Weaning-Induced Alterations to Sulfur Amino Acid Utilization in Pigs

by

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Abstract

Weaning is one of the most stressful events for pigs. It is associated with low and variable feed intake, poor nutrient utilization, and high feed costs. A small percentage of weaned pigs do not ingest feed for several days post-weaning. Cysteine (Cys) is an important sulfur amino acid (SAA) and has key roles in pig production, but how Cys metabolism is modified by weaning is unclear. The objective of this study was to determine how a lack of feed intake alters SAA metabolism. Feed restriction at weaning alters cysteine utilization in a tissue-dependent manner. Specifically, glutathione (GSH) was depleted in the small intestine despite increased endogenous Cys availability from muscle protein breakdown. Impaired intestinal GSH production may negatively affect gut structure and function, leading to poor growth performance and increased morbidity and mortality during the immediate post-weaning period.

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Arg	Arginine
CBS	Cystathionine β-synthase
CDO1	Cysteine dioxygenase 1
CSAD	Cysteinesulfinic acid decarboxylase
CTH	Cystathionine γ-lyase
Cys	Cysteine
GCL	Glutamate cysteine ligase
GIT	Gastrointestinal tract
Glu	Glutamate
GSH	Glutathione
GSS	Glutathione synthetase
HCy	Homocysteine
ISS	Immune system stimulation
LD	Longissimus dorsi
Leu	Leucine
Met	Methionine
MS	Methionine synthase
NO	Nitric Oxide
PWD	Post weaning diarrhea
ROS	Reactive oxygen species
SAA	Sulfur amino acids

SAM	S-adenosylmethionine
SID	Standardized ileal digestibility
Tau	Taurine
Thr	Threonine
Tyr	Tyrosine
VFD	Veterinary Feed Directive
ZnO	Zinc Oxide
γ-GC	γ-glutamylcysteine

Chapter 1:

Literature Review

Weaning stress in pigs

Weaning is one of the most stressful events a pig will undergo during its production cycle. In the United States, the process of weaning piglets begins between 19 and 22 days after birth (Cera et al. 1988). Stressors such as a new environment after removal from the sow, dietary changes, and histological changes in the small intestine effect the immune system and can lead to intestinal dysfunction (Pluske et al. 2018). These stress factors can lead to decreased feed intake which directly effects adequate growth and development of piglets. This critical period is associated with low and variable feed intake that can lead to alterations of the gut. A study conducted by Montagne et al. (2007) evaluated the effects of weaning on the gut during the first two weeks post-weaning by assessing biological markers including gut morphology and enzyme activity. Researchers found that weaning stress results in lower activity of most digestive enzymes, including lactase-phlorizin hydrolase, maltase glucoamylase, amino-peptidase N and dipeptidyl-peptidase 4, in piglets (Montagne et al. 2007). Other stressors such as a new environment after removal from the sow, dietary changes, and histological changes in the small intestine effect the immune system as well as can lead to intestinal dysfunction (Erikson et al. 2022). Overall, these stress factors can lead to decreased feed intake which directly effects adequate growth and development of piglets and therefore the producer profitability as well.

Early weaning is also a problem in the industry that can lead to negative effects. A study conducted by Marion et al. (2005) looked at early weaned pigs and the effects on the small intestine. The study found reduced villous height in the GIT and postponed maturation of

peptidase activity. A common symptom post-weaning is inflammation in the GIT (Frese et al. 2015). In the attempt to reduce inflammation, the pig GIT produces a nitrate-rich environment which is advantageous to certain strains of *E. coli* (Marion et al. 2005). Along with disruption of the microbiota in the intestines, enterotoxigenic *E. coli* thrives and often results in post-weaning colibacillosis (Pluske et al. 2017). Studies conducted post-weaning have found a decrease in *Lactobacillus* spp. bacteria and an increase in facultative anaerobes such as *E. coli* (Gresse et al. 2017). Post-weaning diarrhea (PWD) is one of the biggest problems in the swine industry and causes an average mortality of 20-30% (Rhouma et al. 2017). Symptoms of PWD include dehydration, diarrhea, oxidative stress, nutrient malabsorption, and death. Surviving piglets may suffer negative effects to their overall growth and development (Eriksen et al. 2022).

Multiple factors affect feed intake in newly weaned pigs including weaning age, use of creep feed, as well as housing environment and nutrient balance in the diet (Pluske et al. 2018). Mucosal barrier function is impaired by weaning stress as well (Smith et al. 2010). This reduced feed intake can last up to a week in some pigs and has a cost to producers that results in more money and time devoted to the pigs production cycle (McCracken et al. 1999). On average, 5-10% of piglets will not ingest feed for multiple days after weaning (Kempen, 2014). Weaned pigs require about 300 g of dry feed per day, however, actual feed intake rarely exceeds 200 g during the first week post-weaning (Villagómez-Estrada et al. 2020).

A study conducted by Pie et al. (2004) looked at the influence of diet and stress on proinflammatory cytokines at weaning. Researchers evaluated tissue morphology, enzyme activity, and cytokine mRNA expression. They found that weaning is associated with an early inflammatory response that may contribute to increased gut permeability. New methods for managing these negative effects have emerged in recent years as the inclusion of antibiotics is no

longer recommended or supported. A few of these methods include prebiotics and probiotics in addition to vaccination protocols. A study conducted by Sun et al. (2021) showed how dietary inclusions of probiotics reduced the severity of PWD. The study found that inclusion of multispecies probiotics enhanced growth performance by reducing the pH of digesta, intestinal oxidative stress, and increasing villus height in the small intestine (Sun et al. 2021). Overall, it is key to reduce physiological changes in the small intestine of pigs after weaning as much as possible for ideal growth and production.

Gastrointestinal tract structure, function, and gut health in pigs

Gut health is essential to consider in nutritional programs for pigs because it can affect a multitude of bodily functions and the overall growth of an animal. General nutrition, the mucosal lining of the gut, and microbiota are all key factors that directly affect the gut (Pluske et al. 2018). However, the gastrointestinal tract (GIT) microbiome and the function of the GIT barrier, as well as the interaction between the two, are what should be focused on when considering overall gut health (Bischoff 2011). Multiple factors influence the microbiome of the gut including age of the pig, environment, composition of the diet, stress, feed processing and storage, presence of microbial populations, genetics, and many more. The microbiome serves as a form of protection on the mucosal surface of the gut and is different depending on the specific section. It is dominated by four large groups of bacteria or phyla including *Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria* (Shreiner et al. 2015). The intestinal epithelial barrier works with gut microbiota and the immune system to generate specific immune responses. The GIT epithelium is continually exposed to a harsh luminal environment with toxins, antigens, and pathogens but has multiple barrier mechanisms to protect the gut (Moeser et

al. 2017). These two factors, the GIT microbiome and epithelial barrier, can directly affect the overall health of an animal thus why maintaining gut health can be detrimental if not well maintained. Sow milk specifically has been shown to help shape the initial microbial populations in piglets (Frese et al. 2015). It is also a major factor that influences the abrupt shift in microbiota in piglets due to the diet shifting from a simple to more complex nutrient source (Upadhaya and Kim, 2021).

The small intestine can be separated into three sections with the first being the duodenum, the second being the jejunum, and the last being the ileum that transitions into the large intestine via the ileocecal junction. The small intestine consists of four major layers: the serosa, the muscularis, the submucosa, and the mucosa (Tang et al. 2022). The serosal layer is the outermost layer of the intestinal wall and has a squamous epithelium. The muscular layer has an outer layer of longitudinal fibers and an inner layer of circular fibers that assist with gastrointestinal motility. The submucosa is made up of connective tissue that contains blood vessels, nerves, and lymphatics. Finally, the mucosa consists of three sublayers including the muscularis mucosa, lamina propria, and epithelium, which is a single layer of epithelial cells (Pond et al. 2001). The muscularis mucosa contains different types of muscle fibers that form intestinal folds. The lamina propria contains blood vessels, free lymphocytes, and Peyer's patches which are lymph nodes that monitor intestinal flora and protect against infection. The mucosa is made up of villi and the Crypts of Lieberkuhn, or intestinal glands, shown in Figure 1,1. Villi and subsequent microvilli increase the surface area of the mucosa and tend to increase in size from the duodenum to jejunum then decrease again towards the ileum. Crypts are deepest in the duodenum and jejunum and tend to become shallower towards the ileum. The villus surface has three types of epithelial cells: absorptive, goblet, and enteroendocrine. Villi have

both digestive and absorptive function depending on the section of the stalk. Nutrients are absorbed by enterocytes that line the villi and travel into portal circulation which drains directly into the liver. Enterocytes are continuously shed and renewed as intestinal stem cells located in the crypts proliferate and differentiate as they migrate toward the tip of the villi, while Goblet cells distributed within the villus secrete mucus (Kidder et al. 1978, Lim et al. 2015).

The liver has many functions but in the context of digestion its main purpose is to produce bile to help break down fats in the small intestine. Bile acids are detergent molecules that are stored in the gallbladder until signaled to release into the small intestine. The liver is the only organ that has the enzymes necessary to produce bile. The gut-to-liver axis regulates metabolic homeostasis as well as prevents metabolic diseases (Wahlström et al. 2016). Bile acids regulate the gut microbiota by controlling gut bacteria overgrowth and protecting the epithelial barrier (Chiang et al. 2018). They do so by altering membrane lipid composition, solubilizing membranes, and dissociating integral membrane proteins and damaging membrane integrity. The liver is also the major metabolizing organ for the sulfur amino acids (SAA) cysteine (Cys), methionine (Met), and taurine (Tau), as well as the main site of glutathione (GSH) synthesis (Jung, 2015).

The large intestine, also known as the hindgut, consists of the cecum and the colon. The main function of this organ is absorption of water. The mucosa of the large intestine is made up by columnar epithelial cells with goblet cells interspaced that secrete sulphated carbohydrate-protein to lubricate the colon. The cecum has two sections; the blind end, where material cannot pass through, and another portion connected to the colon where digesta is passed to the rectum and anus to be excreted (Johnson, 2006). Microbial enzyme activity occurs in the large intestine

to form volatile fatty acids (VFA) which are used to assist with the nutrient requirements of the epithelium of the large intestine.

Effects of weaning stress on gut physiology, immunity, and oxidative stress

Weaning stress can lead to a multitude of negative effects pertaining to gut physiology, immunity, and oxidative status. In a study conducted by Xiong et al. (2015), researchers found that stress from weaning caused a downregulation of proteins involved in multiple metabolic pathways including the tricarboxylic acid cycle, beta oxidation, and glycolysis within the villi of the jejunum. Weaning stress has also been found to directly affect villous height and crypt depth within the small intestine, resulting in overall reduced nutrient digestion and absorption. A study conducted Boudry et al. (2004) assessed the intestinal morphology of piglets at weaning. Researchers found that each segment of the intestine reacted differently to decreased feed intake. Villous atrophy was observed shortly after weaning in the duodenum and jejunum but did not occur in the ileum until several days post-weaning. This low absorptive capacity due to villous atrophy could result in osmotic diarrhea. The authors suggest that reducing stressors during weaning as well as promoting higher feed intake in the early post weaning period might help reduce small intestinal structure and function as well as reduce PWD. Brush border enzyme activity is reduced due to the change in diet that occurs during weaning. This results in lactase, sucrase, and maltase being affected 3 to 5 days after weaning (Hedmann et al. 2004) as well as reduced electrolyte absorption and secretion (Nabuurs et al. 1994). Weaning enhances mRNA expression of pro-inflammatory cytokines in the jejunum during the first two days post weaning (Pié et al. 2004). In a study conducted by McCracken et al. (1999), researchers also found that

weaning stress up-regulates matrix metalloproteinase by activating immune cells in the lamina propria which may contribute to villous atrophy.

Stress from weaning can cause inflammation and oxidative stress to occur in piglets. Oxidative stress can be viewed as an imbalance between prooxidants and antioxidants when reactive oxygen species (ROS) accumulate in the body (Lauridsen, 2018). Excessive production of ROS can cause cell death, tissue injury, chronic inflammatory response, and fibrogenesis (Freitas et al. 2016). Excessive ROS can also activate the up-regulation of pro-inflammatory cytokines. Oxidative stress can stem from infectious diseases or nutrient imbalances. Poor nutrition has been known to lead to inflammation as well as oxidative stress which can directly affect the immune system (Gabriele et al. 2017). Young animals, such as weaned piglets, do not have fully mature GIT and have immature immune functions leaving them vulnerable to invading microorganisms. Oxidative stress may also further negatively affect gut permeability and expression of tight junction proteins. (Rao, 2008). Tight junction proteins form an intercellular barrier between the epithelial cells and play many roles such as pro-inflammatory interleukin 17 (Lee et al. 2018). The initial gut inflammatory response includes releasing ROS such as nitric oxide (NO). A study conducted by Gresse et al. (2017) found that, once released into the intestinal lumen, NO is transformed into nitrate and supports an environment for *E. coli* production. Left unmitigated, oxidative stress can directly contribute to the incidence and severity of PWD as well as other enteric infectious diseases, increasing pig morbidity and mortality. Providing the animal with means of obtaining or making antioxidants during this period of stress is crucial in limiting the damage than can result from ROS.

