralleled each other over the times studied. Although the authors suggested post-transcriptional regulation, these data are indicative of transcriptional regulation. Yet in the kidney, the increase in mRNA abundance at 24 d of age was not observed in protein expression and is indicative of post-transcriptional regulation of PepT1 in the kidney. From these data it seems that PepT1 is induced postpartum and again at weaning, and that the colon may be involved in peptide transport early in life.

Dietary Regulation. Most intestinal nutrient transporters are regulated by the dietary levels of their substrates (Ferraris and Diamond, 1989). Generally, transporter activity increases with high dietary substrate levels of nutrients such as sugars, vitamins, and nonessential amino acids. For essential nutrients, such as vitamins and amino acids, low dietary substrate concentrations should upregulate transporter activity. For nutrients

toxic at high concentrations, such as certain minerals, high dietary substrate concentration should downregulate transporter activity. Recently, glucose uptake in the small intestine by SGLT1 was reported to be higher in lambs receiving an abomasal infusion of 500 g/d water plus 35 g casein compared to when only 500 g/d water were infused (Mabjeesh et al., 2001), thus indicating substrate regulation of a nutrient transporter. Additionally, the affinity of SGLT1 remained similar along the length of the small intestine of lambs infused with casein. This was not the case in lambs infused with water only where the greatest affinity was present in the jejunum and the lowest affinity was present in the duodenum. Therefore, it appears that glucose uptake by SGLT1 may be affected by way of SGLT1 affinity at the brush border membrane. This in turn is directly influenced by casein and/or the resulting amino acids and peptides in the small intestine.

Dietary regulation is an important factor in intestinal peptide transport (Ferraris et al., 1988; and Ferraris and Diamond, 1989). Intestinal transport of the dipeptide carnosine was regulated by diet (Ferraris et al., 1988). Carnosine uptake was measured in everted intestinal sleeves from mice receiving different dietary protein levels. Mice receiving the high protein diet (72% protein; 0% sucrose) had a 43% greater uptake than did the mice receiving the low protein diet (18% protein; 54% sucrose). Thus, carnosine uptake was shown to increase with dietary protein level in isolated mouse intestine.

Erickson et al. (1995) provided evidence that dietary protein is a regulator of peptide transport activity. Abundance of PepT1 mRNA in the small intestine as influenced by dietary protein was examined. Rats maintained on a normal diet (17% casein) showed evenly distributed PepT1 mRNA along the longitudinal axis of the small intestine. Conversely, rats that were switched from a low-protein (4% casein) to a high-

protein (50% gelatin) diet showed a 1.5- to 2-fold increase in PepT1 mRNA abundance in the middle and distal small intestine. These results indicate that dietary protein level may have a regulatory role in peptide transport and that the distal small intestine may be the primary site where diet-induced changes occur. Gelatin is much lower in protein quality than casein. Therefore, since gelatin was used to create the high-protein diet, it may be possible that composition of the diets may have influenced transport activity.

In support of the idea that composition of dietary protein may be important, Shiraga et al. (1999) suggested that the upregulation of peptide transport activity by dietary protein was caused by selective amino acids and dipeptides in the diet activating the transcription of the PepT1 gene. Understanding the mechanism by which dietary amino acids or dipeptides induce PepT1 synthesis is very important clinically for nutritional therapy of digestive and absorptive disorders. A series of 5'-deleted fragments ranging from –3119 bp to –13 bp of the rat PepT1 gene promoter were inserted upstream of the luciferase gene and transiently transfected into Caco-2 cells. The ability of amino acids and dipeptides to induce luciferase activity was investigated in transfected cells. Transcription from the –351 clone was enhanced by amino acids (phenylalanine, arginine, and lysine) and dipeptides (Gly-Sar, Gly-Phe, Lys-Phe, and Asp-Lys), indicating the presence of an amino acid and peptide response element with in 351 nucleotides of the transcription start site.

Intestinal amino acid and peptide absorption generally increases during fasting and malnutrition (Ferraris and Carey, 2000). In a recent study, the molecular expression of PepT1 was studied in rats after a 24-h fast (Thamtharan et al., 1999). In the fasted rats, both PepT1 mRNA abundance and protein expression increased three-fold. Due to the

parallel changes in mRNA abundance and protein expression, this is suggestive of transcriptional regulation of PepT1, which is in agreement with the data of Shen et al. (2001).

With the structure and function of PepT1 characterized, it is now possible to study the effects of development and different forms of regulation, such as diet. The purpose of the thesis research reported here was to investigate the effects of age and diet on oPepT1 mRNA abundance and to begin to relate the morphological changes that occur in mucosal structure during the postnatal period with peptide transport.

Chapter III

Ontogenesis of peptide transport and morphological changes in the ovine gastrointestinal tract

ABSTRACT

Nutrient absorption is important in all stages of life. As the diet of an animal changes from birth on, morphological and biochemical adaptation can be anticipated in order to accommodate changing demands. The main focus of the present study was to examine the relationship between age and diet on the potential for peptide transport via PepT1 in the gastrointestinal tract of lambs and to relate changes of peptide transport capability to morphological changes. A 2x4 factorial arrangement of treatments was used with 32 crossbred lambs. Four blocks were created based upon gender, birth type (single or twin), birth weight, and birth date. Lambs were randomly allotted at birth to receive or not to receive a creep diet. All lambs were allowed to nurse. Sampling times of 2, 4, 6, or 8 wk were randomly allotted to lambs. Samples for RNA extraction and histological evaluation were taken from the dorsal rumen, ventral rumen, omasum, duodenum, jejunum, and ileum. Villi were about 7% shorter ($P < 0.09$) in lambs receiving creep feed. Papillary height and width increased linearly ($P < 0.001$ and $P < 0.0001$, respectively) with age. Total and keratinized epithelial cells in the stomach decreased ($P < 0.03$ and $P < 0.004$, respectively) with age and were fewer ($P < 0.0002$ and $P < 0.0001$, respectively) in lambs receiving creep feed. Creep feeding appears to have slightly altered the mucosal structure of the small intestine and it was advantageous in that it

stimulated papillary growth and thus predisposed the rumen for the introduction of feed into the diet. A 2.8 kb oPepT1 mRNA was present in all tissues studied by 2 wk and age did not significantly influence the abundance of oPepT1 mRNA in the small intestine or stomach. In the small intestine, abundance of oPepT1 mRNA was greatest ($P < 0.0007$) in the jejunum. In the stomach, abundance of oPepT1 mRNA was greatest ($P < 0.01$) in the dorsal rumen. In the stomach, particularly in the rumen, a greater abundance of oPepT1 mRNA was observed in lambs not receiving the creep diet. It seems likely that a stimulus for development is coming from the non-luminal direction, possibly blood-borne, and may be involved in the ontogenesis of oPepT1. Peptide transport appears to be a physiologically important process in the young lamb and the rumen appears to be involved in the transport of peptides, particularly in lambs that only nurse.

Key Words: Ovine, Peptide, Transport, Ontogenesis, mRNA, PepT1

Introduction

The PepT1 transports di- and tripeptides as well as some peptidomemetics such as β-lactam antibiotics and angiotensin converting enzyme (ACE) inhibitors (Daniel, 1996). The PepT1 has 12 transmembrane domains with a large extracellular loop between transmembrane domains nine and ten (Fei et al., 1994). Data from our laboratory indicated the presence of peptide transport protein(s) in the forestomach of sheep (Matthews et al., 1996; Pan et al., 1997). Peptide transport protein one (PepT1) was observed to be present in the rumen, omasum, and small intestine of sheep (Chen et al., 1999).

Data on the ontogenesis of peptide transport indicate that the young may have a greater ability to transport peptides than older animals. Influxes of radiolabelled glycyl-L-proline and glycyl-L-phenylalanine were reported to be greater in suckling rabbits than in adults (Rubino and Guandalino, 1977). Using isolated, everted intestinal segments Himukai et al. (1980) reported glycylglycine and glycyl-L-leucine influxes were greatest in sucklings, intermediate in weanlings, and least in adult guinea pigs. A decrease in maximal transport capacity between the suckling and adult guinea pigs (V_{max}; 50 and 20 nmoles·cm^{-2}·min^{-1}, respectively) was attributed to the age-dependent difference observed.

Molecular data on the ontogenesis of PepT1 in laboratory animal species indicate greater mRNA abundance and protein expression during the early postnatal period (Miyamoto, 1996; Shen et al., 2001). Dietary regulation has been implicated as an important factor in the intestinal transport of peptides (Ferraris et al., 1988; Erickson et al., 1995; Shiraga et al., 1999). Therefore, the focus of the present study was to examine the relationship between age and diet on the potential for peptide transport via PepT1 in

the gastrointestinal tract of lambs. It was also of interest to relate changes of peptide transport capability to morphological changes.

Materials and Methods

Materials. All chemicals used were of enzyme, molecular biology, or histological grade. Diethyl pyrocarbonate (DEPC), EDTA, sodium acetate, formaldehyde, chloroform, and isopropanol were purchased from Sigma (St. Louis, MO). Sodium chloride and MOPS were purchased from Fisher Scientific (Fair Lawn, NJ). RNASIN Ribonuclease Inhibitor was purchased from Promega (Madison, WI). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Nylon membranes used were from MSI (Westboro, MA). The [$\propto$-^{32}P]dATP was purchased from ICN Pharmaceuticals (Costa Mesa, CA). DNA polymerase I/DnaseI and the RNA ladder (0.24 – 9.5 Kb) were from Gibco Life Technologies (Gaithersburg, MD). TriReagent was purchased from MRC (Cincinnati, OH).

Experimental Design. A 2 x 4 factorial arrangement of treatments was used with 32 crossbred lambs. Four blocks were created based upon gender, birth type (single or twin), birth weight, and birth date. Lambs were randomly allotted at birth to receive or not to receive a creep diet and all lambs were allowed to nurse. Sampling times of either 2, 4, 6, or 8 wk were randomly allotted to lambs at this time. Ewes and their lamb(s) were placed in their assigned pens on the third day following the birth of the lamb(s). There were 10 sets of twins and 12 singles. Twin lambs were assigned to receive the same diet, but were assigned to different sampling times. Numbers of twins and singles were equalized among treatments.

Animal Management and Diets. Ewes and their lambs were housed in four pens on raised wire mesh flooring. The pens were divided by a barrier that allowed only the lambs access to a portion of the pen. Creep feed was provided in this portion of the pen for the lambs receiving the creep diet. This area was also gated, thus allowing the separation of ewes and lambs while the ewes were being fed.

Diets were formulated to meet NRC requirements (NRC, 1985). Ingredient and chemical composition are presented in Tables 1 and 2, respectively. Diets were analyzed for dry matter (AOAC, 1990), N (Kjeldahl; AOAC, 1990), NDF (VanSoest and Wine, 1967; Goering and VanSoest, 1970), and ADF (VanSoest, 1963; Goering and VanSoest, 1970).

Ewes were fed, based upon average weight (62 kg), to meet requirements for lactating ewes during the first 6 to 8 wk of lactation with suckling twins (NRC, 1985). Ewes were fed at 0630 and 1530 and were allowed 3-h to eat at each feeding. Lambs were fed at 1530 and allowed 24-h access to the creep feed. Refused feed was removed from the bunk, weighed, and recorded after each feeding period.

Tissue Sampling. Final weights of the lambs were obtained and recorded prior to sampling. Lambs were stunned by a captive bolt pistol and killed by exsanguination. The abdomen was opened immediately and the gastrointestinal tract was removed from the caudal end of the esophagus to the ileo-cecal junction. Samples for RNA extraction and histological evaluation were taken from the dorsal rumen, ventral rumen, omasum, duodenum, mid-jejunum, and terminal ileum. Intestinal segments were cut open longitudinally and all tissues were washed in ice-cold 0.9% saline. For RNA extraction, intestinal epithelium was collected by scraping the mucosal surface with a glass slide.

Samples were then wrapped in aluminum foil and frozen in liquid N_2. Whole tissue sections of the dorsal and ventral rumen and omasal plies were collected, wrapped in aluminum foil, and frozen in liquid N_2. All samples were stored at –80°C until further analysis. For histological evaluation, approximately 1 cm^2 sections of tissue were cut and mounted serosal side down on cardboard. In the case of omasal plies, one of the epithelial surfaces was mounted against the cardboard. Samples were fixed in 10% phosphate buffered formalin. Each sample was trimmed, placed in a tissue cassette, processed, and embedded in paraffin wax by routine methodology (Bancroft and Stevens, 1990). Sections (4 μm) were cut in duplicate, mounted on glass slides, and stained with haematoxylin and eosin (Bancroft and Stevens, 1990) for analysis by light microscopy.

Morphometric Measurements. Digital images were obtained at 10X with a Polaroid Digital Microscope Camera, DMCIe (Polaroid, Cambridge, MA). Measurements were made using Sigmascan Pro 5.0 (SPSS, Inc., Chicago, IL). For the duodenum and jejunum, ten intact, well oriented, and transversely cut villi and crypts were measured per animal. Measurements of villus height, villus width, and crypt depth were made as previously described (Kik et al., 1990). Villus width was defined as the distance from the outside epithelial edge to the outside of the opposite epithelial edge determined at mid-height of the villus. Ratios were calculated for villus height:crypt depth and villus height:villus width.

For ruminal tissues, ten intact, well oriented, and transversely cut papillae were measured per animal from both the dorsal and ventral rumen. Papillary height was defined as the distance from the tip of the papillae to the base of the papillae. Papillary width was defined as the distance from the outside epithelial edge to the opposite outside

epithelial edge determined at mid-height of the papillae. The ratio of papillary height:papillary width was calculated. Total epithelial cells, keratinized epithelial cells, and nonkeratinized epithelial cells were determined at 400X. Keratin stains red in haematoxylin and eosin stain, thus allowing for the differentiation of keratinized and nonkeratinized epithelial cells (Bancroft and Stevens, 1990). The ratio of keratinized epithelial cells to nonkeratinized epithelial cells was calculated.

For omasal tissues, total epithelial cells, keratinized epithelial cells, and nonkeratinized epithelial cells were determined at 400X for ten papillae per animal. Keratinized cells were differentiated in the same manner as was performed in the rumen. The ratio of keratinized to nonkeratinized epithelial cell thickness was also determined.

Preparation of Total RNA. Total RNA was extracted from the duodenum, jejunum, and ileum, as well as the dorsal and ventral rumen using TriReagent. Total RNA was recovered per the manufacturer's protocol. Briefly, tissue samples of approximately 0.25 g were homogenized in 3 mL of TriReagent. Next, 200 μL chloroform were added to each homogenate and mixed well. Mixtures were centrifuged at 12,000 x g for 15 min at 4°C and the aqueous layer was transferred to a new tube. This was followed by the addition of 10U of RNASIN Ribonuclease Inhibitor and an equal volume of 100% isopropanol to each tube. All samples were then placed at -80°C for 2-h. Tubes were then centrifuged at 12,000 x g for 15 min at 4°C. Pellets were washed once in DEPC treated 70% ethanol and centrifuged at 12,000 x g for 15 min at 4°C. Total RNA was then recovered by dissolving pellets in 10 m*M* Tris-HCl (pH 7.6), 1m*M* EDTA (DEPC-treated). RNA content and purity was determined by measuring absorption at 260 and 280 nm. Samples were stored at -80°C.

