PROTEIN SYNTHESIS IN MUCIN -PRODUCING TISSUES IS CONSERVED WHEN DIETARY THREONINE IS LIMITING

DONA LALANI LEKHA MUNASINGHE

Protein synthesis in mucin -producing tissues is conserved when dietary threonine is limiting

by

©Dona Lalani Lekha Munasinghe

A thesis submitted to the

School of graduate Studies

in partial fulfillment of requirements for the degree of

Master of Biochemistry

Memorial University of Newfoundland

October, 2013

St. John's Newfoundland

ABSTRACT

Mucins in mucus are particularly rich in an indispensable amino acid, threonine, and their continuous synthesis and secretion represent a major use of dietary threonine. In piglets, the small intestine is particularly sensitive to a low threonine intake resulting in compromised mucin production. Other epithelial tissues also synthesize large amounts of mucin but their impact on threonine requirement is not as well known. Yucatan mini-piglets were fed test diets with different threonine intakes and various tissues were analyzed for protein synthesis. The threonine intake at which maximal protein synthesis was reached was lower for mucin-producing tissues, suggesting mucin-producing tissues are conserved when dietary threonine is limiting, compared to muscle, liver and kidney. So if neonates are on a marginal threonine intake, then growth and the functions of other vital tissues are likely compromised at the expense of maintenance of the mucus layer.

ACKNOWLEDGEMENTS

I highly express my gratitude to Dr. Robert Bertolo, my Supervisor, for giving me the opportunity to conduct this research and providing guidance throughout the whole research. I greatly appreciate him for encouraging and helping me during all my frustrations and the busiest time I have ever had in my life after my first baby's arrival. I express my gratitude to my Co-supervisor and Supervisory Committee Member, Dr. Janet Brunton and to Supervisory Committee Member, Dr. Sukhinder Cheema, for their guidance in conducting this research. My sincerest thanks go to Dr. Elaine Dodge for the assistance given.

I am indebted to Matthew Nosworthy and Bimal Tennakoon for helping me during the surgeries and necropsies. I thank Jason Robinson and Laura McBreairty for helping me to operate the Indirect Calorimetric system. I also wish to thank Luke Richard for completing enzyme assays. I would also like to mention Sophie Gagnon and Nicole Rice for helping me during the sample analysis. I express my gratitude to all other lab mates who have worked with me in the Brunton/Bertolo lab.

I would also like to thank NSERC and the Department of Biochemistry at Memorial University of Newfoundland for providing financial assistance and allowing me to carry out this research without any financial burden. Finally, I should not forget my husband, Chandana Witharana, who has helped me in this endeavor. All my friends who have helped me a lot in different ways in this move are gratefully and lovingly remembered.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Amino acid
AIC	Akaike information criterion
AICc	AIC with a correction
APS	Ammonium persulfate
BIC	Bayesian information criterion
BSA	Bovine serum albumin
CV	Coefficient of variation
DOC	Deoxycholic acid
dpm	Disintegrations per minute
DTT	Dithiothreitol
GIT	Gastrointestinal tract
GuHCl	Guanidium hydrochloride
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
Ks	Rate of tissue protein synthesis
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
Nle	Norleucine
PAS	Periodic acid/schiff's reagent
PDV	Portal drained viscera (intestine, pancreas, spleen, stomach)
Phe	Phenylalanine
PITC	Phenylisothiocyanate

r	Pearson's correlation coefficient
R^2	Regression coefficient
RMSE	Root mean square error
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SI	Small intestine
TCA	Trichloroacetic acid
TDG	Threonine dehydrogenase
TDH	Threonine dehydratase
TEA	Triethylamine
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
Thr	Threonine
TIDT	True ileal digestible threonine

Chapter 1 : LITERATURE REVIEW

1.1 Introduction

Although the gut comprises only 14% of whole body protein (McNurlan & Garlick, 1980), there is substantial amino acid metabolism in the gut (Baracos, 2004). The total amount of protein synthesized per day in liver, stomach, small and large intestine is equal to 43% of the total of whole body (McNurlan & Garlick, 1980). The high growth rate of the newborn infants during their first few weeks of life (Ehrenkranz *et al.*, 1999) puts significant pressure on the intestine to efficiently digest and absorb nutrients. This occurs at a time when the neonatal intestine is adapting to the enteral route of nutrition after a prenatal period in which amino acids were delivered via the umbilical route. Therefore, it may be speculated that a large quantity of amino acids is needed for the growth and maintenance of the premature gut to enable its optimal function and integrity (van der Schoor *et al.*, 2007).

Mucin proteins provide the structural backbone of the mucus gels that provide lubrication and protection from pathogens (Allen *et al.*, 1984; Valle, 2012). The protein cores of these mucins contain large amounts of threonine (Roberton *et al.*, 1991) and therefore threonine is important in the maintenance of the mucus lining of the gut (Bertolo *et al.*, 1998; Lien *et al.*, 1997). In neonatal pigs, the portal drained viscera (PDV), metabolically dominated by the small intestine, accounts for only 3–6% of the total body

mass, but is responsible for 20–35% of the whole-body protein turnover and energy expenditure (Burrin *et al.*, 2001), demonstrating its large contribution to the whole-body threonine metabolism. This PDV extracts 60–90% of dietary threonine on the first pass, whereas extraction of other essential amino acids is limited to about a third (Schaart *et al.*, 2005; Stoll *et al.*, 1997; Stoll *et al.*, 1998b; van der Schoor *et al.*, 2002; Van Goudoever *et al.*, 2000). The vast majority of this threonine is incorporated into mucosal proteins and only 2–9% is oxidized (Schaart *et al.*, 2005). Therefore, adequate dietary threonine is critical in the production of mucus and gut function (Law *et al.*, 2007). This disproportionate requirement for threonine by intestinal tissues has significant nutritional implications (Law *et al.*, 2007).

Because the overall nutrient requirement of the gut is large, it makes sense that the cells of the intestinal mucosa meet their nutrient requirements first (Baracos, 2004). However, no studies have been conducted to identify the gut requirement of dietary threonine. This is important to know because the nutrient requirements of the gut might have a critical impact on the systemic availability of nutrients and a substantial impact on whole-body and individual tissue growth and development in young animals.

1.2 Gastrointestinal physiology and functions

Stomach

The stomach is a muscular sac that functions primarily as a reservoir, controlling the rate of delivery of food to more distal segments of the

gastrointestinal tract (GIT). The mucus layer adhered to the stomach wall protects the gastric mucosa against endogenous agents such as HCl and pepsin and dietary exogenous substances (Dekker *et al.*, 1991).

Anatomically, the stomach is divided into three regions, the cardia, fundus, and antrum which have distinctive structures with specific functions (Barrett, 2006).

The cardia secretes mucus and bicarbonate to protect the surface from the corrosive gastric contents, while the fundus mainly functions to serve as a secretory region. The fundic (or gastric) glands contain specific secretory cells that produce gastric juice, acid from parietal cells and pepsin from chief cells. The antrum (pyloric zone) is involved in extensive motility patterns, mixing the gastric contents and grinding and sieving ingested particles. Finally, the food is gradually emptied into the small intestine via the pylorus (Barrett, 2006).

Folds present in the stomach surface are known as rugae, which can be observed by the naked eye. At the microscopic level, pits further increase the surface area of the stomach and represent the entrances to deep gastric glands. The glands become deeper from the cardia, to the antrum, to the fundus (Barrett, 2006).

Small Intestine

The small intestine (SI) is best known for its role in the digestion and absorption of nutrients. Its innermost layer of the wall is composed of the mucosa, followed by the sub mucosa, the muscle layer and the outermost serosa (Lin *et al.*, 1999). The first segment of the small intestine, the duodenum, begins as a bulb-shaped structure immediately distal to the pylorus. The remainder of the small intestine consists of the jejunum and ileum (Barrett, 2006).

The majority of nutrient absorption in the healthy individual takes place in the jejunum. It has a markedly increased surface area to promote better absorption due to the presence of folds of Kerckring, tall and slender villi and an abundance of microvilli (Barrett, 2006), which are prominent on the epithelium. The epithelium, the innermost layer of mucosa facing the lumen, consists of columnar epithelial cells or enterocytes (Barrett, 2006; Lin *et al.*, 1999). These enterocytes function in digestion, absorption, and secretion (Barrett, 2006; Herlinger, 1999).

Nutrient absorption in the ileum, which has fewer folds and shorter, sparser villi, is low (Barrett, 2006; Lin *et al.*, 1999), with the exception of specific solutes such as conjugated bile acids. However, in impaired jejunal absorption, such as in mal-digestion, the ileum represents an anatomic reserve for absorption.

There are three types of smooth muscle contractions: peristaltic waves, segmentation contractions, and tonic contractions. Peristalsis moves the intestinal contents (chyme) toward the large intestine. Segmentation contractions move the chyme to and from the mucosal surface and increase exposure to it. Tonic contractions are relatively prolonged contractions and isolate one segment of the intestine from another (Barrett, 2006).

Colon

The colon, or large intestine, has a larger diameter than the small intestine with a thicker wall and folds known as haustrations. In the human, the large intestine is divided into several regions: the ascending, transverse, descending, and sigmoid colon (Figure 1.2). In pigs, the proximal portion of the descending colon is arranged in a series of centrifugal and centripetal coils in the left upper quadrant of the abdomen. This structure is known as the spiral colon. The cecum has three longitudinal muscular bands (tenia) and the proximal portion of the spiral colon has two bands, resulting in a series of sacculations (haustra).

The colon stores wastes and indigestible materials prior to their elimination by defecation. In general, the colonic epithelial cells (colonocytes) are not involved in the absorption of conventional nutrients such as monosaccharides, peptides, amino acids, and vitamins, but other luminal constituents can be actively absorbed. For example, although some peptides and amino acids can be broken down by colonic microbes, they cannot be re-

absorbed due to lack of amino acid transporters in the colon. However, the fluid remaining from digestion as well as from the other dietary by-products, such as short chain fatty acids produced by the bacterial fermentation of carbohydrates, including dietary fiber, are recycled at the ascending and transverse colon. Other luminal solutes, such as bile acids and bilirubin, are also modified in the colon by bacterial metabolism (Barrett, 2006).

Transit time from stomach to colon

The transit time of food from stomach into the duodenum depends on factors such as type of food ingested, the osmotic pressure of the material entering the duodenum, and gut contractions. It is fastest with foods rich in carbohydrate and slowest with fatty foods, whereas protein rich foods are moderate (Barrett *et al.*, 2010). Segmentation and tonic contractions reduce transit time in the small intestine and permit longer contact of chyme with enterocytes to increase nutrient absorption (Barrett, 2006).

In humans, the first part of a test meal reaches the cecum in about 4 h in the small intestine, and all the undigested portions will enter the colon in 8-9 h. On average, the first remnants of the meal traverse the first third of the colon in 6 h, the second third in 9 h, and reach the sigmoid colon in 12 h. From the sigmoid colon to the anus, transit is much slower (Barrett *et al.*, 2010).

1.3 Structure and physiological functions of gut mucus/mucin

Mucus is a complex viscous adherent secretion synthesized by goblet cells in the columnar epithelium, which line organs that are exposed to the external environment, including the GIT (Bansil & Turner, 2006). The highly glycosylated proteins (glycoproteins) present in mucus are called "mucins" and consist of a large number of oligosaccharides covalently attached to a protein core.

Classifications of mucins

The mucins are encoded by 12 different mucin genes namely; MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, MUC11 and MUC12 (Silva *et al.*, 2002). Also, mucins can be classified in three distinct subfamilies related to their structure: gel-forming, soluble, and membrane-bound (Moniaux *et al.*, 2001).

Based on the bound carbohydrate side chains, the mucins are further classified into different types. At least two different regions of peptides can be seen within the large glycopeptides present in the native mucin: (1) "naked" segments containing no carbohydrate and (2) the remaining peptide region contains >90% of the carbohydrate attached to a protein core (Wesley *et al.*, 1985).

Composition and structure of mucus/mucin

Mucus is primarily composed of ~95% water, but also contains glycoproteins, salts, and lipids such as fatty acids, phospholipids and cholesterol, as well as proteins with defensive functions (Allen & Snary, 1972). The protein core of mucus, within "mucins", have high molecular weights ranging from 0.5 to 20 MDa (Turner *et al.*, 1999) and are rich in serine, threonine and proline (Table 1.1).

Table 1.1: Amino acid composition of the pig small-intestinal mucus glycoprotein (Mantle & Allen, 1981)

Amino acid	**Composition mol/100mol of protein**
Lysine	2.22
Histidine	1.37
Arginine	2.22
Aspartic acid	4.63
Threonine	**26.53**
Serine	10.40
Glutamic acid	4.20
Proline	15.44
Glycine	5.60
Alanine	3.77
½ Cystine	4.28
Valine	7.15
Methionine	1.25
Isoleucine	3.14
Leucine	4.28
Tyrosine	1.46
Phenylalanine	2.00

The linear or rod-like peptide core with oligosaccharides arranged radially from the central core in the basic mucin structure (Figure 1.1) provides high viscosity to the mucin (Lamont, 1992). The oligosaccharide chains are attached to the protein core by O-glycosidic bonds to the hydroxyl side chains of serine and threonine and arranged in a "bottle brush" configuration about the protein core (Allen & Snary, 1972; Lamont, 1992). These O-glycans are less branched than most N-glycans. The presence of these large numbers of serine and threonine residues in an uncharged and often proline-rich peptide context is responsible for the clustering of O-glycans on mucins (Lamont, 1992). This specific structure contributes to the physico-chemical properties of mucin such as enhancement of polymerization with very high molecular weight, high viscosity contributed by extended rod-like structure, protease resistance due to glycosylated repeating sequences, binding of toxins and bacteria due to availability of branched oligosaccharides, and lipid binding capacity by hydrophobic domains (Lamont, 1992). The carbohydrate constitutes > 65 % of the dry weight (Allen & Snary, 1972; Lamont, 1992) and variations in attached oligosaccharides (length, branching, internal linkages, and specific monosaccharide composition) result in the heterogeneity of mucins. These oligosaccharide chains may contain up to five different monosaccharides, namely: galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid distinguished as either N-acetyl- or N-glycollyl-neuraminic acid (Table 1.2), plus trace quantities of mannose and glucose (Allen & Snary, 1972; Lamont, 1992). Usually, carbohydrates are very hydrophilic and therefore they contribute to the gelling property of mucin

due to their ability to bind water. Also, they protect the peptide core from proteolytic attack from pancreatic and bacterial proteases, and bind pathogenic bacteria, parasites and toxins or sometimes mimic the natural binding sites for pathogens. Furthermore, oligosaccharide side chains provide a stable attachment site for immunoglobulins, lysozyme and digestive enzymes (Lamont, 1992).

Table 1.2: The composition of some gastrointestinal mucus glycoprotein (from Allen & Snary, 1972)

Source	Carbohydrate molar ratios					Protein (% by weight)
	GlcNAc	GalNAc	Gal	Fuc	NeuNAc/ NeuNGl	
Stomach						
Human	2.4	1.0	3.1	2.1	0.2	17
Pig	2.8	1.0	2.9	1.9	0.2	13
SI						
Human	1.0	1.0	2.0	0.6	0.2	12
Rat	1.0	1.0	3.0	1.0	0.8	12-16
Colon						
Human	1.0	1.0	1.1	0.5	0.7	33
Pig	2.9	1.0	2.5	1.5	0.2	13

SI= Small intestine; GlcNAc = N-acetylglucosamine;

GalNAc = N-acetylgalactosamine; Gal=galactose;

Fuc=fucose; NeuNAc=N-acetylneuraminic acid;

NeuNGl= N-glycollylneuraminic acid

Four subunits of 500,000 kDa are linked together to form a mucin monomer (Figure 1.1) and two distinctly different regions can be found in mature mucins. The first region is the protease-sensitive amino- and carboxy-terminals, which are very lightly glycosylated (naked region), with relatively few O-glycosylation and N-glycosylation sites, and a high proportion of cysteine (>10%). These cysteine residues are likely involved in establishing disulfide linkages within and among mucin monomers (Bell *et al.*, 2003; Lamont, 1992). This peptide core is folded like most other globular proteins and allows direct protein-protein interactions, which are critical for aggregation (Lamont, 1992). The second region is the large glycosylated protease-resistant central region (glycosylated region), formed of multiple tandem repeats that are rich in serine, threonine and proline (STP repeats) with > 60% of the amino acids. This area becomes saturated with hundreds of O-linked oligosaccharides. N-linked oligosaccharides are also found on mucins, but much less abundantly (Bell *et al.*, 2003; Lamont, 1992).

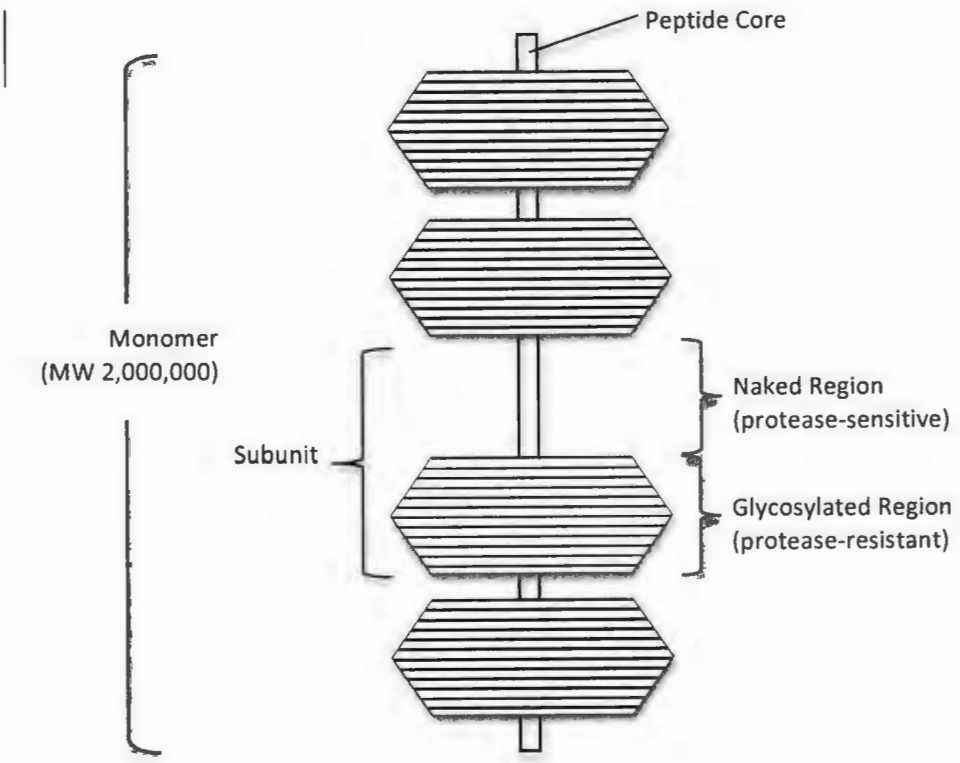


Figure 1.1: Schematic representation of a typical intestinal mucin (adapted from Lamont, 1992)

Functions and biological significance of gut mucus/mucin

The highly viscous mucus gel covering the GIT lubricates the passage of food/chyme and also acts as a permeable gel layer for the exchange of gases and nutrients with the underlying epithelium (Bansil & Turner, 2006). The mucosal defense system, which is composed of pre-epithelial, epithelial, and sub-epithelial elements, provides a three-level barrier. The mucus-bicarbonate-phospholipid layer provides a first line of defense by serving as a physico-chemical barrier to multiple molecules, toxins and pathogens (Allen *et al.*, 1984; Valle, 2012) by protecting underlying mucosal surfaces from abrasion, acid, digestive enzymes and bacterial invasion (Allen *et al.*, 1984). Both the non-stirred water layer and bicarbonate are secreted in a regulated fashion by the gastric-duodenal surface epithelial cells of the mucosa. The water layer of the mucus gel obstructs ion diffusion, including hydrogen ions and molecules such as pepsin, while bicarbonate is secreted into the mucus gel, thus forming a pH gradient of 1-2 at the gastric luminal surface which reaches 6-7 along the epithelial cell surface (Valle, 2012). Additionally, mucus contains lysozyme, immunoglobulins, defensins, growth factors and trefoil factors to defend against pathogens (Bansil & Turner, 2006). The great diversity in mucin O-glycan structures provides optimal protection against a variety of pathogenic agents and microbes in the intestines (Brockhausen, 2004). Further, intestinal mucosa represents a suitable place for beneficial gut microbiota. For example, bacteria are known to adhere specifically to partial oligosaccharide sequences found in mucins, and multiple attachment

sites for these commensal bacteria colonizing the gut may prevent binding for a few pathogenic ones (Brockhausen, 2004).