Feed additives and probiotic use in the swine industry

Antibiotics have long been used in the swine industry to combat issues that stem from weaning stress, such as PWD. However, due to increased antibiotic resistance among intestinal microbes and the Veterinary Feed Directive (VFD), which was put into place to decrease the use of subtherapeutic antibiotics for growth promotion, researchers and producers have been looking into alternatives to combat illnesses in the swine industry. *Zinc oxide (ZnO) is a common additive and alternative to antibiotics in the swine industry for starter pigs but has been banned in the E.U., and will soon be prohibited in Canada, which could potentially affect legality in the U.S* (Sales et al. 2013). *Zinc oxide has been used to effectively reduce PWD in swine. Zinc itself is involved in multiple cellular functions in the body including replication, transcription maintenance of barrier integrity, protection against pathogens, and immunity (Sales, 2013). A study conducted in 1989 by Poulsen et al. found that including pharmacological amounts of ZnO to the diet of weaning piglets resulted in decreased diarrhea and increased growth rates. However, excessive ZnO is an environmental hazard and high Zn intake can lead to Zn toxicity and chronic pancreatitis in pigs (Komatsu et al. 2020). Long et al. (2017) compared the effects of dietary supplementation with low doses of porous and nano ZnO in weaning piglets as a possible alternative to high doses of regular ZnO. Researchers found low doses of both nano and porous ZnO had similar effects as high doses in reducing diarrhea and intestinal morphology changes and inflammation. These results could suggest that including ZnO in smaller amounts than commonly used could result in the same effects while reducing negative environmental hazards.*

Probiotics are another popular suggestion and have been found to both alleviate PWD and promote growth (Reid, 2002). Probiotics can be defined as live microorganisms administered

in sufficient amounts to confer health benefits to the host (Bajagai et al. 2016). Various bacteria and yeast such as *Bifidobacterium* and *Saccharomyces boulardii, respectively, have been used to confer probiotic effects in swine but the most widely used probiotic is Lactobacillus. Past research has found probiotic use swine diets has helped improve growth performance, feed conversion efficiency, intestinal microbiota modulation, nutrient utilization, gut health, and regulation of the immune system (Gareua et al. 2010). Probiotics affect their host by manipulating gut microbes, competing for adhesion sites on the mucosa, strengthening gut epithelial barrier function, and regulating the immune system (Kumar et al. 2015; Corcionivoschi et al. 2010). However, probiotic effects are known to be highly specific dependent on strain, dose, environment, and host. Consequently, there is a level of uncertainty in the scientific community around probiotics. This has led researchers to delve into other areas including nutritional additives that may positively impact the overall health of the pig. Some of these other areas of study include functional amino acids, phytochemicals, antimicrobial peptides, and short chain fatty acids (Xiong et al. 2019).*

Functional Amino Acids

Functional amino acids are known as those amino acids that are not only used to build proteins but are also essential in many metabolic pathways. Functional amino acids, which include amino acids such as threonine (Thr), Met, tryptophan (Trp), arginine (Arg), glutamine (Gln), leucine (Leu), and proline (Pro), play important roles in gene expression, nutrient transport and metabolism, cell signaling, anti-oxidative response, as well as many other processes (Wu, 2013). Threonine, Met, Trp, and Leu are commonly supplemented in swine diets and can help mitigate effects of lower protein in the diet (Sun et al. 2020, Wellington et al.

2018). Dietary supplementation with Glu has been found to maintain gut health and intestinal dysfunction in early weaned piglets (Wu et al. 2011). Arg specifically plays key roles in metabolism, reproduction, neonatal growth, and skeletal muscle protein synthesis (Wu et al. 2009). A study conducted by Dai et al. (2012) investigated the effects of L-arginine on the utilization of amino acids by pure and mixed bacterial strains/cultures from the pig small intestine. The authors suggest that supplementing Arg in the diet could reduce irreversible catabolism of dietary amino acids in the first-pass metabolism of the small intestine and affect the luminal pool of bacterial nitrogenous compounds in the gut.

Sulfur amino acids and gut health

Methionine, cysteine, homocysteine (HCy), and Tau are the four common sulfur-containing amino acids. However, only Met and Cys are incorporated into proteins. Metabolism of SAA plays a key role in cellular function and protein biosynthesis. Methionine, as the precursor for S-adenosylmethionine (SAM), the universal methyl group donor, is necessary for metabolism of polyamines, creatine, and phosphatidylcholine and the synthesis of Cys. Cysteine can then be used for synthesis of GSH, an important intracellular antioxidant, or the amino acid Tau (Martínez et al. 2017). Sulfur amino acids affect cellular metabolism and function by regulating methylation processes and redox states (Elremaly et al. 2016). After weaning, there are three main reasons for low SAA status in piglets: the amount of SAA in the diet does not meet the requirements of the piglet, reduced feed intake results in insufficient SAA intake, and weaning stress lead to increased SAA catabolism resulting in insufficient SAA availability.

Methionine is an essential amino acid and is one of the most hydrophobic amino acids due to the similar electronegativity of sulfur and carbon. Methionine is the initiating amino acid

in eukaryotic protein synthesis and involved in one-carbon metabolism (Clare et al. 2019). These reactions allow the transfer of single carbon moieties for important cellular processes like redox defense (Ducker et al. 2017). Methionine contains a carboxyl group, amino group, and an S-methyl thioether side chain. The main derivative of Met is SAM, the universal methyl group donor. S-adenosylmethionine is implicated in the synthesis or modification of nucleic acids, proteins, lipids, and other metabolites (Pascale et al. 2022). S-adenosylmethionine is made from adenosine triphosphate (ATP) and methionine by the enzyme methionine adenosyl transferase.

Cysteine is either supplied by the diet or is synthesized from Met. Cysteine is a hydrophilic amino acid; however, it has been found to associate with hydrophobic regions of protein (Jiao et al. 2022). Cysteine is present in the catalytic site of many enzymes due to its reactivity and plays an important role in protein structure due to its ability to form disulfide bonds with other Cys residues (Brosnan et al. 2006). Disulfide bonds in proteins are formed by oxidation of the sulfhydryl group of Cys residues and assist with supporting the tertiary structure of a protein. One familiar protein with cystine crosslinking is insulin, which has two separate peptide chains that are connected by a pair of disulfide bonds (Bin et al. 2017). Cysteine also participates in GSH synthesis and can affect oxidative stress and levels of ROS (Go et al. 2011).

Taurine is one of the most abundant free amino acids in tissue and has been proposed to act as an antioxidant (Brosnan et al. 1983; Rakhshandeh et al. 2020). Taurine plays many other essential roles in the body such as contributing to the development of the central nervous system, maintaining structural integrity of cell membranes, modulating protein phosphorylation, osmoregulation, and modulation of calcium signaling (Sturman et al. 1993; Pasantes-Morales et al. 1985; Lombardini et al. 1992). Taurine is also essential for cardiovascular development and function as well as function of skeletal muscle, retinas, and the central nervous system

(Huxtable, 1992). Taurine is a naturally occurring sulfonic acid and a major constituent of bile. It is naturally derived from Cys but can also be synthesized by the cysteine sulfinic acid pathway in the pancreas. In a study conducted by Liu et al. (2014), researchers investigated the effects of dietary Tau supplementation on growth performance and liver and intestinal health in weaned pigs. The experiment used three groups of pigs fed different percentages of Tau within their diet. Tissue and blood samples were collected and analyzed for differences in morphology, enzyme activity, total RNA, and antioxidant capacities. Researchers found that excessive Tau supplementation in the diet was found to have negative effects on growth performance and liver and intestinal health it pigs considering decreased villus height, increased crypt depth, and higher diarrhea index.

Homocysteine is an intermediate metabolite of Met and was discovered in 1932 while studying the sulfur component of insulin (Aubard et al. 2000). Homocysteine functions as the precursor for Cys and other metabolites and as a substrate for remethylation to Met (Finkelstein et al. 2000). Homocysteine is present in plasma as either a free thiol, disulfide-bound to plasma proteins (such as albumin) or combined to itself to form the dimer homocystine or with other thiols as mixed disulfides (Loscalzo et al. 2014).

Sulfur amino acids play a multitude of roles in the pig and contribute to gut health. Sulfur amino acids have been found to directly impact animal growth, intestinal health, and amino acid metabolism (Yan et al. 2018). About 20% of Met in the diet is transmethylated to HCy or continues through transsulfuration to form Cys in the intestine (Song et al. 2017, Reidijk et al. 2007). Approximately 30% of dietary SAA are used for mucosal growth and epithelial cell turnover of the intestine (Bai et al. 2020). Methionine is a dietary indispensable amino acid meaning that it cannot be synthesized in sufficient amounts by the body for proper growth and

instead must be supplied by the diet. While Cys is a dispensable amino acid, its synthesis is solely dependent on the availability of dietary Met intake. The body can synthesize Cys from Met through transmethylation and transsulfuration (Shoveller et al. 2005). Cysteine and GSH can regulate epithelial cell proliferation and differentiation by regulating redox states (Wu et al. 2017). A study conducted by Jiao et al. (2022) investigated the role of Cys in maintaining intestinal integrity and function. Pigs were separated into groups and fed diets with deficient, adequate, or excessive Cys for 28 d. The study found that Cys deprivation resulted in increased ROS and adequate Cys supplied through the diet was essential to maintain intestinal mucosal integrity as well as epithelial cell turnover. Although the liver is considered the primary site of SAA metabolism, the GIT accounts for about 25% of whole body transmethylation and transsulfuration and metabolizes 20% of dietary Met to Cys (Burrin et al. 2007). Deficiency in total SAA intake, while maintaining intake of all other amino acids and energy, suppresses intestinal epithelial cell growth (Bauchart-Thevret et al. 2009). Fewer Goblet cells and an increase in oxidative stress due to diminished concentrations of cellular Cys and GSH in the GIT were also observed in response to deficient total SAA intake. According to Grimble (2006), when the diet is low in SAA, less Met undergoes transsulfuration to Cys, reducing concentrations of Cys and its metabolites GSH and Tau. With weaning stress being a cause for reduced feed intake in piglets, this results in low SAA intake and, thus, reduced antioxidant synthesis and increased ROS or oxidative stress. Another study conducted by Zong et al. (2018) evaluated whether dietary supplementation with SAA could improve intestinal functions of weaned piglets and how dietary SAA supplementation affects intestinal function. The study found that increased dietary supplementation with SAA improved growth and intestinal absorptive functions of weaned piglets. This is most likely due to increased GSH concentrations in the gut resulting in

diminished oxidative stress from weaning. Cysteine is also a key constituent of intestinal mucin proteins that maintain mucosal homeostasis and are responsible for regulatory responses against microorganisms, including pathogens (Bauchart-Thevret et al. 2011). Goblet cell mucins and GSH together represent the major non-oxidative fate of Cys in the gut.

Glutathione

Glutathione is known to have metabolic and regulatory versatility in the body including in antioxidant defense, regulation, and metabolism. As a major antioxidant, GSH scavenges free radicals and other reactive species, removes hydrogen and lipid peroxides, and prevents oxidation of lipids and proteins. Glutathione also regulates intracellular redox status, signal transduction and gene expression, DNA and protein synthesis, cell proliferation and apoptosis, cytokine production and immune response, as well as mitochondrial function and integrity (Sen et al. 2000). In metabolism, GSH plays a role in storage and transport of Cys, synthesis of prostaglandins, and conversion of formaldehyde to formate (Wu et al. 2004). In the context of weaning stress, GSH plays many key roles in protecting cells against oxidative damage and maintaining redox homeostasis. Glutathione is the most abundant low molecular weight thiol in animal cells and the liver is the main site of GSH synthesis. Production of GSH relies on the availability of Cys, glutamate (Glu), and glycine (Gly) and is influenced by the rate of utilization and transport between cells. Muscle proteins can be catabolized in instances of stress or infection for GSH and protein synthesis as well (Forman et al. 2009). Each tissue in the body synthesizes its own GSH and there is rarely any GSH transport from plasma to tissue (Mårtensson et al. 1990).

Past studies have helped determine that GSH serves as the major transport form for Cys in the body and that most Cys used for GSH synthesis in the liver is derived from protein degradation (Lyons et al. 2000; Wu et al. 2004). A study conducted by Rakhshandeh et al. (2019) investigated the effects of immune system stimulation (ISS) on GSH levels and synthesis in tissues and Cys levels in plasma. Researchers found an increase in GSH synthesis in multiple tissues due to ISS in addition to an increase in plasma Cys. Based on their findings, the authors suggest that the increase in Cys utilization during ISS may contribute to increased maintenance SAA requirements in young pigs.

The transsulfuration pathway and glutathione synthesis

The transsulfuration pathway occurs in the liver, kidneys, small intestine, and pancreas (Rizzo et al. 2019). It consists of multiple pathways for the sulfur amino acids, Met and Cys. Methionine can either be used for protein synthesis or production of HCy. Homocysteine is condensed with serine to form cystathionine by cystathionine β-synthase (CBS) which is subsequently lysed by cystathionine γ-lyase (CTH) to generate Cys and α-ketobutyrate (Sbodio et al. 2018). Both enzymes are pyridoxal phosphate-dependent and CBS is also regulated by iron protoporphyrin 4 (Banerjee et al. 2003). Homocysteine is alternatively remethylated to Met using the cobalamin-dependent enzyme methionine synthase (MS). Cysteine has three possible fates after the transsulfuration pathway: synthesis of protein, Tau, or GSH. To synthesize Tau, Cys is oxidized by cysteine dioxygenase 1 (CDO1) to form cysteine sulfinic acid. CDO1 is regulated by the amount of Cys present to form the cysteine-tyrosine cofactor to be able to catalyze Cys (Chen et al. 2022). Cysteine sulfinic acid forms hypotaurine using the enzyme cysteinesulfinic acid decarboxylase (CSAD). Finally, hypotaurine is converted to Tau by hypotaurine dehydrogenase.

Formation of GSH begins with addition of Glu to Cys using the enzyme glutamate cysteine ligase (GCL) to form γ-glutamylcysteine (γ-GC). GCL is comprised of a catalytic (GCLC) and a modifier (GCLM) subunit. The activity of GCLC is greater when it associates with GCLM (Kang et al. 2021) and GCLC can also be upregulated during inflammation and oxidative stress (Fraser et al. 2003). Glycine is then added to γ-GC by the enzyme glutathione synthetase (GSS) to produce GSH (Stipanuk et al. 2020). Glutathione can also be further broken down into cysteinyl glycine and a γ-glutamyl amino acid. Both the synthesis of γ-GC and GSH are ATP-dependent reactions. Glutathione plays a multitude of critical roles in the body including protecting cells from oxidative damage caused by ROS. Taurine has also been shown to act as an indirect antioxidant by suppressing alterations in membrane permeability caused by oxidative stress (Jafri et al. 2019; Baliou et al. 2021).