Abundance of oPepT1 mRNA. Twenty micrograms of total RNA were electrophoresed through a 1% agarose gel containing 2.2 *M* formaldehyde and gels were stained with 0.05 mg/mL ethidium bromide. The RNA was transferred to a nylon membrane by downward capillary transfer with 10 m*M* NaOH, 5X SSC (3 *M* NaCl, 0.3 *M* sodium-citrate pH 7.0) and crosslinked with UV light for 30 s. Membranes were probed with oPepT1 cDNA (Pan et al., 2001). One hundred nanograms of probe were labeled with [$\propto$-^{32}P]dATP by nick translation and purified using Sephadex G-50 spin column chromatography. Prehybridization was conducted for 2-to 2.5-h at 42°C in a solution containing 50% deionized formamide, 5X Denhardt's solution, 6X saline-sodium phosphate-EDTA (SSPE), 0.2% sodium lauryl sulfate (SDS), and 10 μg/mL tRNA. Hybridization was conducted at 42°C overnight. Following hybridization, membranes were washed: twice at room temperature for 30 min in 5X SSPE, 0.5% SDS; twice at 42°C for 30 min in 1X SSPE, 0.5% SDS; and twice at 65°C for 30 min in 0.1X SSPE and 1% SDS. Membranes were exposed to Kodak XAR-5 film at -80°C. Next, membranes were stripped in a solution containing 50% deionized formamide, and 6X SSPE for 30 min at 65°C and washed twice in 2X SSPE. Membranes were then probed with 18S rRNA as an internal control using the same hybridization conditions as were used for oPepT1.

Sizes of mRNA bands were determined using an RNA ladder (0.24 kb to 9.5 kb). Northern blots were scanned with a ScanPrisa 640U (Acer, Inc., Taipei, Taiwan) scanner. Densitometric analyses of the scans were preformed using Gelworks 1D Image Analysis Software (UVP, Inc., Upland, CA) to quantify the bands on the northern blots.

Statistical Analysis. Data were analyzed with one-way analysis of variance using the General Linear Models procedure (SAS, 1999). The model included tissue, age, diet, block and all two-way interactions. The age main effect was further tested for linear and quadratic fit using orthogonal contrast statements in the General Linear Models procedure. Significant differences in tissue effects were further separated using Tukey's HSD.

Results

Lamb Growth. As lambs increased in age, dry matter intake increased (Table 3). Weight gain (Figure 1) increased linearly ($P < 0.0001$) in lambs sampled at 2, 4, 6, and 8 wk of age. However, differences in weight gain due to dietary treatment were not significant.

Morphometric Measurements. In the small intestine, villus height (Figure 2) in the jejunum was approximately 25% greater ($P < 0.0001$) than in the duodenum. Lambs that received that creep diet had villi approximately 7% shorter ($P < 0.09$) than lambs not receiving the creep diet, whereas differences observed in age were not significant. Villi were approximately 25% wider ($P < 0.0001$) in the jejunum (Figure 3) than the duodenum. Villus width increased ($P < 0.02$) linearly with age. However, a tissue*age interaction ($P < 0.01$) was observed. Generally, villus width tended to decrease in the duodenum (except at 8 wk) and increase with age in the jejunum. Diet did not influence villus width. Differences observed between tissues in crypt depth (Figure 4) were not significant. Crypt depth increased linearly ($P < 0.04$) with age, and a tissue*age interaction ($P < 0.02$) was observed. There was a general tendency for crypt depth to increase with age in the jejunum, while crypt depth remained unchanged in the

duodenum. Differences between dietary treatments were not significant. The ratio of villus height to villus width (Figure 5) did not differ between tissues or diets or among ages. The tissue*age interaction ($P < 0.07$) that was observed appears to be largely due to the ratio remaining relatively constant in the jejunum, but in the duodenum the ratio was greater at wk 4 and 6 than at wk 2 and 8. The ratio of villus height to crypt depth was approximately 18% greater ($P < 0.0005$) in the jejunum than in the duodenum (Figure 6). Differences observed in dietary treatment were not significant. However, a tissue*diet interaction ($P < 0.07$) was observed, and it appears that the increased ratio from lambs that did not receive the creep diet at 2 wk of age in the jejunum may be responsible for this interaction. Although differences observed in age were not significant, a tissue*age interaction ($P < 0.02$) was observed. The ratio of villus height to crypt depth tended to decrease in the jejunum, whereas the ratio increased slightly in the duodenum. The difference between dietary treatments at 2 wk in the jejunum is most likely responsible for the diet*age interaction ($P < 0.09$) which was observed. The increased difference in dietary treatments at wk 8 in the duodenum, in comparison to the jejunum, may have also contributed to the diet*age interaction.

In the rumen, differences observed in papillary height (Figure 7) were not significant between tissues, and papillary height was observed to increase linearly ($P < 0.0001$) with age. A tissue*age interaction ($P < 0.08$) was observed, and most likely results from a relatively lower rate of increase in papillary height in the dorsal rumen compared with the ventral rumen. Differences observed due to dietary treatment were not significant. Differences observed in papillary width (Figure 8) were not significant between tissues or dietary treatments. Papillary width increased linearly ($P < 0.0001$)

with age. A tissue*age interaction ($P < 0.10$) was observed. Generally, papillary width in the dorsal rumen increased steadily with age, but in the ventral rumen, papillary width did not begin to increase until wk 6. The ratio between papillary height and papillary width (Figure 9) did not differ between tissues or diets. The ratio of papillary height to papillary width increased linearly ($P < 0.04$) with age. A tissue*age interaction ($P < 0.10$) was observed. Generally, the ratio of papillary height to papillary width remained relatively constant in the dorsal rumen, but increased with age in the ventral rumen.

Omasal plies were collected for histological evaluation. Epithelial cells were counted as in the rumen, but measurements of papillae height and width were not taken due to the orientation of the tissue. Mounting one of the epithelial surfaces of the omasal ply to cardboard distorted the opposite epithelial surface.

In the dorsal rumen, ventral rumen, and omasum, total epithelial cells (Figure 10) did not differ among tissues. The total number of epithelial cells was approximately 12% greater ($P < 0.0002$) in lambs that did not receive the creep diet. Total epithelial cells decreased linearly ($P < 0.03$) with age. A tissue*diet interaction ($P < 0.07$) was observed in total epithelial cells. Generally, lambs that did not receive the creep diet had a greater number of total epithelial cells in the rumen, whereas the number of total epithelial cells remained relatively constant between diets in the omasum. Differences in keratinized epithelial cells (Figure 11) among tissues were not significant. There were approximately 21% fewer ($P < 0.0001$) keratinized epithelial cells observed in lambs that received the creep diet. However, this was more prominent in the dorsal and ventral rumen than in the omasum, which appears to have led to the tissue*diet interaction ($P < 0.07$). Keratinized epithelial cells decreased linearly ($P < 0.004$) with age. Differences in the numbers of

nonkeratinized epithelial cells (Figure 12) among tissues were not significant. Nonkeratinized epithelial cells were approximately 6% greater ($P < 0.08$) in lambs that did not receive the creep diet. Nonkeratinized epithelial cells decreased linearly ($P < 0.005$) with age. A tissue*age interaction ($P < 0.03$) was observed with nonkeratinized epithelial cells. Nonkeratinized epithelial cell numbers were similar among ages in the ventral rumen, while numbers decreased in both the dorsal rumen and omasum. Additionally, the decline in number of nonkeratinized epithelial cells was greater in the dorsal rumen than omasum. The ratio of keratinized to nonkeratinized epithelial cells was approximately 13% greater ($P < 0.001$) in lambs that did not receive the creep diet, and the ratio was observed to increase linearly ($P < 0.001$) with age. A tissue*age interaction ($P < 0.04$) was observed, and appears to result from the many differences among tissues at the different ages. There is no clear pattern to these changes.

Abundance of oPepT1 mRNA. The 2.8 kb mRNA that hybridized to the oPepT1 cDNA probe (Pan et al., 2001) was present in all tissues by 8 wk of age. In the small intestine (Figure 14), abundance of oPepT1 mRNA was greatest ($P < 0.0007$) in the jejunum. Abundance was approximately 56% greater than the duodenum and approximately 46% greater than the ileum. However differences observed between the duodenum and ileum were not significant. Diet did not influence the abundance of oPepT1 mRNA. In the stomach (Figure 15), the abundance of oPepT1 mRNA was approximately 68% greater ($P < 0.01$) in the dorsal rumen than the omasum. Abundance of oPepT1 mRNA in the ventral rumen did not differ significantly from abundance in the dorsal rumen or omasum. A lower ($P < 0.004$) abundance of oPepT1 mRNA was observed in lambs that received the creep diet in comparison to lambs that did not receive

the creep diet. A tissue*diet interaction ($P < 0.02$) was observed, and appears to be due largely to the abundance of oPepT1 mRNA being greater in the dorsal rumen of lambs of all ages and the ventral rumen of 4-wk-old lambs not receiving the creep diet. Abundance of oPepT1 mRNA remained relatively constant among diets in the omasum. Differences observed in abundance of oPepT1 mRNA among stomach tissues due to age were not significant. However, although a tissue*age interaction was not present, we speculate that there is a difference in the ventral rumen, where the abundance of oPepT1 mRNA was 63% times greater in 4-wk-old lambs than in older lambs (6 and 8 wk).

Discussion

Nutrient absorption is important at all stages of life in an animal. As the diet of an animal changes from birth on, morphological and biochemical adaptation can be anticipated in order to accommodate changing demands. The main focus of the present study was to examine the relationship between age and diet on the potential for peptide transport via oPepT1 in the gastrointestinal tract of lambs and to relate changes of peptide transport capability to morphological changes.

Lamb Growth. Creep feeding is the practice of providing supplemental feed to nursing lambs in an area that cannot be accessed by the dams (NRC, 1985). As consumption of creep feed increases, milk intake will begin to decline (Holloway et al., 1975; Lusby et al., 1976; NRC, 1985). Lambs are generally be expected to consume an average of nearly 700 g creep/d from 10 to 120 d of age (SID, 1987). Positive responses in lamb growth have been reported with respect to creep feeding (Perry et al., 1958; Matthew and Madsen, 1960; Jordan and Gates, 1961). In contrast, Glimp (1971) reported no effect of creep feeding on pre-weaning or post-weaning lamb growth, which was

attributed to a low creep intake of about 300 g/d. In order for an increase to be seen in lamb performance, creep intake needs to average 227 g/d by 20 d of age (SID, 1987). Lambs in the present study were not consuming 227 g/d until 8 wk of age. We expected creep feed intake by the lambs in the present study to be about 450 to 700 g/d at 40 to 50 d of age (SID, 1987). Therefore, the lack of influence of dietary treatment on weight gain is very likely attributable to the lower than anticipated level of creep intake.

Morphometric Measurements. The importance of the prevention of infection through mucosal surfaces is critical, therefore there can be considerable prevalence of lymphoid tissue at these surfaces. Extensive aggregates of lymphoid nodules, or Peyer's patches, were present in the ileum of lambs in the present study. Peyer's patches play a critical role in the intestinal immunological system (Tizard, 1996), but can cause alterations of the mucosal surface, thus altering villus height (Frappier, 1998). Therefore, although samples were collected from the ileum for morphometric observations, measurements were not taken.

The jejunum is the site where most nutrients are absorbed (Pond et al., 1995). In the present study, the jejunum had the longest and widest villi, which indicates an increased surface area available for nutrient absorption. Additionally, numeric increases in villus height were accompanied by linear increases in villus width in both the duodenum and jejunum, but the differences observed in the ratio of villus height to villus width were not significant. These results indicate that the villi of the small intestine tended to increase proportionally with age. Increased villus height is contradictory to previously reported results. Moon and Joel (1975) reported that villi of newborn lambs were longer than those of sheep at 3 wk, 3 mo, and > 1 yr of age, and the authors stated

that all animals were clinically normal. However, the authors failed to mention if the lambs had been weaned. This may be of interest because in the present study, lambs that did not receive the creep diet had longer villi than lambs that received the creep diet.

The crypts of Leiberkuhn act as a progenitor region for undifferentiated cells (Klein, 1989). In the present study, crypt depth increased with age in conjunction with increasing villus height and villus width, particularly in the jejunum where the largest villi were located. This may indicate that the depth of the crypt may be important in determining the ability of the crypt to support increases in villus height and width, as well as maintain villus structure. Moon and Joel (1975) reported that crypt depth was lowest in newborn lambs and increased during the first few weeks postnatal and that the greatest ration of villus height to crypt depth was in the jejunum, as found in our study.

Kelly et al. (1990) reported that creep feeding pigs did not alter the mucosal structure or function of the small intestine. However, results from the present study indicate that lambs receiving the creep diet had shorter villi than lambs that were only nursing. Therefore, it appears that creep feeding may have slightly altered the mucosal structure of the small intestine.

The mucosal surface of the rumen is covered by many papillae that vary in size from region to region. In sheep, these papillae began to develop at 100 d of fetal life, and by 8 wk postnatal extensive development was observed in papillary height and width (Wardrop, 1961a). In the present study, papillary height and width were observed to increase linearly with age. This indicates that age was a factor in the development of ruminal papillae. Lambs that received the creep diet tended to have longer papillae than lambs that did not receive that creep diet. The stimulation for papillary development

occurs in parallel with the establishment of ruminal fermentation and the resulting production of VFA. Creep feeding stimulates the hydrolysis of carbohydrates and protein to ammonia and VFA in the rumen (Poe, 1971). However, data from the present study do not indicate a diet effect on papillary development. It is possible that this may be a reflection of the lower than anticipated level of creep intake (Table 3). Numerically, papillae in the ventral rumen of lambs receiving the creep diet were longer than those of lambs not receiving the creep diet. In addition, the height of papillae in the dorsal rumen appeared to level off by 8 wk of age. It is possible that the greater exposure to digesta in the ventral rumen provided a greater stimulation for papillary growth. The ratio of papillae height to papillae width increased linearly with age, primarily in the ventral rumen. This indicates that the stimulation for papillary growth is greater for height than width, and the response was greater in the ventral than in the dorsal rumen.

Wardrop (1961b) suggested that depth of the keratinized epithelial cell layer is determined by the amount of mechanical wear that occurs against the papillae. In adult sheep, this is most prominent in the center of the dorsal and ventral rumen. The greater number of total and keratinized epithelial cells observed in lambs not receiving the creep diet was more prominent in the rumen than in the omasum. This indicates that the ruminal papillae are subject to more mechanical wear from digesta. Additionally, in lambs not receiving the creep diet, little to no milk would have entered the rumen as a result of the reticular groove. Therefore, the lack of mechanical wear from digesta resulted in the number of total and keratinized cells to be greater in lambs not receiving the creep diet. In addition, the number of keratinized cells decreased as age increased. This likely indicates that increasing intake produced additional wear on the keratinized

cell layer. These results therefore indicate that the keratinized epithelial cell layer is important in protection of the papillae against wear from the digesta. Additionally, the cell thickness of this layer appears to be related to the amount of solid feed consumption. The decline in nonkeratinized epithelial cell number with age in the dorsal rumen and omasum is a reflection of a change in the cell turnover rate.