In human intestinal mucin, the composition of naked segments, which are sensitive to attack by proteases, is constant (Wesley *et al.*, 1985) and these segments provide a major antigenic effect (Mantle *et al.*, 1984). Oligosaccharides present in the remaining peptide region play a main role in maintaining the structure and conformation of mucins (Veluraja *et al.*, 2011), and provide considerable water-holding capacity and also make them resistant to proteolysis by digestive enzymes. The high molecular weight mucins are responsible for the viscoelastic properties of the mucus barrier (Gendler & Spicer, 1995) and mucins promote gelation by cross-linking in a solution by disulfide bonding (Marth, 1999). However, the glycoprotein constituents and thickness of the gel differ from region to region within the gut (Allen *et al.*, 1984).

1.4 Threonine and its requirements

Proteins are vital to health because they support the growth and repair of structural and functional components of the body. Amino acids serve as the building blocks for these proteins and their requirements can be affected by many factors, including age, body weight, sex, genotype, environment (climate, microbial, social), and dietary factors (Defa *et al.*, 1999). With these multiple factors, it is rather difficult and impractical to conduct experiments to determine the amino acid requirements for each individual group and

situation. Therefore, estimates of amino acid requirements have large variations.

Threonine is an indispensable amino acid for poultry (Kidd & Kerr, 1996), pigs and humans (Defa *et al.*, 1999; Le Floc'h *et al.*, 1996), and its oxidation represents an irreversible loss for body protein because it cannot be synthesized de novo by mono-gastric animals (Le Floc'h *et al.*, 1996). Moreover, threonine is the second limiting amino acid in common cereals used in swine diets including, sorghum (Cohen & Tanksley, 1976), barley (Fuller *et al.*, 1979) and wheat (Allee & Hines, 1971), and the third limiting amino acid in corn (Grosbach *et al.*, 1985).

In pigs, threonine has been identified as the first limiting amino acid for maintenance (Fuller *et al.*, 1989) and its utilization for processes other than muscle protein synthesis and deposition is high (Le Floc'h & Seve, 2005). Growing animals have a higher threonine requirement and their total requirement must depend on the relative contributions of maintenance and tissue protein accretion to its total needs (Fuller *et al.*, 1989). Therefore, an adequate supply of dietary threonine is important to maintain protein synthesis and deposition (Le Floc'h *et al.*, 1996).

Threonine is also critical for the production of gut mucins and also contributes to collagen, elastin, and tooth enamel formation (Vlaardingerbroek *et al.*, 2011). As threonine has quantitatively important

functions in the gut, the threonine requirement is different according to the mode of dietary intake. For example, the threonine requirement for piglets ~8 days of age was 0.20 $g.kg^{-1}.day^{-1}$ when fed intravenously and 0.55 $g.kg^{-1}.day^{-1}$ when fed intragastrically (Bertolo *et al.*, 1998), indicating a three times higher requirement when the gut is healthy and intact.

Imbalances of threonine intake (both deficiency and excess) can cause detrimental effects, especially during the neonatal period. Wang *et al.* (2007) found that protein synthesis rate was reduced in the small intestine of piglets receiving either 50% or more than 100% of daily threonine recommendations. A high threonine intake increases serum and brain threonine concentration (Kang-Lee & Harper, 1978) and leads to a decrease of food intake and growth failure (Muramatsu *et al.*, 1971) in animals. On the other hand, feeding young piglets a threonine deficient diet (70% of recommendations) for 2 weeks induced villous atrophy in the ileum (Hamard *et al.*, 2007) and a 40% reduction in dietary threonine in rats led to reduced fractional synthesis rate of intestinal mucins without any effect on total mucosal protein synthesis rate (Faure *et al.*, 2005). Recently, Hamard *et al.* (2010) demonstrated that a 30% reduced threonine supply for 2 weeks induced paracellular permeability and reduced glucose absorption capacity in weaning piglets. A similar threonine deficient diet also resulted in reduced total threonine contents of carcass, colon and liver by 4%, 7% and 11%, respectively. These authors assumed that the reduction in threonine content could be associated with a deposition of proteins less rich in threonine. For

example, the reduction in threonine content in the colon, also described in other studies (Law *et al.*, 2007; Wang *et al.*, 2007), could be ascribed to a reduction in mucin synthesis and content.

A deficiency in a particular amino acid induced by an excess of one or more dietary amino acids can also cause amino acid imbalance. For example, a threonine deficiency can be caused by a slight excess of methionine in rats (Salmon, 1958). As dietary methionine increased, the growth rate decreased and additional dietary threonine alleviated the decrease in growth. Castagné *et al.* (1993) showed that a high dietary content of threonine (5.8g/100g diet, i.e. 15 times the normal levels) can change the amino acid balance by elevating glycine levels in the brain. However, diets containing only 2 to 4 times the normal levels of threonine are insufficient to modify brain glycine, in spite of inducing a weak hyperthreoninemia. The brain neurotransmitters, catecholamines, serotonin, and histamine, are dependent on the supply of their precursor amino acids, phenylalanine/tyrosine, tryptophan, and histidine, respectively. High plasma threonine concentration due to high dietary threonine intake can reduce the entry into the brain of the other neutral amino acids through competition at the level of transport systems at the blood-brain-barrier, thereby disturbing the levels of those neurotransmitters in the brain. If the plasma threonine concentration is sufficient to compete and depress the brain supply of indispensable amino acids, then protein synthesis might also be impaired (Castagne *et al.*, 1993; Wu, 1998). Yoshida *et al.* (1966) found that dietary amino acid imbalance in

rats caused by the addition of an amino acid mixture with limited threonine or histidine causes a decrease in plasma levels of these indispensable amino acids due to more efficient incorporation of them into tissues. It has also been suggested (Kidd & Kerr, 1996) that there is a homeostatic mechanism in rats which holds body lysine levels constant while body threonine fluctuates depending upon the amount present in the diet. The lack of such a homeostatic mechanism regulating threonine may result in a more variable requirement estimate. When dietary lysine levels are lower than the requirement, all absorbed lysine which is not used for maintenance or not oxidized will go to skeletal muscle accretion (Kidd & Kerr, 1996).

1.5 Threonine utilization in the gut

As an indispensable amino acid in mammals, functions involving threonine are dependent on an adequate supply of dietary threonine. Dietary threonine is absorbed in the small intestine and then used by the peripheral tissues mainly for protein deposition associated with growth and maintenance (Hamard *et al.*, 2009). During this intestinal absorption, more than one half of orally supplied threonine is extracted on first-pass by the small intestine of young pigs (Le Floc'h & Seve, 2005; Stoll *et al.*, 1998b; van der Schoor *et al.*, 2002). Further, many studies (Schaart *et al.*, 2005; Stoll *et al.*, 1998b; van der Schoor *et al.*, 2002; Van Goudoever *et al.*, 2000) have demonstrated that the portal-drained viscera (PDV; the intestine, pancreas, spleen, and stomach), metabolically dominated by the small intestine, extracts 60–90% of dietary threonine on the first pass (Stoll *et al.*, 1998b), whereas extraction of other

essential amino acids is limited to about a third. In preterm infants, the splanchnic tissues retain dietary threonine to a similar degree (~75%), regardless of the amount of enteral threonine delivery (van der Schoor *et al.*, 2007). This high obligatory requirement for threonine by the portal-drained viscera may be due to its abundance in mucosal proteins (Schaart *et al.*, 2005). Interestingly, additional data demonstrated a substantial portion of the oral threonine intake by piglets is used by the healthy gut and is not required when the gut is relatively inactive or atrophied, as during parenteral feeding (Bertolo *et al.*, 1998). The colon and the carcass utilize amino acids from blood whereas the small intestine uses mostly luminal amino acids for protein synthesis (Schaart *et al.*, 2005; Le Floc'h & Seve, 2005), which helps explain the disproportionate requirement and utilization of dietary threonine by small intestinal tissues. Furthermore, this high threonine extraction by the small intestine on first-pass is nutritionally important, because it determines threonine availability for non-digestive tissues that depend on threonine supplied by the blood.

A threonine deficient diet results in GIT-related consequences such as diarrhea, reduced intestinal protein synthesis (Wang *et al.*, 2007), ileal villous hypotrophy (Hamard *et al.*, 2007), and reduced mucin synthesis and content (Faure *et al.*, 2005; Law *et al.*, 2007; Wang *et al.*, 2007). Mucin has a threonine content of 13-26% of total AA (Lien *et al.*, 1997; Mantle & Allen, 1981; Piel *et al.*, 2004). Therefore, the dietary, rather than systemic, threonine is preferentially utilized for protein (Cid *et al.*, 1999; Schaart *et al.*, 2005) and

mucin (Faure *et al.*, 2005; Law *et al.*, 2007; Wang *et al.*, 2007) synthesis in the small intestinal mucosa. Hence, the reduced protein synthesis due to a threonine deficient diet may specifically reflect reduced mucin synthesis. However, it should be noted that others have not observed similar results. For example, Hamard *et al.* (2007) found that a low dietary threonine supply (6.5 g/100 g diet) to piglets did not alter intestine growth and architecture of the proximal small and large intestine. Additionally, a marginally threonine deficient diet did not affect protein metabolism of the small intestine, whereas modifications in protein threonine content in the carcass, the liver and the colon of piglets were observed (Hamard *et al.*, 2009). On the other hand, both dietary threonine restriction and excess have been shown to reduce fractional synthesis rate of small intestinal mucosal protein and mucins (Van Goudoever *et al.*, 2000; Wang *et al.*, 2007) but had no effect on total mucosal protein synthesis (Faure *et al.*, 2005). A possible explanation for the same outcome due to excess of threonine may be a reduction in the uptake of neutral amino acids by the intestinal mucosa due to their sharing the same transport systems, thereby limiting the local synthesis of proteins (Castagne *et al.*, 1993; Wu, 1998). The uptake of amino acids across the small intestinal epithelium occurs in two stages, 1) uptake across the brush-border membrane, and 2) exit into the blood across the basolateral membrane. Transport across the basolateral membrane occurs via the classical transport systems, namely the Na-independent system L and the Na-dependent systems A and ASC (Mircheff *et al.*, 1980). Simple diffusion of amino acids across a membrane also contributes to their net movement. The basolateral

membrane of the enterocyte is much more permeable to amino acids than in non-epithelial membranes or the brush-border membrane (Stevens *et al.*, 1984). The leaky nature of this membrane allows accumulated amino acids to diffuse from the enterocyte to the blood. However, when gut luminal amino acid concentrations are low, the carrier-mediated pathways are important for amino acid uptake into the enterocyte. Amino acid transport across the brush-border membrane also can be Na-independent and Na-dependent processes. Large neutral amino acids and cationic amino acids in the small intestine share Na-dependent and Na-independent transport systems (Harvey *et al.*, 1993; Munck & Schultz, 1969; Wolfram *et al.*, 1984) and brush-border membrane vesicles from fetal and adult small intestine also demonstrate the presence of Na-dependent and Na-independent transport systems for neutral amino acids (Lucke *et al.*, 1977). Threonine transport is usually depressed more by large neutrals, rather than small neutrals (Tovar *et al.*, 1988).

Therefore, in young pigs, the small intestine may be very sensitive to dietary threonine levels with regard to tissue protein synthesis.

1.6 Intestinal threonine metabolism

Amino acids can be utilized for 3 main metabolic purposes after absorption: 1) protein synthesis, 2) conversion to other amino acids, metabolic substrates and biosynthetic intermediates, and 3) complete oxidation to CO_2.

The gastrointestinal tissues have an important impact on the whole-body metabolism of animals due to their relatively high protein turnover rate and high oxygen consumption (Burrin *et al.*, 2001). Although the small intestine represents less than 5% of whole body mass, it accounts for 20–50% of total body protein turnover and energy expenditure in pigs. Further, the fractional rate of protein turnover in intestinal tissues exceeds that of peripheral tissues (Burrin *et al.*, 2001). This high metabolic activity partially explains the high amino acid requirements of the gut. The small intestine can use amino acids from either the lumen or artery and the contribution of these two sources can be changed according to the nutritional status of the animal and the amino acids (Stoll *et al.*, 1997) However, in normal conditions, the small intestine uses mostly luminal amino acids for protein synthesis (Le Floc'h & Seve, 2005; Schaart *et al.*, 2005) .

Since threonine is the first limiting amino acid for maintenance in pigs (Fuller *et al.*, 1989), most of the maintenance requirement could be attributed to the incorporation of threonine in digestive tract protein synthesis (Le Floc'h & Seve, 2005). Because there is no significant threonine recycling from mucosal proteins (van der Schoor *et al.*, 2002), it seems that these mucosal proteins are very resistant to digestion. Alternatively, recycled threonine might be immediately reincorporated into mucosa protein instead of being absorbed or lost in the lumen (Le Floc'h & Seve, 2005; van der Schoor *et al.*, 2007). In either event, it appears that the threonine that is extracted by the gut is

irreversibly metabolized, either by being incorporated into digestion-resistant mucin proteins, or by catabolism.

Three enzymes participate in threonine catabolism, namely, threonine dehydratase (TDH), threonine dehydrogenase (TDG), and threonine aldolase (Figure 1.2). In pigs, more than 80% of threonine catabolism occurs through the TDG pathway to produce glycine and acetyl CoA *in vivo* (Le Floc'h *et al.*, 1996). However, in adult humans, only 7-11% of threonine catabolism occurs via TDG pathway (Darling *et al.*, 2000) and over 80% is via TDH. TDH (also known as serine dehydratase) uses pyridoxal-5'-phosphate to degrade threonine to 2-ketobutyric acid and ammonia, and this reaction becomes important only during fasting in pigs (Kidd & Kerr, 1996) and the contribution of TDH to whole body threonine catabolism during the fed state is probably less than 20% (Ballevre *et al.*, 1990; Ballevre *et al.*, 1991). Threonine degradation to glycine and acetaldehyde by threonine aldolase is considered very minor in both pigs and humans (Kidd & Kerr, 1996). *In vivo*, threonine catabolism depends on the amount of active enzyme and the availability of threonine as substrate. Plasma threonine concentration, as well as threonine transport into the tissue, is the major factor controlling the flux of threonine catabolism through the TDG pathway (Le Floc'h & Seve, 2005).

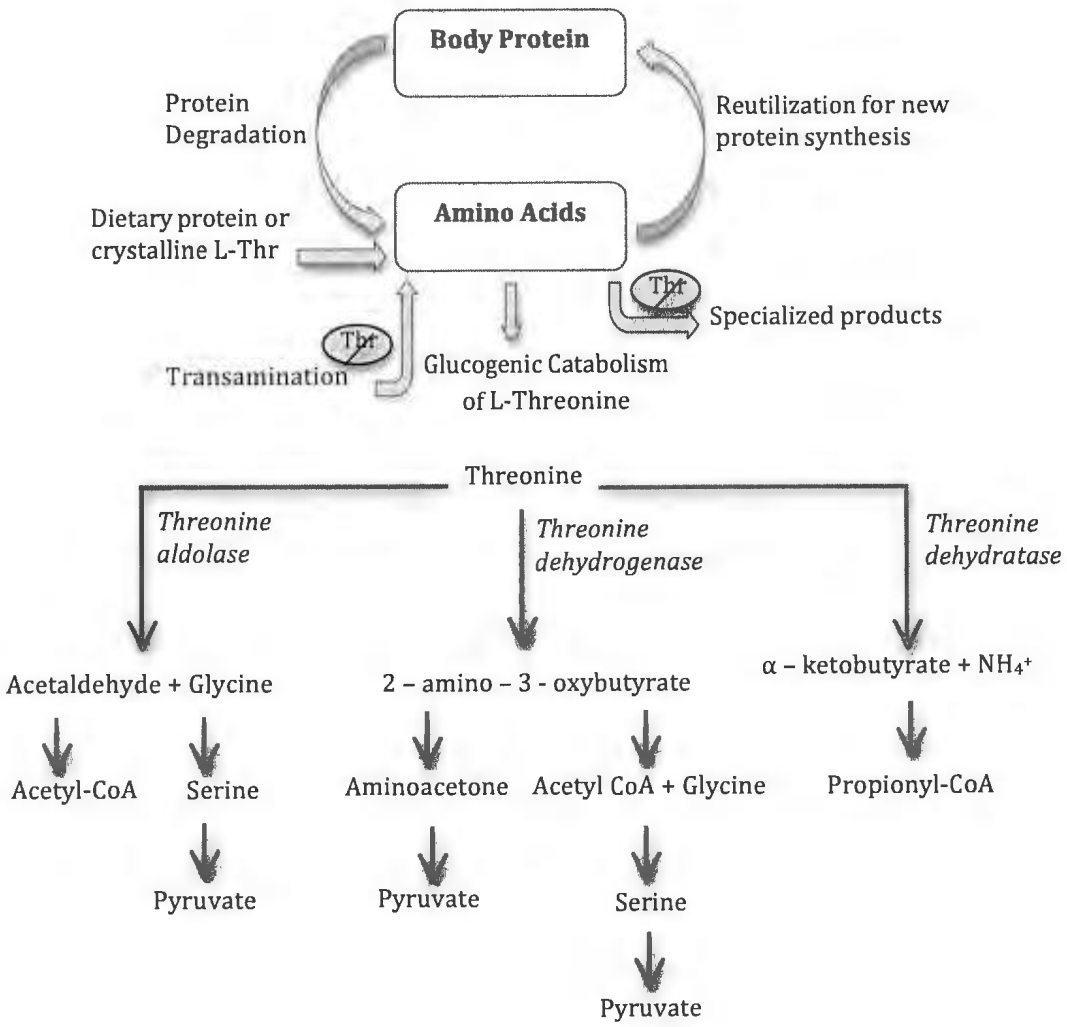


Figure 1.2: Schematic representation of threonine catabolism (Used with permission of The Journal of Applied Poultry Research; Kidd & Kerr, 1996, vol 5)

If threonine degradation occurs in the gut, an increase in intestinal threonine catabolism associated with an increase in dietary threonine could partially explain the gut's high requirement for threonine. However, similar to most of the essential amino acids, threonine catabolism in pigs occurs mainly in the liver, but also in the pancreas (Le Floc'h *et al.*, 1997); no TDG enzymatic activity to degrade threonine has been detected in the small intestine, colon and associated lymphatic nodes in pigs (Le Floc'h & Seve, 2005; Le Floc'h *et al.*, 1997; Wu, 1998) . Hence, the precursor pool for threonine oxidation is likely represented by intrahepatic threonine. Nevertheless, when dietary threonine supply is limiting, the main part of threonine oxidation could be extrahepatic (Le Floc'h & Seve, 2005). Although the highest specific activity of TDG occurs in the pancreas, the activities of TDH and threonine aldolase are highest in liver and muscle in pigs (Davis & Austic, 1994a in Kidd & Kerr, 1996). This lack of detectable enzyme activity in the intestine (Wu, 1998) could be the consequence of the high protease activity in the intestinal mucosa (Le Floc'h & Seve, 2005), complicating its measurement. However, other methods have demonstrated that most of the dietary threonine extracted by the small intestine is incorporated into mucosal proteins synthesis and only 2–9% is oxidized (Schaart *et al.*, 2005). It has also been found that the plasma threonine concentration is directly proportional to the dietary true ileal digestible threonine (TIDT) level (Yamashita & Ashida, 1971). Glycine is a product of threonine catabolism in mammals, including pigs (Le Floc'h *et al.*, 1995), and therefore, increasing dietary levels of TIDT from 0.37 to 0.74% increased plasma concentrations of glycine. However, a

further increase in dietary threonine level beyond 0.74% TIDT does not cause further increase in plasma glycine concentration, suggesting a limited threonine catabolism capacity through hepatic TDG pathway (Wang *et al.*, 2007). It is unknown whether intestinal threonine catabolism is upregulated at such high dietary threonine intakes to enhance the overall whole body threonine oxidation capacity.