Current amino acid requirements for weaning piglets

According to the National Research Council, amino acids for swine can be separated into three sections: indispensable, dispensable, and semi-indispensable. Semi-indispensable means it is an amino acid that must be consumed in an adequate amount to prevent the use of essential amino acids to synthesize it. Table 1, shown below, identifies each amino acid separated into its correct classification. For the purposes of this thesis, only the current amino acid requirements of Met and Cys for growing and finishing swine will be presented. Recommended inclusion of standardized ileal digestible (SID) lysine for 5-12 kg piglet diets is 1.18% and SID SAA:Lys is between 50-60%. (Chung et al. 1992; Gaines et al. 2005; Dean et al. 2007; NRC, 2012). Sulfur amino acid requirements for growing and finishing swine have been extensively studied. However, these amino acid requirements have often been incorrectly extrapolated to starter pigs.

A study conducted by Capozzalo et al. in 2017 investigated optimum SID SAA:Lys ratios in weaned pigs challenged with enterotoxigenic *E. coli* (ETEC). Researchers found that higher supplementations of SAA than recommended by NRC (2012) maximized average daily gain in piglets challenged with ETEC. The authors suggest that this may indicate the need for higher SID SAA:Lys ratios than originally recommended by the NRC in the diet of weaned piglets that are challenged with ETEC. Another study by Kahindi et al. (2017) investigated optimal SID SAA:Lys ratio for weaned piglets in clean and unsanitary conditions. Ratios included 52%, 56%, 60%, 64%, and 68%. Higher SID SAA:Lys mitigated the reduction in villus height suggesting that SAA requirements for gut development could be higher in piglets in poor sanitary conditions.

While these papers suggest updated SID SAA:Lys ratios, they are in response to inflicted bacterial challenges. Low feed intake at weaning itself may alter total SAA requirements and the ratio of Met to Cys. Both studies also increased SID SAA:Lys by increasing SID Met content in the diet. The issue with this approach is that low SAA intake results in reduced Met transsulfuration to Cys. Increased Met conservation for methylation reactions and protein synthesis could limit Cys availability and reduce the production of important antioxidants such as GSH and Tau. The aim of this research is to observe the effects weaning-induced reductions in feed intake on SAA metabolism in pigs.

Tables and Figures

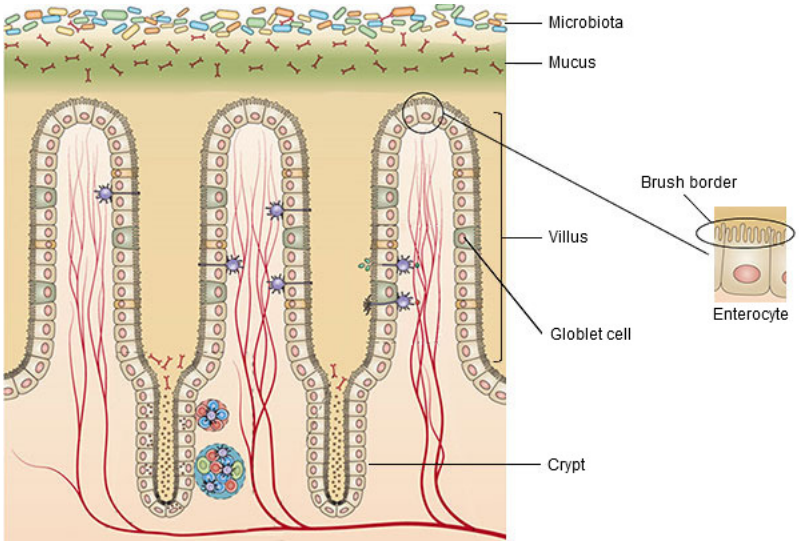

Figure 1.1: Structure of swine small intestine mucosa. Figure adapted from Vallespin (2016) and Sommar and Bäckhed (2013)

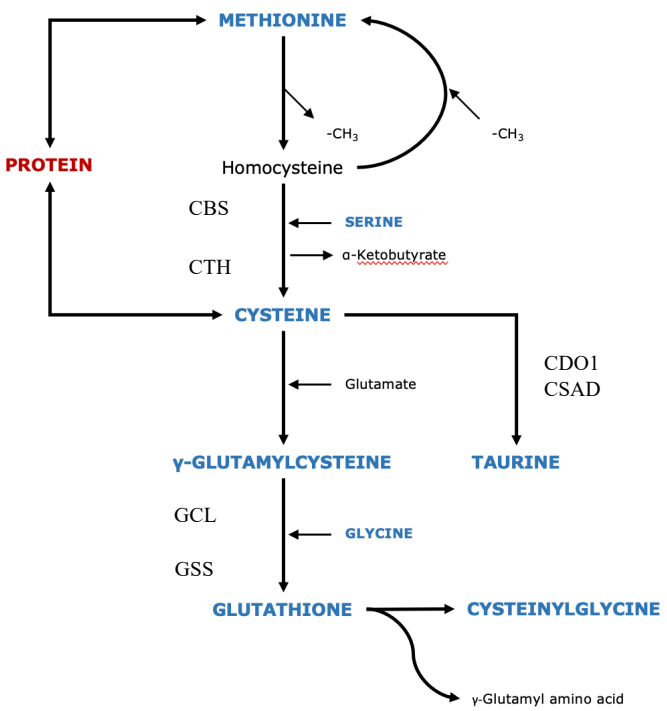

Figure 1.2: Major pathways of sulfur amino acid metabolism.

Table 1.1. List of dispensable, indispensable, and semi-dispensable amino acids in pigs.

Indispensable	Dispensable	Semi-indispensable
Histidine	Alanine	Arginine
Isoleucine	Asparagine	Cysteine
Leucine	Aspartate	Glutamine
Lysine	Glutamate	Proline
Methionine	Serine	Tyrosine
Phenylalanine		Glycine
Threonine		
Tryptophan		
Valine		

Chapter 2:

Feed restriction at weaning alters cysteine utilization in a tissue-dependent manner

Objective

The general objective of the present thesis is to investigate the lack of feed intake on cysteine metabolism in the liver, small intestine, and skeletal muscle in pigs.

Specific aims

1. Determine how gut morphology is affected by a lack of feed intake
2. Determine how plasma and tissue amino acids and amino thiols are affected by a lack of feed intake
3. Determine how hepatic and intestinal enzymes for transsulfuration and GSH synthesis are affected by a lack of feed intake

Abstract

One of the most stressful events a pig undergoes during its production cycle is weaning. Weaning is associated with low and variable feed intake, poor nutrient utilization, and high feed costs. A small percentage of weaned pigs do not ingest feed for several days post-weaning. Cysteine (Cys) is an important sulfur amino acid (SAA) and has key roles in pig production, but how Cys metabolism and requirements are affected by weaning should be better defined. The objective of this study was to determine how a lack of feed intake changes SAA metabolism. Pigs (initial BW = 6.88 ± 0.70 kg) were either weaned at 21 d of age and euthanized at 23 d of age (W; n = 9) or remained with the sow and euthanized at 23 d age (NW; n = 9). Weaned pigs were fasted but had free access to water. At the time of euthanasia, blood, liver, jejunum, ileum, and longissimus dorsi (LD) tissues were collected. Plasma and tissue amino acids and amino thiols were analyzed by high-performance liquid chromatography following pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and 4-fluoro-7-aminosulfonylbenzofurazan, respectively. The abundance of proteins central to tissue Cys utilization were determined by immunoblot and enzyme activity. Body weight decreased in W pigs by d 3 of the study (6.42 vs. 7.41 ± 0.25 kg, $P = 0.01$). Villi of the jejunum were shorter in W pigs (370 vs. 246 ± 15 μm, $P < 0.001$), and branched chain amino acids in LD muscle increased in the W group (0.090 versus 0.244 ± 0.019 nmol/mg, $P < 0.001$). Glutathione (GSH) concentrations were lower in jejunum (1.72 vs..38 ± 0.07 nmol/mg, $P < 0.01$) and ileum (1.74 versus 1.50 ± 0.07 nmol/mg, $P = 0.033$) of W pigs. However, liver GSH concentrations were not different between W and NW pigs ($P > 0.10$). While Cys in jejunum and ileum were not affected by weaning *(*$P > 0.10$*)*, γ-glutamylcysteine, the immediate precursor of GSH, declined in

jejunum (0.014 versus 0.010 ± 0.001 nmol/mg; $P = 0.001$) and in ileum (0.011 versus 0.006 ± 0.001 nmol/mg; $P < 0.001$) in W pigs. In liver, Cys concentration was higher in W compared to NW pigs (0.417 versus 0.298 ± 0.030 nmol/mg; $P = 0.021$), but γ-GC was not different between groups ($P > 0.10$). Cysteine was the only dispensable amino acid in plasma of W pigs (192 vs. 271 ± 19.1 μmol/L, $P < 0.001$). Western blot analysis of the catalytic subunit of glutamate cysteine ligase (GCLC) showed a decrease in liver and ileum of W pigs (GCLC; $P < 0.05$). While there was no change in the modifier subunit of glutamate cysteine ligase (GCLM) in ileum, jejunum, and LD muscle (GCLM; $P > 0.10$), GCLM was lower in liver of W pigs (1.95 versus 1.47 ± 0.17 AU, $P = 0.057$). While plasma taurine was lower in NW compared to W pigs (125.5 versus 63.0 ± 6.9 μmol/L, $P < 0.001$), liver taurine was higher in W pigs (2.25 versus 4.73 ± 0.47 nmol/mg; $P < 0.001$). However, liver abundance of cysteine dioxygenase 1 and cysteine sulfinic acid decarboxylase, which irreversibly oxidize cysteine to taurine, was not different between W and NW pigs ($P > 0.10$). Despite an increase endogenous and liver availability of Cys, the rate-limiting substrate for GSH synthesis, small intestine GSH concentrations declined. This suggests that endogenous Cys may be less available for gut GSH production in pigs after weaning and could impair gut redox status and intestinal epithelial cell turnover.

Introduction

One of the most stressful events a pig is subject to is weaning. Weaning stress can have a multitude of negative effects on piglets including increased incidence of enteric disease, including PWD, reduced feed intake and impaired nutrient digestion and absorption, and oxidative stress (Moeser et al. 2017). Recent research has shown how including additional SAA supplementation in starter diets have helped reduce these negative effects caused by weaning stress. Starter pigs with higher SAA supplementation exhibited increased villus height in the jejunum and decreased crypt depth in the duodenum (Zong et al. 2018). This was paired with enhanced intestinal nutrient digestion and absorption and growth performance. Another recent study found supplementation of Cys in drinking water of weaned pigs did not increase GSH levels suggesting that Cys availability is not the first or only rate limiting factor for GSH synthesis in the early post weaning phase (Degroote et al. 2019). Environment can also play an important role in SAA requirements of weaning piglets. Higher SID SAA:Lys ratios for pigs in unsanitary conditions mitigated reduction in jejunal villus height (Kahindi et al. 2017).

This research poses many questions for us to still answer about SAA in the diet of weaned piglets and how they are utilized. Past studies have also only looked into SID SAA:Lys by supplementing Met in the diet and not Cys which poses a problem since low SAA intake results in reduced Met transsulfuration to Cys. Also, increased Met conservation for methylation reactions and protein synthesis could limit Cys availability and overall reduced production of important antioxidants such as GSH and Tau. In the context of weaning, this could result in decreased synthesis of antioxidants and increased oxidative stress. Approximately 10% of piglets

weaned do not ingest feed for the first few days after weaning (Kempen, 2014). The aim of this research is to determine how a lack of feed intake affects Cys metabolism in weaned piglets.

Materials and Methods

Animals and Design. Purebred Yorkshire piglets were randomly assigned to two treatment groups across two blocks at 21 d of age (initial body weight = 6.88 ± 0.70 kg). Treatments included the weaned group (W; n = 9) and the non- weaned group (NW; n = 9). The W group was weaned at 21 d of age and housed in 12.2 m^2 pens at the Auburn University Swine Research and Education Center (SREC) in Auburn, Alabama for the duration of the experiment. The W group was fasted for 48 hours and had *ad libitum* access to water. The NW group remained in farrowing crates with their sow and litter for the duration of the study. All piglets from both treatment groups were weighed daily until the end of the experiment.

Sample Collection. All piglets from both treatment groups were euthanized at 23 d of age by intracardiac injection of KCl under isoflurane anesthesia. Once euthanized, blood, liver, jejunum, ileum, and longissimus dorsi (LD) muscle tissues were collected and snap frozen in liquid nitrogen. Samples were then stored at -80°C until analysis. A second sample of jejunum and ileum tissue for histological analysis was collected and fixed in neutral-buffered formalin for 48 h. Intestinal tissue was then transferred to 70% ethanol and stored until processing and analysis.

Jejunum and ileum morphology. Jejunum and ileum tissue collected for histological analysis was placed in 10 % neutral-buffered formalin (NBF) for 48 h and transferred to 70% v/v ethanol until processing. Samples were trimmed into 1-mm thick slices and placed into a cassette. Samples were dehydrated in 70% EtOH for 1 h, 80% EtOH for 1 h, 95 % EtOH for 3 h (3 incubations × 1 h/incubation), 100 % EtOH for 3 h (3 incubations × 1 h/incubation), and StatLab XS-3 xylene substitute for 2 h (2 incubations × 1 h/incubation) with a carousel-style Leica TP1020 automatic benchtop tissue processor. Dehydrated samples were embedded in paraffin (StatLab ParaPro 360) with a Sakura Tissue Tek TEC-6 embedding station. Tissue blocks were sectioned at 4 µm using a Leica 2125 rotary microtome and sections placed on Superfrost slides (VWR, Radnor, PA). Slides were air dried at room temperature overnight. Prior to staining, the slides were baked in an incubator at 60°C for 10 minutes. The slides were then rehydrated and manually stained using the solutions listed in Table 2.1. Following staining, the slides were immediately cover slipped with 24 × 50mm coverslips and Richard Allen CytoSeal XYL mounting medium. Stained tissue sections were imaged with an Olympus VS200 Research Slide Scanner. Villus heights and crypt depths were measured with QuPath v0.4.3 (Bankhead et al. 2017). An average of 12 well-oriented villus-crypt axis were analyzed per tissue.