Abundance of oPepT1 mRNA. The size of the mRNA transcript that hybridized to the oPepT1 cDNA probe (Pan et al., 2001), 2.8 kb, is in agreement with previous research from our laboratory (Chen et al., 1999). Data from the present study are also in keeping with previous observations in our laboratory showing the greatest abundance of oPepT1 mRNA in the jejunum (Chen et al., 1999). The abundance of oPepT1 was greatest in the jejunum and dorsal rumen for the small intestine and stomach, respectively. For all of the northern blots, equal quantities of RNA were loaded onto the gels. There was, however, a slightly greater recovery of RNA from intestinal epithelium than from stomach tissues (191 vs 156 μg/0.25 g tissue, respectively). For cross-tissue comparisons, oPepT1 mRNA abundance must be adjusted for this difference. With this adjustment, it still appears that the jejunum is the primary site for peptide absorption. It must be kept in mind that RNA was extracted from whole tissue samples from stomach sections and from epithelial scrapings in the intestine. In the small intestine, PepT1 has been localized in the brush border membrane of the enterocyte (Ogihara et al., 1996). If oPepT1 is localized in stomach epithelium, then relative abundance may be quite higher in stomach epithelial cells than in intestinal epithelial cells. Additionally, if abundance of oPepT1 mRNA is indicative of oPepT1 protein expression, then it is likely that the jejunum is the predominant site for peptide absorption in the intestine of lambs up to 8 wk of age. For

lambs that only nurse, the dorsal rumen is likely the predominant site of peptide absorption in the stomach.

Rubino and Guandalini (1977) originally reported age-dependent differences in intestinal peptide transport in rabbits. Uptake of glycyl-L-proline increased from 25 d gestation and peaked shortly after birth. Uptake then remained constant for 6 d postnatal, declined steadily, and reached adult levels by 50 d. More recently, abundance of PepT1 mRNA was reported to be greatest at 4 d of age in the jejunum of rats, and then decreased until reaching adult levels by 28 d of age (Miyamoto et al., 1996). Shen et al. (2001) reported PepT1 mRNA abundance was maximal in all segments of the small intestine 3 to 5 d after birth. In the present study, sampling times did not begin until 2 wk of age, but the mRNA transcript that hybridized to our probe was present in all tissues studied by that time. These data provided evidence for the existence of peptide transport proteins in the tissues studied, as well as providing evidence for peptide transport being a physiologically important process in the young lamb.

Abundance of oPepT1 mRNA for lambs not receiving the creep diet was greater in the rumen, but abundance of oPepT1 mRNA was relatively constant between diets in the omasum. In the case of the rumen, it is likely that the stimulation for the development of the oPepT1 is not coming from the luminal side. Due to the reticular groove, little to no milk should enter the rumen, thus providing the transporter with little to no substrate. Additionally, abundance of oPepT1 was also greater in the dorsal rumen, which has the least exposure to digesta of the two areas sampled in the rumen. Therefore, it seems more likely that a stimulus from another direction, possibly blood-borne, may be involved in the ontogenesis of oPepT1, particularly in the rumen.

In conclusion, creep feeding appears to have slightly altered the mucosal structure of the small intestine. However, it did provided an advantage to stimulating papillary growth and thus, predisposing the rumen for the introduction of feed into the diet. A 2.8 kb oPepT1 mRNA was present in all tissues studied by 2 wk of age, although age did not significantly influence the abundance of oPepT1 mRNA in the small intestine of stomach. In the stomach, particularly the rumen, lambs that did not receive the creep diet had a greater abundance of oPepT1 mRNA. Thus, it appears that a stimulus from the non-luminal direction, possibly blood-borne, may be involved in the ontogenesis of oPepT1. If mRNA abundance is indicative of oPepT1 protein expression, it is likely that the jejunum is the predominant site of peptide transport in the small intestine of lambs up to 8 wk of age. For lambs that only nurse, the dorsal rumen is likely the predominate site of peptide transport in the stomach. Together these results indicate that peptide transport is a physiologically important process in the young lamb and that the rumen is involved in peptide transport, particularly in nursing lambs up to 8 wk of age.

Implications

To our knowledge, the present study was the first to report data on the ontogenesis of a peptide transporter in a ruminant species. The results obtained will contribute to future investigations into the developmental regulation of peptide transporters. Studying the ontogenesis of peptide transporters will provide us with information that will contribute to understanding the regulation of peptide transport. With this information, we may be able to develop nutritional strategies that will allow us to better formulate diets to enhance animal production and efficiency.

Table 1. Ingredient composition of diets.

	Diet	
Item	Ewes	Lambs
Ingredient, % DM		
Alfalfa hay	34.0	---
Ground corn	---	36.5
Cracked corn	30.0	---
Orchardgrass hay	20.0	---
Dehydrated alfalfa meal	---	20.0
Soy mill feed	---	15.0
Soybean meal (44% CP)	8.0	10.0
Wheat midds	---	10.0
Molasses	5.0	---
Distillers dried grain w/ soluables	---	4.5
Ground limestone	---	1.0
Dicalcium phosphate	---	0.5
Plain salt	0.5	1.0
Ammonium chloride	---	0.5
Cocci control (1X) crumbles [a]	2.5	---
Pellet binder	---	0.6
Aureo 50	---	0.05
Premix[b, c]	0.3	0.5

[a] Southern States, Richmond, VA
[b] Akey Inc., Lewisburg, OH
[c] Premix contained (as fed): Ca, 13.3 %; Na, 0.0198%; K, 0.0198%; S, 1.4%; Zn, 20 g/kg; Fe, 8.0 g/kg; Mn, 20.0 g/kg; 0.25 g/kg; Co, 0.16 g/kg; Se, 0.21 g/kg; Vitamin A, 3319 KIU/kg; Vitamin D, 662 KIU/kg; Vitamin E, 13 KIU/kg; Menadione, 73 mg/kg; Vitamin B12, 2640 mcg/kg; Riboflavin, 440 mg/kg; Pantothenic acid, 1760 mg/kg; Niacin, 2640 mg/kg.

Table 2. Chemical composition of diets.

	Diet	
Item	Ewes	Lambs
Chemical composition, %		
DM	91.1	91.0
CP	12.4	13.9
ADF	21.3	15.5
NDF	37.8	27.9

Table 3. Dry matter intake of ewes and lambs

Animal	Diet[b]	Period[a]				
		1[c]	2	3	4	5
		DMI, $g \cdot h^{-1} \cdot d^{-1}$				
Lambs	C	2	22	129	219	227
Lambs	NC	----	----	----	----	----
Ewes	C	2,392	2,655	2,674	2,757	2,737
Ewes	NC	2,576	2,769	2,447	2,858	2,218

[a]1 = 03/09/00 to 04/05/00; 2 = 03/23/00 to 04/19/00; 3 = 04/06/00 to 05/03/00; 4 = 04/20/00 to 05/16/00; 5= 05/04/00 to 05/06/00

[b]C=Creep; NC=No Creep

[c]Average ages of lambs: 1 = 11.6 d; 2 = 13.3 d; 3 = 20.2 d; 4 = 37.9 d; 5 = 52.3 d

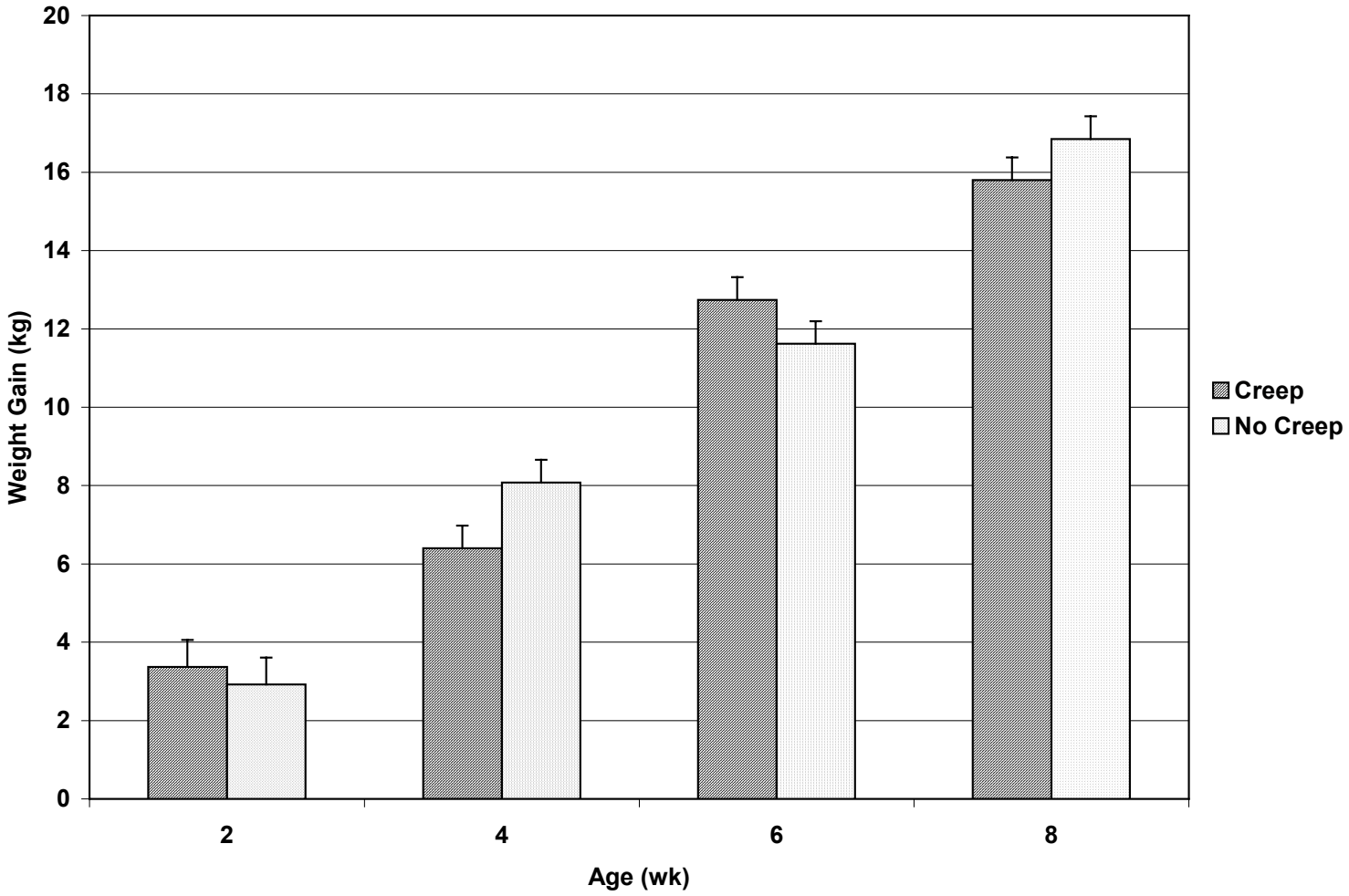


Figure 1. Weight gain (kg) of lambs sampled during the first 8-wk postnatal. Results are expressed as means ± SE, n = 3, 2 wk; n = 4, 4 wk, 6 wk, 8 wk. Linear age effect, $P < 0.0001$.

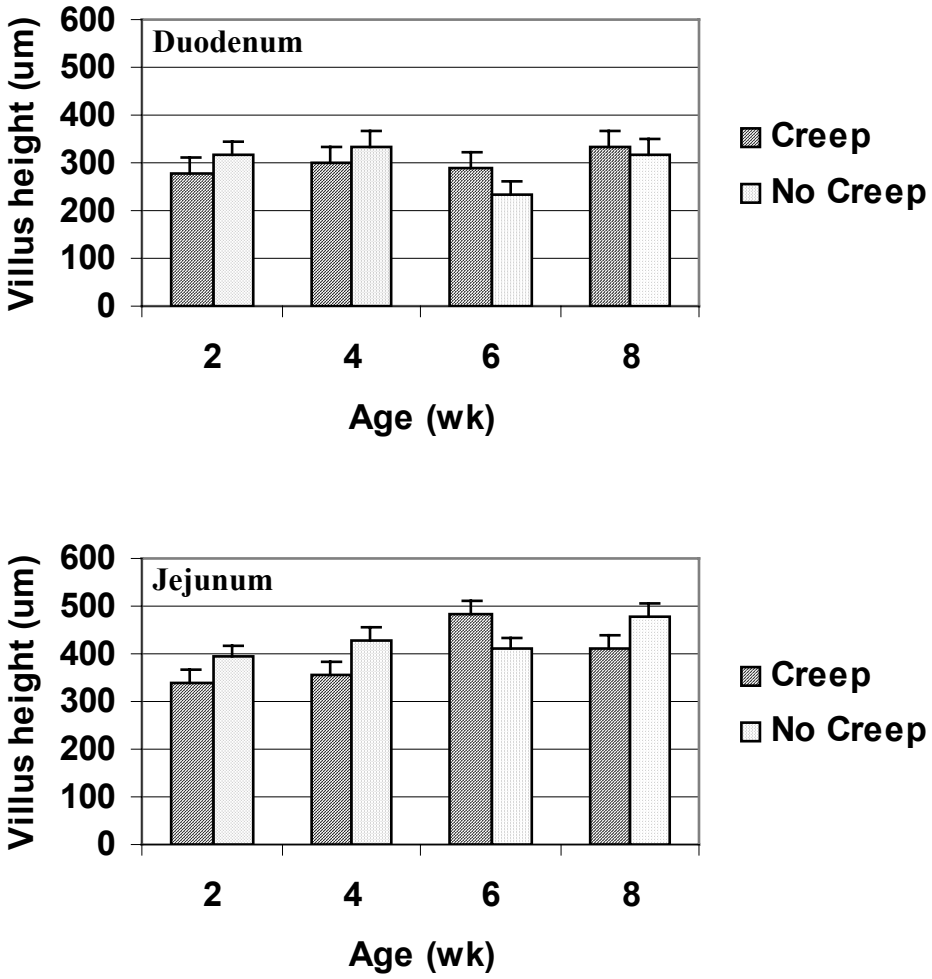


Figure 2. Measurements of villus height in the small intestine of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact, well oriented, and transversely cut villi, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera, DMCIe (Polaroid, Cambridge, MA). Measurements of villi were made using Sigmascan Pro 5.0 (SSPS, Inc., Chicago, IL). Results are expressed as means ± SE, n = 4. Duodenum differed from jejunum, $P < 0.0001$. Diets differed, $P < 0.09$.