1.7 Swine as an animal model for metabolic studies

The pig is an increasingly important animal model in many aspects of biomedical research, including embryology, teratology, nutrition, metabolism and immunology (Book & Bustad, 1974; Miller & Ullrey, 1987; Nunoya *et al.*, 2007). There are over 50 breeds of miniature pigs (minipigs) that can be found worldwide, but only a few of them are important in biomedical research (Michael & Smith, 2008) including Yucatan, Hanford, Sinclair and Göttingen (Murtaugh *et al.*, 1996; Nunoya *et al.*, 2007). These minipigs have been established from naturally occurring populations and from the genetic selection of two or more existing breeds. Yucatan minipigs, who are essentially hairless and have a docile temperament, were developed at Colorado State University from foundation animals imported from the Yucatan peninsula in 1960 (Nunoya *et al.*, 2007). They have a mean birth weight of 0.74 kg and mean weaning weight at 60 days of 6.47 kg (Nunoya *et al.*, 2007).

Piglets are the best animal models for research involving the physiology of digestion and metabolism compared with most of the other commonly used research animals such as rodents, dog, cat, goat and sheep. The piglet is very similar to a newborn human infant, especially with respect to anatomical, physiological, gastrointestinal, respiratory, renal, cardiovascular and hematologic systems (Book & Bustad, 1974; Miller & Ullrey, 1987; Nunoya *et al.*, 2007). Therefore, nutrient requirements of the pig resemble the human in more ways than any other non-primate mammalian species allowing the use of the pig in human nutritional studies including calorie-protein malnutrition, and nutrient absorption and metabolism (Miller & Ullrey, 1987). However, there are some significant anatomical differences in the large intestine (Michael & Smith, 2008), including a large cecum and a spiral colon.

Minipigs have special advantages: easily handled because of its inherently smaller size even at full maturity, easily housed, economical, easily treated, and they also have few health problems (Book & Bustad, 1974; Michael & Smith, 2008; Miller & Ullrey, 1987; Nunoya *et al.*, 2007). Other advantages include rapid growth rate (Miller & Ullrey, 1987; Nunoya *et al.*, 2007), controlled genotype (Nunoya *et al.*, 2007), large litter size and the two to three litters per sow per year (Book & Bustad, 1974; Nunoya *et al.*, 2007). Also, they enable collection of larger volumes of multiple samples of body fluids or biopsies compared to rodents (Nunoya *et al.*, 2007). Moreover, they are more tractable than the domestic breeds raised in an agricultural setting

allowing successful performance of experiments under unstressed conditions (Nunoya *et al.*, 2007).

Compared to a human infant, the piglet has a lower birth weight, higher metabolic rate, higher body temperature and smaller fat reserve (Miller & Ullrey, 1987). However, there are some similarities in the portions of total life to reach chemical maturity and the chemical compositions of fat-free body tissue at common stages of life for humans and pigs (Table 1.3). However, the piglet grows very rapidly and therefore has more strict nutritional requirements than a human infant. In the first 6 weeks of life, the domestic piglet increases its birth weight by 1000% (from 1,200 to 12,000 g) compared to the human infant which increases its birth weight by about 50% (from 3400 to 5000 g; Miller & Ullrey, 1987).

Table 1.3: Age and chemical development comparison of man and pig (Miller & Ullrey, 1987 in Moulton, 1923).

	Human	**Pig**
Length of gestation (days)	285	114
Approximate length of life (years)	80	20
Age at chemical maturity (days)	1285	270-420
Portion of total life to reach chemical maturity (%)	4.4	4.6
Composition at birth (fat-free basis)		
• Water (%)	82	82
• Protein (%)	14	13
• Ash (%)	3	3
Composition at 3 months (fat-free basis)		
• Water (%)	81	77
• Protein (%)	16	19
• Ash (%)	3	4
Composition at maturity (fat-free basis)	(33 y)	(3 y)
• Water (%)	69	72
• Protein (%)	21	23
• Ash (%)	9	4

Chapter 2 : EXPERIMENTAL RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1 Principle of tissue-specific requirement breakpoint analysis

In the flooding dose technique, a large dose of phenylalanine is injected with a tracer dose of phenylalanine. This fixed phenylalanine specific radioactivity rapidly equilibrates with all free phenylalanine pools in the body. The isotopic steady state from the dose is maintained and then the incorporation of label into protein is measured. From these data, the rate of protein synthesis of individual tissues can be calculated.

When threonine is deficient, protein synthesis will be reduced even when all other essential amino acids are available. As threonine intake increases, rate of protein synthesis also will be increased. When the maximal fractional rate of protein synthesis is reached, the plot of rate of protein synthesis against threonine intake will follow a plateau (Figure 2.1). This inflection point is the "breakpoint" and the threonine intake at this breakpoint is the "threonine requirement" of that particular tissue. Similarly, different breakpoints or threonine requirements can be identified for each individual tissue. Biologically, protein dynamics are not linear when amino acids are limiting. However, beyond the amino acid requirement, protein dynamics should be a plateau. Therefore, even if this relationship is curvilinear, breakpoints can be identified using two linear regressions.

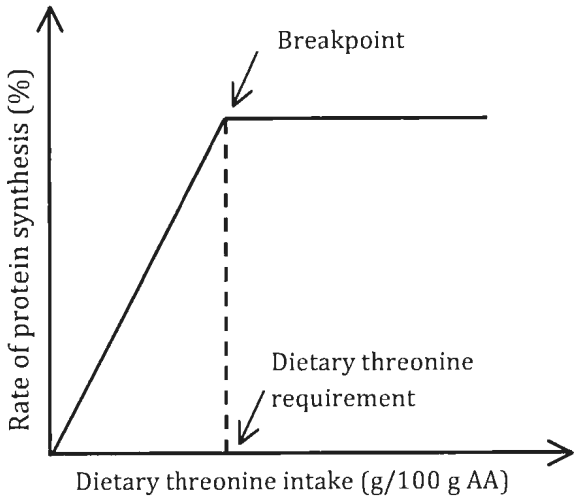


Figure 2.1: Theoretical plot of protein synthesis over increasing threonine intakes showing the 'breakpoint'

Flooding dose technique:

A very large dose of unlabelled amino acid along with a tracer sufficient to flood all possible precursor pools will be given intravenously. This flood ensures that all tissues have nearly the same specific radioactivity, which is then maintained almost constant during the period of incorporation into protein (Garlick *et al.*, 1980). For example, when a large dose of unlabelled phenylalanine is given with labelled phenylalanine, then the specific radioactivity of phenylalanine in liver and small intestine rise rapidly to a value close to that in plasma and then fall slowly, but linearly. This allows the average specific radioactivity of free phenylalanine during a short period of labelling to be estimated from a tissue. The main advantage of this technique is its ability to measure the rate of protein synthesis in tissues with very rapid protein turnover, such as liver and gut (McNurlan *et al.*, 1979) due to its ability reach isotopic steady state rapidly. This short duration also allows more accurate definition of the metabolic state of the animal (Garlick *et al.*, 1973). Use of phenylalanine is preferable as it is an essential biochemical requirement in mammals, it has higher solubility which allows giving a larger dose, the pool of free phenylalanine in tissues is smaller, it has a reasonably uncomplicated and non-reversible metabolism, it has a comparatively low expense, and it has more convenient, rapid, sensitive and reliable analytical quantification methods (Garlick *et al.*, 1980; Schaefer & Scott, 1993). Also, perhaps most importantly, a large dose of phenylalanine injected does not in itself influence rates of protein synthesis in tissues (Garlick *et al.*, 1980). However, use of this technique for large animals requires a large volume of

unlabeled amino acid that has to be given over minutes or hours with sufficient label, which needs more sensitive analytical techniques and also increases the cost of the experiment (Schaefer & Scott, 1993).

2.2 Rationale

When dietary threonine is limiting, intestinal tissues will sequester a greater proportion of threonine to meet their needs, especially for mucin production, at the expense of extra-intestinal tissues. As threonine intake increases above the gut requirement, other tissues will increase protein synthesis until their needs are met. By comparing these tissue-specific breakpoint requirements, we can determine which tissues are prioritized or spared when threonine is limiting.

2.3 Hypothesis

Mucin-producing tissues, especially gut, sequester limited dietary threonine for protein synthesis at the expense of other tissues.

2.4 Objectives

1. To determine the threonine requirement of different tissues for protein synthesis.
2. To identify whether mucin-producing tissues sequester dietary threonine for protein and/or mucin synthesis compared with other tissues when dietary threonine is limiting.
3. To identify the effect of dietary threonine intake on threonine dehydrogenase activity in the liver.
4. To determine the rate of mucin synthesis in mucin-producing tissues with varying levels of dietary threonine intake.
5. To determine the total body threonine requirement using plasma threonine response curve.

Chapter 3 : METHODOLOGY

3.1 Animals and surgery

The Animal Care Committee of Memorial University of Newfoundland approved the animal utilization protocol for this study.

Twenty Yucatan suckling miniature piglets (age: 15±1 d; weight: 3.1±0.3 kg) were removed from the sows at the Vivarium Pig Breeding Station of Memorial University and delivered to Biotechnology building for surgery. Upon arrival, piglets were weighed and pre-anaesthetized with an intramuscular injection of acepromazine (0.5 mg/kg) plus ketamine (22 mg/kg). The piglet was given an IM injection of atropine (0.05 mg/kg) to reduce airway secretions and had an endotracheal tube put in place to maintain the airway. The piglet then received gas anaesthesia consisting of isoflurane (1-2%) delivered with oxygen (1.5 L/min), was restrained in the supine position, and kept warm with a heating pad placed under a sterile surgical towel. Animals were fitted with 2 venous catheters, one for blood sampling (femoral vein advanced to inferior vena cava) and one for intravenous infusions (jugular vein advanced to superior vena cava). They were also fitted with a gastric catheter using a Stamm gastrostomy for intragastric feeding of the experimental diets. Each venous catheter was flushed with heparinized saline (20 U/mL) to avoid any possible blockages due to blood clots.

3.2 Post-surgery care

Immediately following surgery, piglets were given an intramuscular injection of Temgesic (0.03 mg/kg of buprenorphine hydrochloride). This analgesic was repeated twice daily until Day 3 of the post-op, as required. Immediately following surgery, piglets were also given an intravenous injection of 0.5 mL of the antibiotic mixture Borgal (40 mg/mL trimethoprim and 200 mg/mL sulfadoxine). Thereafter, 0.5 mL of the same antibiotic mixture Borgal was diluted to 10 mL with non-heparinized saline and administered through both femoral and jugular catheters (half into femoral and half into jugular) daily until Day 5.

3.3 Animal housing

The piglets were housed in circular metabolic cages (0.5 m height, 1 m diameter) that allow visual and aural contact with each other. Each cage was fitted with heat lamps for additional heat. Each piglet was fitted with an adjustable clean cotton jacket with an anchoring button attached to a tether so that piglets have free movement inside the cage without tangling catheters. Toys were provided to each piglet for stimulation.

3.4 Diets

Piglets received a continuous infusion through the tether-swivel system of a complete, elemental sterile diet that provided all nutrients required (Bertolo *et al.*, 1998). The diet's amino acid profile was based on human milk protein and met 120% of each amino acid's requirement for neonatal piglets

(NRCSoS, 1998); for threonine, the requirement determined by Bertolo *et al.* (1998) was used (i.e., 100% = 0.42 g threonine/kg/d; Table 3.1). This diet was infused intravenously immediately post-surgery (Day 0) at a volume that provided 50% of the piglets' full infusion rate. On the morning of Day 1, the diet was infused intragastrically at 50%; the rate was increased to 75% in the middle of the day; and that evening, diet infusion was increased to full rate (13.5 mL/kg/h). Each morning, piglets were weighed and the rate of diet infusion was adjusted according to the daily weight. The blood sampling catheter was flushed daily with heparinized saline and blood samples (5 mL) were collected once daily until Day 4. Plasma was separated and stored at -80°C for later analysis.

Table 3.1: Amino acid composition of the complete diet

Amino acid (AA)	g AA/kg body weight/d
Alanine	1.66
Arginine	0.94
Aspartic acid	0.94
Cysteine	0.22
Glutamic acid	1.63
Glycine	0.48
Histidine	0.48
Isoleucine	0.72
Leucine	1.62
Lysine-HCl	1.61
Methionine	0.30
Phenylalanine	0.82
Proline	1.29
Serine	0.87
Taurine	0.08
Threonine	0.50
Tryptophan	0.33
Tyrosine	0.12
Valine	0.82

The complete diet was fed until the evening of Day 4 when test diets were begun. Each pig randomly received one of 20 test diets, each containing a different threonine concentration (ranging between 0.54-5.99 g/100 g AA or 20% - 220% of requirement: 100% = 0.42 g threonine/kg/d as identified in Bertolo *et al.* (1998)). All diets were made isonitrogenously by manipulating alanine content. Composition of all other amino acids and nutrients were the same as the complete diet.

3.5 Flooding dose infusion

Nearly 24 hours after starting the experimental diet, a flooding dose of 1 mCi/kg body weight of labeled L-[^{3}H]phenylalanine mixed with unlabeled phenylalanine (150 mmol/L, 10 mL/kg body weight) was infused intravenously over 5 min (Brunton *et al.*, 2012). Blood (1.5 mL) was sampled in heparinized saline tubes at 30 min after the initiation of isotope infusion; plasma was separated by centrifugation at 4^{o}C and stored at -80^{o}C for later analysis.

3.6 Sample collection and processing

Immediately following the last blood sample, piglets were anaesthetized with halothane/oxygen by mask and a mid line incision was made to expose the intestine. Sixty centimeters of intestine was removed at 15 cm and 300 cm distal to the ligament of Treitz and labeled as proximal jejunum and mid jejunum, respectively. Another 60 cm segment of small intestine was removed at thirty centimeters from the ileo-cecal valve and labeled as the

ileum. The intestinal sections of gut were flushed with ice cold sterile 0.9% saline and cut lengthwise to expose the lumen. On a glass plate on ice, mucosa was collected by firmly scraping along the gut section recovering the entire mucosal tissue leaving only muscularis. All tissues and mucosa were weighed, snap frozen in liquid nitrogen and stored at -80oC for later analysis. The distal one third of the stomach (pylorus), distal colon, gastrocnemius muscle, longissimus dorsi muscle, right kidney, left liver lobe and right lung were also rapidly removed, weighed, snap frozen and stored at -80°C for later analysis. The piglets were killed under anaesthesia via exsanguination after removal of tissues.

3.7 Rate of tissue protein synthesis

The methods for the measurement of tissue free and bound amino acids were adapted from those of Garlick *et al.* (1980) with some modifications.

Tissue free amino acid quantification

Tissue free amino acids were extracted by homogenizing 1 g of tissue in 3 mL of cold 2% perchloric acid (PCA) and centrifuged at 4000xg for 15 min. Supernatant was separated and the pellet was homogenized two more times making the total volume of the supernatant 9 mL. The pellet of precipitated protein was stored at -20°C for tissue bound amino acid determination. Fifty µL of 0.5 µCi ^{3}H-leucine and 100 µL of 25 mM norleucine were added to the supernatant as internal standards and stored at -20°C for further analysis.

Acid present in the 3 mL of supernatant was neutralized with 375 µL of 2 M K_2CO_3 and centrifuged at 4500xg for 3 min. One milliliter of the supernatant was freeze-dried overnight and stored at -20°C until derivatization.

Tissue bound amino acid quantification

Protein pellets were re-dissolved with 8 mL of 1 M NaOH solution and solubilized in 37°C water bath for 1-3 hours until clear. Fifty µL of the suspension was stored at -20°C for total protein analysis using the Biuret method. Protein was recovered by adding 4 mL of ice cold 20% PCA and kept in an ice bath for 20 min. Precipitated protein was centrifuged at 3000xg for 15 min and the supernatant was discarded. Two hundred µL of 0.5 µCi ^{3}H-leucine and 400 µL of 25 mM norleucine were added as internal standards to the tube with the pellet and hydrolyzed with 10 mL of 6 M HCl for 24±0.5 hours at 110°C. After allowing hydrolysates to cool overnight in the fumehood, the hydrolyzed sample was diluted up to 25 mL with HPLC-grade water and filtered using a filter paper (size 54). One mL was dried down overnight under vacuum oven to evaporate the HCl and was subsequently derivatized.