Plasma amino acid analysis. To prepare the plasma samples, 100 µL plasma is added to a 1.5 mL microcentrifuge tube along with 100 µL of 10% w/v trichloroacetic acid (TCA). This mixture is then incubated on ice for 10 min and centrifuged at 12,000 × g for 10 min at 4°C. The deproteinized plasma supernatant is then transferred to a clean 1.5 mL tube and stored at -20°C until analysis.

Plasma amino acids were analyzed by high performance liquid chromatography (HPLC) following pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. To a clean 1.5-mL microcentrifuge tube, 10 µL deproteinized plasma, 10 µL norvaline internal standard (250 pmol/µL), 10 µL 0.35 mol/L NaOH, and 50 µL sodium tetraborate buffer (125 mmol/L, pH 9.5) were added. Next, 20 µL of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization reagent (3 mg/mL in acetonitrile) was added to each tube. A calibration standard was prepared by adding to a clean 1.5-mL microcentrifuge tube 10 µL calibration standard solution (containing amino acids, including citrulline, ornithine, and the norvaline internal standard at 250 pmol/µL and cystine at 125 pmol/µL), 10 µL 0.35 mol/L NaOH, and 50 µL sodium tetraborate buffer (125 mmol/L, pH 9.5), and 20 µL 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization reagent. Plasma samples and calibration standards were incubated at room temperature for 1 min and then heated at 55°C for 10 min before transferring contents to an HPLC autosampler vial.

After a 1-µL injection, amino acids were separated under gradient reverse-phase chromatography conditions on an Acquity UPLC BEH C18, 1.7 µm, 2.1 × 100 mm column (Waters, Milford, MA) on an Acquity UPLC H-Class PLUS system (Waters, Milford, MA). The mobile phases consisted of 20 mmol/L formic acid (pH = 2.8) and acetonitrile; flow rate was 0.3 mL/min. Amino acids were detected with fluorescence (excitation wavelength, 266 nm; emission wavelength, 473 nm). The ratio of the area under of the curve (AUC) of each amino acid to the norvaline internal standard in the calibration standard was compared to the ratio of the AUC of each amino acid to the norvaline internal standard in each plasma sample to calculate plasma amino acid concentrations.

Tissue amino acid analysis. All tissues were stored at -80°C until analysis. The norvaline internal standard and all calibration standards were prepared in the same way as for plasma amino acid analysis. Tissues (100 mg) were weighed in 5-mL polypropylene culture tubes, homogenized with TCA (10% w/v; 1.5 mL TCA per 100 mg tissue), and incubated on ice for 10 min. Tissue homogenate (1000 µL) was transferred to a clean 1.5-mL microcentrifuge tube and centrifuged at 12000 x g for 10 min at 4°C. Deproteinized tissue homogenate was transferred to a clean 1.5-mL microcentrifuge tube and stored at -20°C until HPLC analysis.

Preparation and derivatization of tissue samples and calibration standards was identical as described above for plasma amino acid analysis by HPLC. Tissue amino acids are expressed per mg wet tissue weight.

Plasma and tissue amino thiol analysis. Plasma and tissue amino thiols were analyzed by HPLC according to the method described by Rudar et al. (2023). Calibration curves were constructed from increasing concentrations of Cys, CysGly, HCy, γGC, and GSH. Each calibration standard and plasma or tissue sample contained a known amount of the 2-mercaptopropionylglycine internal standard. Peak area ratio of each amino thiol to the internal standard was calculated for each calibration curve and plotted against the amino thiol concentration. Amino thiol calibration curves were generated by weighted (1/x) linear regression analysis with the regression procedure of SAS (version 9.4; SAS Institute, Cary, NC).

Western blot analysis. Tissue (100 mg) was homogenized in RIPA lysis buffer (150 mM NaCl; 1% NP-40; 0.5% Na deoxycholate; 0.1% SDS; 50 mM Tris (pH 8.0); 1 mM Na pyrophosphate; 1 mM β-glycerophosphate; 5 mM NaF; 1 mM Na_3VO_3; 1% v/v mammalian

protease inhibitor). Chilled lysis buffer was added to each tissue sample (1500 µL per 100 mg tissue), tissue was homogenized with a rotor-stator homogenizer, and the lysate was incubated on a platform rocker at 4°C for 60 minutes. Following incubation, the lysate was centrifuged at 17,709 × g at 4°C for 20 min. Final lysate protein concentrations were equalized by diluting the lysate with additional lysis buffer and adding a target amount of 4× Laemmli sample loading buffer, depending on the initial lysate protein concentrations determined by the bicinchoninic acid (BCA) protein assay. The lysate was then incubated at 70°C for 10 min to denature protein and centrifuged at 17709 × g at room temperature for 2 minutes.

An SDS-PAGE gel (8% to 15% resolving gel, depending on the molecular weight of the target protein; 4% stacking gel), containing 2,2,2-trichloroethanol (0.5% v/v), was prepared (SE640 wide-mini vertical protein electrophoresis unit; Hoefer, Holliston, MA). Lysate, containing 20-40 µg protein, was added to wells and separated by electrophoresis (100 V for 2 hours). Protein separation was monitored by assessing the migration of a pre-stained protein ladder (10-180 kDa, PL0001; Proteintech Group, Inc., Rosemont, IL) and bromophenol blue dye front. After electrophoresis, the gels were exposed to UV light and visualized on a ChemiDoc XRS+ imager (Bio-Rad, Hercules, CA). The 2,2,2-trichloroethanol enables fluorescent detection of proteins by modifying tryptophan residues upon UV exposure (Ladner et al. 2004).
After electrophoresis and gel imaging, the proteins are transferred from the gel to a hydrophobic polyvinylidene fluoride (PVDF) membrane (pore size, 0.2 µm), activated in methanol and equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol v/v). After the transfer cassette was assembled, it was put into the transfer apparatus (TE42; Hoefer, Holliston, MA) and filled with transfer buffer. The proteins in the gel were coated in SDS, making them negatively charged so within the chamber they will be on the negatively charged side. The

membrane was positioned on the positively charged side so that when current was run through it the proteins moved towards the positive charge and transfer onto the membrane. This transfer ran for 16 h at room temperature at 90 mA.

After the protein transfer was complete, the transfer cassette was removed from the chamber, and the membrane was removed and quickly washed with distilled water. Proteins labelled with 2,2,2-trichloroethanol were imaged on the membrane to quantify total protein transferred. After imaging, the membranes were stained with a 0.1% w/v Ponceau S for 5 min at room temperature to visualize protein and trim the membranes to fit in custom blotting boxes (19 cm × 4.5 cm). Next, the membrane was washed in distilled water to remove the stain and then washed in Tris-buffered saline with Tween20 (0.1% v/v; TBS-T) for 5 min three times. Membranes were blocked by incubating them in milk (5% w/v dry milk powder in TBS-T) for 1 h at room temperature. After incubation, the membrane is washed three more times with TBS-T for 10 min.

Primary and corresponding secondary antibodies were applied to the membrane. The primary and secondary antibodies are listed in Table 2.1. The primary antibody was reconstituted in 5% w/v BSA and 0.02% sodium azide in TBS-T, applied to the membrane, and incubated on a platform rocker for 16 h at 4°C. After primary antibody incubation, the primary antibody solution was removed and the membrane was membrane was washed three times with TBS-T for 10 min. The secondary antibody was diluted in 5% w/v non-fat dry milk in TBS-T, applied to the membrane, and incubated for 1 hour at room temperature. After secondary antibody incubation, the membrane was washed three times with TBS-T for 10 min and proteins were detected by chemiluminescence. Enhanced chemiluminescent detection reagents (Amersham ECL Prime Western Blotting Detection Reagent; Cytiva, Marlborough, MA) were applied to the membrane,

incubated for 180 sec, and imaged with the ChemiDoc XRS+ imager. A corresponding colorimetric image of the protein ladder was obtained at the same time to match the position of the protein band with an approximate molecular weight.

The total volume of target protein bands and total lane protein was determined with the built-in imaging software (Image Lab v6.0; Bio-Rad, Hercules, CA). The target protein band density was divided by the lane normalization factor (LNF). The LNF was calculated by dividing the total lane volume by the largest total lane volume of all the samples measured. The value that resulted was the normalized volume and was divided by the minimum volume value to find the final normalized abundance of each protein within the tissue.

Tissue enzyme activity. The enzyme activity of GCL and GSS in liver, jejunum, ileum, and LD muscle were found by measuring the reaction products by HPLC. Tissue (100 mg) was homogenized in lysis buffer (20 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, and 1% v/v protease inhibitor; 1500 µL lysis buffer was used per 100 mg tissue). After homogenization, the homogenate was transferred to a clean 1.5-mL microcentrifuge tube and centrifuged at 3000 × g for 10 minutes at 4°C. Tissue homogenate supernatant was then transferred to a second clean 1.5-mL microcentrifuge tube and spun at 15,000 × g for 30 min at 4°C. The clarified supernatant was transferred to a third clean 1.5-mL microcentrifuge tube and analyzed immediately; a subsample was taken to determine protein concentrations with the BCA assay. Two enzyme reaction tubes were prepared with 250 µL of reaction buffer, containing 200 mM Tris, 40 mM MgCl2, 4 mM EDTA, 300 mM KCl, and 4 mg/L acivicin. To each reaction, 100 µL of 50 mM ATP and, depending on the enzyme, either 50 µL of 50 mM L-Cysteine and 100 mM L-Glutamate (for GCL) or 50 µL of 50 mM γ-GC and 100 mM L-Glycine (for GSS)

were added. Reaction tubes were then vortexed and incubated at 37°C for 5 min. After pre-heating, 50 µL of clarified tissue homogenate was added to each enzyme reaction tube and put back onto the dry block heater at 37°C. One tube was incubated for 15 min while the other tube was incubated for 45 min. After the corresponding incubation period, 100 µL of 30% w/v trichloroacetic acid (TCA) was added to the tube, the tube was held on ice for 15 min, and the tube was centrifuged at 15,000 × g for 15 minutes at 4°C. The deproteinized supernatant was then transferred to autosampler vials for HPLC analysis. The HPLC method to quantify enzyme activity was quantified using the same method as plasma and tissue amino thiols.

Statistical analysis. Data were analyzed with SAS version 9.4 (PROC GLIMMIX; SAS Institute, Cary, NC). Treatment was considered a fixed effect, and pig and block were considered random effects. Data are presented as least squares means ± standard error of the mean (SEM). Results were considered significant at $P < 0.05$ and a trend at $0.05 \leq P < 0.10$.

Results

Body weight. Initial body weight was not different between groups (Figure 2.1; *d 1*; $P > 0.10$). Final body weight was lower in W than in NW pigs (*d 3*; 6.42 vs. 7.41 ± 0.25 kg, $P = 0.01$).

Jejunum and ileum morphology. Villus and crypt morphology of the mid-jejunum and distal ileum was analyzed from all pigs and is shown in Figure 2.2 and 2.3. The crypts of jejunum (140 vs. 122 ± 18.6 µm, $P = 0.480$) and ileum (125 vs. 127 ± 15.5 µm, $P = 0.920$) were

not different between NW and W pigs. While the villi of the ileum were not different between treatment groups (257 vs. 215 ± 27 μm, $P = 0.270$), the villi of the jejunum were significantly shorter in W piglets (370 vs. 246 ± 15 μm, $P < 0.001$).

Plasma amino acid and amino thiol concentrations. Plasma amino acids are shown in Table 2.3. In plasma, both indispensable amino acids (1777 vs. 1559 ± 67 μmol/L, $P = 0.040$) and dispensable amino acids (4382 vs. 2693 ± 160 μmol/L, $P < 0.001$) were significantly lower in the W group. Of the amino acids relevant to SAA and GSH metabolism, Tau (125.5 vs 63.0 ± 6.9 μmol/L, $P < 0.001$), Glu (242.0 vs. 92.3 ± 18.1 μmol/L, $P < 0.001$), and Met (78.8 vs. 52.2 ± 4.1 μmol/L, $P = 0.003$) decreased in W pigs. Glycine was not different between groups (1024 vs. 1165 ± 59 μmol/L, $P = 0.110$).

Table 2.4 shows the amino thiols measured in plasma of the piglets. In plasma, γ-GC (2.69 vs. 3.80 ± 0.22 μmol/L, $P = 0.002$) was higher in W pigs, whereas GSH (2.74 vs. 2.08 ± 0.46 μmol/L, $P = 0.019$) and CysGly (30.3 vs. 20.8 ± 2.3 μmol/L, $P = 0.010$) were higher in NW pigs. Among all dispensable amino acids, Cys was the only one to increase in plasma of W pigs (192 vs. 271 ± 19 μmol/L, $P < 0.001$).

Tissue amino acid and amino thiol concentrations. Table 2.5 shows the amino acids measured in liver of NW and W pigs. In liver, there was no difference in dispensable and indispensable amino acids between treatment groups (21.6 vs 20.1 ± 0.13 nmol/mg, $P = 0.460$, 1.96 vs. 1.92 ± 0.10 nmol/mg, $P = 0.730$). Among amino acids important to SAA and GSH metabolism, Tau (2.25 vs. 4.73 ± 0.47 nmol/mg, $P < 0.001$) and Gly (1.98 vs. 3.70 ± 0.25

nmol/mg, $P < 0.001$) increased in W pigs. Methionine (0.04 vs. 0.03 ± 0.01 nmol/mg, $P = 0.189$) and Glu did not change between treatment groups (3.17 vs. 2.56 ± 0.36 nmol/mg, $P = 0.250$).