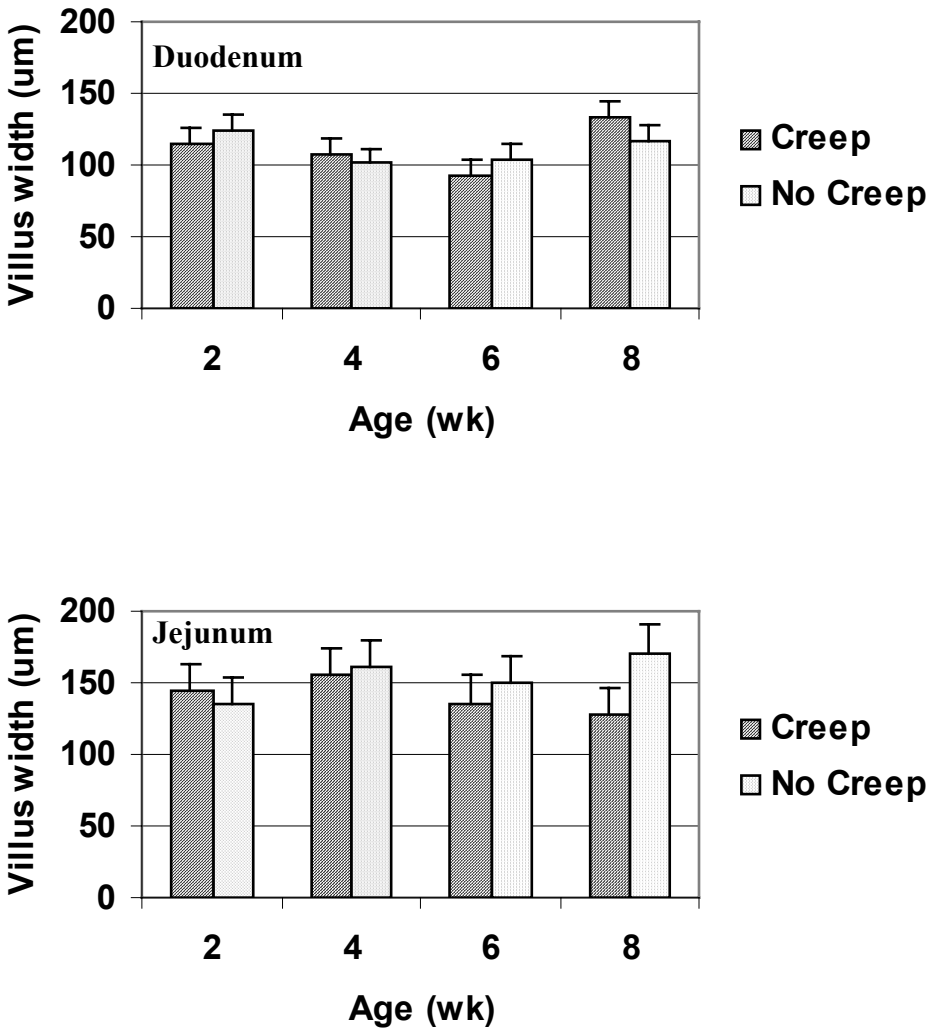


Figure 3. Measurements of villus width in the small intestine of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact, well oriented and transversely cut villi, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera, DMCIe (Polaroid, Cambridge, MA). Measurements of villi were made using Sigmascan Pro 5.0 (SSPS, Inc., Chicago, IL). Results are expressed as means ± SE, n = 4. Duodenum differed from jejunum, $P < 0.0001$. Linear age effect, $P < 0.02$. Tissue*age interaction, $P < 0.01$

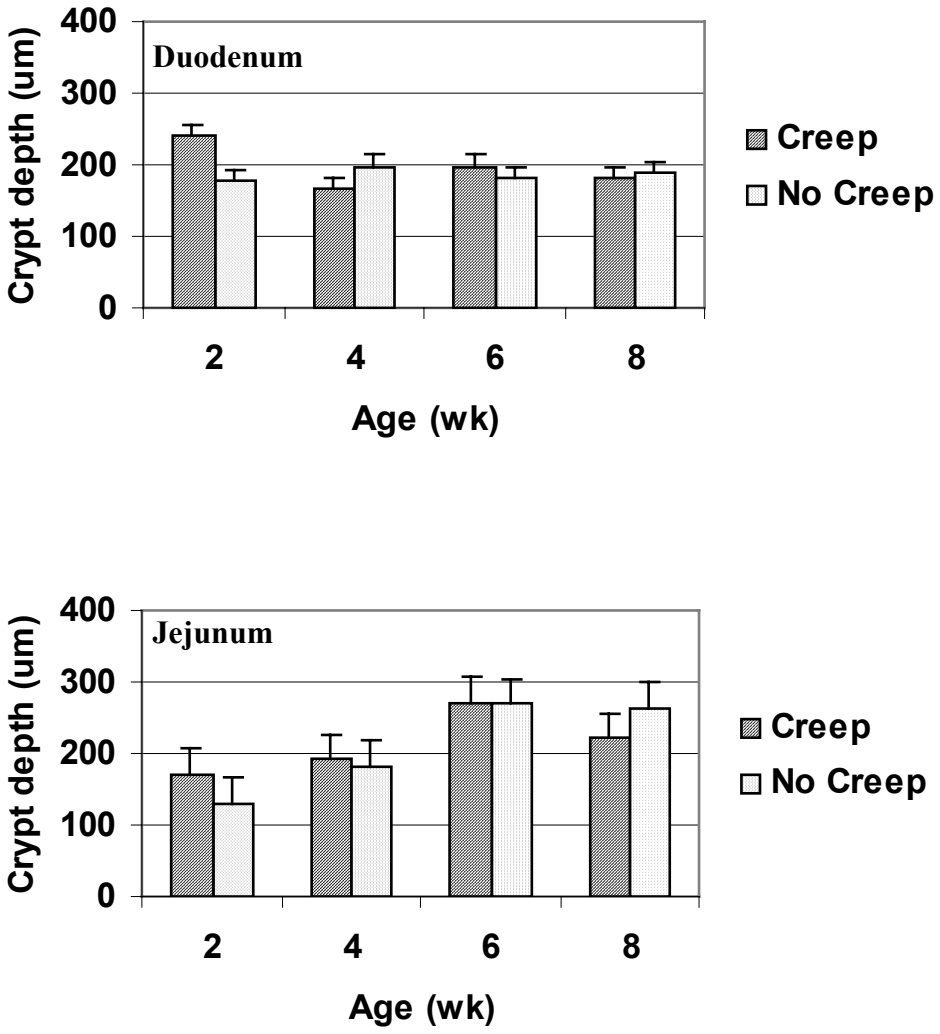


Figure 4. Measurements of crypt depth in the small intestine of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact and transversely cut crypts, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera, DMCIe (Polaroid, Cambridge, MA). Measurements of crypts were made using Sigmascan Pro 5.0 (SSPS, Inc., Chicago, IL). Results are expressed as means ± SE, n = 4. Linear age effect, $P < 0.04$. Tissue*age interaction, $P < 0.02$.

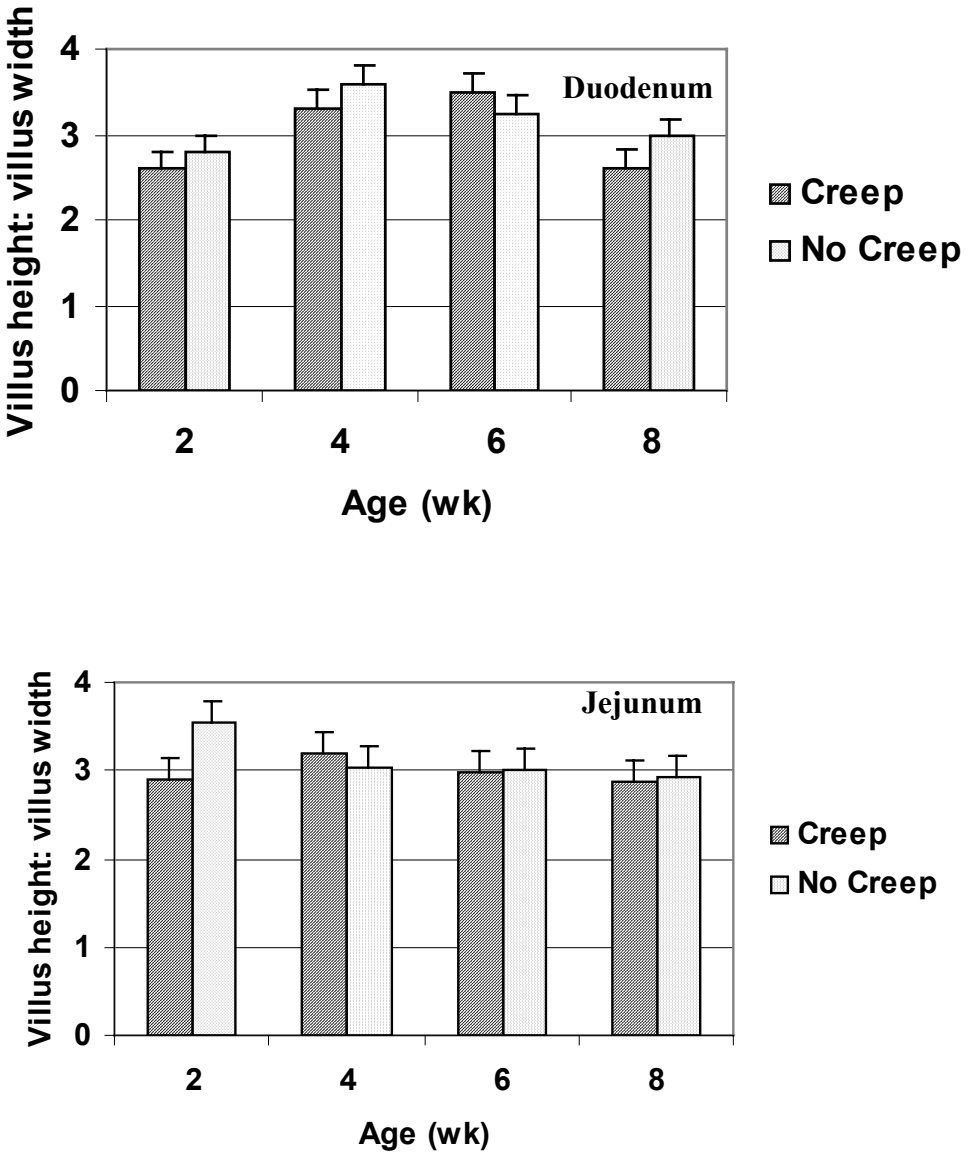


Figure 5. Measurements of villus height:villus width in the small intestine of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact, well oriented, and transversely cut villi, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera, DMCIe (Polaroid, Cambridge, MA). Measurements of villi were made using Sigmascan Pro 5.0 (SSPS, Inc., Chicago, IL). Results are expressed as means ± SE, n = 4. Tissue*age interaction, $P < 0.07$.

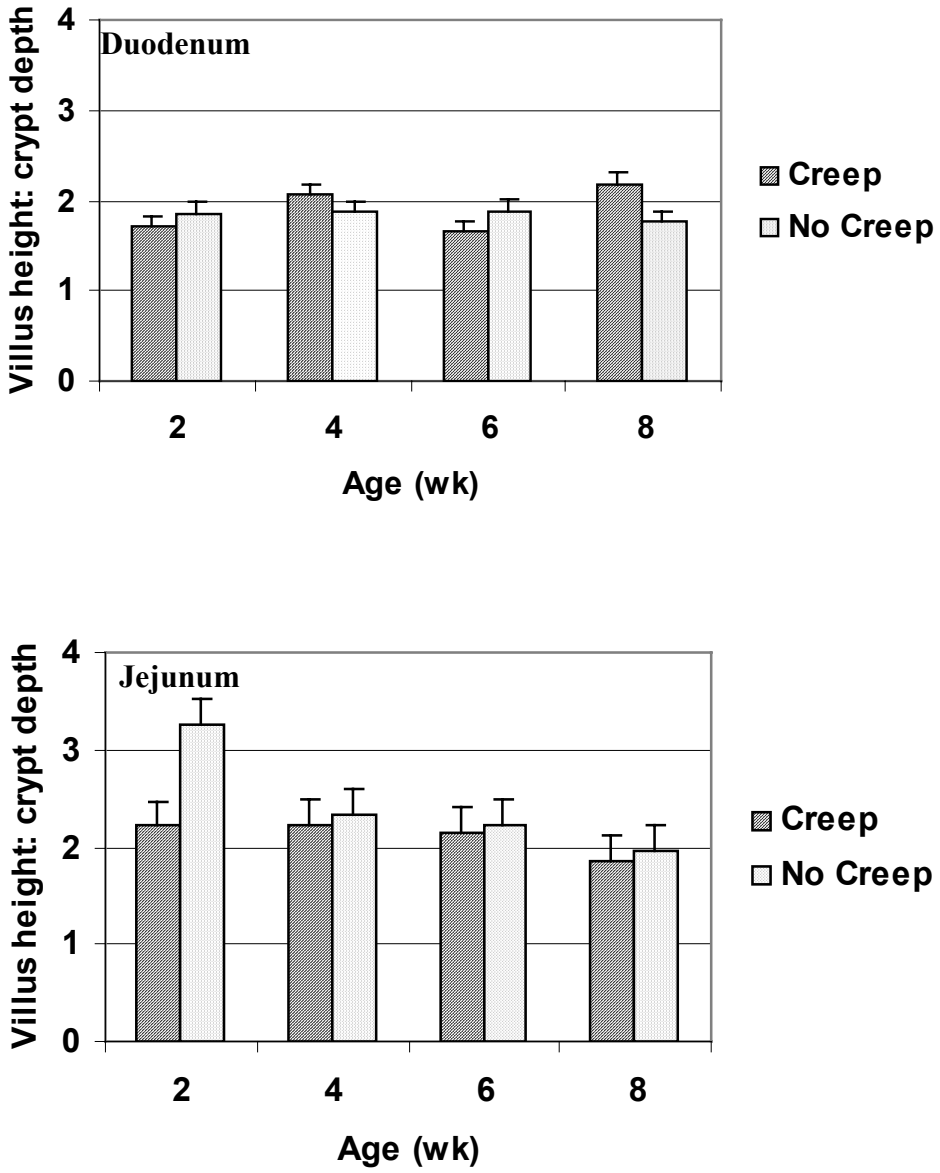
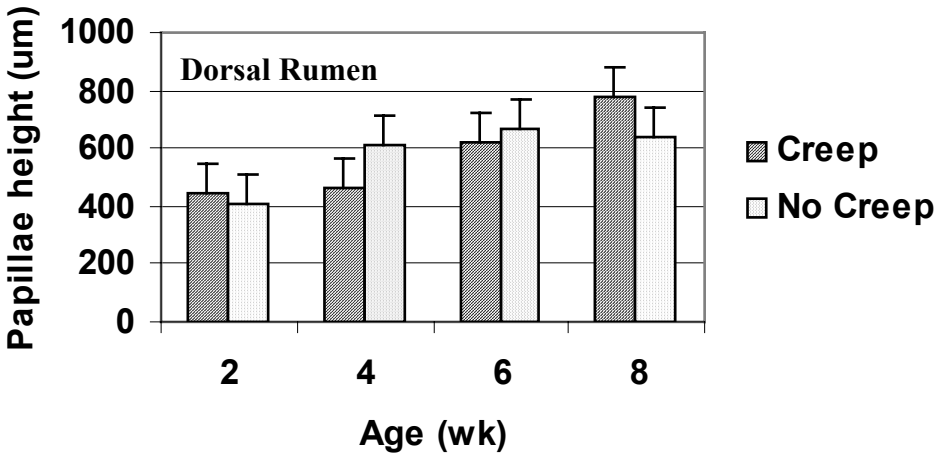


Figure 6. Measurements of villus height:crypt depth in the small intestine of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact, well oriented, and transversely cut villi and crypts, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera, DMCIe (Polaroid, Cambridge, MA). Measurements of villi and crypts were made using Sigmascan Pro 5.0 (SSPS, Inc., Chicago, IL). Results are expressed as means ± SE, n = 4. Duodenum differed from jejunum, $P < 0.0005$. Tissue*diet interaction, $P < 0.07$. Tissue*age interaction, $P < 0.02$. Diet*age interaction, $P < 0.09$.