Plasma amino acid composition

One hundred µL of the last plasma sample (taken just before the flooding dose infusion) was mixed with 20 µL of 2.5 mM norleucine. Protein was precipitated in 1 mL of 0.5% trifluoroacetic acid (TFA) in methanol and

centrifuged at 5000xg for 5 min. Separated supernatant was freeze-dried overnight and derivatized.

Amino acid concentration in plasma was calculated as: (AUC amino acid / AUC norleucine) x (μmol norleucine added / volume of plasma).

Phenylisothiocyanate (PITC) derivatization and amino acid identification

Samples were first made basic by adding 100 μL (for tissue bound and tissue free amino acid samples) or 50 μL (for plasma samples) of freshly prepared triethylamine (TEA) : methanol : water solution in a 1:1:3 volume ratio and freeze-dried for 1 hour to form deprotonated amines, which are more reactive to PITC. Samples were then derivatized by adding 50 μL (for tissue bound and tissue free amino acid samples) or 20 μL (for plasma samples) of freshly prepared derivatizing solution (water : TEA : methanol: PITC in 1:1:7:1 ratio). After a 35 min reaction, samples were frozen in liquid nitrogen and freeze-dried overnight. Derivatized samples were re-suspended in 300 μL (for tissue bound and tissue free amino acid samples) or 200 μL (for plasma samples) of sample diluent (710 mg of Na_2HPO_4 in 1 L was titrated to pH 7.4 with 10% H_3PO_4 and mixed with 5% of total volume with acetonitrile). Supernatants were separated by centrifuging at 5000xg for 5 min and re-centrifuged again if necessary.

Samples were injected (30 μL for tissue bound amino acid analysis, 10 μL for tissue free amino acid analysis and 40 μL for plasma amino acid analysis) and

amino acids were separated by reverse-phase HPLC on a C18 column (Waters). Fractions (3 min) corresponding to tyrosine, leucine and phenylalanine peaks were collected from tissue bound and tissue free amino acid samples. The radioactivity associated with each amino acid was measured as disintegrations per minute (dpm) using a liquid scintillation counter with added scintillant (10 mL of Scintiverse in 3 mL fractions). Counting error was kept below 5%.

Calculations

The rate of tissue protein synthesis (Ks) was calculated using the equations below as in Garlick *et al.* (1980).

$$\text{Specific radioactivity (SRA)} = \frac{^{3}\text{H-Phe (dpm/g)}}{\text{Phe (µmol/g)}}$$

$$\text{Phe in dpm/g} = \frac{\text{Max. AUC Nle}}{\text{AUC Nle}} \times \frac{\text{dpm Phe}}{\text{weight of the tissue injected (g)}}$$

$$\text{Phe in µmol/g} = \frac{\text{AUC Phe}}{\text{AUC Nle}} \times \frac{\text{µmol Nle in sample}}{\text{weight of the tissue injected (g)}}$$

$$\text{Ks (\%/d)} = \frac{\text{SRA of tissue bound protein}}{\text{SRA of tissue free AA}} \times \frac{100}{\text{Time between } ^{3}\text{H-}\textit{Phe} \text{ injection and tissue freezing in days}}$$

AUC – area under the curve

dpm – disintegrations per minute

Max. AUC Nle – the maximum AUC of norleucine among samples in a set; this was used to correct for loss of sample during processing to determine dpm recovery

3.8 Muco-protein (mucin) synthesis

This method was adapted from Nichols & Bertolo (2008) and Faure *et al.* (2002). Mucin synthesis was measured for the ileum, colon and stomach samples.

Protease digestion

Approximately 300 mg of tissue was homogenized for 45 s in 2 mL of ice-cold 50 mM Tris (pH 7.5) using a Polytron homogenizer at 30%. A solution containing 20 mg of Flavourzyme (a fungal complex of exopeptidases and endoproteases; Novozymes) was added to the homogenate and left to incubate under agitation in a 37°C water bath overnight. After 18 h, the enzymatic activity of Flavourzyme was stopped by placing the homogenate on ice for 10 min. Guanidium hydrochloride and dithiothreitol were added to 4 M and 10 mmol/L concentrations, respectively, and the mixture was left at room temperature for 2 h under agitation. Finally, 25 mM iodoacetamide was added to the homogenate and left overnight at room temperature under agitation.

Gravity gel filtration, dialysis and radioactivity measurement

Mucins were purified by gravity gel filtration chromatography using columns (PD10 columns, BioRad) filled with 10 mL of Sepharose CL-4B resin (Sigma, wet bead diameter 40-165 μm). The resin was equilibrated with 20 mL (or two times the volume of the column) of 0.05 M Tris/HCl buffer, pH 7.5 containing 2 M guanidinium hydrochloride (38.21 g GuHCl in 500 mL of 0.05

M Tris). One mL of homogenate was loaded onto the columns and eluted as the first fraction. Another 11 fractions were eluted separately in the void volume of the column by adding 1 mL of 0.05 M Tris/HCl buffer, pH 7.5 containing 2 M guanidinium hydrochloride each time.

Homogenate samples from each tissue (ileum, colon and stomach) were measured from piglets with the lowest, medium and highest threonine intakes. The muco-protein-containing fractions were dialyzed against deionized water for 48 h at 4°C using Spectra/Por molecular porous membrane tubing (12,000–14,000 molecular weight cutoff; Fisher Scientific). Initially, fractions were run on SDS-PAGE in order to identify the fractions from the column with mucin (described below). From those results, the first 5 fractions were pooled for each tissue and radioactivity in 1 mL of the pooled fractions was measured using a liquid scintillation counter with 5 mL of Scintiverse.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and PAS staining

The following reagents were prepared.

Tris-HCl	1.5 M (pH 8.8)	18.2 g/100 mL; adjust pH with HCl
	1.0 M (pH 6.8)	12.1 g/100 mL; adjust pH with HCl
10% Sodium dodecyl sulfate (SDS)		10 g/100 mL
80% glycerol		80 g/100 mL
1 M dithiothreitol (DTT)		1.545 g/10 mL
1.5% ammonium persulfate (APS)		0.15 g/10 mL

Stacking gel and resolving gel were prepared using the recipe below.

	Stacking gel (4%)	**Separating gel (7.5%)**
Deionized water	3.34 mL	3.1 mL
30% Acrylamide	0.665 mL	1.875 mL
1.0 M Tris-HCl (pH 6.8)	0.57 mL	-
1.5 M Tris-HCl (pH 8.8)	-	1.9 mL
10% SDS	45 µL	75 µL
1.5% APS	330 µL	500 µL
Tetramethylethylenediamine (TEMED)	5 µL	5 µL

Three μL of 80% glycerol and 2 μL of DTT were mixed with 20 μL of each fraction and boiled for 3 min. Twenty μL of boiled fractions and 7 μL of thawed molecular weight marker were applied to SDS–PAGE. Mucins were electrophoresed for 30 min at 10 mA and then for 2 h at 20 mA. Proteins in gels were removed and immediately stained with periodic acid/Schiff's reagent (PAS).

Calculations

Phenylalanine tracer incorporation into mucin was calculated for ileum, colon and stomach as dpm per milligram tissue per day and dpm per gram of protein per day.

3.9 Threonine dehydrogenase activity

TDG catalyzes the oxidation of L-threonine, yielding 2-amino-3-oxobutyric acid (2-amino-3-ketobutyrate), which is then decarboxylated to give aminoacetone or glycine and acetyl CoA when TDG is coupled with 2-amino-3-oxobutyrate CoA ligase (or aminoacetone synthetase). The TDG activity is measured as the rate of aminoacetone formation at 533 nm. This method was adapted from Le Floc'h *et al.* (1994).

The following reagents and buffers were prepared.

0.2 M Tris		24.22 g/L
0.15 M KCl		11.2 g/L
0.5 M L-threonine		59.55 g/L
25 mM Nicotinamide adenine dineucleotide (NAD)		16.58 g/L
3 mM aminoacetone		33 mg/100 mL
Sodium acetate	0.5 M	41.02 g/L
	3 M	246.09 g/L
1 N NaOH		40 g/L
Acetylacetone		
3 M Trichloracetic acid (TCA)		490.24 g/L
Ehrlich Reagent		1 g dimethylaminobenzaldehyde (DMAB) + 8 mL 70% perchloric acid (PCA) + 50 mL glacial acetic acid
Buffer 1		0.2 M Tris + 0.15 M KCl pH 7.6 (with HCl)
Buffer 2		0.2 M Tris pH 8.4

Preparation of tissue homogenate

Approximately 1 g of tissue (liver and mid jejunum) was homogenized in 5 mL of ice-cold Buffer 1. The homogenate was kept on ice for the duration of assay. The amount of homogenate to use in the assay was determined by conducting a homogenate dilution curve using a range of 5 to 100 µL; the final homogenate concentration used was within the linear portion of this curve.

Determination of the activity of TDG

The standards of aminoacetone with concentrations of 0, 75, 150, 300, 450, 600 μM were prepared as in Table 3.2 and run in duplicate.

The reaction mixture was prepared in triplicate for the samples and in duplicate for controls as in Table 3.3.

Table 3.2: Reaction mixture for the standards of aminoacetone

Aminoacetone (μM)	3 mM Aminoacetone (μL)	Water (μL)	Buffer 2 (μL)	25 mM NAD (μL)
0	0	200	500	100
75	25	175	500	100
150	50	150	500	100
300	100	100	500	100
450	150	50	500	100
600	200	0	500	100

Table 3.3: Reaction mixture for the samples and controls of TDG assay

	Sample (µL)	Control (µL)
Buffer 2	500	500
NAD 25 mM	100	100
Homogenate	20	0
Water	180	200

After a 5 min pre-incubation at 37°C, 200 µl of 0.5 M L-threonine was added to the samples and the controls (instead 200 µl of water was added to the standards in order to correct the volume) and the final mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 1 mL of 3 M TCA and centrifuged for 5 min at 5000xg.

Assay of aminoacetone

One mL of supernatant was mixed with 250 mL of 0.5 M Na acetate and 150 mL of 1 N NaOH. One hundred µL of this mixture was taken and mixed with 400 µL of 3 M sodium acetate (pH 4.6) and 50 µL of acetylacetone. The mixture was kept 10 min at 100°C and then let to cool on ice. After adding 500 µl of Ehrlich's reagent, samples were incubated for 6 - 20 min at room temperature, and aminoacetone formation was measured at 553 nm.

Calculations

The concentration of aminoacetone formed (C) in µmol/mL was calculated by linear regression using the standard curve. The average concentration of the controls was subtracted from the average sample concentration and the protein concentration of the liver homogenate was identified from the Biuret assay. Activity per gram of tissue, activity per milligram of protein, total activity and activity per kilogram of body weight were calculated using the following equations.

$$\text{Activity per g of tissue } (\mu\text{mol/min/g tissue}) = \frac{(C \times \text{initial volume of homogenate})}{(\text{incubation time} \times \text{portion of homogenate used} \times \text{weight of ground tissue})}$$

$$\text{Activity by mg of protein } (\mu\text{mol/min/mg protein}) = \frac{C}{(\text{incubation time} \times \text{portion of homogenate used} \times \text{protein conc. of homogenate})}$$

Total Activity (μmol/min) = Activity per g of tissue x weight of the liver

$$\text{Activity per kg of body weight } (\mu\text{mol/min/kg}) = \frac{\text{total activity}}{\text{body weight of the animal}}$$

3.10 Total tissue protein analysis using Biuret method

Biuret reagent was prepared by dissolving 1.59 g of $CuSO_4.5H_2O$ in ~500 mL deionized water; and adding 6 g of sodium potassium tartrate and 300 mL of 10% NaOH with constant swirling. Then, the reagent was made up to 1 L with deionized water.

The reaction mixture was prepared in individual tubes by adding the order of deoinized water, 10 mg/mL of bovine serum albumin (BSA) or tissue sample and 5% sodium deoxycholic acid (DOC) as in Table 3.4. Tissue samples for the total protein analysis were taken from the tissue bound AA preparations after solubilizing the re-suspended pellets in section 3.7. Therefore, 1 M NaOH used to re-suspend the pellet was used to prepare the reaction mixture for the reagent blank.

Table 3.4: Reaction mixture for the BSA standards and samples

	Water (µL)	BSA/sample/NaOH (µL)	DOC (µL)
Blank	800	0	200
BSA 10	790	10 (BSA)	200
BSA 20	780	20 (BSA)	200
BSA 30	770	30 (BSA)	200
BSA 50	750	50 (BSA)	200
Tissue Sample	750	50 (sample)	200
Reagent blank	750	50 (NaOH)	200

Reaction mixtures were incubated at room temp for 10 min and 4 mL of the Biuret reagent was added to each tube. After 30 min at room temperature, the absorbance was read at 550 nm. Total protein was expressed as milligram of total protein per gram of tissue.

3.11 Statistical analyses

Individual tissue dietary threonine requirements were identified using breakpoint analysis using one-slope, dual linear regression and quadratic fit. Then the best-fit model comparison was done by considering highest adjusted R^2, R^2 and lowest CV/RMSE/BIC/AIC/AICc. All these analyses were done in SAS version 9.0 and significance level was maintained at 5%. Individual tissue dietary threonine requirements were described as mean ± SE.

Whole body threonine requirement was identified using plasma threonine response curve (plasma threonine concentration vs dietary threonine intake) with breakpoint analysis using one-slope, dual linear regression. This analysis was done in SAS version 9.0 and significant level was maintained at 5%.

Pearson Product Moment Correlations were calculated for the individual plasma amino acids vs plasma threonine concentrations.

Chapter 4 : RESULTS

4.1 Health and performance of the animals

All 20 Yucatan miniature piglets (age: 15 ± 1.1 days; body weight: 3.14 ± 0.30 kg) were apparently healthy at the time of surgery and up to the time of the introduction of the experimental diet 5 days later (3.87 ± 0.35 kg); during the first 5 days of complete diet, pigs experienced an average rate of weight gain of 188 g/d, indicating normal weight gain throughout the study comparable to sow-fed piglets in this herd. They actively played with the toys provided and only 4 animals had transient diarrhea for a maximum of one day at day 2-3, which was considered negligible.

4.2 Plasma amino acid profile after experimental diet

The plasma amino acid profile was analyzed after providing the 24-h experimental diet with different threonine levels (Appendix 1). Plasma threonine concentrations increased significantly with dietary threonine intakes greater than 3.0 g/100 g amino acid (Figure 4.1). Plasma concentrations of glycine, histidine, methionine and serine were all positively correlated with dietary threonine intakes; in contrast, plasma 3-methyl histidine was negatively correlated with dietary threonine and plasma tryptophan appeared to demonstrate a breakpoint response (Appendix 1). According to the Pearson Product Moment Correlation, only plasma glycine ($r = 0.695$; $p = 0.0014$) and serine ($r = 0.702$; $p = 0.0008$) concentrations were positively correlated with plasma threonine concentrations (Appendix 2). 3-

Methyl histidine concentration, which reflects muscle protein breakdown, was constant across plasma threonine concentrations.

The plasma threonine concentration response curve (Figure 4.1) over increasing dietary threonine intakes follows a breakpoint model. Plasma threonine concentrations are low and constant when threonine is below requirement because extra threonine is immediately incorporated into protein. Above requirement, extra threonine accumulates in the plasma pool as it is transported to the liver for oxidation (Bertolo *et al.*, 2005). Therefore, the plasma threonine breakpoint of 3.04 ± 0.60 g/100 g AA reflects the whole body requirement. This value is consistent with the threonine requirement of 8 d old piglets identified by the indicator amino acid oxidation technique, i.e. 2.8 ± 0.6 g/100 g AA (Bertolo *et al.*, 1998).

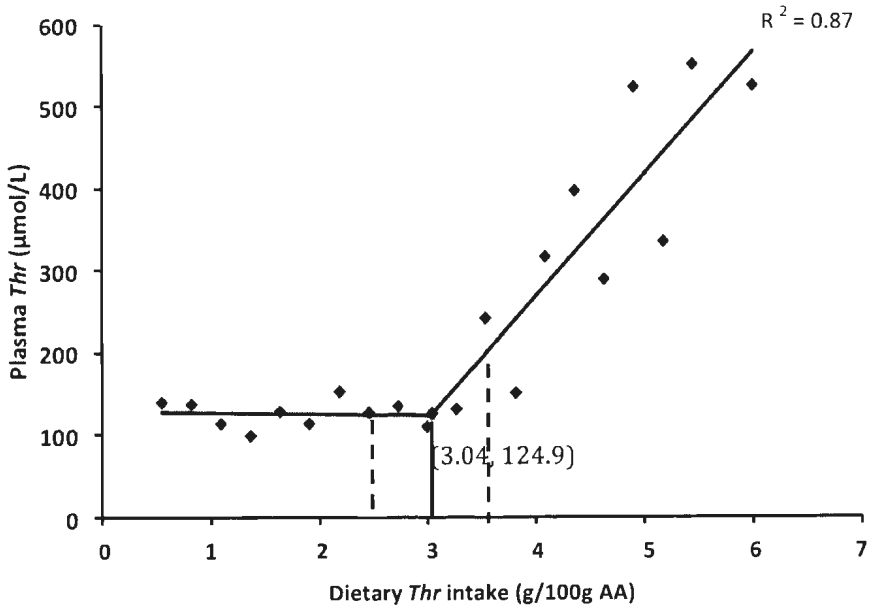


Figure 4.1: Plasma threonine response curve. Each point represents data from individual pigs (n=20); data are regressed using dual linear regression with breakpoint analysis. Solid vertical line reflects breakpoint mean threonine requirement; dashed lines refer to ± SE of the breakpoint estimate.

4.3 Individual tissue dietary threonine requirements and maximal fractional rate of protein synthesis

The rates of fractional protein synthesis (K_s) using breakpoint analysis in non-mucus producing tissues (Figure 4.2) such as kidney, liver, gastrocnemius muscle and longissimus dorsi muscle, as well as mucus producing tissues (Figure 4.3) like lung, stomach, proximal jejunum, mid jejunum, ileum and colon, were plotted against different levels of dietary threonine intakes.

All breakpoints were analyzed using one-slope / dual linear regression and quadratic fit models. One-slope, dual linear regression was selected as the best model considering highest adjusted R^2, R^2 and lowest CV/RMSE/BIC/AIC/AICc; the results are presented in Figures 4.2 and 4.3.