Table 2.6 shows the amino acids measured in the jejunum of NW and W pigs. In jejunum, dispensable amino acids were significantly lower in W pigs (15.1 vs. 10.5 ± 0.7 nmol/mg, $P < 0.001$), whereas indispensable amino acids were higher in NW pigs (3.29 vs. 1.58 ± 0.20 nmol/mg, $P = 0.047$). Among amino acids that are important to SAA and GSH metabolism, Gly was higher in NW pigs (3.30 vs. 2.44 ± 0.15 nmol/mg, $P = 0.001$), whereas Glu (3.25 vs. 2.65 ± 0.19 nmol/mg, $P = 0.047$) and Met (0.08 vs. 0.04 ± 0.01 nmol/mg, $P = 0.026$) were higher in the NW group. Taurine was not different between groups (3.71 vs. 3.18 ± 0.22 nmol/mg, $P = 0.111$).

Table 2.7 shows amino acids measured in ileum tissue. In ileum, dispensable amino acids trended lower in W pigs (20.1 vs. 17.9 ± 0.75 nmol/mg, $P = 0.070$) and indispensable amino acids were not different between treatment groups (2.00 vs. 1.84 ± 0.11 nmol/mg, $P = 0.320$). Among amino acids important to SAA and GSH metabolism, Tau (2.07 vs. 1.76 ± 0.10 nmol/mg, $P = 0.058$) and Met (0.054 vs. 0.047 ± 0.004 nmol/mg, $P = 0.270$) were not different between treatment groups. Glutamate was higher in NW pigs (6.63 vs. 5.46 ± 0.25 nmol/mg, $P = 0.005$) and Gly was higher in W pigs (3.76 vs. 4.77 ± 0.30 nmol/mg, $P = 0.031$).

In LD muscle, all branched chain amino acids were higher in W pigs including isoleucine (0.03 vs. 0.12 ± 0.02 nmol/mg, $P < 0.001$), leucine (0.09 vs. 0.22 ± 0.02 nmol/mg, $P < 0.001$), and valine (0.15 vs. 0.33 ± 0.03 nmol/mg, $P < 0.001$) shown in Figure 2.9.

Table 2.8 shows amino thiol levels in all tissues. In liver, γ-GC (0.03 vs. 0.02 ± 0.003 nmol/mg, $P = 0.270$) and GSH (2.92 vs. 2.83 ± 0.245 nmol/mg, $P = 0.749$) were not different between groups but Cys was higher in W pigs (0.298 vs. 0.417 ± 0.030 nmol/mg, $P = 0.021$) and

was the only dispensable amino acid to increase in the liver of weaned pigs. For jejunum, Cys was not different between groups (0.158 vs. 0.138 ± 0.031 nmol/mg, $P = 0.349$) but γ-GC (0.014 vs. 0.010 ± 0.001 nmol/mg, $P = 0.001$) and GSH (1.72 vs. 1.38 ± 0.07 nmol/mg, $P = 0.003$) were both higher in NW piglets. In ileum, GSH was lower in W pigs than in NW pigs (1.74 vs. 1.50 ± 0.07 nmol/mg, $P = 0.030$) and γ-GC was significantly higher in the NW group (0.011 vs. 0.006 ± 0.001 nmol/mg, $P < 0.001$) while Cys was not different between treatment groups (0.14 vs. 0.13 ± 0.02 nmol/mg, $P = 0.507$). Finally, in LD muscle, Cys (0.21 vs. 0.43 ± 0.03 nmol/mg, $P < 0.001$) and GSH (0.89 vs. 1.07 ± 0.04 nmol/mg, $P = 0.010$) were both significantly higher in W pigs.

Tissue CBS, CTH, GCLC, GCLM, GSS, CSAD, and CDO1 protein abundance. Western blot analysis was used to measure the abundance of CBS, CTH, GCLC, GCLM, GSS in jejunum, ileum, liver, and LD muscle shown in Table 2.9. The enzymes CDO1 and CSAD were also measured in liver tissue. Ileum GCLC decreased in W pigs (2.15 vs. 1.48 ± 0.20 AU, $P = 0.030$) and CTH was lower in the jejunum of W pigs (3.32 vs. 1.69 ± 0.30 AU, $P = 0.001$). Liver GCLC (2.21 vs. 1.40 ± 0.22 AU, $P = 0.020$) and GCLM (1.95 vs. 1.47 ± 0.20 AU, $P = 0.060$) decreased in W pigs, but there was no difference in abundance of CBS, CTH, CSAD, or CDO1in the liver. There was no difference in GCLC (1.40 vs. 1.67 ± 0.16 AU, $P = 0.270$) or GCLM (4.32 vs. 6.28 ± 0.93 AU, $P = 0.160$) abundance in jejunum or LD muscle of NW and W pigs.

GCL and GSS enzyme activity. The activity of GCL and GSS enzymes in liver, jejunum, ileum, and LD muscle is shown in Table 2.10. The activity of GCL was significantly lower in the liver in W pigs than in NW pigs (4.15 vs. 2.49 ± 0.22 nmol γ-GC/[mg protein • min], $P < 0.001$).

The activity of GSS was also significantly lower in the liver in W pigs than in NW pigs (1.92 vs. 1.54 ± 0.06 nmol GSH/[mg protein • min], $P < 0.001$). The activity of GCL and GSS was not different in jejunum, ileum, or LD muscle.

Discussion

General nutrition is possibly the most important factor in maintaining proper growth and gut health of pigs (Bischoff, 2011). With weaning being one of the most stressful events for pigs, it can result in many negative effects such as reduced feed intake, enteric disease leading to PWD, and oxidative stress (Moeser et al. 2017). Sulfur amino acids have been found to directly impact animal growth, intestinal health, and amino acid metabolism as well as help alleviate negative impacts of weaning stress (Yan et al. 2018). Sulfur amino acid requirements for growing and finishing swine have been extensively studied. However, these requirements are often incorrectly extrapolated to starter pigs; moreover, how weaning and low feed intake after weaning impacts these requirements is unclear. On average, 5-10% of piglets weaned do not ingest feed for the first few days after weaning (Kempen, 2014). The present study aimed to define the effects of reduced feed intake on SAA metabolism. In order to study the effects of weaning on SAA utilization, one set of pigs was weaned with access to water but not feed, and the other group remained on the sow. It is important to note that this degree of nutritional stress is not typical of weaned pigs in a commercial setting. However, some pigs at weaning do not eat or consume limited amounts of feed for the first 48 h (Bruininx et al. 2001).

Weaning stress can cause changes to the immune system and gut morphology. Low feed intake results in alteration of gut integrity (Rhouma et al. 2017) which can be defined as

shortening of villus height, lengthening of crypt depth, increased mucosal permeability, decreased digestive enzymatic activity, and decreased nutrient absorptive capacity (Pluske et al. 1997). Changes to the villi height and crypt depth are key measurements for the assessment of intestinal development and the effect of nutrition interventions on gut structure and function (Peng et al. 2016). Research conducted by McCracken et al. (1999) evaluated local inflammatory responses and their relationship with morphological changes in the intestine of weaned piglets. Researchers found that inadequate feed intake during the immediate post weaning period contributed to intestinal inflammation and compromised villus-crypt structure and function. Another study by Montagne et al. (2007) examined the effects of weaning on gut morphology during the first two weeks after pigs are weaned. Sections of intestines were taken from pigs at different time points over 14 d. Intestines were then analyzed for villus height, crypt depth, as well as analysis of enzyme activities. Within the first few days, researchers found decreases in villus length and brush border enzyme activity and increased gut permeability, which were all linked to low feed intake caused by weaning stress. In concurrence with this research, the present study showed a significant decrease in villi height between groups in the jejunum. The average height of jejunal villi was shorter in weaned pigs (euthanized two days post-weaning) than in NW pigs, but crypt depth was not affected. Villi height and crypt depth were also not different in the ileum of the two treatment groups. Research conducted by Hedemann et al. (2003) also found that villi height decreased in the proximal portions of the small intestine (duodenum and jejunum) but did not change in the distal portion (ileum) during the first three days after weaning. Hedemann et al. 2003 also found that crypt depth did not increase until d 5 post-weaning. Other past research on gut morphology after weaning in piglets shows that villi height in the jejunum tends to decrease two to three days post-weaning whereas villi height in other

sections of the small intestine along with crypt depth throughout the small intestine does not show change until at least five days after weaning (Hampson, 1986, Cera et al. 1988, Pasick et al. 2014, Aubry et al. 2017, Spreeuwenberg et al. 2001, Murugesan et al. 2015). Reduced villi height post weaning is a result of increased cell loss and reduced crypt cell production. Consequently, there is a loss of mature enterocytes which leads to decreased nutrient absorption (Hedemann et al. 2003).

Along with changes in gut morphology there were many other pieces of evidence that indicated the impact of weaning on the pigs in the current study. There was a decrease in body weight of W pigs between d 1 and d 3 of the study as well as an increase in branched chain amino acids in the LD muscle of the W group compared to the NW group. This indicates that muscle protein breakdown was increased in W pigs due to reduced feed intake since branched-chain amino acids account for a substantial fraction of indispensable amino acids that comprise muscle protein (Mahan et al. 1998). Gut fill also accounts for about 5% of body weight and does not fully account for the reduced body weight in the W group (Arthur et al. 2008). Plasma and liver alanine concentrations were also significantly lower in W pigs which indicates that these amino acids are likely used in the glucose-alanine cycle, which uses muscle degradation-derived amino acids as substrates for hepatic gluconeogenesis (Felig, 1973). Citrulline plays many regulatory roles in gut modulation (Allerton et al. 2018) and is a functional biomarker of gut barrier dysfunction (Dey et al. 2020). Plasma, jejunum, and ileum concentrations of citrulline were lower in W pigs, which aligns with gut morphology results that there was gut modulation in the intestines of the W group.

Amino acids are commonly known as the building blocks of proteins and play many vital roles in the pig. Different amino acid requirements in young piglets have been well studied.

However, the effects of weaning stress on SAA requirements have not been investigated thoroughly (Mou et al. 2019). Sulfur amino acids and the transsulfuration pathway are extremely important to the overall health and immunity of piglets and have several key roles in gut health (Kahindi et al. 2017).

Methionine is an essential amino acid and participates in transmethylation to HCy. Homocysteine can be re-methylated into Met using the methionine synthase (MTR) enzyme which links the folate cycle with HCy metabolism. Homocysteine is condensed with serine by CBS to form the intermediate cystathionine; cystathionine in turn is lysed by CTH to yield Cys and α-ketobutyrate (Brosnan et al. 2006, Sbodio et al. 2018). Methionine supplementation has been shown to alleviate intestinal injury and improve jejunal epithelial barrier function in weaning piglets which implicates the importance of Met in intestinal growth and function beyond its role as a precursor for protein synthesis (Chen et al. 2014). Cysteine also contributes to gut health through the synthesis of important antioxidants, Tau and GSH, and intestinal mucin proteins (Bauchart-Thevret, 2011). The present study showed decreased levels of plasma and jejunal Met in W pigs but increased levels of plasma, liver, and LD muscle Cys in the W group. Serine was also lower in the plasma and tissues of W pigs which could indicate that it is potentially limiting synthesis of Cys from Met through transsulfuration. HCy was not different in plasma or tissues except for LD muscle which had a lower concentration in the W group. In enzymes needed for transsulfuration, CBS and CTH enzyme abundances were not different between groups in both liver and ileum; however, CTH was lower in jejunum of W pigs. Although jejunum Cys was not different between groups, a specific reduction in transsulfuration activity in jejunum suggests that Met is conserved in jejunum for one-carbon metabolism or protein synthesis. Based on these results, increased plasma, liver, and LD muscle Cys does not

appear to be coming from the transsulfuration of Met and could instead be due to muscle protein breakdown from a lack of feed intake. This hypothesis is supported by the decrease in body weight in W pigs from day 1 to day 3 and increase in branched chain amino acids in LD muscle of W pigs due to muscle breakdown.

Taurine plays many key roles in the body, but in relation to weaning stress, it plays a protective role against oxidative stress (Baliou et al. 2021). Taurine is found in high concentrations in all tissues, and it serves as a regulator of mitochondrial protein synthesis and protects the mitochondria against ROS (Jong et al. 2012). Taurine is a dispensable amino acid that is synthesized from Cys oxidation via the enzymes CDO1 and CSAD. Biosynthesis of Tau primarily occurs in the liver and kidney and its synthesis relies on the other SAA, Cys and Met. Taurine also plays an important role in reducing lipid peroxidation products to protect cells from further tissue damage from ROS (Goodman et al. 2009). Taurine enhances the activity of antioxidant enzymes to preserve redox levels and GSH stores (Oudit et al., 2004). The present study showed decreased levels of Tau in plasma and ileum of W pigs but increased levels of Tau in liver of the W group. These results indicate that excess Cys in plasma, liver, and LD muscle is likely being used for hepatic Tau synthesis despite similar abundances of CDO1 and CSAD enzymes between groups. High rates of Cys oxidation to Tau may effectively limit Cys availability, in the liver, small intestine, or other tissues, for protein synthesis or GSH production.