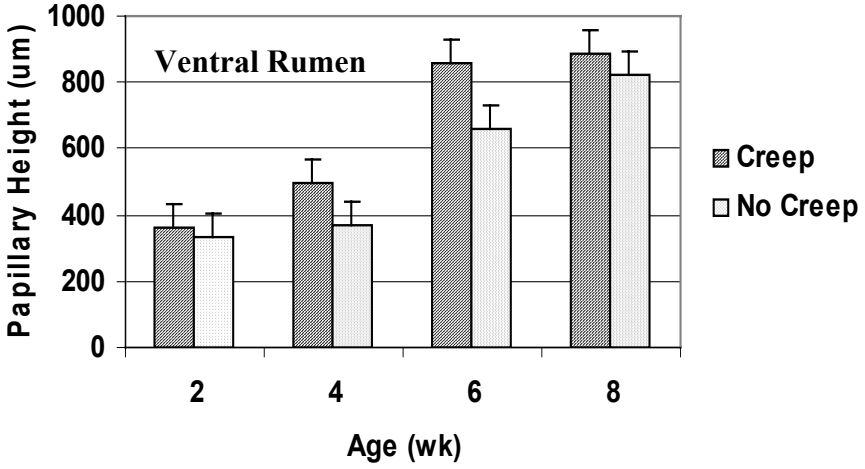


Figure 7. Measurements of papillae height in the rumen of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact, well oriented, and transversely cut papillae, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera DMCIe (Polaroid, Cambridge, MA). Measurements of papillae were made using Sigmascan 5.0 (SSPS, Inc, Chicago, IL). Results are expressed as means ± SE, n = 4. Linear age effect, $P < 0.0001$. Tissue*age interaction, $P < 0.08$.

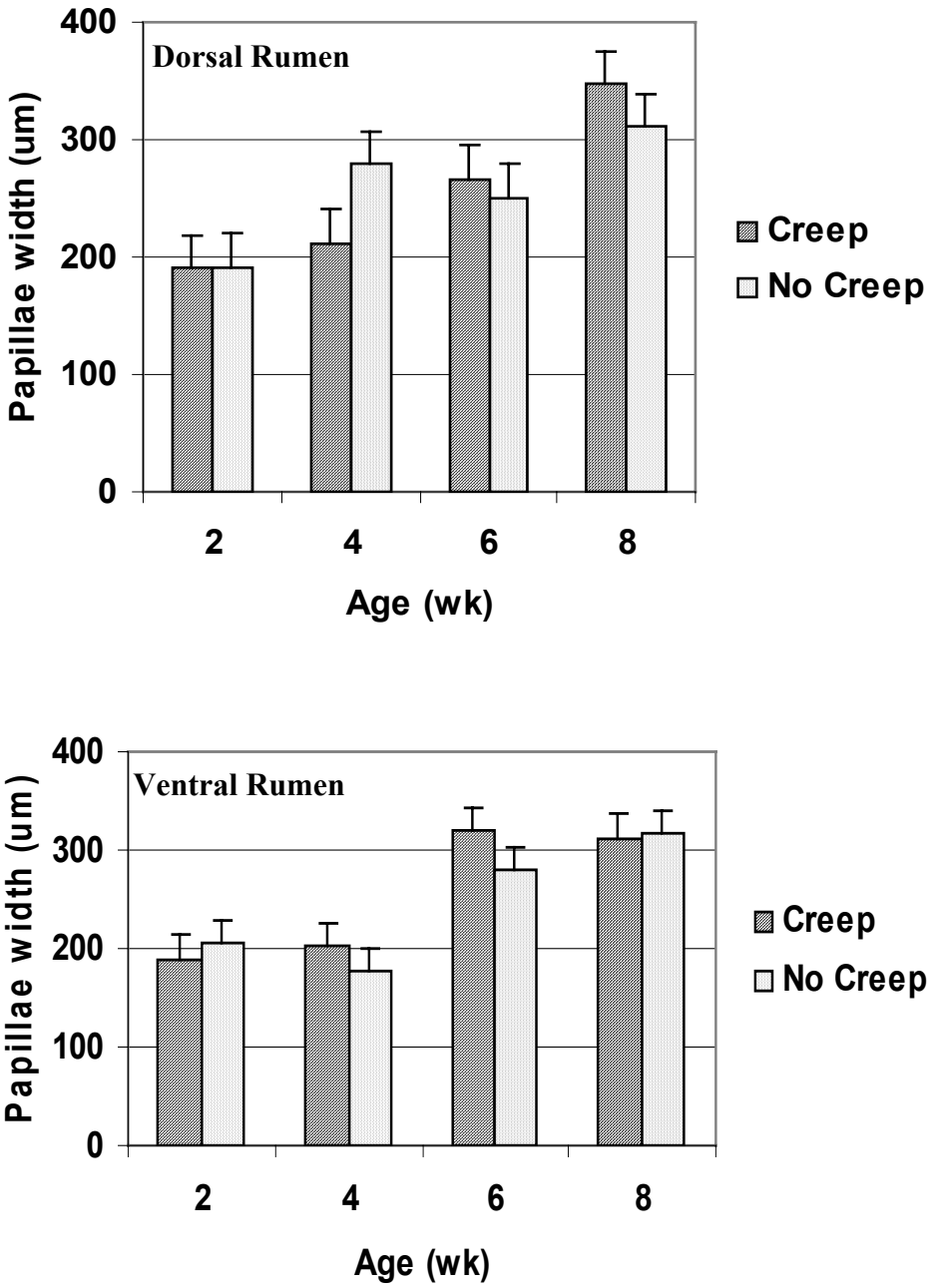


Figure 8. Measurements of papillae width in the rumen of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact, well oriented, and transversely cut papillae, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera DMCIe (Polaroid, Cambridge, MA). Measurements of papillae were made using Sigmascan 5.0 (SSPS, Inc, Chicago, IL). Results are expressed as means ± SE, n = 4. Linear age effect, $P < 0.0001$. Tissue*age interaction, $P < 0.10$.

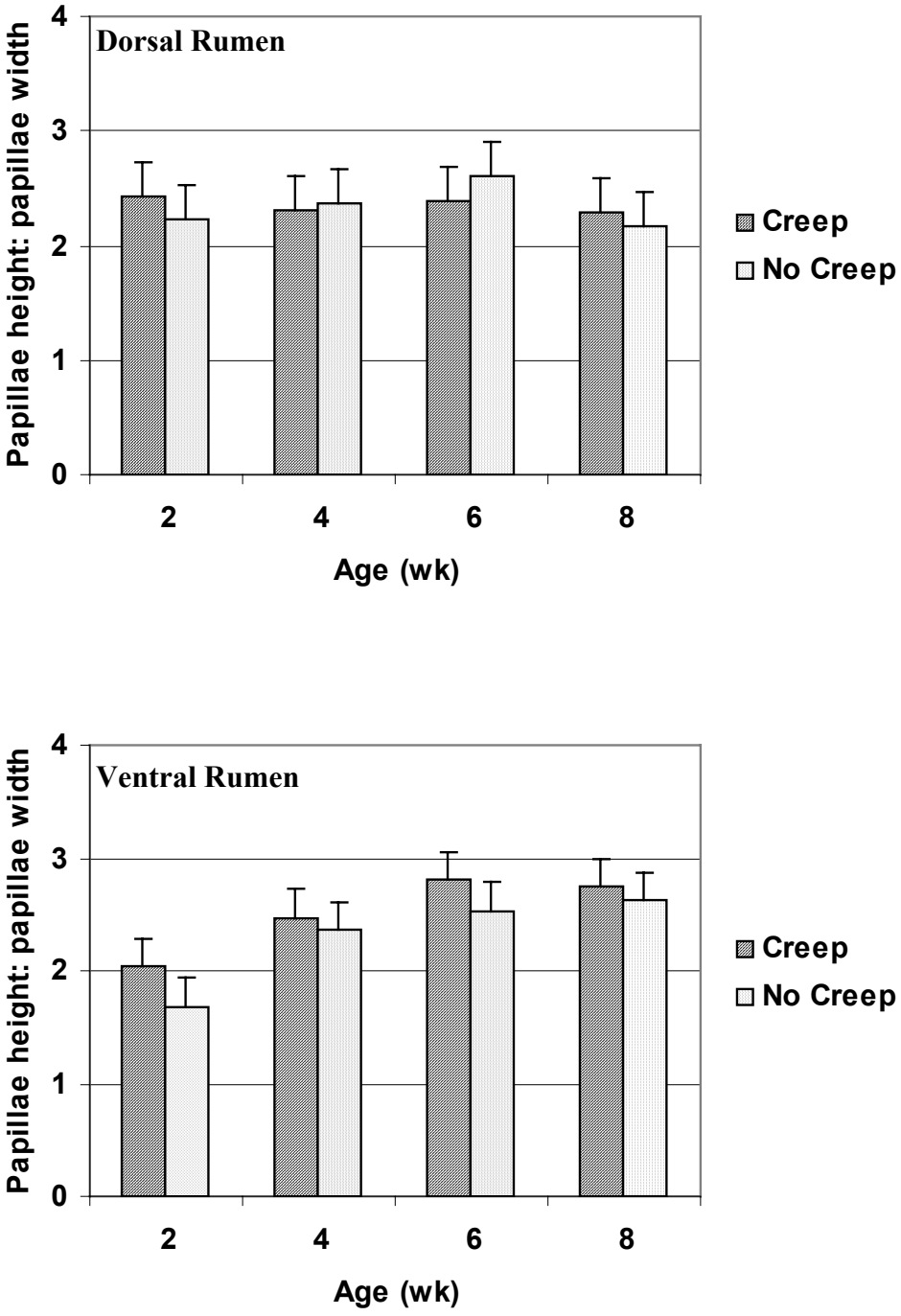


Figure 9. Measurements of papillae height:papillae width in the rumen of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Digital images of ten intact, well oriented, transversely cut papillae, selected at random, were obtained at 10X with a Polaroid Digital Microscope Camera DMCIe (Polaroid, Cambridge, MA). Measurements of papillae were made using Sigmascan 5.0 (SSPS, Inc, Chicago, IL). Results are expressed as means ± SE, n = 4. Linear age effect, $P < 0.04$. Tissue*age interaction, $P < 0.10$.

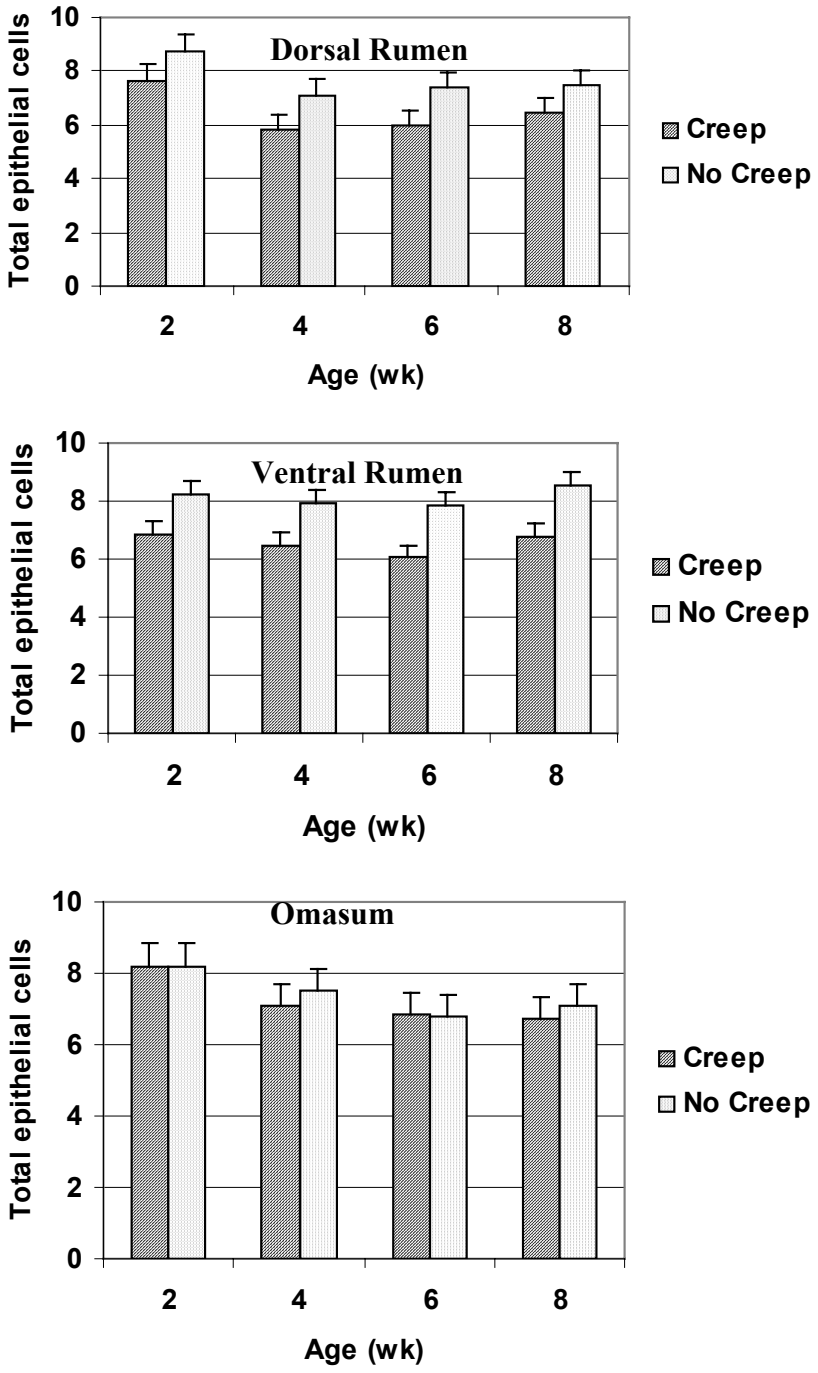


Figure 10. Total epithelial cell counts from ruminal and omasal papillae of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Ten intact and well oriented papillae were selected at random, and epithelial cells were counted at 400X under a light microscope. Results are expressed as means ± SE, n = 4. Diets differed, $P < 0.0002$. Linear age effect, $P < 0.03$. Tissue*diet interaction, $P < 0.07$.