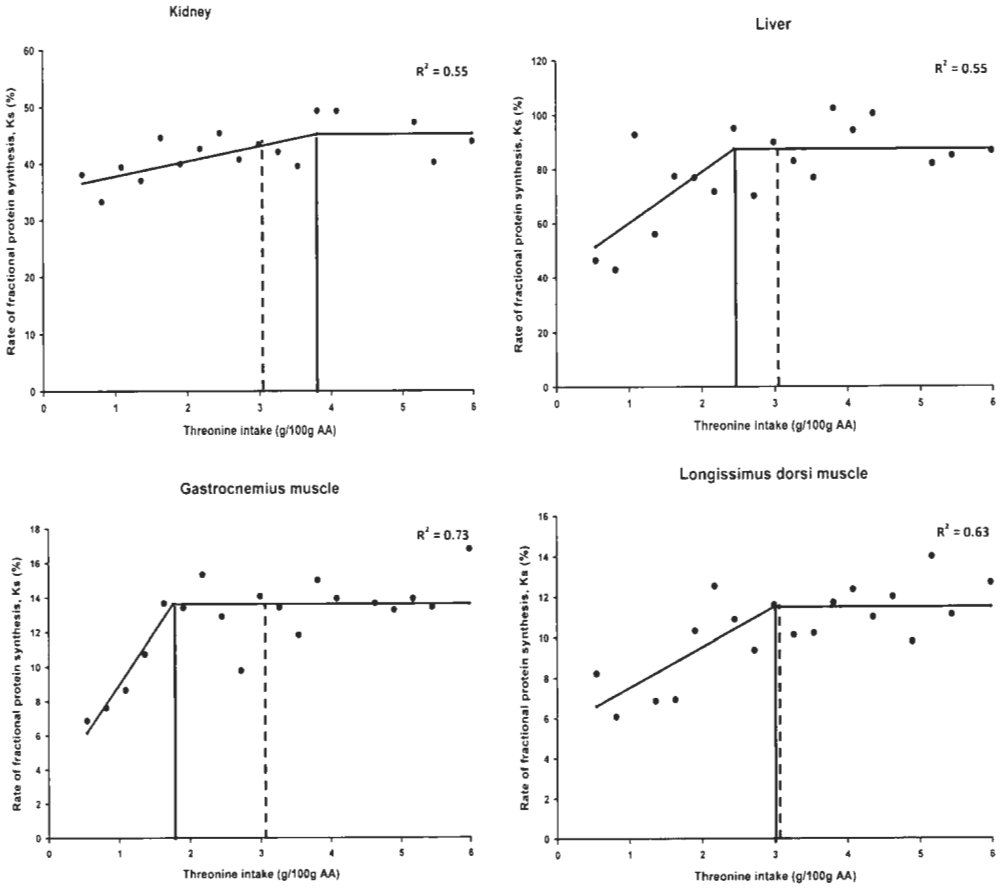


Figure 4.2: Rates of fractional protein synthesis (K_S) of non-mucus producing tissues. Each point represents data from individual pigs; data are regressed using dual linear regression with breakpoint analysis. Solid vertical lines – dietary threonine requirement; dashed lines – whole body threonine requirement determined from plasma threonine concentrations.

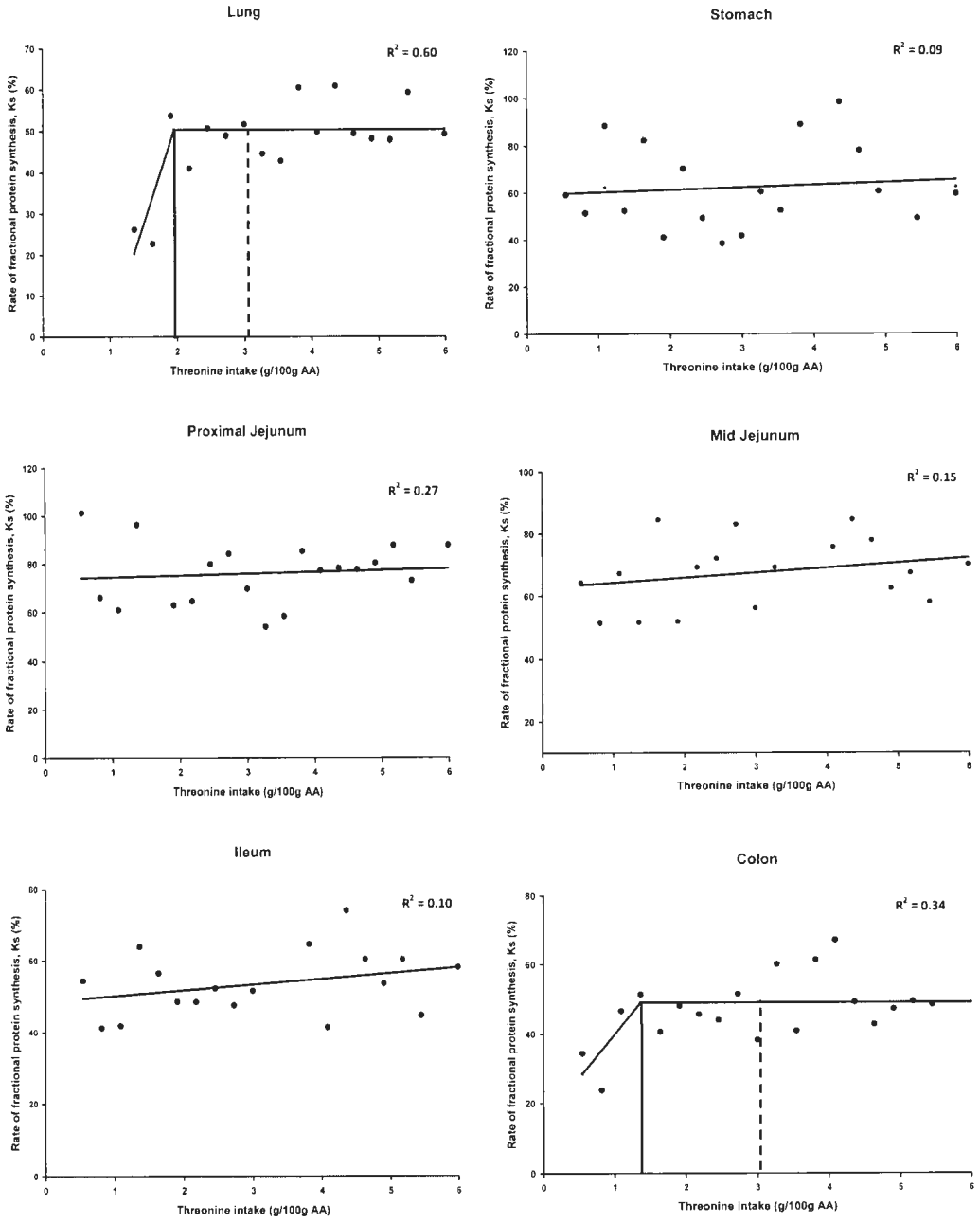


Figure 4.3: Rates of fractional protein synthesis (K_S) of mucus producing tissues. Each point represents data from individual pigs; data are regressed using dual linear regression with breakpoint analysis. Solid vertical lines – dietary threonine requirement; dashed lines – whole body threonine requirement determined from plasma threonine concentrations.

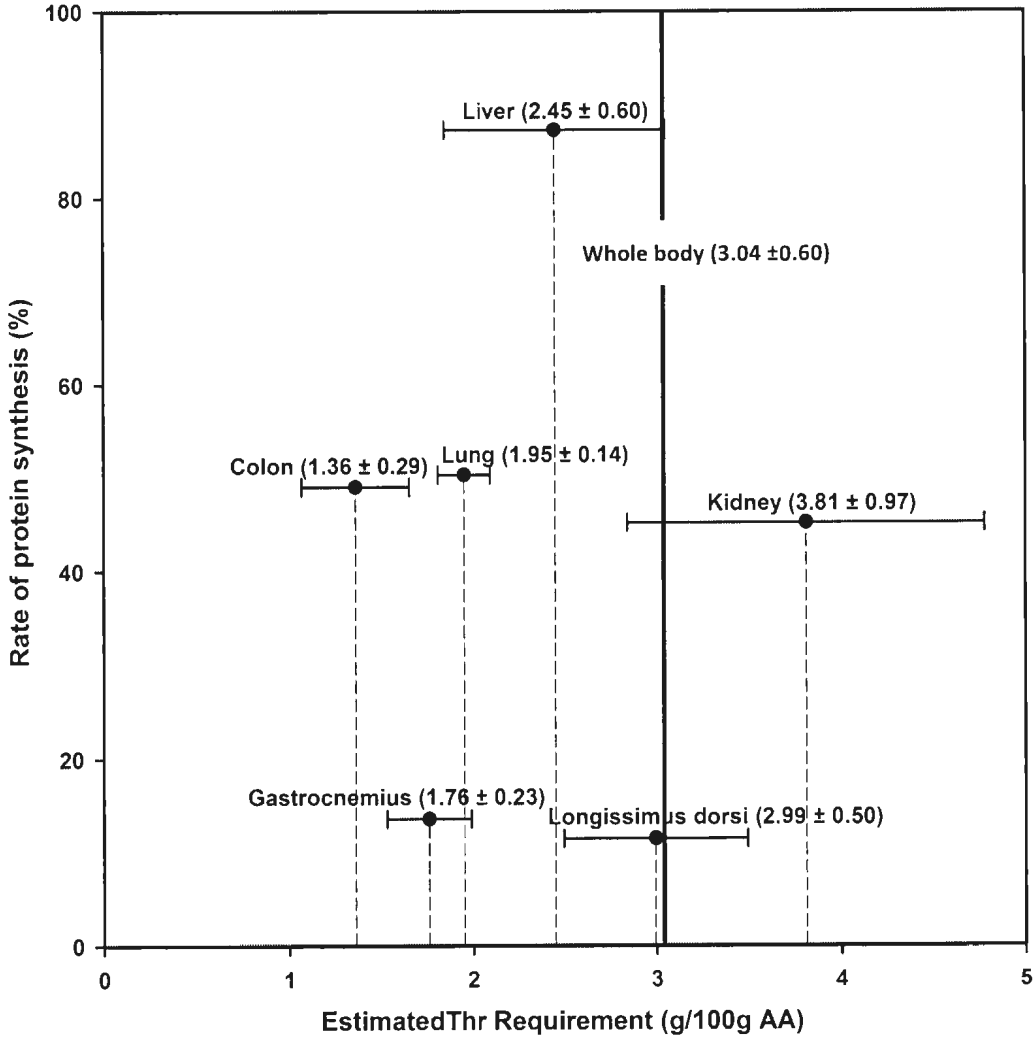


Figure 4.4: Estimated threonine requirement (±SE) of different tissues. Dashed dotted lines – threonine requirement of different tissues; solid vertical line – whole body threonine requirement determined from plasma threonine concentrations.

Breakpoints were identified in non-mucus producing tissues (kidney, liver and muscle) and some of the mucus producing tissues (lung and colon). The other mucus producing tissues (stomach, proximal jejunum, mid-jejunum, ileum) did not have discernible breakpoints and with the exception of the ileum, the data were not different across threonine intakes, suggesting the data were at plateau ($p>0.05$). This suggests that for these tissues, the breakpoints might have existed at a dietary threonine level lower than the lowest threonine level fed, i.e. < 0.5 g/100 g AA.

Of the tissues with breakpoints (Figure 4.2 and 4.3), lung, gastrocnemius muscle and colon had dietary threonine requirements that were lower than the whole body threonine requirement identified by plasma threonine response curve. Liver, longissimus dorsi muscle and kidney all had breakpoints with SE that overlapped the whole body threonine requirement SE and so were not considered different (Figure 4.4).

The dietary threonine requirement of proximal and mid-jejunum and stomach were considered lowest and less than 0.5 g/100 g AA. The lowest observed breakpoint requirement was for colon (1.36 ± 0.29 g/100 g AA) followed by gastrocnemius muscle (1.76 ± 0.23 g/100 g AA), lung (1.95 ± 0.14 g/100 g AA), liver (2.45 ± 0.60 g/100 g AA), longissimus dorsi muscle (2.99 ± 0.50 g/100 g AA) and then kidney (3.81 ± 0.97 g/100 g AA).

After reaching the maximal fractional rate of protein synthesis at the breakpoint, the rate of fractional protein synthesis (K_s) follows a plateau. For data with a significant breakpoint, the plateau was regressed with a forced slope of zero; for data without a breakpoint, all data were linearly regressed but only ileum had a slope different than zero (Figure 4.5).

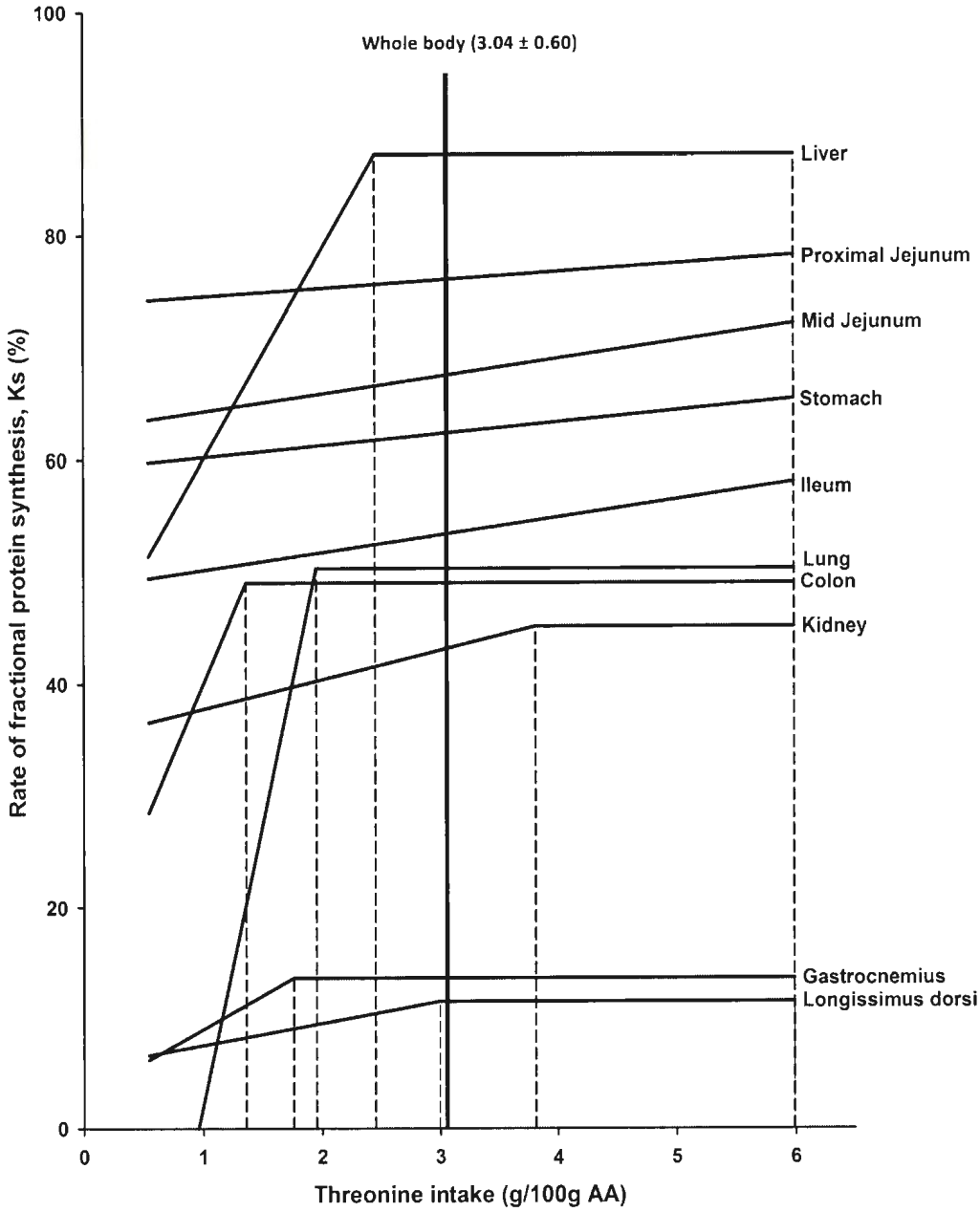


Figure 4.5: Breakpoint regression lines of different tissues of piglets receiving graded levels of dietary threonine. Solid vertical line shows the whole body threonine requirement determined from plasma threonine concentrations. Dashed vertical lines represent breakpoints of respective tissues.

4.4 Dietary threonine required to maximize muco-protein synthesis

The results for mucin isolation are consistent with those of Faure *et al.* (2002), indicating that high molecular weight muco-proteins have only slightly migrated into the separating gel (Figure 4.6), and mucin was eluted primarily in the first three fractions. Therefore, the first five fractions were pooled to analyze mucin content.

Dietary threonine required to maximize mucin synthesis in ileum and colon was 4.54 ± 1.50 g/100 g AA and 3.20 ± 4.70 g/100 g AA, respectively (Figure 4.7), whereas, data for stomach did not have a breakpoint. Therefore, it was assumed that stomach needs less than 0.5 g/100 g AA of dietary threonine to maximize its muco-protein synthesis.

After reaching the maximal fractional rate at the breakpoint, muco-protein synthesis reached a plateau. The maximal amount of muco-protein synthesis (y value at the breakpoint) in ileum is 47.53 dpm Phe/mg of tissue and 47.26 dpm Phe/mg of tissue for the colon; interestingly, the maximal fractional rate for stomach was similar at around 42 dpm Phe/ mg of tissue.

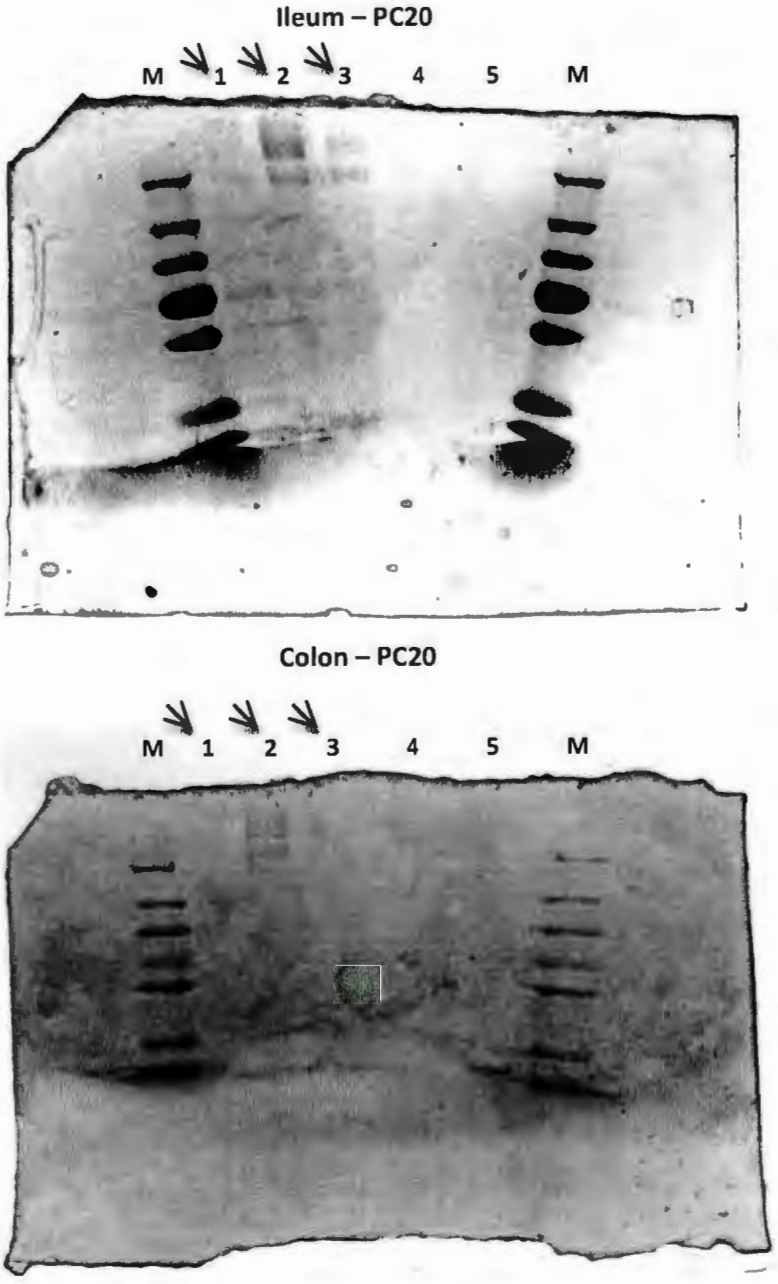


Figure 4.6: SDS-PAGE analysis of fractions 1-5 (20 µL) eluted from the Sepharose CL-4B column. Only ileum and colon of the piglet with the highest threonine intake are displayed. 1, 2, 3, 4, 5 indicate the fractions eluted from the column with the order of elution; M indicates the marker.