Glutamate is an important functional amino acid in cell metabolism and signaling (Wu, 2013). Within GSH synthesis, Glu is added to Cys using GCL to form γ-GC. Glycine is a multifunctional dispensable amino acid that is involved in metabolism, immunity, and development (Stipanuk et al. 2020). In the GSH synthesis pathway, Gly is added to γ-GC by the

enzyme GSS to form GSH. Past research has found Glu to be a critical energy substrate for the intestinal mucosa and a precursor to GSH (Burrin et al. 2009, Reeds et al. 1997, Reeds et al. 2000). A study conducted by Lin et al. (2015) found that Glu supplementation improved intestinal integrity and increased expression of tight junction proteins, including occluding and zonula occludens protein 1, in the intestinal epithelium of weaned piglets. Glutamate decreased in plasma and jejunum, but not ileum, of W pigs. While γ-GC was lower in the ileum and jejunum of W pigs, γ-GC was higher in the plasma of W pigs. As for the enzymes used in GSH synthesis that require Glu, GCLC abundance decreased in the ileum and liver of W pigs and GCLM abundance decreased only in liver tissue of W pigs. The activity of GCL was only decreased in the liver of weaned pigs, and, as noted previously, Cys was higher in plasma and liver of W pigs. Since GCL is the rate-limiting enzyme and Cys is the rate-limiting substrate for GCL, these results further show that excess Cys, in plasma or in liver, is not being channeled for γ-GC or GSH production in the small intestine.

Glycine and Cys are involved in reducing oxidative stress by acting as rate-limiting substrates for synthesis of GSH and facilitating the maintenance of redox homeostasis (McCarty et al. 2018). A study conducted by Ji et al. (2022) found that supplementation of Gly in the diet of weaned piglets resulted in suppression of inflammatory response along with improvement of mucosal immune function and composition of the microbiome in the gut. While Gly was not different in the plasma between groups, it was higher in liver and ileum of weaned pigs. The activity of GSS was lower in the liver of W pigs but not different in any other tissue. This is an interesting result since GSH was not different in liver, GSH was lower in ileum, jejunum, and plasma of W pigs, but GSH was higher in LD of W pigs. These results indicate that, despite sufficient Cys and Gly, GSH is not being synthesized as effectively in the gut of W pigs; instead,

the excess may be stored in LD muscle as GSH. This would suggest that GCL and GSS activity should be higher in LD but this was not the case. One explanation for this is that, as muscle protein is broken down, Cys builds up in the muscle and, despite that GCL and GSS activity are not higher in LD, the availability of substrates, including Cys and Gly, are. This would result in the excess GSH concentrations in muscle. Despite increased plasma and liver Cys, jejunum and ileum γ-GC and GSH concentrations were lower in W compared to NW pigs. These data suggest that Cys is less available for GSH production in the gut of weaned pigs. Cysteine and GSH can regulate epithelial cell proliferation and differentiation by regulating redox states (Wu et al. 2017). Diminished concentrations of Cys and GSH in the gut may also result in fewer Goblet cells, lower intestinal mucin protein synthesis, and increased oxidative stress (Bauchart-Thevret, 2009, 2011).

Overall, a lack of feed intake leads to decreased body weight and increased branched chain amino acids in LD muscle of W pigs which indicates increased muscle protein breakdown. Alanine is lower in liver and plasma of W pigs and is likely being used for hepatic gluconeogenesis while decreased citrulline in W pigs may reflect impaired gut function. Cysteine and GSH concentrations were increased in LD muscle of W pigs, suggesting that excess Cys here is most likely being stored as GSH. Cysteine was also increased in liver of W pigs but it appears to be oxidized to Tau rather than used for GSH production since the abundance and activity of enzymes needed for GSH synthesis were lower in the W group. In the intestine, Cys and Tau were not different between groups but GSH was lower in W pigs. This depletion of GSH in the intestine is important to note as it could lead to impaired gut function as well as increased oxidative stress in the gut. Less Cys in the intestine is produced from Met through transsulfuration in the jejunum based on lower abundance of CTH in W pigs as well as decreased

Met and Ser substrates. This suggests that Met is conserved for one-carbon metabolism or protein synthesis in the W group.

In conclusion, a lack of feed intake directly affects Cys metabolism in the liver, small intestine, and skeletal muscle in pigs. Reduced feed intake elicits muscle protein breakdown, increases endogenous Cys, yet paradoxically decreases GSH levels across the small intestine. Without the production of important antioxidants like GSH in the gut, the effects of oxidative stress and ROS worsen and may increase gut permeability and the incidence of infectious enteric diseases, leading to increased morbidity and mortality (Gresse et al. 2017). The long-term goal of this research is to generate more accurate recommendations for SID Cys to SID total SAA and SID Met to SID Lys for starter pig rations. Further research is needed to determine appropriate nutritional strategies to enhance gut GSH production in weaned pigs to prevent the negative health and production effects caused by reduced feed intake from weaning stress.

Table 2.1. Primary and secondary antibodies used in western blot analysis of liver, ileum, jejunum, and LD tissue in weaned and non-weaned pigs

Antibody	Size (kDa)	Primary antibody dilution	Secondary antibody dilution	Vendor and catalog number
Rabbit anti-CBS	61	1:2000 (jejunum, ileum) 1:10000 (liver)	1:20000 GAR	Proteintech 14787-1-AP
Rabbit anti-CTH	40-45	1:2000 (jejunum, ileum) 1:5000 (liver)	1:20000 GAR	Proteintech 12217-1-AP
Mouse anti-GSS	38	1:5000 (jejunum, ileum, LD muscle) 1:5000 (liver)	1:20000 GAM	Proteintech 67598-1-Ig
Rabbit anti-GCLC	73	1:20000 (jejunum, ileum, LD muscle) 1:20000 (liver)	1:20000 GAR	Proteintech 12601-1-AP
Mouse anti-GCLM	31	1:20000 (jejunum, ileum, LD muscle) 1:20000 (liver)	1:20000 GAM	Proteintech 66808-1-Ig
Rabbit anti-CDO1	23	ND 1:10000	1:20000 GAR	Proteintech 12589-1-AP

Abbreviations used: GAM, goat anti-mouse; GAR, goat anti-rabbit; CBS, cystathionine β-synthase; CTH, cystathionine γ-lyase; GSS, glutathione synthetase; GCLC, glutamate cysteine ligase, catalytic; GCLM, glutamate cysteine ligase, modifier; CDO1, cysteine dioxygenase 1; CSAD, cysteine sulfinic acid decarboxylase.

Table 2.2. Outline of jejunum and ileum tissue rehydration, staining with hematoxylin and eosin, and dehydration.

Step	Reagent	Duration
1	Hemo-De (d-Limonene)	8 min
2	Hemo-De (d-Limonene)	8 min
3	Hemo-De (d-Limonene)	8 min
4	100% Ethanol	2 min
5	100% Ethanol	2 min
6	95% Ethanol	2 min
7	95% Ethanol	2 min
8	80% Ethanol	2 min
9	Water, running	2 min
10	Hematoxylin	5 min
11	Water	2 min
12	Bluing	8-15 dips
13	Water	2 min
14	80% Ethanol	2 min
15	Eosin	2 min
16	95% Ethanol	2 min
17	95% Ethanol	2 min
18	100% Ethanol	2 min
19	100% Ethanol	2 min
20	Hemo-De (d-Limonene)	2 min
21	Hemo-De (d-Limonene)	2 min

Table 2.3. Plasma amino acid concentrations in non-weaned and weaned pigs.[1]

Amino Acid (µmol/L)	NW	W	SEM	*P*-value
Histidine	125.4	73.8	5.0	<0.001
Asparagine	107.3	57.6	5.8	<0.001
Arginine	243.3	158.6	15.3	0.001
Glutamine	706.1	403.9	48.4	<0.001
Aspartate	23.5	8.0	1.5	<0.001
Citrulline	118.1	79.0	9.9	0.013
Threonine	257.5	230.1	26.2	0.47
Alanine	1049.8	387.5	76.5	<0.001
Proline	662.6	231.8	59.0	<0.001
Ornithine	85.7	50.4	6.3	0.001
Cystine	38.4	55.6	7.4	0.12
Lysine	337.3	214.4	26.4	0.004
Tyrosine	157.7	75.8	8.3	<0.001
Methionine	78.8	52.2	4.1	0.003
Valine	282.2	361.1	16.8	0.004
Isoleucine	109.6	176.6	10.0	<0.001
Leucine	221.2	194.9	9.3	0.062
Phenylalanine	89.9	97.8	3.1	0.093

[1]Values are least-squares means ± SEM. Significance is measured at $P < 0.05$. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

Table 2.4. Plasma amino thiol concentrations in non-weaned and weaned pigs.[1]

Amino thiol (μmol/L)	NW	W	SEM	*P*-value
γ-Glutamylcysteine	2.69	3.80	0.22	0.0018
Glutathione	2.74	2.08	0.46	0.019
Cysteinylglycine	30.3	20.8	2.30	0.007
Homocysteine	32.6	33.6	3.43	0.852

[1]Values are least-squares means ± SEM. Significance is measured at $P < 0.05$. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

Table 2.5. Liver amino acid concentrations in non-weaned and weaned pigs.[1]

Amino Acid (nmol/mg)	NW	W	SEM	*P*-value
Histidine	0.49	0.38	0.03	0.022
Asparagine	1.19	1.47	0.12	0.119
Arginine	0.32	0.19	0.02	<0.001
Glutamine	10.06	9.16	0.74	0.404
Aspartate	1.75	1.39	0.26	0.334
Citrulline	0.04	0.04	0.004	0.346
Threonine	0.27	0.27	0.04	0.900
Alanine	1.94	0.49	0.15	<0.001
Proline	0.67	0.42	0.05	0.003
Ornithine	0.16	0.19	0.02	0.432
Cystine	0.15	0.17	0.01	0.017
Lysine	0.20	0.22	0.02	0.460
Tyrosine	0.12	0.09	0.01	0.022
Valine	0.22	0.30	0.01	0.002
Isoleucine	0.11	0.17	0.01	<0.001
Leucine	0.25	0.25	0.02	0.882
Phenylalanine	0.09	0.10	0.004	0.066

[1]Values are least-squares means ± SEM. Significance is measured at $P < 0.05$. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

Table 2.6. Jejunum amino acid concentrations in non-weaned and weaned pigs.[1]

Amino Acid (nmol/mg)	NW	W	SEM	*P*-value
Histidine	0.04	0.03	0.01	0.177
Asparagine	2.33	2.33	0.15	0.972
Arginine	0.24	0.13	0.02	0.009
Glutamine	0.72	0.31	0.11	0.018
Aspartate	0.75	0.54	0.05	0.015
Citrulline	0.38	0.15	0.03	<0.001
Threonine	0.40	0.29	0.02	0.008
Alanine	1.94	0.77	0.13	<0.001
Proline	0.91	0.35	0.09	0.001
Ornithine	0.10	0.04	0.01	<0.001
Cystine	0.16	0.05	0.04	0.081
Lysine	0.41	0.21	0.06	0.032
Tyrosine	0.23	0.11	0.02	0.003
Valine	0.34	0.31	0.03	0.596
Isoleucine	0.23	0.19	0.03	0.375
Leucine	0.41	0.26	0.04	0.043
Phenylalanine	0.16	0.12	0.02	0.133

[1]Values are least-squares means ± SEM. Significance is measured at $P < 0.05$. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

Table 2.7. Ileum amino acid concentrations in non-weaned and weaned pigs.[1]

Amino Acid (nmol/mg)	NW	W	SEM	*P*-value
Histidine	0.05	0.04	0.004	0.079
Asparagine	3.53	3.62	0.13	0.628
Arginine	0.32	0.27	0.03	0.304
Glutamine	0.65	0.54	0.04	0.093
Aspartate	1.66	1.45	0.12	0.237
Citrulline	0.14	0.08	0.01	0.003
Threonine	0.42	0.36	0.04	0.321
Alanine	1.61	0.77	0.08	<0.001
Proline	0.73	0.33	0.05	<0.001
Ornithine	0.06	0.03	0.003	<0.001
Cystine	0.06	0.04	0.003	<0.001
Lysine	0.32	0.26	0.01	0.002
Tyrosine	0.18	0.10	0.01	<0.001
Valine	0.28	0.31	0.02	0.223
Isoleucine	0.15	0.19	0.01	0.010
Leucine	0.29	0.26	0.01	0.096
Phenylalanine	0.12	0.11	0.01	0.606

[1]Values are least-squares means ± SEM. Significance is measured at $P < 0.05$. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

Table 2.8. Amino thiol concentrations in liver, jejunum, ileum, and LD muscle in non-weaned and weaned pigs.[1]

Tissue	Amino thiol (nmol/mg)	NW	W	SEM	*P*-value
Liver	γ-GC	0.026	0.021	0.003	0.271
	GSH	2.921	2.825	0.245	0.749
	CysGly	0.0171	0.0180	0.005	0.685
	HCy	0.004	0.005	0.001	0.322
Jejunum	γ-GC	0.014	0.010	0.001	0.0012
	GSH	1.72	1.38	0.07	0.003
	CysGly	0.006	0.005	0.001	0.214
	HCy	0.007	0.007	0.001	0.601
Ileum	γ-GC	0.011	0.006	0.001	<0.001
	GSH	1.74	1.50	0.07	0.033
	CysGly	0.003	0.003	0.0003	0.780
	HCy	0.006	0.005	0.001	0.068
LD muscle	Cys	0.21	0.43	0.003	<0.001
	γ-GC	ND	ND	-	-
	GSH	0.89	1.07	0.04	0.009
	CysGly	0.004	0.005	0.0012	0.038
	HCy	0.005	0.004	0.001	0.003

[1]Values are least-squares means ± SEMs. Significance is measured at $P < 0.05$. Abbreviations used: LD, longissimus dorsi; Cys, cysteine; γ-GC, γ-glutamylcysteine; GSH, glutathione; CysGly, cysteinylglycine; HCy, homocysteine. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

Table 2.9. Protein abundance of transsulfuration and GSH synthesis enzymes in ileum, jejunum, liver, and LD muscle in weaned and non-weaned pigs.[1]