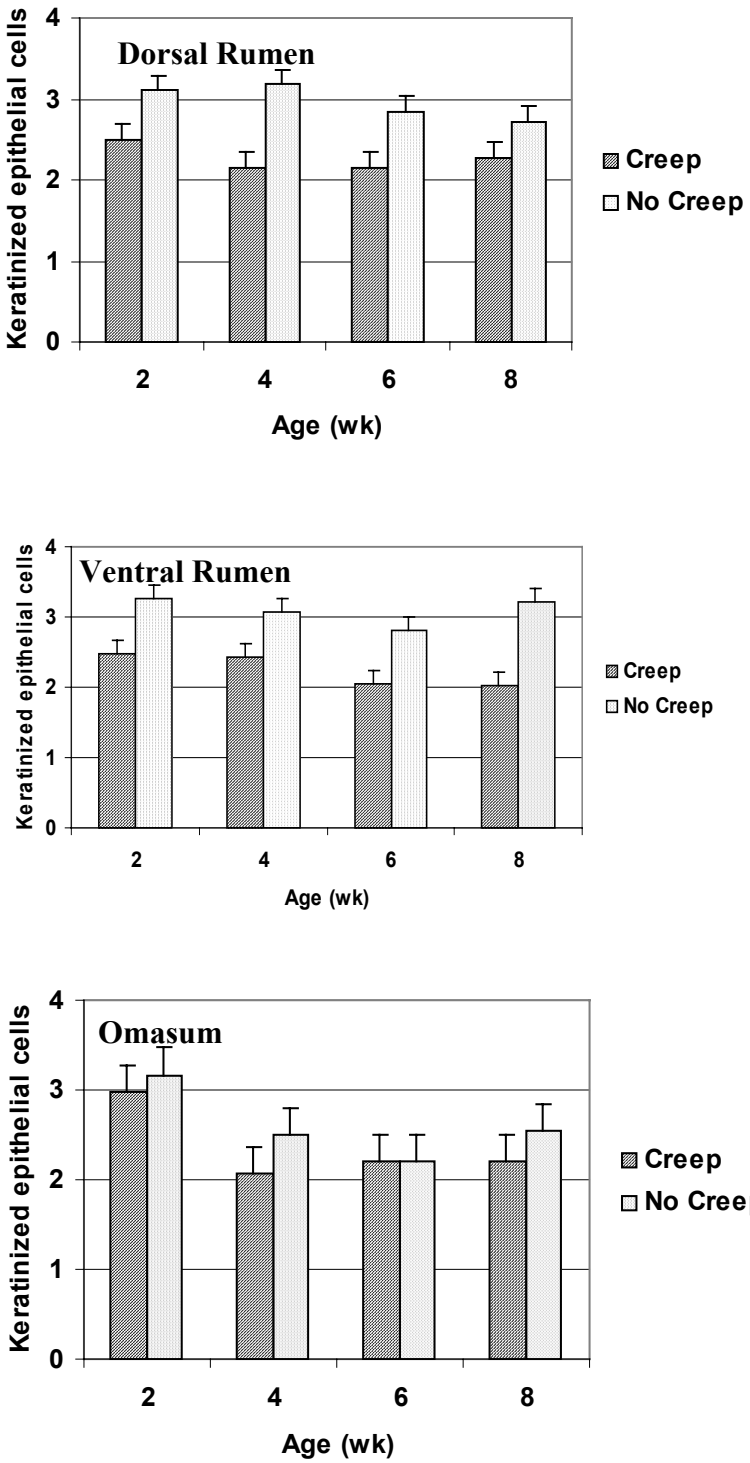


Figure 11. Keratinized epithelial cell counts from ruminal and omasal papillae of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Ten intact and well oriented papillae were selected at random, and epithelial cells were counted at 400X under a light microscope. Results are expressed as means ± SE, n = 4. Diets differed, $P < 0.0001$. Linear age effect, $P < 0.004$. Tissue*diet interaction, $P < 0.07$.

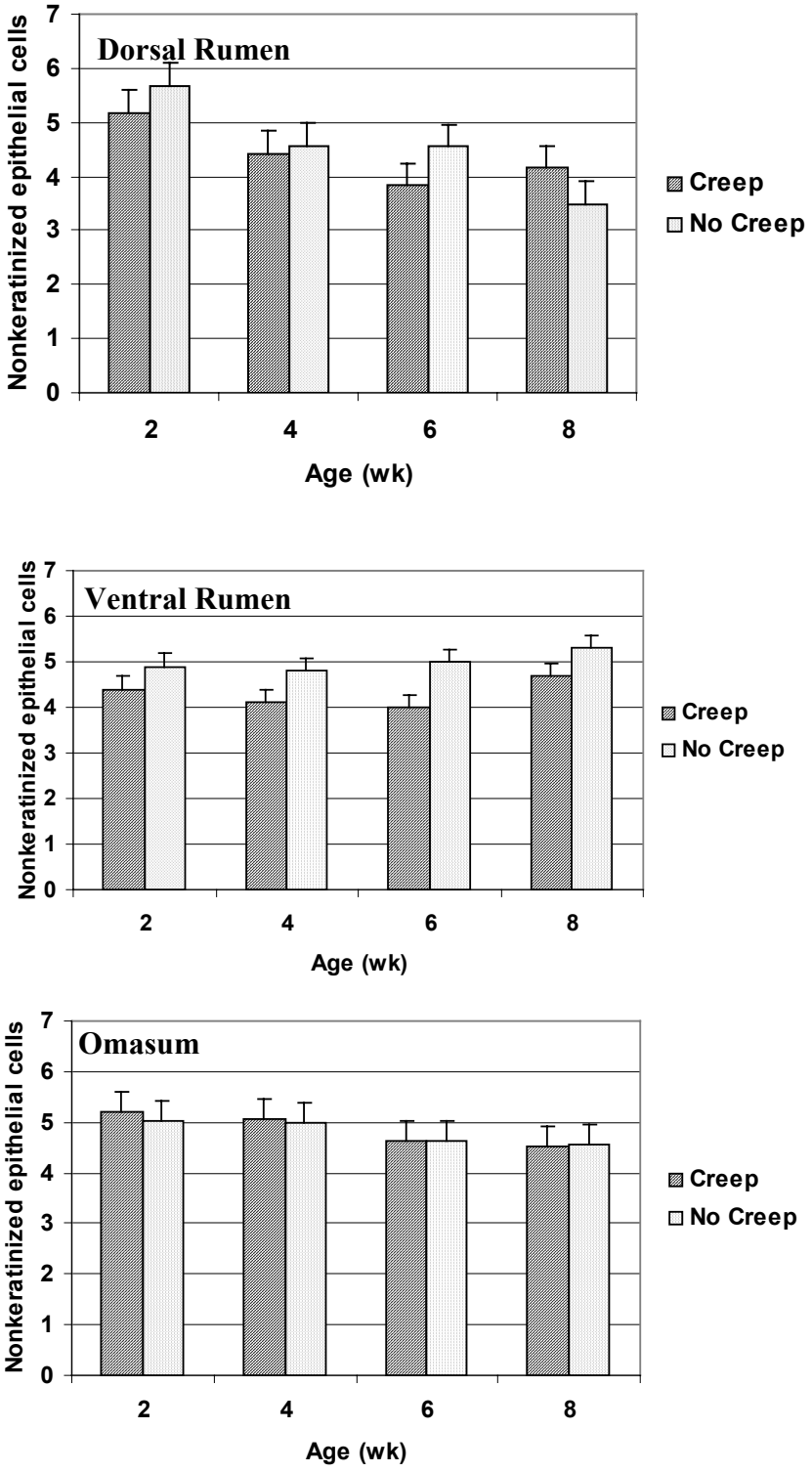


Figure 12. Nonkeratinized epithelial cell counts from ruminal and omasal papillae of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Ten intact and well oriented papillae were selected at random, and epithelial cells were counted at 400X under a light microscope. Results are expressed as means ± SE, n = 4. Diets differed, $P < 0.08$. Linear age effect, $P < 0.005$. Tissue*age interaction, $P < 0.03$.

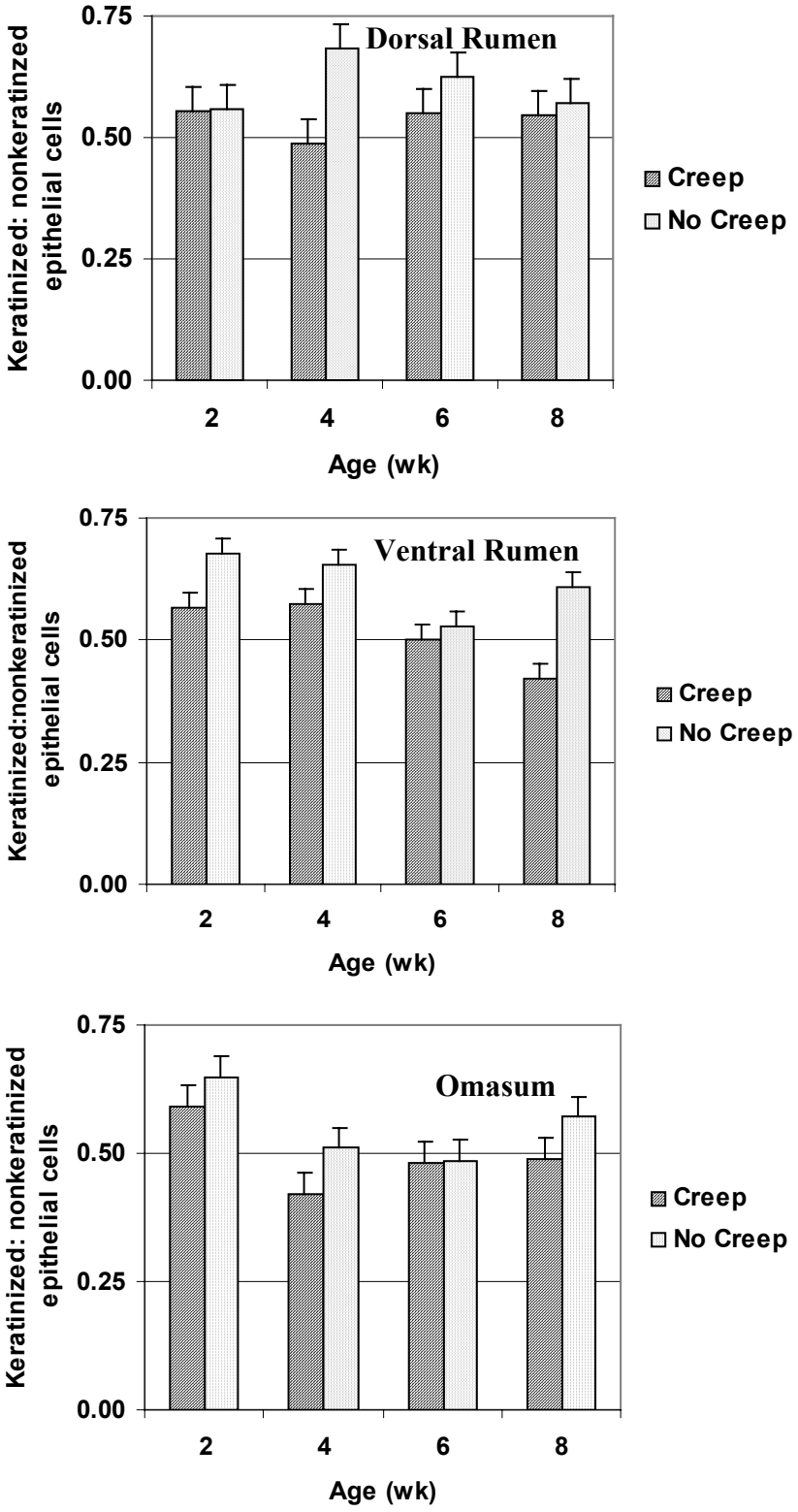


Figure 13. Keratinized:nonkeratinized cell ratios from ruminal and omasal papillae of lambs sampled during the first 8-wk postnatal. Tissue was prepared and stained with hematoxylin and eosin by routine methodology (Bancroft and Stevens, 1990). Ten intact and well oriented papillae were selected at random, and epithelial cells were counted at 400X under a light microscope. Results are expressed as means ± SE, n = 4. Diets differed, $P < 0.001$. Linear age effect, $P < 0.001$. Tissue* age interaction, $P < 0.04$.

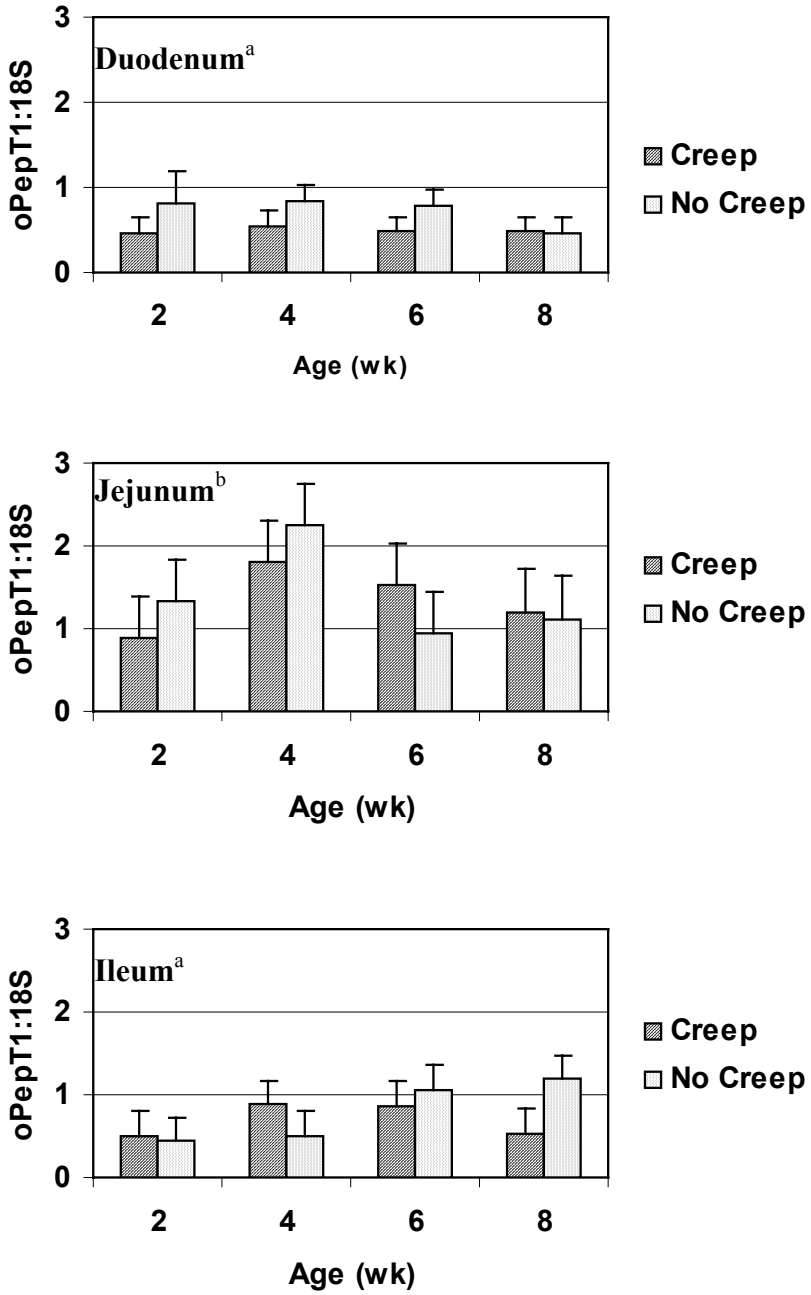


Figure 14. Abundance of oPepT1 mRNA small intestinal epithelium of lambs sampled during the first 8-wk postnatal. Abundance of oPepT1 mRNA was determined by hybridization with a full length oPepT1 cDNA as a probe. The same membranes were rehybridized with radiolabelled 18S as a reference probe. Results are expressed as means ± SE, n = 4. [a,b]Tissues lacking common subscripts differ, $P < 0.0007$.