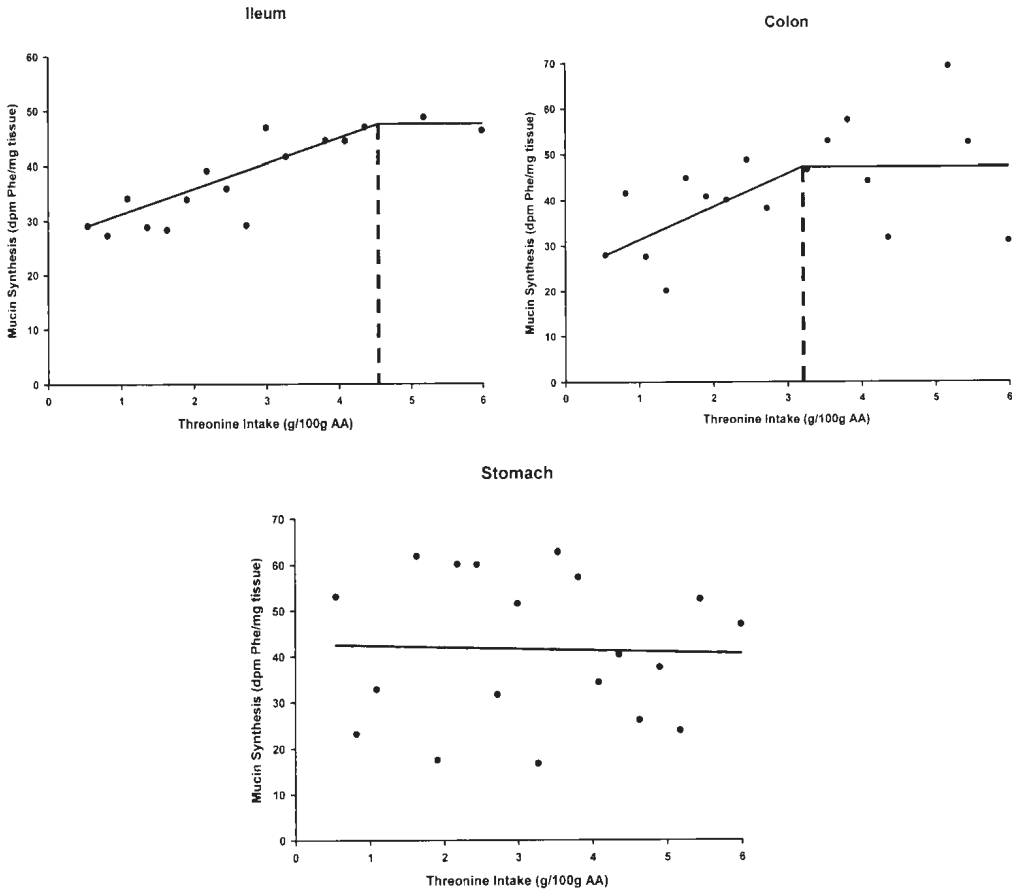


Figure 4.7: Mucin synthesis of ileum, colon and stomach. Mucin synthesis as incorporation of Phe tracer into mucin protein per unit weight of tissue. Each point represents data from individual pigs; data are regressed using dual linear regression with breakpoint analysis.

4.5 Liver and gut threonine dehydrogenase (TDG) activity

Dietary threonine that is not incorporated into protein is oxidized to glycine or ketobutyrate. Therefore, increased dietary threonine intake could potentially induce threonine catabolism and potentially mask the true breakpoint. In other words, increased threonine oxidation could limit availability of dietary threonine for protein synthesis. Hence, TDG activity, the main enzyme involved in threonine catabolism in pigs, was measured in order to ensure that the dietary threonine intake is directly proportional to protein synthesis.

In vivo, catabolism depends on the amount of active enzyme, but also on the availability of threonine for the enzyme itself. This means, plasma concentration as well as threonine transport in the tissue are the main factors controlling the flux of threonine catabolism through the TDG pathway. Liver is the main site of threonine catabolism and >80% of threonine is catabolized by hepatic TDG. Therefore, activity of TDG in liver was also plotted against dietary threonine, plasma threonine, and liver free threonine levels.

The TDG assay was conducted under the optimal conditions using 100 μM threonine as the substrate. However, tissue free threonine in the piglets of this study was <0.5 μM, indicating enzyme's capacity is much higher than the in vivo availability of threonine for oxidation. Therefore, 100% threonine in the liver of the sampled piglets would be catabolized by TDG.

No studies have been done to identify TDG activity in the gut and in order to compare gut TDG activity with the liver, gut TDG activity was also measured using three samples of the mid jejunum.

Liver maximal TDG activity did not change across plasma, liver free or dietary threonine concentrations (Figure 4.8), indicating TDG catabolism of threonine does not change with intake over the short adaptation period of this study. The mean TDG activity per gram of wet liver was 8.56 ± 1.35 μmol/min/g and this value is comparable to the liver TDG activity identified in a previous study using the same analytical method, i.e. 5.62 μmol/min/g (Le Floc'h & Seve, 2005). TDG activity of the jejunum was 0.25-1.15 μmol/min/g and this value was considered negligible (0.4-2.7% of total activity in both gut and liver) when compared with the liver (97.3-99.6% of total activity).

The tissues of the GI tract require threonine to sustain their own functions and growth, whereas the liver is mainly involved in the catabolism of excess amino acid.

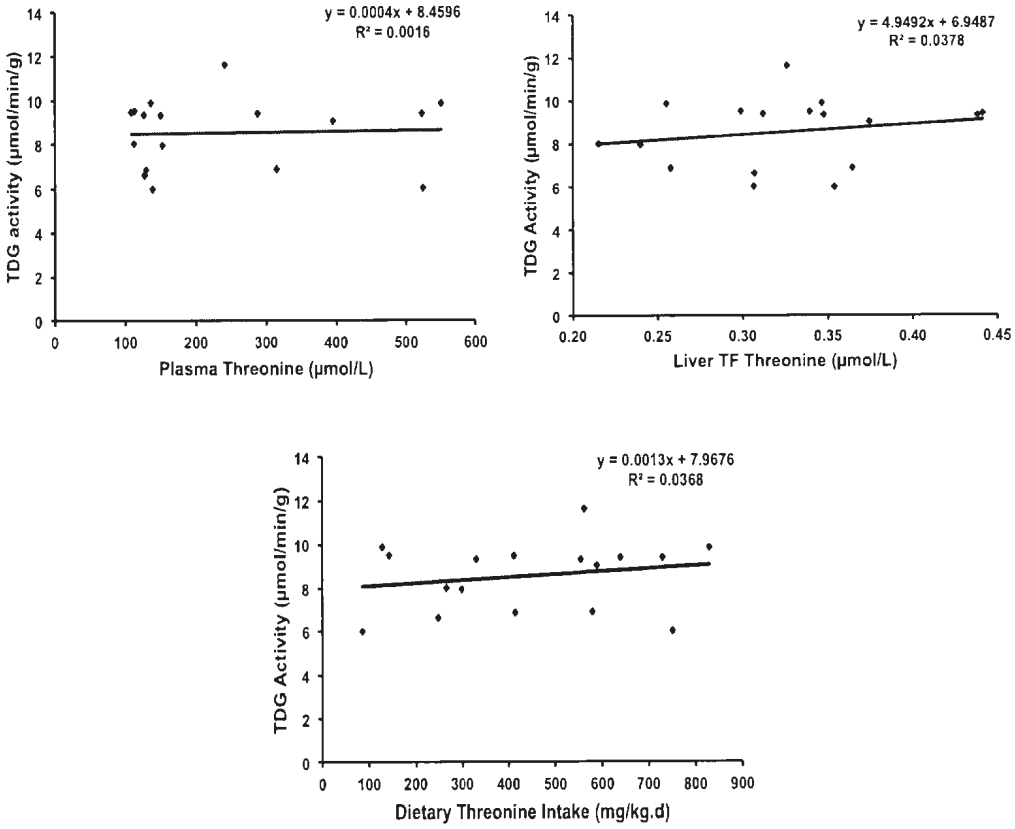


Figure 4.8: Liver TDG specific activity per gram of tissue. Each point represents data from individual pigs; data are regressed using linear regression. TF: tissue-free.

Chapter 5 GENERAL DISCUSSION AND CONCLUSION

Results of the current study indicate that dietary threonine is preferentially utilized for protein synthesis in the gastrointestinal tract tissues of piglets. Interestingly, the mucin-producing tissues have been reported to have a high obligatory requirement for threonine due to their abundance of threonine-rich muco-proteins (Mantle & Allen, 1981). Therefore, protein synthesis in these tissues is maintained when dietary threonine is limiting. If neonates are on a marginal dietary threonine intake, then muscle growth and the functions of other vital tissues are likely compromised at the expense of maintenance of the mucus layer. Moreover, TDG activity is not markedly affected by changes in the level of 24-h dietary threonine intake indicating that dietary threonine reflects threonine available for protein synthesis.

The plasma threonine response (Figure 4.1) was typical of that observed in other experiments designed to determine amino acid requirements (Mitchell *et al.*, 1968; Young *et al.*, 1972). Plasma threonine concentrations were low and constant when dietary threonine was deficient and increased with dietary threonine intake after the breakpoint.

Threonine is critical for the production of gut mucins (Vlaardingerbroek *et al.*, 2011) and the mucus layer provides a first line of defense to multiple molecules, toxins and pathogens (Allen *et al.*, 1984; Valle, 2012). A novel and important finding from the present study is that the protein synthesis in mucin-producing

tissues (Figure 4.3) does not change with lower dietary threonine content, unlike in non-mucin-producing tissues (Figure 4.2), suggesting that mucin-producing tissues extract limiting threonine at the expense of other tissues for protein synthesis, especially at lower dietary threonine intakes. Therefore, mucin-producing tissues will not be affected by deficient dietary threonine. Because the small intestine extracts dietary threonine on first pass (Le Floc'h & Seve, 2005; Stoll *et al.*, 1998a; Stoll *et al.*, 1998b), it is anatomically positioned to extract dietary threonine first to meet its own requirements. This in turn decreases threonine availability for the other vital organs and muscle when dietary threonine is deficient. Moreover, in addition to the likely first pass extraction of limited dietary threonine, the intestine also likely extracts arterial threonine to maintain protein synthesis; it is also possible that the intestinal tissues are simply more efficient at protein turnover during dietary deficiency so little threonine is lost and/or more is recycled.

The concept that dietary threonine extraction by the small intestine may reduce threonine availability for the other tissues when young piglets are fed a diet marginally deficient in threonine has been discussed before (Hamard *et al.*, 2009). Similar to our data, Hamard *et al.* (2009) found that moderate reductions in dietary threonine supply to piglets did not modify the fractional rates of mucosal protein synthesis in the proximal jejunum, the ileum and the colon. Similarly, in adult rats, the mucosal protein synthesis (Schaart *et al.*, 2005; van der Schooler *et al.*, 2002) was not impaired in the small and large intestines whatever the level of threonine deficiency (Faure *et al.*, 2005). Contradictorily, a previous study (Wang

et al., 2007) indicated that the fractional synthesis rate of small intestinal mucosal protein and mucins of young pigs was reduced by an imbalanced intake of dietary threonine. The response of protein synthesis to a threonine deficient diet could be influenced by several factors such as the tissue and organ, the degree of threonine deficiency and the animal species and age (Hamard *et al.*, 2009).

Both small intestine and stomach did not have discernible breakpoints. Moreover, for the ileum, the data were either slightly increasing or at plateau. To help clarify the response of these tissues, we decided to determine the response of mucin synthesis to dietary threonine to see if the most abundant threonine-rich protein is more sensitive to dietary threonine supply. Although protein synthesis in the ileum did not have a breakpoint over the dietary threonine range, the mucin synthesis response did display a distinct breakpoint. Moreover, the breakpoint for colonic mucin was much greater than that for total protein ynthesis. These data suggest that these intestinal tissues are sensitive to dietary threonine supply and that mucin protein synthesis specifically is reduced with lower dietary threonine. It is possible that the reduced mucin synthesis is responding to luminally delivered threonine whereas total protein synthesis is maintained from arterial threonine extraction. Whether other secretory proteins are similarly affected by limiting luminal threonine is unknown but it appears individual proteins might be prioritized within intestinal tissues in the face of threonine deficiency. Similar to breakpoint analysis for total protein, the stomach did not have a breakpoint when purified mucin was analyzed; these data suggest that perhaps the stomach is particularly resistant to changes in dietary threonine. The maximal synthesis rates

of total protein and mucin were similar to those in small intestinal tissues, which suggests that the stomach does utilize significant amounts of threonine, but apparently does not respond to dietary threonine in the same manner as the lower GIT.

Unlike non-mucin-producing tissues, protein synthesis of intestines and stomach do not respond to dietary threonine suggesting these tissues maintain synthesis of total protein at the expense of other tissues when dietary threonine is deficient. The muscle and kidney are the most sensitive organs for protein synthesis when dietary threonine is limiting, as these tissues maximized protein synthesis at higher dietary threonine levels compared with other tissues. Therefore, the growth and functions of the kidney and muscle are most likely to be compromised at even moderately deficient dietary threonine intake. However, the reason why the kidney is the most sensitive organ to dietary threonine intake is unknown; although it should be noted that the difference in the rate of kidney protein synthesis from plateau and the lowest rate was smaller (~25%) than that in other tissues (~50%). In the current study, dietary deficiency of threonine results in lower rates of protein synthesis in skeletal muscle and liver. Wang *et al.*, (2007) found that young pigs fed a 0.37% true ileal digestible threonine (TIDT) diet had a 20% lower weight gain compared with pigs fed control (0.74 % TIDT) diets. This was considered due to impaired muscle growth due to the threonine deficient diet but they also suggested that the rate of protein synthesis in skeletal muscle is more responsive to changes in dietary intake of nutrients, including amino acids, than in the liver (Wang *et al.*, 2007). In another study (Hamard *et al.*, 2009), protein

metabolism in the small intestine seemed to be preserved while the liver appeared more sensitive to a moderate dietary threonine deficiency than the other body compartments, such as colon and muscle. In the current study, the liver was more sensitive to dietary threonine compared to all GIT tissues but less so than kidney and longissimus dorsi muscle.

The gastrocnemius muscle is a very powerful superficial pennate muscle that is in the back part of the lower leg, and is involved in standing, walking, running and jumping, whereas, the longissimus is the muscle lateral to the semispinalis, and involved in respiratory movements as well as helping to move the neck. The fast and slow muscle fibres alter the activity of myosin ATPase, which is directly related to the intrinsic speed of contraction of a muscle, and the number of fibres low in myosin ATPase activity increases with growth (Davies, 1972). All these differences result in different distribution of the muscle fibre types; for example, the gastrocnemius contains about 50% slow twitch oxidative fibres (Edgerton *et al.*, 1975) that use oxidative metabolism to generate ATP, whereas longissimus has 30.5% slow twitch fibres (Davies, 1972). There may be differences in amino acid composition of fast and slow-twitch muscle fibres and so it may be possible to have differences in the amino acid composition of the muscle types. This might help explain why the two muscle groups responded differently to dietary threonine content. Alternatively, gastrocnemius muscle is more active in young pigs compared with longissimus dorsi and therefore blood circulation may be higher in gastrocnemius muscle to meet its increased demand for oxygen and other substrates during movements. Therefore, it is also possible that the gastrocnemius

muscle extracts more dietary threonine via the blood than longissimus dorsi, perhaps explaining why the latter muscle is more sensitive to diminishing threonine supply.

When comparing maximal fractional rates of protein synthesis (Figure 4.5), the highest maximal fractional rate was found for the liver and lowest for the muscle. This was hypothesized, as liver is a highly metabolically active organ, whereas muscle is least metabolically active. The second highest maximal synthesis rate was for the small intestine indicating its importance as a metabolically active organ.

In pigs, TDG is the main enzyme involved in threonine catabolism (Kidd & Kerr, 1996; Le Floc'h *et al.*, 1995; Le Floc'h *et al.*, 1996) and it mainly occurs in the liver (Le Floc'h *et al.*, 1995; Le Floc'h *et al.*, 1997). However, extrahepatic threonine oxidation may also be associated with the decrease in total oxidation when dietary supply of threonine is reduced (Le Floc'h *et al.*, 1995). If TDG oxidation is increased as dietary threonine is increased, then dietary threonine may not be available for protein synthesis, which might change the shape of the breakpoint curves. For example, if most of each increment of additional dietary threonine is oxidized by the gut and liver, then the protein synthesis curve would increase only slowly, if at all. So to address this potential confounder, we measured TDG activity in gut and liver but found no differences across dietary threonine content.

As in previous observations (Le Floc'h *et al.*, 1995; Wang *et al.*, 2007), plasma concentrations of glycine, a product of oxidation of threonine in mammals, including pigs, was positively correlated with plasma threonine concentrations (results not shown; $r = 0.695$; $p = 0.0014$). However, Wang *et al.* (2007) identified that further increases in dietary threonine beyond 0.74% true ileal digestible threonine did not additionally increase plasma glycine, suggesting a limit in threonine degradation via the hepatic TDG pathway. However, in our study, deficient diets were only fed for 24 hours, so induction of oxidative enzymes might be more limited than in chronic feeding studies; but threonine oxidation to glycine also takes place in extrahepatic tissues and probably only partly in the liver (Le Floc'h *et al.*, 1995). Indeed, although we did not detect any correlation of hepatic TDG activity with dietary threonine, plasma glycine was positively correlated with dietary threonine intake perhaps suggesting there is some extrahepatic oxidation induction as dietary threonine is increased.