Tissue	Protein	NW	W	SEM	*P*-value
Ileum	CBS	2.02	1.71	0.20	0.287
	CTH	2.82	2.95	0.42	0.830
	GCLC	2.15	1.48	0.20	0.031
	GCLM	1.65	1.87	0.19	0.440
	GSS	1.32	1.17	0.06	0.102
Jejunum	CBS	5.43	3.57	0.88	0.154
	CTH	3.32	1.69	0.30	0.001
	GCLC	2.47	2.30	0.27	0.669
	GCLM	1.43	1.69	0.11	0.130
	GSS	4.32	3.52	0.61	0.369
Liver	CBS	2.68	2.59	0.32	0.841
	CTH	1.94	2.03	0.17	0.733
	GCLC	2.21	1.40	0.22	0.018
	GCLM	1.95	1.47	0.17	0.057
	GSS	1.84	1.50	0.18	0.207
	CDO1	7.47	3.98	1.96	0.227
	CSAD	1.54	1.50	0.16	0.877
LD muscle	CBS	ND	ND	-	-
	CTH	ND	ND	-	-
	GCLC	1.40	1.67	0.16	0.264
	GCLM	4.32	6.28	0.93	0.156
	GSS	ND	ND	-	-

[1]Values are least-squares means ± SEM. Significance is measured at $P < 0.05$. Abbreviations used: AU, arbitrary unit; ND, not determined; LD, longissimus dorsi; CBS, cystathionine β-synthase; CTH, cystathionine γ-lyase; GSS, glutathione synthetase; GCLC, glutamate cysteine ligase, catalytic; GCLM, glutamate cysteine ligase, modifier; CDO1, cysteine dioxygenase 1; CSAD, cysteine sulfinic acid decarboxylase. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

Table 2.10. Enzyme activity of GCL and GSS in liver, ileum, jejunum, and LD muscle in non-weaned and weaned pigs.[1]

Tissue	NW	W	SEM	*P*-value
		GCL (nmol γ-GC/[mg protein • min])		
Liver	4.15	2.49	0.22	<0.001
Ileum	2.54	2.39	0.50	0.746
Jejunum	3.64	2.91	0.38	0.16
LD muscle	0.20	0.19	0.03	0.94
		GSS (nmol GSH/[mg protein • min])		
Liver	1.92	1.54	0.06	<0.001
Ileum	2.37	2.99	0.36	0.175
Jejunum	5.80	5.64	0.72	0.881
LD muscle	0.98	1.17	0.17	0.438

[1]Values are least-squares means ± SEM. Significance is measured at $P < 0.05$. Abbreviations used: LD, longissimus dorsi; GCL, glutamate cysteine ligase; γ-GC, γ-glutamylcysteine; GSS, glutathione synthetase; GSH, glutathione. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9).

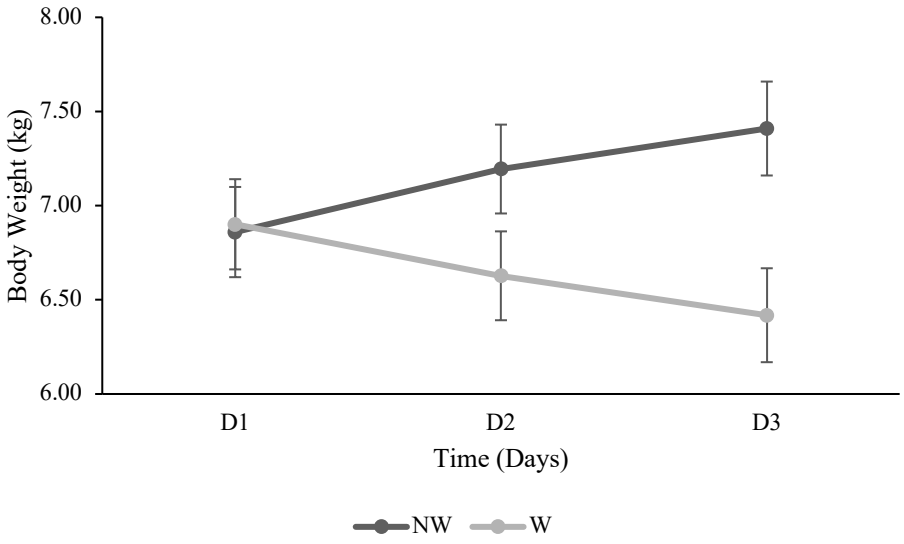

Figure 2.1. Pig body weight over time in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

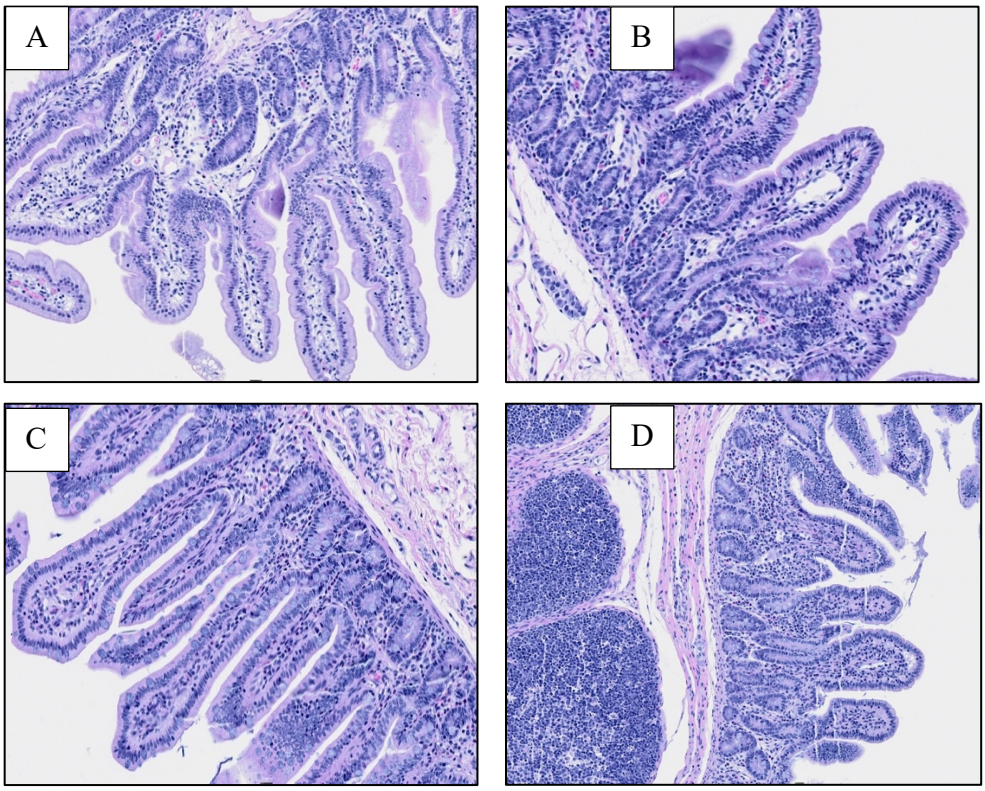

Figure 2.2: Representative images of (A), NW jejunum (B), W jejunum (C), NW ileum (D), W ileum crypts and villi.

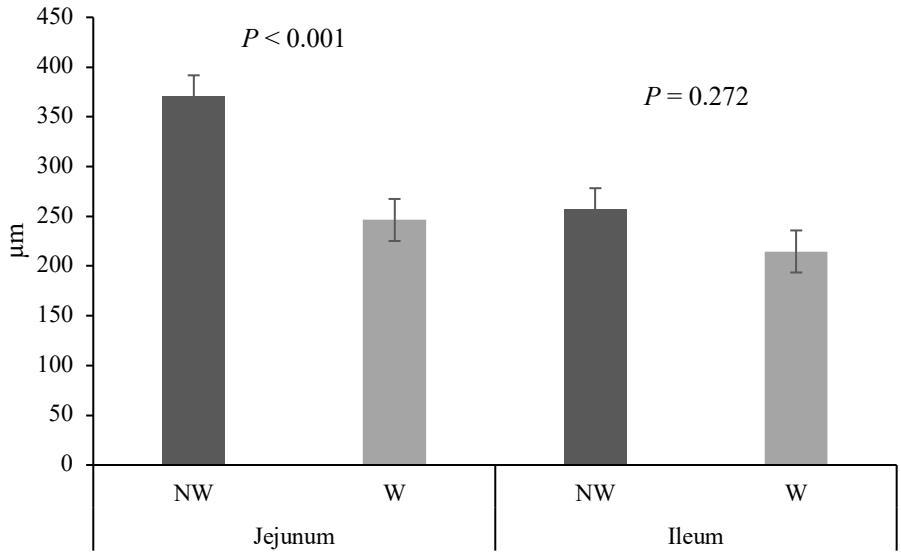

Figure 2.3. Villus height in jejunum and ileum mucosa in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

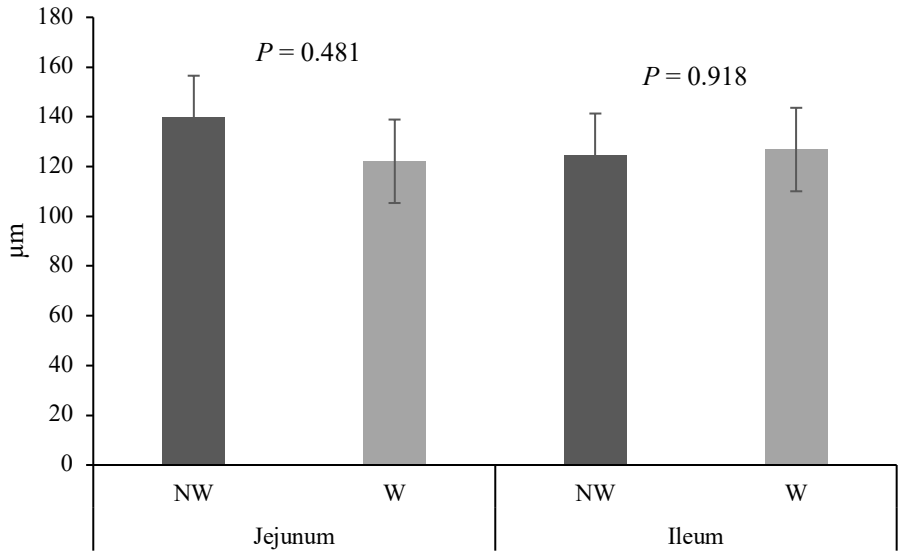

Figure 2.4. Crypt depth in jejunum and ileum mucosa in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

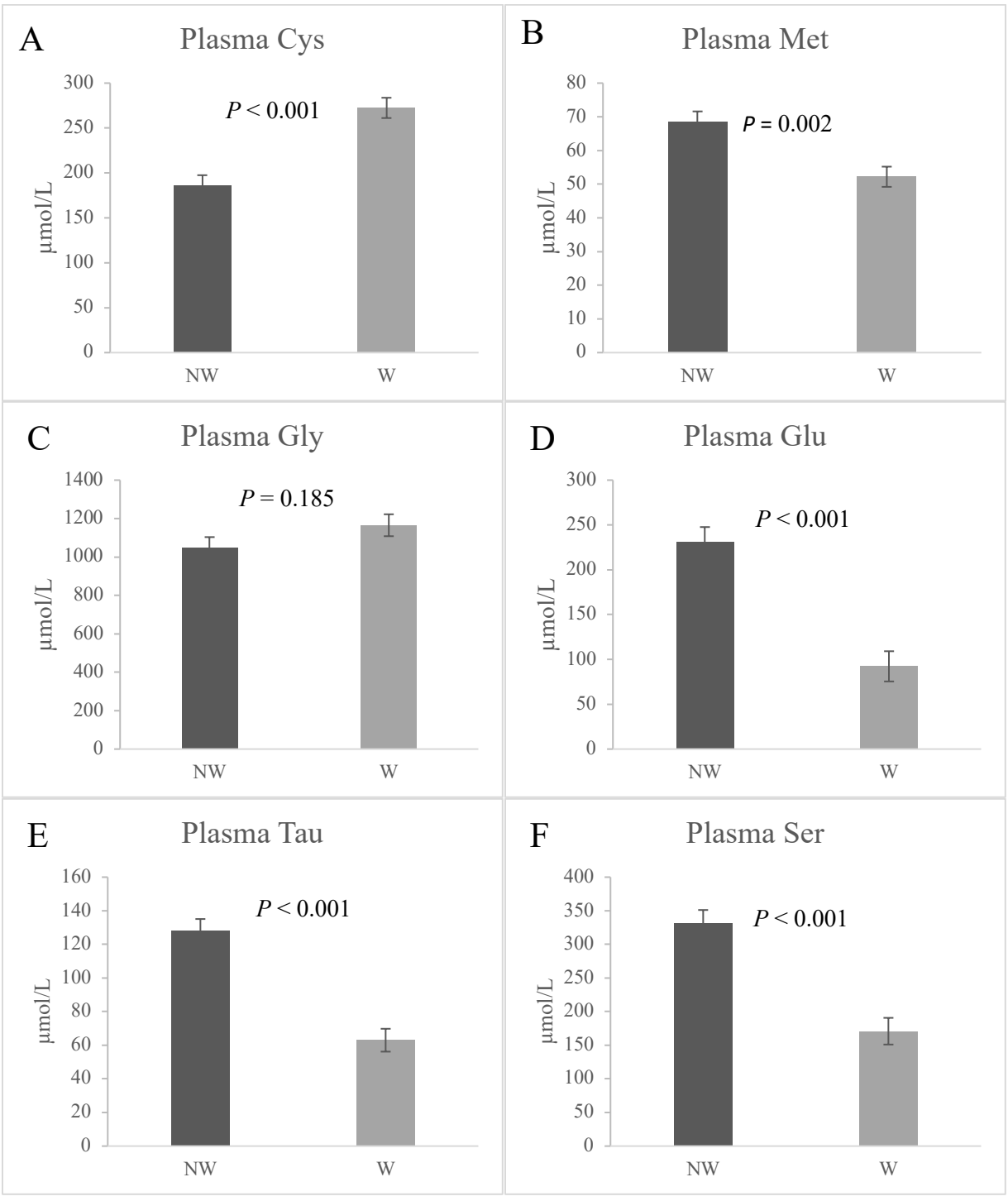

Figure 2.5: Plasma cysteine (A), methionine (B), glycine (C), glutamate (D), taurine (E), and serine (F) in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