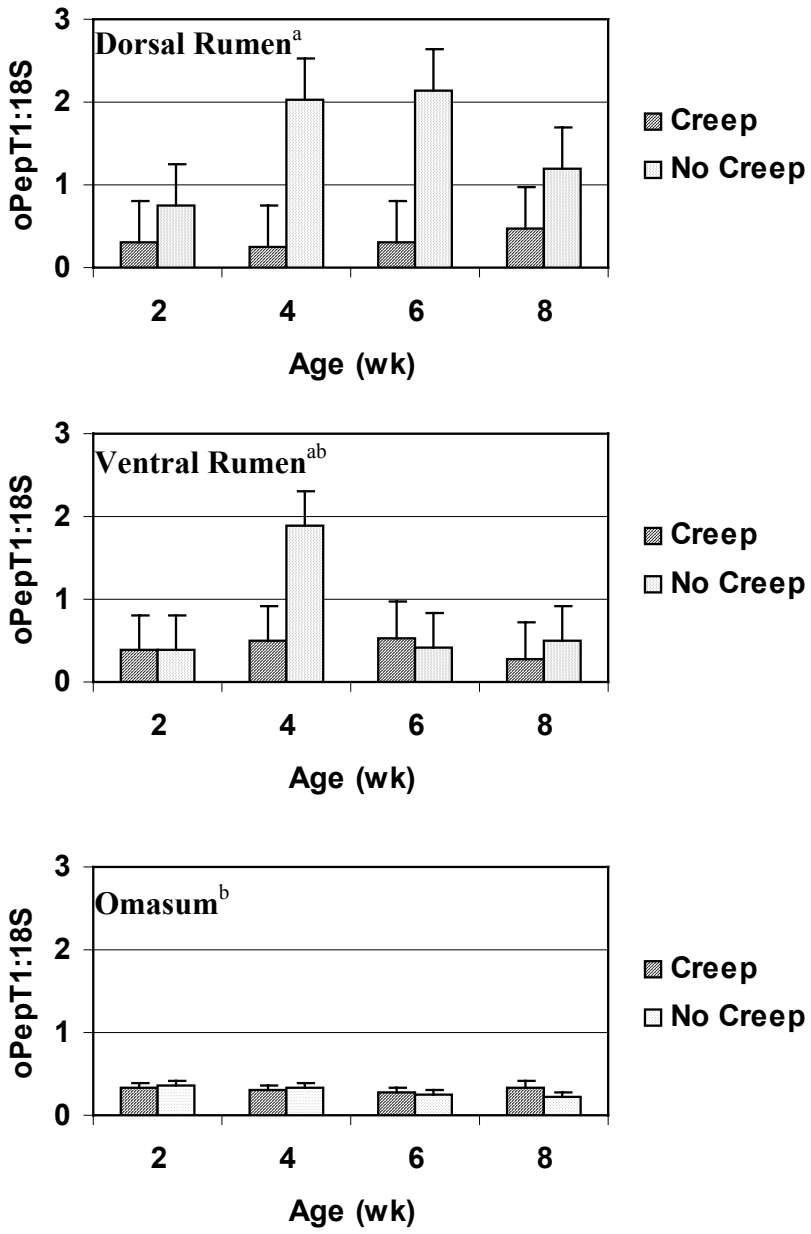


Figure 15. Abundance of oPepT1 mRNA in forestomach epithelium of lambs sampled during the first 8-wk postnatal. Abundance of oPepT1 mRNA was determined by hybridization with a full length oPepT1 cDNA as a probe. The same membranes were rehybridized with radiolabelled 18S as a reference probe. Results are expressed as means ± SE, n = 4. [a, b] Tissues lacking common superscripts differ, $P < 0.01$. Diets differed, $P < 0.004$. Tissue*diet interaction, $P < 0.02$.

Chapter IV Epilogue

If abundance of oPepT1 mRNA is indicative of expression the oPepT1 protein, the present study provides evidence for peptide transport being a physiologically important process in the young lamb. If this is the case, the next logical step in studying the ontogenesis of oPepT1 will be to investigate the age at which this transporter begins to be expressed. Initial appearance of nutrient transporters varies among species (Buddington and Diamond, 1989). In rats, PepT1 mRNA and protein was present in the small intestine beginning at 17 d of embryonic life (Shen et al., 2001). Recently, it was suggested that the colon is a physiologically important tissue in peptide transport early in life (Shen et al., 2001). It will therefore be critical to analyze both mRNA abundance and protein expression of the colon, in addition to the small intestine and forestomach, if ontogenic studies of PepT1 are going to continue.

The half-life of mRNA is a balance between the rate of transcription and intracellular degradation. Regulation of PepT1 has been reported to be transcriptional (Thamtharan et al., 1999; Shen et al., 2001). Data in the jejunum from the present study may indicate that the transcription of oPepT1 is induced during the first few weeks postnatal. It is possible that the act of suckling or something in the milk may be inducing transcription of oPepT1 mRNA. Nuclear run-off transcription assays would allow us to test this hypothesis by measuring the rates of transcription (Rapley and Manning, 1998). In this assay, nuclei are isolated from tissue or cultured cells. Elongation of nascent mRNA chains is conducted in the presence of ribonucleotides, one of which is radiolabelled. Total nuclear RNA, containing the newly synthesized and labeled RNA, is then extracted. Next, hybridization is conducted with the appropriate cDNA probe. The membrane is then washed, and bound mRNA are hydrolyzed

from the membrane and counted in a scintillation counter. If oPepT1 transcription is being induced, we would expect to observe greater nuclear transcription rates in younger animals.

The effects of weaning and early weaning on oPepT1 mRNA abundance and protein expression might be another step in the study of the ontogenesis of oPepT1. At the time of weaning in rats, PepT1 mRNA abundance and protein level increased, particularly in the ileum (Shen et al., 2001). Although lambs were not weaned in the present study, data indicate that lambs that were not allowed access to creep feed had increased PepT1 mRNA abundance in the duodenum and dorsal rumen. If this is the case, investigating the affects of weaning and early weaning will be particularly important in the duodenum and dorsal rumen.

In the future, dietary regulation of oPepT1 needs to be further investigated. Data from this study indicate that dry matter intake and/or protein intake, and protein quality may affect mRNA abundance of oPepT1. Therefore, diets composed of protein sources varying in quality, specific blends of selected protein sources, and specific mixes of hydrolyzed peptides could be fed at varying levels to determine their effects on PepT1. Epithelial samples would then be collected from the rumen, omasum, duodenum, jejunum, ileum, and colon and analyzed for mRNA abundance and protein expression. These data would then provide insight into the dietary regulation of PepT1.

Data from our laboratory indicate the presence of peptide transport protein(s) in the forestomach and small intestine of sheep (Matthews et al., 1996; Pan et al., 1997; Chen et al., 1999). It will be important to localize the transport protein in these tissues. Although Ogihara et al. (1996) localized PepT1 protein to the brush border member of enterocytes in the duodenum, jejunum, and ileum; PepT1 protein has not been localized within the four strata of the rumen or omasum. Localizing PepT1 in the rumen and omasum will provide insight into the function of

peptide transport in these tissues. Our laboratory has recently obtained a rabbit anti-sheep PepT1 antibody raised against the 16-carboxy terminal amino acids of oPepT1. If able to detect PepT1 protein, these antisera could be used to detect the presence of oPepT1 protein in these tissues. Samples from the lambs in the present study were collected for immunoflourescence analysis. It will be particularly interesting to determine whether a lower villus height has an effect on the expression of oPepT1. The antibody would also be used to analyze oPepT1 protein in western blot analysis.

From our data, it is likely that the jejunum is the predominant site of peptide transport in the intestine of lambs up to 8 wk of age. For lambs that only nurse the dorsal rumen is likely the predominant site of peptide transport in the stomach. We have demonstrated that an oPepT1 mRNA transcript is present in the dorsal rumen, ventral rumen, omasum, duodenum, jejunum, and ileum by 2 wk of age indicating that peptide transport is a physiologically important process in the young lamb. These data will facilitate future research into the ontogenesis and dietary regulation of peptide transport.

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Appendix

Appendix Table 1. Morphometric measurements of duodenal tissue from individual sheep

				Morphometric measurements[a,b]				
ID[c]	Diet[d]	Block	Age[e]	VH:CD	VH:VW	VH	VW	CD
2310	C	1	2	2.05	3.40	0.31	0.09	0.16
2309	C	1	4	1.93	3.21	0.31	0.10	0.17
2304	C	1	6	1.73	3.92	0.31	0.09	0.19
2308	C	1	8	1.33	2.27	0.20	0.09	0.16
2307	NC	1	2	2.05	3.40	0.31	0.09	0.16
2303	NC	1	4	2.18	3.39	0.36	0.11	0.21
2301	NC	1	6	2.29	4.12	0.44	0.10	0.20
2302	NC	1	8	1.91	3.03	0.30	0.10	0.17
2317	C	2	2	1.43	2.40	0.22	0.10	0.16
2312	C	2	4	1.95	2.98	0.25	0.10	0.15
2311	C	2	6	1.64	4.06	0.24	0.06	0.18
2316	C	2	8	2.44	2.06	0.34	0.15	0.15
2313	NC	2	2	1.97	3.40	0.32	0.10	0.17
2318	NC	2	4	1.69	3.30	0.33	0.11	0.21
2306	NC	2	6	1.58	3.85	0.30	0.08	0.18
2314	NC	2	8	1.54	2.63	0.25	0.10	0.17
2325	C	3	2	1.37	1.90	0.23	0.13	0.44
2327	C	3	4	2.07	3.86	0.33	0.12	0.19
2326	C	3	6	1.53	2.58	0.29	0.12	0.21
2320	C	3	8	2.94	2.85	0.44	0.17	0.20
2323	NC	3	2	1.67	2.09	0.28	0.13	0.19
2321	NC	3	4	1.64	3.54	0.28	0.08	0.18
2319	NC	3	6	2.03	2.38	0.30	0.13	0.18
2322	NC	3	8	1.62	2.62	0.31	0.14	0.19
2328	C	4	2	1.98	2.66	0.36	0.14	0.19
2330	C	4	4	2.30	3.18	0.31	0.11	0.16
2336	C	4	6	1.73	3.41	0.33	0.10	0.20
2329	C	4	8	2.07	3.24	0.36	0.13	0.21
2334	NC	4	2	1.77	2.29	0.35	0.17	0.20
2333	NC	4	4	1.96	4.12	0.37	0.10	0.20
2337	NC	4	6	1.66	2.67	0.24	0.11	0.17
2335	NC	4	8	2.01	3.60	0.42	0.12	0.22

[a] All data are expressed as means of 10 individual observations
[b] VH:CD, villus height:crypt depth; VH:VW, villus height:villus width; VH, villus height, mm; VW, villus width, mm; CD, crypt depth, mm
[c] Individual animal identification
[d] C: creep; NC: no creep
[e] All ages expressed in weeks

Appendix Table 2. Morphometric measurements of jejunal tissue from individual sheep

				Morphometric measurements[a, b]				
ID[c]	Diet[d]	Block	Age[e]	VH:CD	VH:VW	VH	VW	CD
2310	C	1	2	1.58	3.05	0.37	0.12	0.25
2309	C	1	4	3.01	2.93	0.36	0.12	0.12
2304	C	1	6	1.52	3.04	0.47	0.16	0.42
2308	C	1	8	1.92	2.79	0.45	0.17	0.24
2307	NC	1	2	3.93	4.44	0.46	0.11	0.12
2303	NC	1	4	2.52	3.13	0.38	0.12	0.15
2301	NC	1	6	2.47	3.10	0.38	0.13	0.16
2302	NC	1	8	2.28	2.71	0.54	0.19	0.24
2317	C	2	2	2.68	3.16	0.33	0.11	0.13
2312	C	2	4	1.65	2.84	0.40	0.14	0.25
2311	C	2	6	2.04	2.31	0.48	0.22	0.26
2316	C	2	8	1.46	2.87	0.42	0.15	0.21
2313	NC	2	2	3.63	3.87	0.37	0.10	0.11
2318	NC	2	4	1.86	2.96	0.41	0.15	0.23
2306	NC	2	6	3.01	3.03	0.37	0.27	0.38
2314	NC	2	8	2.13	2.63	0.32	0.12	0.16
2325	C	3	2	1.81	2.26	0.24	0.11	0.17
2327	C	3	4	2.24	3.58	0.35	0.13	0.21
2326	C	3	6	2.83	3.03	0.51	0.17	0.18
2320	C	3	8	2.02	2.71	0.36	0.13	0.19
2323	NC	3	2	2.93	2.88	0.33	0.15	0.15
2321	NC	3	4	2.28	3.63	0.45	0.13	0.18
2319	NC	3	6	1.52	2.53	0.41	0.17	0.30
2322	NC	3	8	1.83	3.13	0.47	0.16	0.26
2328	C	4	2	2.80	3.08	0.41	0.14	0.14
2330	C	4	4	1.98	3.43	0.32	0.09	0.18
2336	C	4	6	2.22	3.55	0.48	0.14	0.22
2329	C	4	8	2.00	3.10	0.43	0.14	0.25
2334	NC	4	2	2.51	3.03	0.41	0.12	0.14
2333	NC	4	4	2.64	2.42	0.48	0.22	0.17
2337	NC	4	6	2.12	3.42	0.47	0.14	0.24
2335	NC	4	8	1.62	3.23	0.59	0.21	0.41

[a] All data are expressed as means of 10 individual observations
[b] VH:CD, villus height:crypt depth; VH:VW, villus height:villus width; VH, villus height, mm; VW, villus width, mm; CD, crypt depth, mm
[c] Individual animal identification
[d] C: creep; NC: no creep
[e] All ages expressed in weeks

Appendix Table 3. Morphometric measurements of dorsal rumen tissue from individual sheep