Le Floc'h & Seve (2005) reported that the specific TDG activity in liver and pancreas was 5.62 and 1.46 μmol/min/g, respectively, compared with 8.56 ± 1.35 μmol/min/g found for liver in the current study. TDG activity has not been detected in the small intestine in pigs (Le Floc'h & Seve, 2005; Le Floc'h *et al.*, 1997; Wu, 1998) and our current data confirm this conclusion of negligible specific TDG activity (< 2.78% of the hepatic activity, based on grams of tissue) in the intestine. Moreover, the activity of TDG in the gut relative to the hepatic activity identified in the current study is even lower than that of pancreas (< 10%), brain (10%) and kidney (30%) in pigs identified by previous studies (Green & Elliott,

1964 in Le Floc'h *et al.*, 1995). Therefore, intestinal threonine catabolism does not help explain why intestinal tissues had no breakpoint across dietary threonine intakes. In other words, the first-pass dietary threonine extracted by the intestine is mostly available for protein synthesis. Pancreas oxidation could be more responsive to a decrease in plasma threonine concentration when compared with liver (Le Floc'h *et al.*, 1995). However, the current study did not investigate TDG activity in the pancreas, as we hypothesized that given the enormous size difference between gut and pancreas, gut TDG activity would be more significant.

Le Floc'h *et al.* (1995) also could not find any effect of dietary threonine on liver TDG specific activity in pigs. Moreover, when the dietary supply of essential amino acids is low, in vivo oxidation of these acids seems to be limited to preserve their use for protein synthesis (Le Floc'h *et al.*, 1995). Also, threonine seems to be poorly taken up by the liver compared with other amino acids in pigs (Rerat *et al.*, 1992), and therefore, one of the reasons for a relatively low hepatic oxidation of threonine could be a lower uptake (Le Floc'h *et al.*, 1995). Further, according to Michaelis Menten kinetic parameters, the hepatic enzyme in pigs has high value of K_m, i.e. 5 mM (Tressel *et al.*, 1986) and therefore, threonine oxidation into glycine should occur at a level dependent on tissue threonine concentration (Le Floc'h *et al.*, 1995). However, liver TDG activity did not change according to the change in plasma threonine concentration or liver tissue free threonine level in our study, further indicating dietary threonine is mainly used for the protein synthesis and was not catabolized via the TDG pathway (Figure 4.8). Moreover, it is important to note that in the current study, the hepatic free threonine availability (i.e., <0.5 μM

is much lower than the hepatic enzyme capacity. Therefore capacity exceeded available threonine and any changes within our study parameters would not be measurable without tracer kinetics applications.

Conclusion

By analyzing breakpoints in individual tissues, we were able to evaluate the partitioning of dietary threonine to target organs for protein synthesis. In other words, as threonine is removed from the diet, we were able to determine which tissues reduced protein synthesis thereby sparing threonine for other tissues. It also helped further define the requirement of dietary threonine to achieve optimal growth as marginal dietary threonine intake can adversely affect growth and functions of certain vital tissues except the gut. It is obvious that not all tissues require the same dietary threonine level and our data help explain why growth is limited before intestinal function. Dietary threonine is obviously very important to maintain the mucus layer of the gut, which is the first barrier protection against p thogens and anti-nutritional dietary components. By determining individual dietary threonine "requirements" by breakpoint, we can estimate the amount of dietary threonine necessary to maintain protein synthesis in each tissue. In the future, it would be interesting to investigate the changes to these dietary threonine "requirements" of individual tissues in the presence of different mucin secretagogues such as fibre. Such data would help define the therapeutic levels of threonine necessary to maintain function in specific tissues.

BIBLIOGRAPHY

1. Allee G, Hines R: **Amino acid supplementation of wheat and milo for the finishing pig.** In *Swine Day.* Manhattan: Kansas State University. Agricultural Experiment Station and Cooperative Extension Service 1971:1-6.
2. Allen A, Snary D: **The structure and function of gastric mucus.** *Gut* 1972, **13:**666-672.
3. Allen A, Cunliffe W, Pearson J, Sellers L, Ward R: **Studies on gastrointestinal mucus.** *Scandinavian Journal of Gastroenterology Supplement* 1984, **93:**101-113.
4. Ballevre O, Cadenhead A, Calder A, Rees W, Lobley G, Fuller M, Garlick P: **Quantitative partition of threonine oxidation in pigs: effect of dietary threonine.** *American Journal of Physiology-Endocrinology and Metabolism* 1990, **259:**E483-E491.
5. Ballevre O, Houlier ML, Prugnaud J, Bayle G, Bercovici D, Seve B, Arnal M: **Altered partition of threonine metabolism in pigs by protein-free feeding or starvation.** *American Journal of Physiology-Endocrinology and Metabolism* 1991, **261:**E748-E757.
6. Bansil R, Turner BS: **Mucin structure, aggregation, physiological functions and biomedical applications.** *Current Opinion in Colloid & Interface Science* 2006, **11:**164-170.
7. Baracos VE: **Animal models of amino acid metabolism: a focus on the intestine.** *The Journal of Nutrition* 2004, **134:**1656S-1659S.

8. Barrett KE: **Functional Anatomy of the GI Tract and Organs Draining into It.** In *Gastrointestinal Physiology.* Edited by Barrett KE. New York: McGraw-Hill; 2006.

9. Barrett KE, Boitano S, Barman SM, Brooks HL: **Transport & Metabolic Functions of the Liver.** In *Ganong's Review of Medical Physiology.* 23 edition. USA: McGraw-Hill Medical; 2010.

10. Bell SL, Xu G, Khatri IA, Wang R, Rahman S, Forstner JF: **N-linked oligosaccharides play a role in disulphide-dependent dimerization of intestinal mucin Muc2.** *Biochemical Journal* 2003, **373:**893-900.

11. Bertolo RFP, Chen CZL, Law G, Pencharz PB, Ball RO: **Threonine requirement of neonatal piglets receiving total parenteral nutrition is considerably lower than that of piglets receiving an identical diet intragastrically.** *The Journal of Nutrition* 1998, **128:**1752-1759.

12. Bertolo RFP, Pencharz PB, Ball RO: **Role of Intestinal First-pass Metabolism on Whole Body Amino Acid Requirements.** In Biology of Growing Animals. Volume 3: Biology of Metabolism in Growing Animals. Edited by Burrin DG, MersmannHJ: Elsevier Science, London; 2005: 127-156.

13. Book SA, Bustad LK: **The fetal and neonatal pig in biomedical research.** *Journal of Animal Science* 1974, **38:**997-1002.

14. Brockhausen I: **Intestinal candyfloss.** *The Biochemical Journal* 2004, **384:**e3-e5.

15. Brunton JA, Baldwin MP, Hanna RA, Bertolo RF: **Proline supplementation to parenteral nutrition results in greater rates of protein synthesis in the muscle, skin, and small intestine in neonatal Yucatan miniature piglets.** *The Journal of Nutrition* 2012, **142**:1004-1008.

16. Burrin D, Stoll B, Van Goudoever J, Reeds P: **Nutrient Requirements for Intestinal Growth and Metabolism in the Developing Pig.** In: Digestive Physiology of Pigs. Edited by Lindberg J, Ogle B: CABI Publishing; 2001: 75-88.

17. Castagne V, Moennoz D, Finot PA, Maire JC: **Effects of diet-induced hyperthreoninemia. I) amino acid levels in the central nervous system and peripheral tissues.** *Life Sciences* 1993, **53**:1803-1810.

18. Cid MC, Esparza J, Schnaper HW, Juan M, Yague J, Grant DS, Urbano-Marquez A, Hoffman GS, Kleinman HK: **Estradiol enhances endothelial cell interactions with extracellular matrix proteins via an increase in integrin expression and function.** *Angiogenesis* 1999, **3**:271-280.

19. Cohen R, Tanksley T: **Limiting amino acids in sorghum for growing and finishing swine.** *Journal of Animal Science* 1976, **43**:1028-1034.

20. Corfield AP, Myerscough N, Longman R, Sylvester P, Arul S, Pignatelli M: **Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease.** *Gut* 2000, **47**:589-594.

21. Darling PB, Grunow J, Rafii M, Brookes S, Ball RO, Pencharz PB: **Threonine dehydrogenase is a minor degradative pathway of threonine catabolism in adult humans.** *American Journal of Physiology-Endocrinology and Metabolism* 2000, **278:**E877-E884.

22. Davies A: **Postnatal changes in the histochemical fibre types of porcine skeletal muscle.** *Journal of Anatomy* 1972, **113:**213.

23. Defa L, Changting X, Shiyan Q, Jinhui Z, Johnson E, Thacker P: **Effects of dietary threonine on performance, plasma parameters and immune function of growing pigs.** *Animal Feed Science and Technology* 1999, **78:**179-188.

24. Dekker J, Aelmans PH, Strous GJ: **The oligomeric structure of rat and human gastric mucins.** *The Biochemical Journal* 1991, **277 (Pt 2):**423-427.

25. Edgerton VR, Smith J, Simpson D: **Muscle fibre type populations of human leg muscles.** *The Histochemical Journal* 1975, **7:**259-266.

26. Ehrenkranz RA, Younes N, Lemons JA, Fanaroff AA, Donovan EF, Wright LL, Katsikiotis V, Tyson JE, Oh W, Shankaran S: **Longitudinal growth of hospitalized very low birth weight infants.** *Pediatrics* 1999, **104:**280-289.

27. Faure M, Moennoz D, Montigon F, Mettraux C, Breuille D, Ballevre O: **Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats.** *The Journal of Nutrition* 2005, **135:**486-491.

28. Faure M, Moennoz D, Montigon F, Fay LB, Breuille D, Finot PA, Ballevre O, Boza J: **Development of a rapid and convenient method to purify mucins and determine their in vivo synthesis rate in rats.** *Analytical Biochemistry* 2002, **307:**244-251.

29. Fuller M, Livingstone R, Baird BA: **The optimal amino acid supplementation of barley for the growing pig.** *British Journal of Nutrition* 1979, **41:**321-331.

30. Fuller M, McWilliam R, Wang T, Giles L: **The optimum dietary amino acid pattern for growing pigs.** *British Journal of Nutrition* 1989, **62:**255-267.

31. Garlick PJ, McNurlan MA, Preedy VR: **A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [3H] phenylalanine.** *Biochemical Journal* 1980, **192:**719.

32. Garlick PJ, Millward D, James W: **The diurnal response of muscle and liver protein synthesis in vivo in meal-fed rats.** *Biochemical Journal* 1973, **136**:935-945.

33. Gendler S, Spicer A: **Epithelial mucin genes.** *Annual Review of Physiology* 1995, **57:**607-634.

34. Green ML, Elliott W: **The enzymic formation of aminoacetone from threonine and its further metabolism.** *Biochemical Journal* 1964, **92:**537.

35. Grosbach D, Lewis A, Peo Jr E: **An evaluation of threonine and isoleucine as the third and fourth limiting amino acids in corn for growing swine.** *Journal of Animal Science* 1985, **60:**487-494.

36. Hamard A, Seve B, Le Floc'h N: **Intestinal development and growth performance of early-weaned piglets fed a low-threonine diet.** *Animal* 2007, **1:**1134-1142.

37. Hamard A, Seve B, Le Floc'h N: **A moderate threonine deficiency differently affects protein metabolism in tissues of early-weaned piglets.** *Comparative Biochemistry and Physiology-Part A: Molecular & Integrative Physiology* 2009, **152:**491-497.

38. Hamard A, Mazurais D, Boudry G, Le Huerou-Luron I, Seve B, Le Floc'h N: **A moderate threonine deficiency affects gene expression profile, paracellular permeability and glucose absorption capacity in the ileum of piglets.** *The Journal of Nutritional Biochemistry* 2010, **21:**914-921.

39. Harvey CM, Muzyka WR, Yao S, Cheeseman CI, Young JD: **Expression of rat intestinal L-lysine transport systems in isolated oocytes of Xenopus laevis.** *American Journal of Physiology-Gastrointestinal and Liver Physiology* 1993, **265**:G99-G106.

40. Herlinger H: **Anatomy of the Small Intestine.** In *Clinical Imaging of the Small Intestine.* 2nd edition. Edited by Hans Herlinger DDTM, Bernard A. Birnbaum. New York: Springer-Verlag New York, Inc.; 1999

41. Kang-Lee YA, Harper AE: **Threonine metabolism in vivo: effect of threonine intake and prior induction of threonine dehydratase in rats.** *The Journal of Nutrition* 1978, **108:**163.

42. Kidd M, Kerr B: **L-threonine for poultry: A review.** *The Journal of Applied Poultry Research* 1996, **5:**358-367.

43. Lamont JT: **Mucus: the front line of intestinal mucosal defense.** *Annals of the New York Academy of Sciences* 1992, **664:**190-201.

44. Law GK, Bertolo RF, Adjiri-Awere A, Pencharz PB, Ball RO: **Adequate oral threonine is critical for mucin production and gut function in neonatal piglets.** *American Journal of Physiology Gastrointestinal and Liver Physiology* 2007, **292:**G1293-G1301.

45. Le Floc'h N, Seve B: **Catabolism through the threonine dehydrogenase pathway does not account for the high first-pass extraction rate of dietary threonine by the portal drained viscera in pigs.** *British Journal of Nutrition* 2005, **93:**447-456.

46. Le Floc'h N, Obled C, Seve B: **In vivo threonine oxidation rate is dependent on threonine dietary supply in growing pigs fed low to adequate levels.** *The Journal of Nutrition* 1995, **125:**2550-2562.

47. Le Floc'h N, Obled C, Seve B: **In vivo threonine oxidation in growing pigs fed on diets with graded levels of threonine.** *British Journal of Nutrition* 1996, **75:**825-837.

48. Le Floc'h N, Seve B, Henry Y: **The addition of glutamic acid or protein to a threonine-deficient diet differentially affects growth performance and threonine dehydrogenase activity in fattening pigs.** *The Journal of Nutrition* 1994, **124:**1987-1995.

49. Le Floc'h N, Thibault JN, Seve B: **Tissue localization of threonine oxidation in pigs.** *British Journal of Nutrition* 1997, **77:**593-603.

50. Lien K, Sauer W, Fenton M: **Mucin output in ileal digesta of pigs fed a protein-free diet.** *Zeitschrift fur Ernahrungswissenschaft* 1997, **36**:182-190.

51. Lin JH, Chiba M, Baillie TA: **Is the role of the small intestine in first-pass metabolism overemphasized?** *Pharmacological Reviews* 1999, **51**:135-158.

52. Lucke H, Haase W, Murer H: **Amino acid transport in brush-border-membrane vesicles isolated from human small intestine.** *Biochemical Journal* 1977, **168**:529-532.

53. Mantle M, Allen A: **Isolation and characterization of the native glycoprotein from pig small-intestinal mucus.** *Biochemical Journal* 1981, **195**:267-275.

54. Mantle M, Forstner GG, Forstner JF: **Antigenic and structural features of goblet-cell mucin of human small intestine.** *Biochemical Journal* 1984, **217**:159-167.

55. Marth J: **O-Glycans.** In *Essentials of Glycobiology.* 2 edition. Edited by Varki A CR, Esko J, Freeze H, Hart G, Marth J. New York: Cold Spring Harbor 1999.

56. McNurlan M, Garlick P: **Contribution of rat liver and gastrointestinal tract to whole-body protein synthesis in the rat.** *Biochemical Journal* 1980, **186**:381-383

57. Michael SM, Smith AC: **Swine in Biomedical Research.** *Sourcebook of Models for Biomedical Research* 2008:233-239.

58. Miller ER, Ullrey DE: **The pig as a model for human nutrition.** *Annual Review of Nutrition* 1987, **7**:361-382.

59. Mircheff AK, van Os CH, Wright EM: **Pathways for alanine transport in intestinal basal lateral membrane vesicles.** *The Journal of membrane biology* 1980, **52**:83-92.

60. Mitchell Jr J, Becker D, Jensen A, Harmon B, Norton H: **Determination of amino acid needs of the young pig by nitrogen balance and plasma-free amino acids.** *Journal of Animal Science* 1968, **27**:1327-1331.

61. Moniaux N, Escande F, Porchet N, Aubert JP, Batra SK: **Structural organization and classification of the human mucin genes.** *Frontiers in Bioscience : A Journal and Virtual Library* 2001, **6**:D1192-1206.

62. Munck B, Schultz SG: **Lysine transport across isolated rabbit ileum.** *The Journal of general physiology* 1969, **53**:157-182.

63. Muramatsu K, Odagiri H, Morishita S, Takeuchi H: **Effect of excess levels of individual amino acids on growth of rats fed casein diets.** *The Journal of Nutrition* 1971, **101**:1117-1125.

64. Murtaugh MP, Monteiro-Riviere NA, Panepinto L: **Swine Research Breeds, Methods, and Biomedical Models.** In *Advances in Swine in Biomedical Research. Volume* 2. Edited by Tumbleson ME, School LB. New York: Plenum Publishing Corporation; 1996.

65. Nichols NL, Bertolo RF: **Luminal threonine concentration acutely affects intestinal mucosal protein and mucin synthesis in piglets.** *The Journal of Nutrition* 2008, **138**:1298-1303.

66. NRCSoS N: *Nutrient requirements of swine.* 10 edn: National Academies Press; 1998.

67. Nunoya T, Shibuya K, Saitoh T, Yazawa H, Nakamura K, Baba Y, Hirai T: **Use of miniature pig for biomedical research, with reference to toxicologic studies.** *Journal of Toxicologic Pathology* 2007, **20:**125-132.

68. Piel C, Montagne L, Salgado P, Lalles JP: **Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods.** *Reproduction Nutrition Development* 2004, **44:**419-435.

69. Rerat A, Simoes-Nunes C, Mendy F, Vaissade P, Vaugelade P: **Splanchnic fluxes of amino acids after duodenal infusion of carbohydrate solutions containing free amino acids or oligopeptides in the non-anaesthetized pig.** *British Journal of Nutrition* 1992, **68:**111-138.

70. Roberton AM, Rabel B, Harding CA, Tasman-Jones C, Harris PJ, Lee S: **Use of the ileal conduit as a model for studying human small intestinal mucus glycoprotein secretion.** *American Journal of Physiology-Gastrointestinal and Liver Physiology* 1991, **261:**G728-G734.

71. Salmon WD: **The significance of amino acid imbalance in nutrition.** *The American Journal of Clinical Nutrition* 1958, **6:**487-494.

72. Schaart MW, Schierbeek H, van der Schoor SRD, Stoll B, Burrin DG, Reeds PJ, van Goudoever JB: **Threonine utilization is high in the intestine of piglets.** *The Journal of Nutrition* 2005, **135:**765-770.

73. Schaefer A, Scott S: **Amino acid flooding doses for measuring rates of protein synthesis.** *Amino Acids* 1993, **4**:5-19.