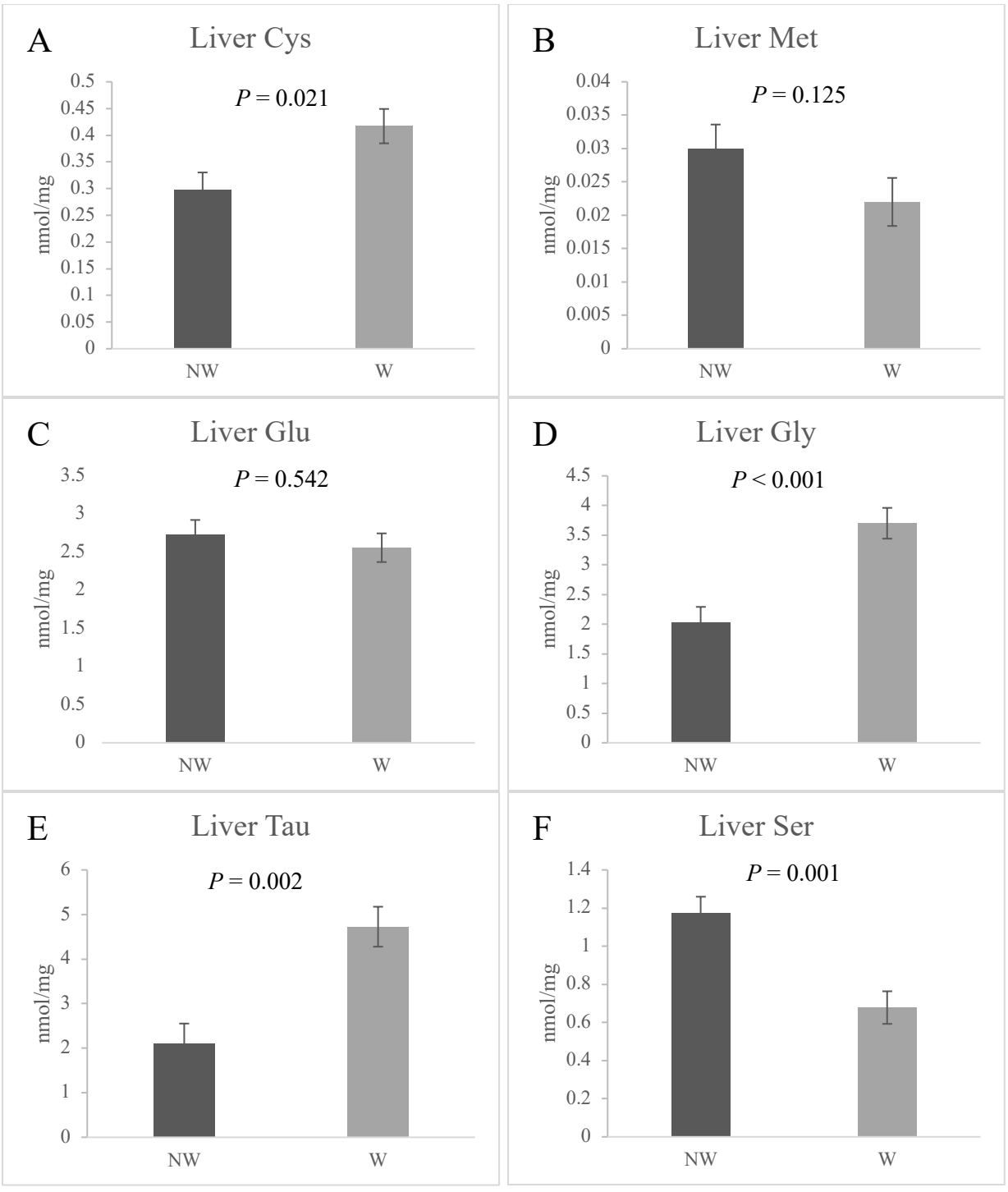

Figure 2.6: Liver cysteine (A), methionine (B), glycine (C), glutamate (D), taurine (E), and serine (F) in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

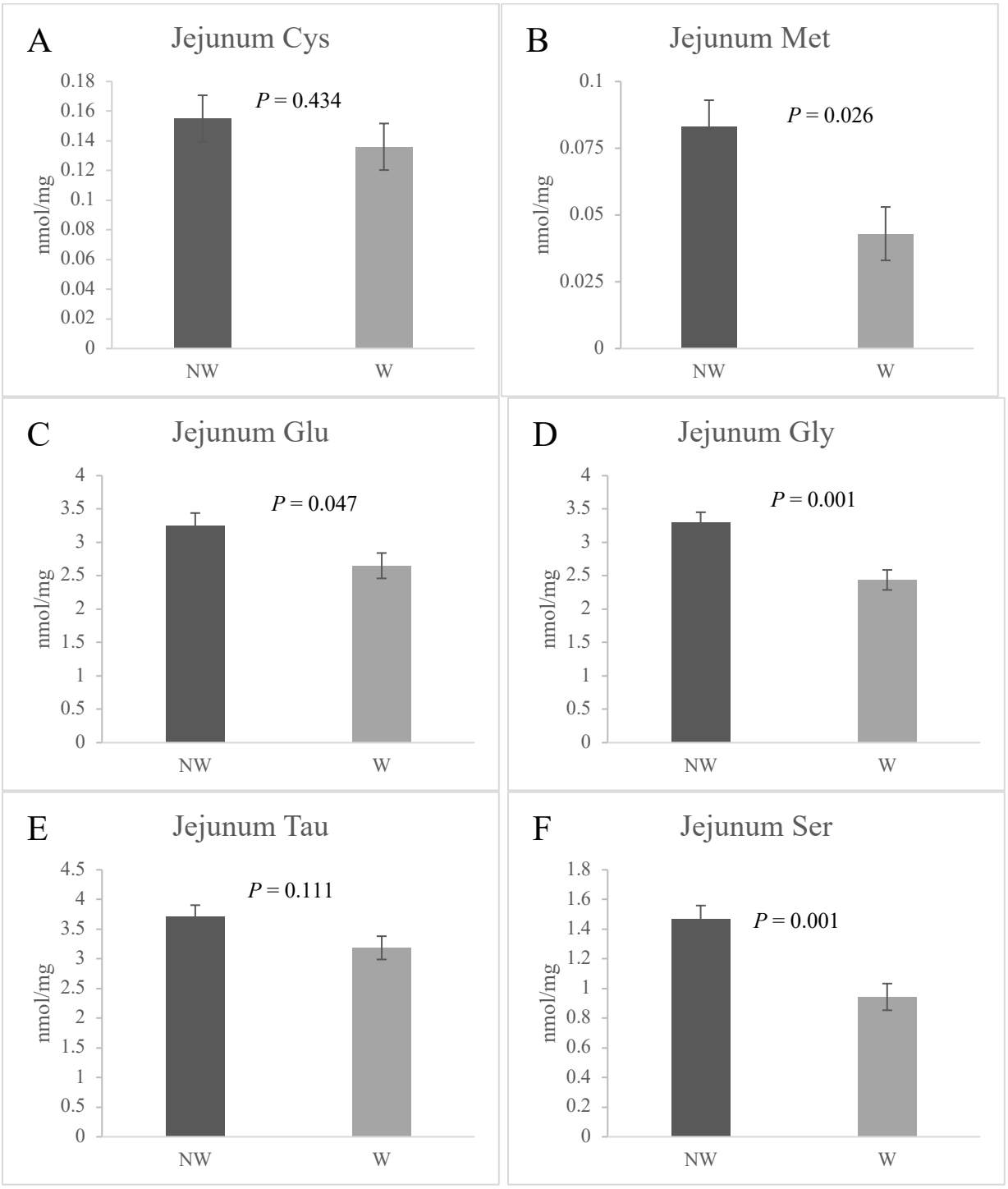

Figure 2.7: Jejunum cysteine (A), methionine (B), glycine (C), glutamate (D), taurine (E), and serine (F) in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

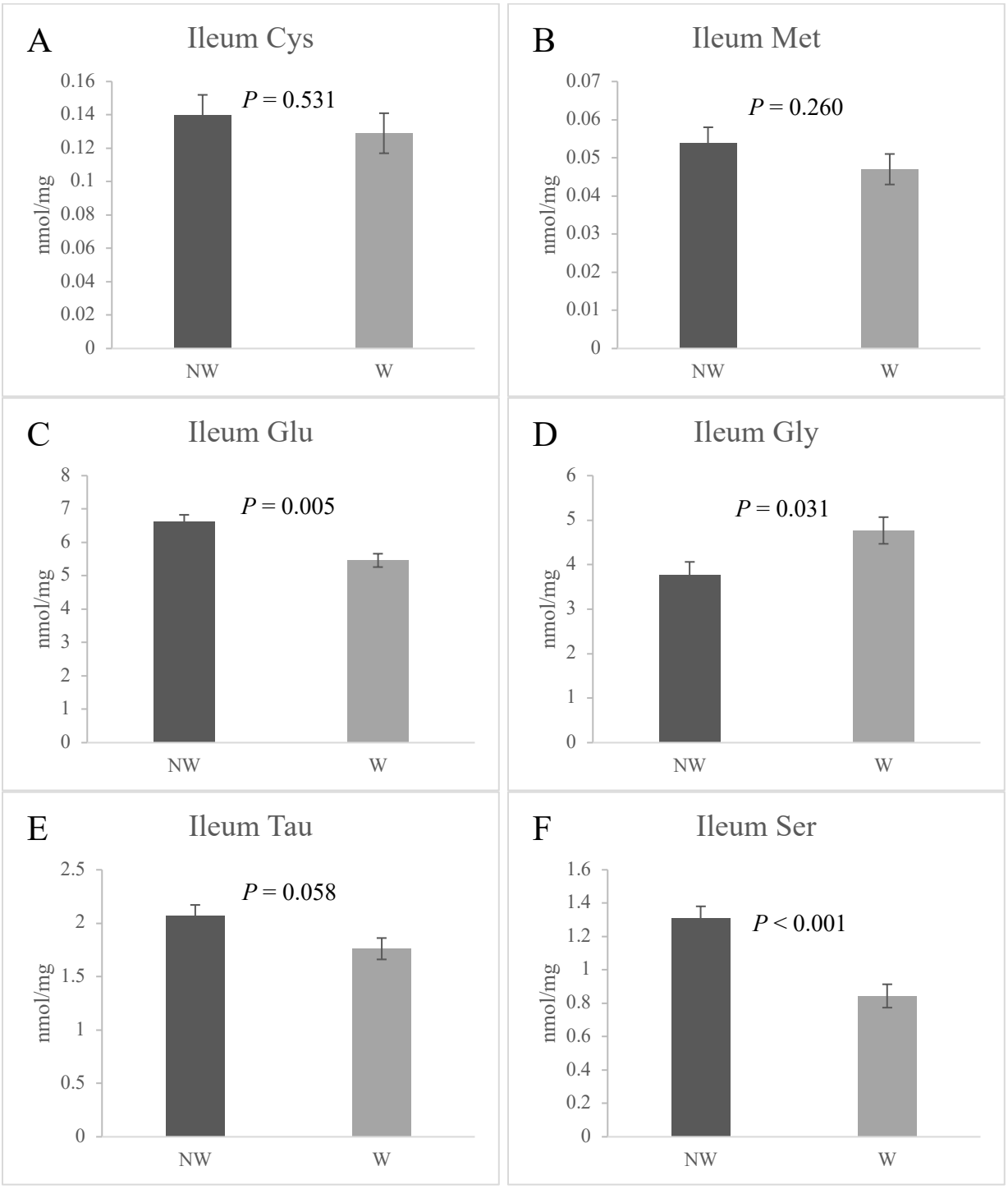

Figure 2.8: Ileum cysteine (A), methionine (B), glycine (C), glutamate (D), taurine (E), and serine (F) in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

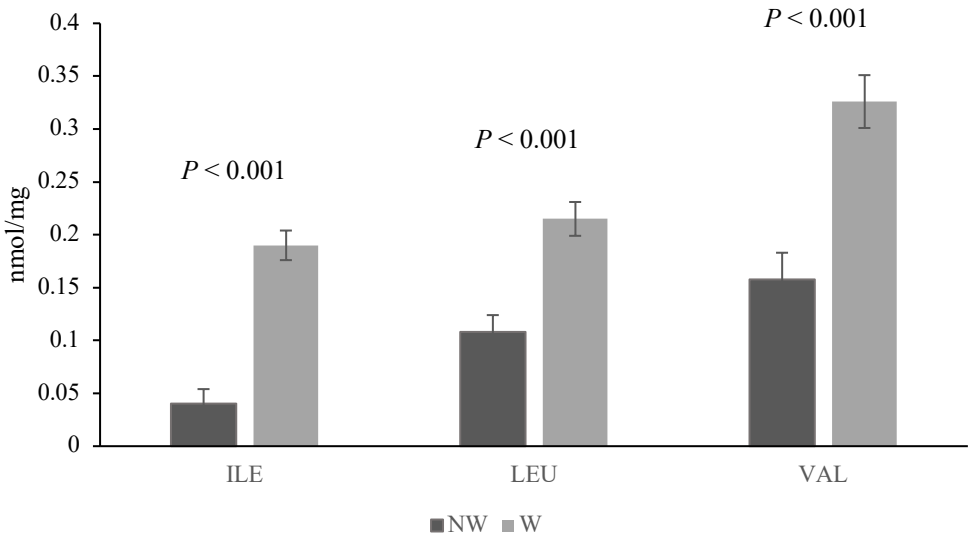

Figure 2.9: Longissimus dorsi isoleucine (Ile), leucine (Leu), and valine (Val) concentrations in non-weaned and weaned pigs. Treatments: NW, non-weaned (n = 9); W, weaned (n = 9). Values are least-squares means ± SEM; significance is measured at $P < 0.05$.

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