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Diet Induced Alterations and Distribution of Indices of Lysine Catabolism

Stephanie K. Gatrell

Dissertation submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in
Animal and Food Science

Kenneth P. Blemings, Ph.D., Chair
Matthew E. Wilson, Ph.D.
Kimberly Barnes, Ph.D.
Jianbo Yao, Ph.D.
Daniel Panaccione, Ph.D.

Division of Animal and Nutritional Sciences

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ABSTRACT

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Stephanie K. Gatrell

Lysine is thought to be oxidized primarily by lysine α -ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH), which are restricted to the mitochondrial matrix. Lysine is presumably transported through the plasma membrane by isoforms(s) of a cationic amino acid transporter (CAT). Although the mechanism(s) of lysine transport through the inner mitochondrial membrane is not known, it is presumptively transported by one or both mitochondrial "ornithine transporters" (ORC-1/ORC-2). Tissue distribution of LKR and the mechanism(s) responsible for alterations in hepatic lysine catabolism in swine are unclear. Also, the roles of the alternative pathways of lysine degradation, the L-amino acid oxidase-dependent (LAAO), in poultry, and the lysyl oxidase-dependent pathway (LO), in mammals and poultry, have not been established. Genetic effects on lysine degradation were evaluated throughout the production-cycle in 2 strains of commercial turkey. Hepatic LKR activity ($P<0.05$), LKR mRNA ($P<0.01$), SDH activity ($P<0.05$), lysine oxidation (LOX, $P<0.0001$), LAAO activity ($P<0.05$), LO activity ($P<0.01$) L-amino acid oxidase ($P<0.0001$), and CAT1 mRNA abundance ($P<0.05$) differed throughout the production cycle. Differences in indices of lysine catabolism due to strain in these 2 commercial lines of turkey were not detected. The LAAO and LO activities represented only 0.21 and 0.03% of the activity of enzymes involved in the saccharopine-dependent pathway. These data support that the saccharopine-dependent pathway is the predominant pathway of lysine degradation in turkey liver and that indices of hepatic lysine catabolism vary throughout the production cycle. Next, studies were conducted in swine to characterize the tissue distribution and evaluate possible mechanisms of alterations in lysine degradation. In growing pigs, LKR activity ($P<0.001$) was highest in liver, intestine and kidney samples and SDH activity ($P<0.0001$) and LKR ($P<0.0001$) and SDH mRNA ($P<0.0001$) were highest in liver. Interestingly, tissue distribution of LKR activity was correlated with ORC-1 and ORC-2 mRNA ($r^2=0.32$, $P<0.05$ and $r^2=0.41$, $P<0.05$, respectively). Average LO activity across tissues represented only 0.5% of the activity of enzymes involved in the saccharopine-dependent pathway. These data indicate that extra-hepatic tissues play a role in whole-body lysine degradation, ORC transporters may play a role in the transport of lysine into the mitochondrial matrix for its catabolism, and the saccharopine-dependent pathway is the predominant pathway of lysine degradation in pig tissues. To further investigate the role of ORC in lysine catabolism and to discern the mechanisms responsible for alterations of lysine catabolism, weanling pigs ($n=35$) were fed either a control (C), high protein (HP), low protein (LP), high lysine (HL) or low lysine (LL) diet. Liver LKR activity ($P<0.05$) and AASS protein expression ($P<0.01$) were reduced in pigs consuming the LL diet compared to C. Liver SDH mRNA expression was reduced ($P<0.08$) with the consumption of the LL diet compared to C, and AASS mRNA was reduced ($P<0.05$) with the consumption of the LL diet compared to the HP and HL diets. No significant dietary alterations in lysine catabolism were detected in heart or kidney. There was an increase ($P<0.05$) in liver ORC-1 mRNA expression with the consumption of the HL diet and a dramatic decrease in ORC-1 expression when treated with the LL diet. From these data it can be concluded that diet-induced alterations in lysine catabolism do occur in pig liver and the response of the ORC-1 mRNA to low lysine diets implicates that transporter as an important molecular basis of lysine conservation.

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Literature Review

History of Protein Nutrition

Although the importance of protein in nutrition had been appreciated for some time, the first detailed report of the capacity of various nitrogenous substances and their components to support growth and maintenance was published in 1911 (Osborne and Mendel). Rose (1938) created a method for recognizing the nutritive importance of the recognized components of protein, amino acids. In growing rats, ten amino acids were found to be indispensable for growth; therefore amino acids were classified as essential or nonessential. It was first proposed that lysine was an essential amino acid after rats ceased to grow while consuming diets containing lysine-free protein sources (Osborne and Mendel, 1914). The authors concluded that construction of new tissues is limited by the supply of lysine; however lysine was not required for maintenance. The latter conclusion was questioned by others and it was reported that lysine was essential for maintenance in 1945 (Neuberger and Webster). When rats consuming a lysine-devoid diet continuously lost body weight, while those consuming the same diet supplemented with lysine maintained or gained weight. Overall, it became evident that amino acids, lysine included, were essential for growth, maintaining nitrogen balance and other physiological roles.