74. Silva E, Teixeira A, David L, Carneiro F, Reis CA, Sobrinho-Simoes J, Serpa J, Veerman E, Bolscher J, Sobrinho-Simoes M: **Mucins as key molecules for the classification of intestinal metaplasia of the stomach.** *Virchows Archiv* 2002, **440**:311-317.

75. Stevens BR, Kaunitz JD, Wright EM: **Intestinal transport of amino acids and sugars: advances using membrane vesicles.** *Annual Review of Physiology* 1984, **46**:417-433.

76. Stoll B, Burrin DG, Henry J, Jahoor F, Reeds PJ: **Phenylalanine utilization by the gut and liver measured with intravenous and intragastric tracers in pigs.** *American Journal of Physiology-Gastrointestinal and Liver Physiology* 1997, **273**:G1208-G1217.

77. Stoll B, Burrin DG, Henry J, Yu H, Jahoor F, Reeds PJ: **Dietary amino acids are the preferential source of hepatic protein synthesis in piglets.** *The Journal of Nutrition* 1998a, **128**:1517-1524.

78. Stoll B, Henry J, Reeds PJ, Yu H, Jahoor F, Burrin DG: **Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets.** *The Journal of Nutrition* 1998b, **128**:606-614.

79. Tovar A, Tews JK, Torres N, Harper AE: **Some Characteristics of Threonine Transport Across the Blood-Brain Barrier of the Rat.** *Journal of Neurochemistry* 1988, 51:1285-1293.

80. Tressel T, Thompson R, Zieske L, Menendez M, Davis L: **Interaction between L-threonine dehydrogenase and aminoacetone synthetase and mechanism of aminoacetone production.** *The Journal of Biological Chemistry* 1986, **261**:16428-16437.

81. Turner BS, Bhaskar KR, Hadzopoulou-Cladaras M, LaMont JT: **Cysteine-rich regions of pig gastric mucin contain von Willebrand factor and cystine knot domains at the carboxyl terminal.** *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1999, **1447:**77-92.

82. Valle JD: **Peptic Ulcer Disease.** In *Harrison's Princicles of Internal Medicine.* 18th edition. Edited by Dan L. Longo ASF, Dennis L. Kasper, Stephen L. Hauser, J. Larry Jameson, Joseph Loscalzo. New York: McGraw-Hill Companies, Inc; 2012.

83. van der Schoor SRD, Wattimena DL, Huijmans J, Vermes A, van Goudoever JB: **The gut takes nearly all: threonine kinetics in infants.** *The American Journal of Clinical Nutrition* 2007, **86:**1132-1138.

84. van der Schoor SRD, Reeds PJ, Stoll B, Henry JF, Rosenberger JR, Burrin DG, van Goudoever JB: **The high metabolic cost of a functional gut.** *Gastroenterology* 2002, **123:**1931-1940.

85. Van Goudoever J, Stoll B, Henry J, Burrin D, Reeds P: **Adaptive regulation of intestinal lysine metabolism.** *Proceedings of the National Academy of Sciences* 2000, **97:**11620-11625.

86. Veluraja K, Vennila KN, Umamakeshvari K, Jasmine A, Velmurugan D: **Structural investigation of porcine stomach mucin by X-ray fiber diffraction and homology modeling.** *Biochemical and Biophysical Research Communications* 2011, **406:**570-573.

87. Vlaardingerbroek H, van den Akker CHP, de Groof F, Hogewind-Schoonenboom JE, Huang L, Riedijk MA, van der Schoor SRD, Huang Y: **Amino Acids for the Neonate: Search for the Ideal Dietary Composition.** *Neoreviews* 2011, **12**:e506-e516.

88. Wang X, Qiao S, Yin Y, Yue L, Wang Z, Wu G: **A deficiency or excess of dietary threonine reduces protein synthesis in jejunum and skeletal muscle of young pigs.** *The Journal of Nutrition* 2007, **137**:1442-1446.

89. Wesley A, Mantle M, Man D, Qureshi R, Forstner G, Forstner J: **Neutral and acidic species of human intestinal mucin. Evidence for different core peptides.** *The Journal of Biological Chemistry* 1985, **260**:7955-7959.

90. Wolfram S, Giering H, Scharrer E: **Na+-Gradient dependence of basic amino acid transport into rat intestinal brush border membrane vesicles.** *Comparative Biochemistry and Physiology Part A: Physiology* 1984, **78**:475-480.

91. Wu G: **Intestinal mucosal amino acid catabolism.** *The Journal of Nutrition* 1998, **128**:1249-1252.

92. Yamashita K, Ashida K: **Effect of excessive levels of lysine and threonine on the metabolism of these amino acids in rats.** *The Journal of Nutrition* 1971, **101**:1607-1613.

93. Yoshida A, Leung PMB, Rogers Q, Harper A: **Effect of amino acid imbalance on the fate of the limiting amino acid.** *The Journal of Nutrition* 1966, **89**:80-90.

94. Young VR, Tontisirin K, Ozalp I, Lakshmanan F, Scrimshaw NS: **Plasma amino acid response curve and amino acid requirements in young men: valine and lysine.** *The Journal of Nutrition* 1972, **102:**1159-1169.

APPENDIX 1: Plasma amino acid profile against dietary threonine

Figures 1 to 20: Each point represents data from individual pigs; data are regressed using simple linear regression or dual linear regression (for Fig. 1 and 16) with breakpoint analysis. Dashed lines in Fig. 1 and 16 represent their break points. Fig. 18 has a sigmoidal curve. Regression co-efficient (R^2) and p-value for each linear regression are shown.

APPENDIX 1: Plasma amino acid profile

Fig. 1: Alanine

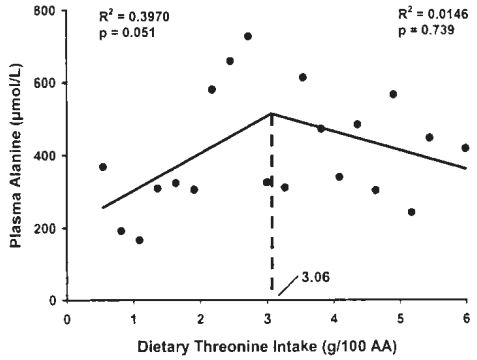


Fig. 2: Arginine

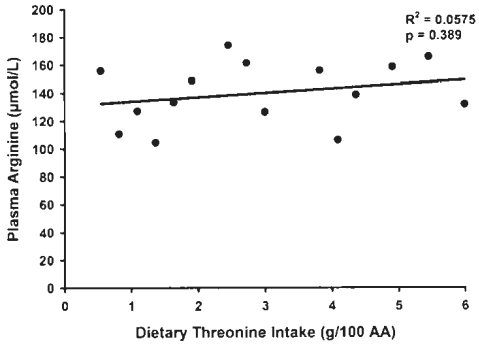


Fig. 3: Aspartic Acid

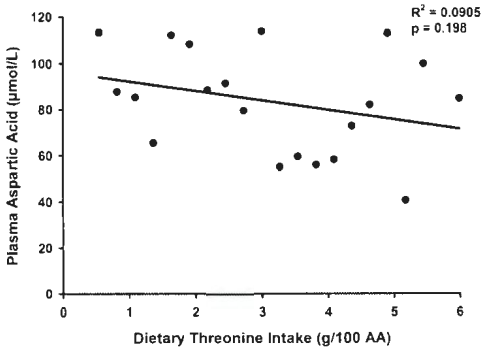


Fig. 4: Cysteine

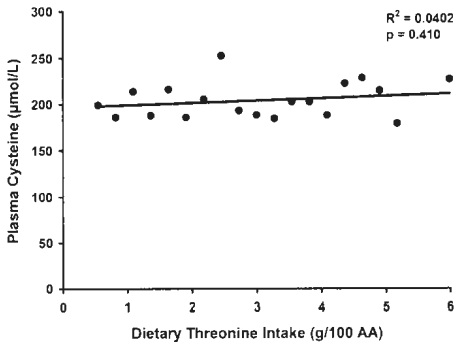


Fig. 5: Glycine

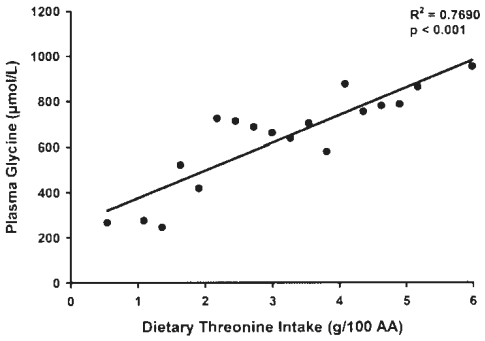


Fig. 6: Glutamic Acid

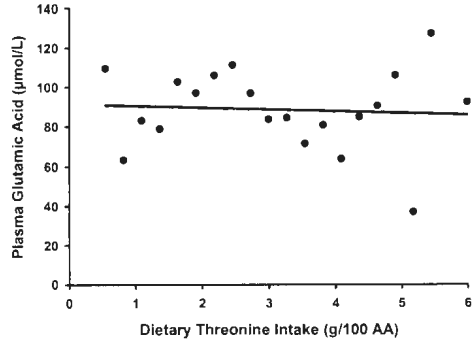


Fig. 7: Histidine

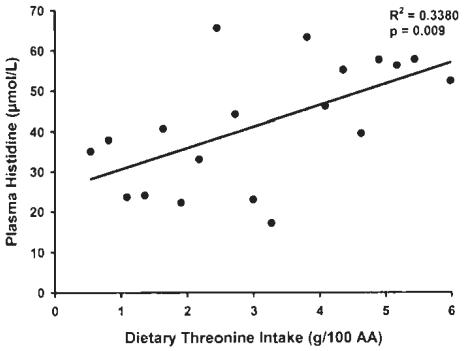


Fig. 8: Isoleucine

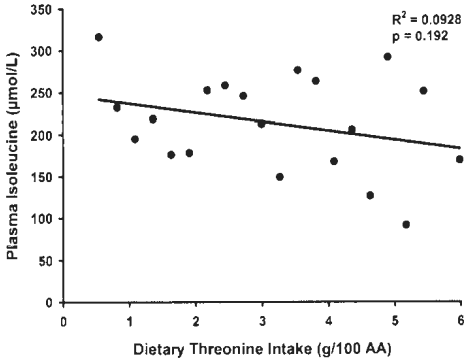


Fig. 9: Leucine

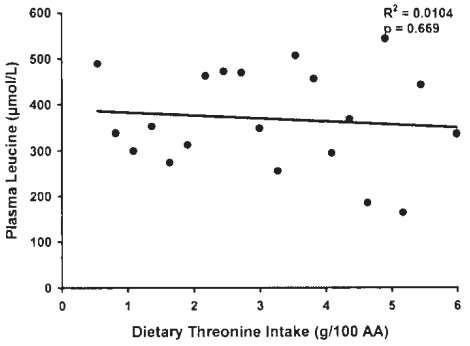


Fig. 10: Lysine

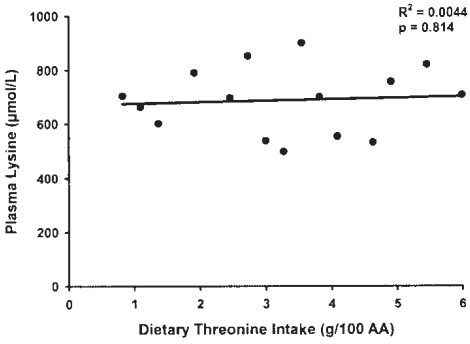


Fig. 11: Methionine

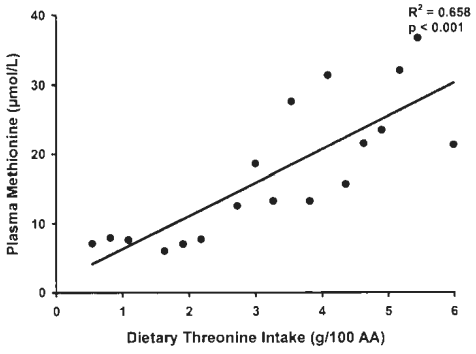


Fig. 12: Phenylalanine

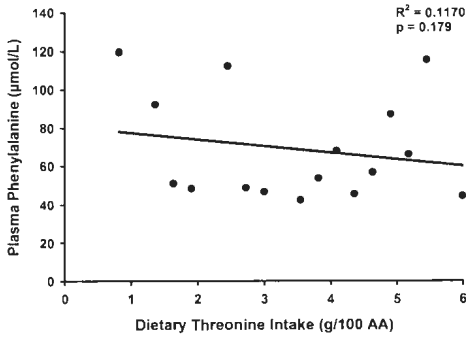


Fig. 13: Proline

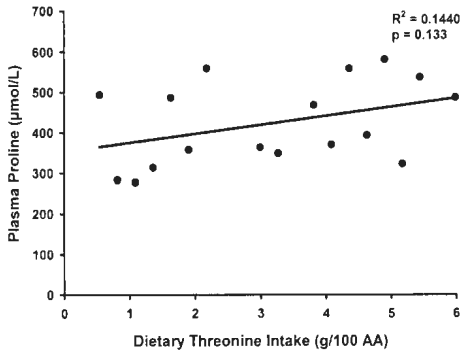


Fig. 14: Serine

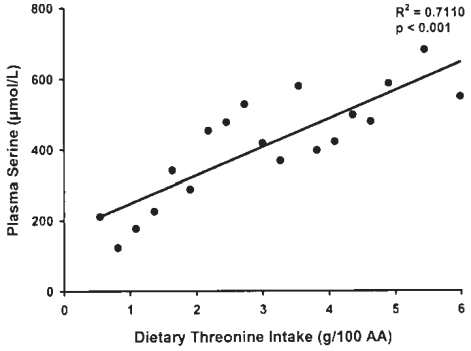


Fig. 15: Taurine

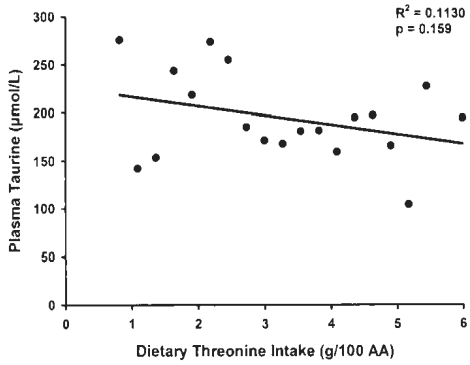


Fig. 16: Threonine

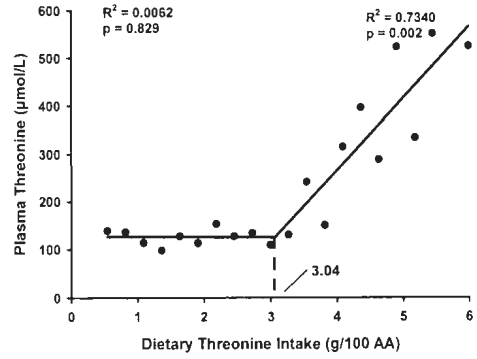


Fig. 17: 3-Methyl Histidine

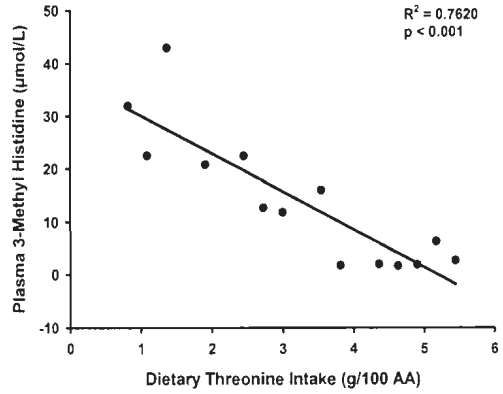


Fig. 18: Tryptophan

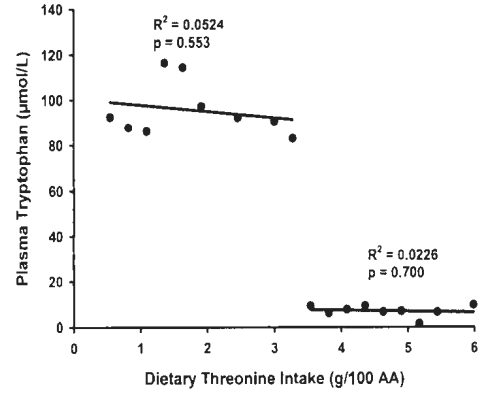


Fig. 19: Tyrosine

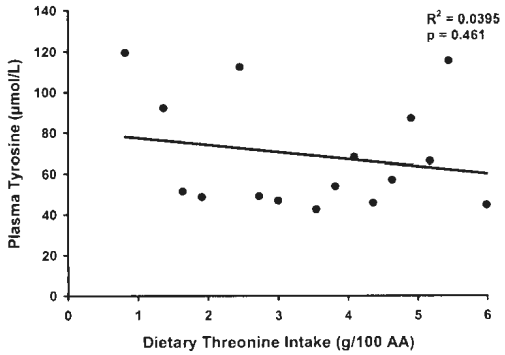
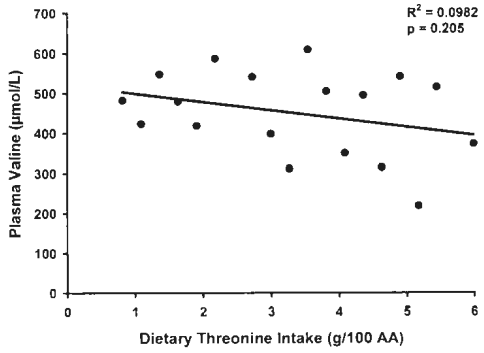


Fig. 20: Valine



APPENDIX 2: Plasma glycine and serine against plasma threonine

Figures 1 and 2: Each point represents data from individual pigs; data are regressed using simple linear regression. Pearson Product Moment Correlation co-efficient (r) and p-value for each correlation are shown.

Fig. 1: Plasma Glycine

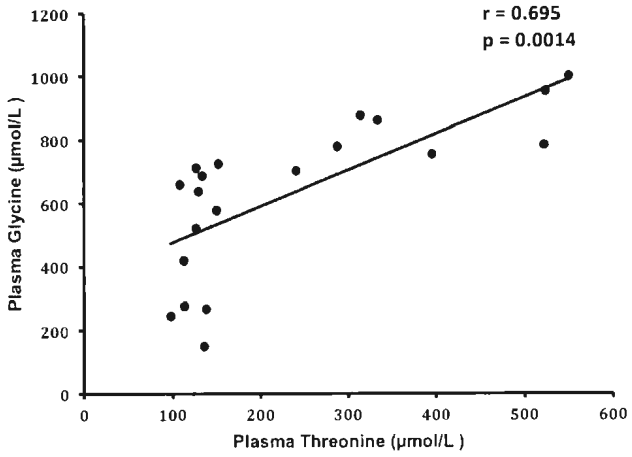


Fig. 2: Plasma Serine