				Morphometric measurements[a, b]						
ID[c]	Diet[d]	Block	Age[e]	PH	PW	PH:PW	Ker	NKer	Ker:Nker	Tot
2310	C	1	2	0.27	0.16	1.81	2.5	5.2	0.78	7.7
2309	C	1	4	0.47	0.18	2.73	1.8	3.7	0.47	2.5
2304	C	1	6	0.78	0.26	3.05	1.8	3.6	0.50	5.4
2308	C	1	8	0.99	0.28	3.41	1.9	3.8	0.49	5.7
2307	NC	1	2	0.41	0.20	2.16	3.1	6.2	0.52	9.3
2303	NC	1	4	0.46	0.25	2.12	3.5	5.9	0.60	9.4
2301	NC	1	6	0.47	0.23	2.10	2.9	4.6	0.63	7.5
2302	NC	1	8	0.34	0.20	1.70	3.5	5.6	0.63	9.1
2317	C	2	2	0.46	0.22	2.08	2.4	5.3	0.45	7.7
2312	C	2	4	0.48	0.24	2.01	2.5	5.0	0.50	7.5
2311	C	2	6	0.77	0.31	2.54	1.7	3.3	0.52	5.0
2316	C	2	8	0.89	0.41	2.19	2.1	3.8	0.57	5.9
2313	NC	2	2	0.38	0.17	2.30	3.4	5.6	0.62	9.0
2318	NC	2	4	0.39	0.17	2.37	3.9	4.0	0.88	7.9
2306	NC	2	6	0.49	0.23	2.44	2.9	4.7	0.61	7.6
2314	NC	2	8	0.57	0.30	1.94	3.0	0.4	0.56	8.4
2325	C	3	2	0.47	0.19	2.64	2.0	4.5	0.44	6.5
2327	C	3	4	0.53	0.24	2.29	2.1	4.0	0.54	6.1
2326	C	3	6	0.51	0.26	2.13	1.7	3.2	0.53	4.9
2320	C	3	8	0.82	0.43	1.92	1.9	3.8	0.50	5.7
2323	NC	3	2	0.48	0.22	2.28	3.2	5.6	0.60	8.8
2321	NC	3	4	0.69	0.28	2.57	3.2	5.0	0.65	8.2
2319	NC	3	6	0.36	0.24	1.53	3.6	5.4	0.68	9.0
2322	NC	3	8	0.83	0.46	1.97	2.8	4.8	0.58	7.6
2328	C	4	2	0.60	0.19	3.18	3.1	5.7	0.55	8.8
2330	C	4	4	0.38	0.18	2.20	2.2	5.0	0.44	7.2
2336	C	4	6	0.40	0.24	1.84	3.4	5.2	0.65	8.6
2329	C	4	8	0.42	0.27	1.59	3.2	5.2	0.62	8.4
2334	NC	4	2	0.38	0.18	2.18	2.7	5.3	0.51	8.0
2333	NC	4	4	0.92	0.42	2.41	2.1	3.4	0.62	2.9
2337	NC	4	6	1.34	0.31	4.33	2.0	3.5	0.59	5.5
2335	NC	4	8	0.82	0.29	3.04	1.6	3.2	0.50	4.8

[a] All data are expressed as means of 10 individual observations

[b] PH, papillary height, mm; PW, papillary width, mm; PH:PW, papillary height:papillary width; Ker, keratinized epithelial cells expressed in cell number; Nker, nonkeratinized epithelial cells expressed in cell number; Ker:NKer, keratinized:nonkeratinized epithelial cells; Tot, total epithelial cells expressed in cell number

[c] Individual animal identification

[d] C: creep; NC: no creep

[e] All ages are expressed in weeks

Appendix Table 4. Morphometric measurements of ventral rumen tissue from individual sheep

				Morphometric measurements[a,b]						
ID[c]	Diet[d]	Block	Age[d]	PH	PW	PH:PW	Ker	Nker	Ker:NKer	Tot
2310	C	1	2	0.42	0.23	1.92	2.3	3.6	0.64	5.9
2309	C	1	4	0.55	0.19	2.87	2.2	3.4	0.59	5.6
2304	C	1	6	1.02	0.26	3.88	1.6	3.2	0.50	4.8
2308	C	1	8	0.85	0.29	2.21	1.7	4.4	0.39	6.1
2307	NC	1	2	0.28	0.19	1.48	3.7	5.7	0.68	9.4
2303	NC	1	4	0.31	0.20	2.12	3.8	5.1	0.75	8.9
2301	NC	1	6	0.44	0.23	2.03	3.1	4.9	0.63	8.0
2302	NC	1	8	0.56	0.30	2.00	3.1	4.9	0.64	8.0
2317	C	2	2	0.23	0.13	1.89	1.6	4.0	0.40	5.6
2312	C	2	4	0.56	0.24	2.37	2.0	3.7	0.54	5.7
2311	C	2	6	0.90	0.32	2.53	1.8	3.9	0.44	5.7
2316	C	2	8	1.10	0.35	3.36	2.0	5.3	0.38	7.3
2313	NC	2	2	0.30	0.20	1.61	3.4	4.5	0.76	7.9
2318	NC	2	4	0.35	0.15	2.35	3.0	4.5	0.68	7.5
2306	NC	2	6	0.51	0.22	2.82	3.0	5.4	0.56	8.4
2314	NC	2	8	0.60	0.29	3.46	3.3	5.0	0.66	8.3
2325	C	3	2	0.48	0.18	2.74	2.7	4.3	0.63	7.0
2327	C	3	4	0.55	0.21	2.62	2.0	3.7	0.54	5.7
2326	C	3	6	0.82	0.27	3.09	1.6	3.9	0.41	5.5
2320	C	3	8	0.94	0.30	3.12	1.3	3.5	0.38	4.8
2323	NC	3	2	0.43	0.26	1.70	3.3	4.6	0.72	7.9
2321	NC	3	4	0.31	0.20	1.57	3.8	6.0	0.71	9.8
2319	NC	3	6	0.73	0.34	2.15	3.5	5.6	0.63	9.1
2322	NC	3	8	0.77	0.30	2.72	3.4	5.7	0.60	9.1
2328	C	4	2	0.31	0.23	1.59	3.3	5.7	0.60	9.0
2330	C	4	4	0.32	0.16	2.02	3.5	5.6	0.63	9.1
2336	C	4	6	0.71	0.43	1.73	3.2	5.0	0.65	8.2
2329	C	4	8	0.66	0.31	2.33	3.1	5.8	0.54	8.9
2334	NC	4	2	0.31	0.17	1.92	2.7	5.0	0.56	7.7
2333	NC	4	4	0.50	0.15	3.40	1.7	3.7	0.48	5.4
2337	NC	4	6	0.98	0.33	3.12	1.7	4.2	0.30	5.9
2335	NC	4	8	1.35	0.39	2.33	3.1	5.8	0.54	8.9

[a] All data are expressed as means of 10 individual observations

[b] PH, papillary height, mm; PW, papillary width, mm; PH:PW, papillary height:papillary width; Ker, keratinized epithelial cells expressed in cell number; NKer, nonkeratinized epithelial cells expressed in cell number; Ker:NKer, keratinized:nonkeratinized epithelial cells; Tot, total epithelial cells expressed in cell number

[c] Individual animal identification

[d] C: creep: NC: no creep

[e] All ages expressed in weeks

Appendix Table 5. Morphometric measurements of omasum tissue from individual sheep

				Morphometric measurements[a, b]			
ID[c]	Diet[d]	Block	Age[e]	Ker	NKer	Ker:NKer	Tot
2310	C	1	2	2.5	3.5	0.74	6.0
2309	C	1	4	2.8	6.2	0.47	9.0
2304	C	1	6	2.3	5.1	0.45	7.4
2308	C	1	8	2.0	4.0	0.50	6.0
2307	NC	1	2	4.5	4.7	0.99	9.2
2303	NC	1	4	2.8	5.0	0.57	7.8
2301	NC	1	6	2.3	4.5	0.54	6.8
2302	NC	1	8	1.9	3.9	0.49	5.8
2317	C	2	2	4.5	6.9	0.68	11.4
2312	C	2	4	2.2	4.2	0.53	6.2
2311	C	2	6	2.0	4.9	0.42	6.9
2316	C	2	8	2.5	4.5	0.56	7.0
2313	NC	2	2	3.2	5.2	0.63	8.4
2318	NC	2	4	2.7	4.9	0.56	7.6
2306	NC	2	6	2.4	5.1	0.48	7.5
2314	NC	2	8	2.5	4.5	0.58	7.0
2325	C	3	2	2.3	4.5	0.51	6.8
2327	C	3	4	1.5	4.6	0.34	6.1
2326	C	3	6	2.2	4.2	0.53	6.4
2320	C	3	8	2.4	5.2	0.46	7.6
2323	NC	3	2	2.3	4.5	0.51	6.8
2321	NC	3	4	2.3	4.9	0.48	7.2
2319	NC	3	6	1.9	4.6	0.42	6.5
2322	NC	3	8	3.1	4.9	0.65	8.0
2328	C	4	2	2.6	6.0	0.44	8.6
2330	C	4	4	1.8	5.3	0.35	7.1
2336	C	4	6	2.3	4.3	0.53	6.6
2329	C	4	8	1.9	4.4	0.44	6.3
2334	NC	4	2	2.7	5.7	0.47	8.4
2333	NC	4	4	2.2	5.2	0.44	7.4
2337	NC	4	6	2.2	4.4	0.51	6.6
2335	NC	4	8	2.7	4.9	0.56	7.6

[a] All data expressed as means of 10 individual observations

[b] Ker, keratinized epithelial cells expressed as cell number; NKer, nonkeratinized epithelial cells expressed as cell number; Ker:NKer, keratinized:nonkeratinized epithelial cells; Tot, total epithelial cells expressed as cell number

[c] Individual animal identification

[d] C: creep; NC: no creep

[e] All ages expressed in weeks

Appendix Table 6. Example of analysis of variance for effect of age, diet, and block on total gain

Genereal Linear Model Procedure

Dependent variable: totalgain (kg)

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	23	722.0934363	31.3953668	47.08	<.0001
Error	6	4.0013937	0.6668989		
Corrected Total	29	726.0948300			

R-Square	Coeff Var	Root MSE	totalgain Mean
0.994489	8.103987	0.816639	10.07700

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Age	3	294.9075999	98.3025333	147.40	<.0001
Diet	1	4.7396729	7.7396729	7.11	0.0372
Birthweight	10	23.8168008	2.3816801	3.57	0.0664
Block	3	22.2662456	7.4220819	11.13	0.0073
Diet*Block	3	19.6903163	6.5634388	9.84	0.0098
Diet*Age	3	4.4450330	1.4816777	2.22	0.1863

Tests of linear and quadratic fit for Age using orthogonal contrasts

Linear	1	279.6387178	279.6387178	419.31	<.0001
Quadratic	1	0.1643870	0.1643870	0.25	0.7328

Appendix Table 7. Example of analysis of variance for effect of age, diet, and block on villus height in tissues of the small intestine.

General Linear Model Procedure

Dependent variable: vrpw (mm)

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	21	0.29222031	0.01391525	4.11	<.0001
Error	42	0.14202812	0.00338162		
Corrected total	63	0.43424844			

R-Square	Coeff Var	Root MSE	vrpw Mean
0.672933	16.03494	0.058152	0.362656

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Tissue	1	0.16301406	0.16301406	48.21	<.0001
Diet	1	0.01025156	0.01025156	3.03	<0.0890
Age	3	0.01515469	0.00505156	1.49	0.2301
Tissue*Diet	1	0.00035156	0.00035156	0.10	0.7487
Tissue*Block	3	0.01774219	0.00591406	1.75	0.1717
Tissue*age	3	0.00931719	0.00310573	0.92	0.4402
Diet*block	3	0.03465469	0.01155156	3.42	0.0258
Diet*age	3	0.00440369	0.00143490	0.42	0.7366

Tests of linear and quadratic fit for Age using orthogonal contrasts

Linear	1	0.00472781	1.40	0.2437
Quadratic	1	0.00472781	1.40	0.2437
2 vs older	1	0.00004219	0.01	0.9116
4 vs older	1	0.00271938	0.80	0.3858
6 vs 8	1	0.01240313	3.67	0.0623

Estimates for values of the contrasts

Parameter	Estimate	Standard	t Value	Pr > |t|
2 vs older	0.00187500	0.01678695	0.11	0.9116
4 vs older	0.01593750	0.01780525	0.90	0.3758
6 vs 8	0.03937500	0.02055974	1.92	0.0623

Least squares means

Adjustment for multiple comparisons: Tukey

Tissue	vh LSMEAN	Standard Error	HO: LSMEAN = 0 Pr > |t|	HO: LSMEAN1 = LSMEAN2 Pr > |t|
1	0.31218750	0.01207987	<.0001	<.0001
2	0.41312500	0.01027987	<.0001	

1=duodenum

2=jejunum

Appendix Table 8. Example of analysis of variance for effect of age, diet, and block on abundance of oPepT1 mRNA in tissues of the small intestine

General Linear Model Procedure

Dependent variable: mRNA (oPepT1:18S)

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Model	38	53.51561042	1.40830554	3.42	<.0001
Error	57	23.50435521	0.41235711		
Corrected Total	95	77.01996563			

R-Square	Coeff Var	Root MSE	mRNA Mean
0.694828	70.30042	0.642150	0.913438

Source	DF	Sum of squares	Mean squares	F value	Pr > F
Tissue	2	10.79595625	5.39797812	13.09	<.0001
Diet	1	0.40950937	0.40950937	0.99	0.3232
Block	3	14.75745313	4.91915104	11.93	<.0001
Age	3	2.11413646	0.70471215	1.71	0.1754
Tissue*Diet	2	0.14089375	0.07044687	0.17	0.8434
Tissue*Block	6	7.05104375	1.17517396	2.85	0.0170
Tissue*Age	6	3.70638542	0.61773090	1.50	0.1953
Diet*Block	3	6.62907813	2.20969271	5.36	0.0025
Age*Block	9	7.68532604	0.85392512	2.07	0.0475
Diet*Age	3	0.22582812	0.07527604	0.18	0.9078

Tests of Age using orthogonal contrasts

Linear	1	0.00697687	0.00697687	0.02	0.8970
Quadratic	1	0.00697687	0.00679687	0.02	0.8970

Least squares means

Adjustment for multiple comparisons: Tukey

Tissue	mRNA LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0.61062500	0.11351722	<.0001	1
2	1.38093750	0.11351722	<.0001	2
3	0.74875000	0.11351722	<.0001	3

Least squares means for effect of tissue

Pr < |t| for HO: LSMEAN (i) = LSMEAN (j)

Dependent variable: mRNA

i/j	1	2	3
1		<.0001	0.6672
2	<.0001		0.0007
3	0.6672	0.0007	

1 = duodenum, 2 = jejunum, 3 = ileum

VITA

Catherine Ann Poole, daughter of Thomas A. Poole and the late Kathleen T. Poole, was born in Framingham, MA on February 5, 1977. She has a younger brother, Richard, and a golden retriever, J. J.

She moved Milford, MA in 1978, and then to Sandwich, MA in 1983. After graduating from Sandwich High School in 1995, she attended the University of Massachusetts Amherst where she received a Bachelor of Science degree in Veterinary and Animal Sciences in 1999. She was awarded the John Lee Pratt Fellowship in Animal Nutrition, and in August 1999 moved to Blacksburg, Virginia to begin her graduate study in the Department of Animal and Poultry Sciences in the College of Agriculture and Life Sciences at Virginia Polytechnic Institute and State University.

She is a member of The American Society of Animal Science, The Honor Society of Phi Kappa Phi, Gamma Sigma Delta The Honor Society of Agriculture, and Golden Key National Honor Society.

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Catherine Ann Poole