Lysine metabolism

In animals, lysine is an essential amino acid. Lysine is found as a main component in body proteins, and comprises about 6% of total body protein by weight. Lysine is required for a variety of structural, functional and metabolic roles throughout the body, as well as required for protein synthesis. Cross-linking of peptide-bound lysine is crucial for the structural integrity of

proteins. The enzyme lysyl oxidase initiates the covalent crosslinking of collagen and elastin in the extracellular space by oxidizing lysine residues (Kagan and Li, 2003). The crosslinking stabilizes and precipitates polymeric collagen and elastin fibers in the extracellular matrix (Smith-Mungo and Kagan, 1998). Lysine residues also have functional roles; reversible acetylation of lysine residues is essential to histone formation, and ultimately gene expression, replication and nuclear division (Megee et al., 1990).

Circulating lysine is also involved in a variety of biological processes. Lysine is a strictly ketogenic amino acid and may be metabolized to TCA cycle intermediates for energy (Noda and Ichihara, 1976). Protein-bound lysine is used for the biosynthesis of carnitine, an important mediator of fatty acid transport across the mitochondrial membrane for oxidation (Tanphaich and Broquist, 1973). The degradation of lysine also results in the release of glutamate, the main excitatory neurotransmitter in the mammalian central nervous system. Up to 33% of glutamate found in the brain is potentially derived from lysine degradation (Papes et al., 2001).

As one considers lysine from the perspective of a nutritional biochemist, three broad and overlapping areas come into focus. These three areas are the focus of the volume of this introductory chapter. First, what is known about the role of lysine in nutrition and growth? A perspective that is focused on the whole animal. Second, what is known about lysine degradation and how it is regulated? A perspective that is more focused on the molecular level. Third, and finally, how do perturbations in these pathways of lysine catabolism impact humans? A perspective that goes from molecular back to the whole animal.

Dietary Lysine, Growth and Metabolism

Lysine is not only an essential amino acid, but it is frequently limiting. A limiting amino acid is one found in the diet below its requirement. A first limiting amino acid is present in the diet in the lowest amount compared to the requirement; therefore the growth of the animal is dependent on the availability of the amino acid. A second limiting amino acid is present in the diet next shortest of its requirement. Lysine is typically first limiting in swine (Mavromichalis et al., 1998) and human (Young and Pellett, 1990) diets. Lysine is also typically second limiting in production diets for poultry (Fernandez et al. 1994) and fish (Cheng et al., 2003). Consuming diets which contain lysine below the requirement may result in compromised growth. Therefore, it is imperative for producers to supplement grain-based feed with lysine to avoid any losses due to limitations of a limiting amino acid. For animals, this supplementation occurs through protein sources rich in lysine and with feed-grade lysine.

During growth, the requirement for individual nutrients must correlate with the rate of gain (Schwartz et al., 1958). The requirement for lysine is affected by growth rate and age. In growing chicks, the initially high lysine requirement (1.1% lysine) decreases 0.05% per week after week 4 of life, until a level of 0.8% lysine is reached by week 8 (Schwartz et al., 1958). Similar trends occur for swine; the lysine requirement for weanling piglets (5 kg) is 1.45%, which is reduced to 0.6% in market-weight animals (100 kg, NRC, 1998). Therefore, it is crucial to meet the requirement of dietary lysine at all stages of growth in production animals to avoid the negative results of lysine deficiency.

Young rats consuming a lysine deficient diet displayed cessation of growth, muscle wasting, hypoproteinaemia, decreased subcutaneous fat and reduced bone calcification (Harris et al., 1943). Chickens consuming a lysine deficient diet had decreased body weight, a 30-40%

reduction in growth rate and increased feed conversion ratio (FCR, feed/gain) compared to chicks consuming a lysine-adequate diet (Tesseraud et al., 1999). Interestingly, lysine deficiency in chickens led to a reduction in muscle weight, yet an increased muscle protein fractional synthesis rate, capacity for protein synthesis (muscle RNA:Protein) and fractional breakdown rates (Tesseraud et al., 2001). These data indicate the chickens consuming a lysine deficient diet have an increased rate of muscle turnover. However, since muscle weights are reduced, whole body protein synthesis is also reduced with a lysine deficiency.

Swine fed a lysine deficient diet consumed less feed, had about a 50% reduction in weight gain and reduced muscle weights (Rivera-Ferre et al., 2005). Contradictory to evidence in poultry, pigs consuming a lysine deficient diet had a reduction in fractional protein synthesis rate, absolute rate of protein synthesis, capacity for protein synthesis, and translational efficiency (protein synthesized:RNA, Rivera-Ferre et al., 2005). The authors hypothesized, based on the work of Waterlow (1999), the reduction in protein synthesis may be explained by a disintegration of polysomes, hindering the initiation of translation, therefore reducing translational efficiency and protein synthesis rates (Rivera-Ferre et al., 2005). The differences in metabolic responses of chickens and swine to lysine deficiency indicate that responses to dietary amino acid deficiencies may be species specific.

Relationships between genetics, growth and lysine requirements have been reported. Tesseraud et al. (1999) demonstrated that a line of broilers selected for improved carcass quality had increased body and muscle weights compared to a control line while consuming a lysine-deficient diet. Sterling et al. (2006) showed a three-way interaction between dietary protein, lysine levels and genotype for weight gain and FCR. Acar et al. (1991) demonstrated interactions between strain and dietary lysine for abdominal fat, breast fillet and tender yield. A

potential explanation for the interaction between strain and lysine requirement could be differences in lysine catabolism.

A unique aspect of lysine deficiency is the notion of conservation. Given that animals need all essential amino acids to synthesize proteins, one would hypothesize that when a single essential amino acid was lacking in the diet, the animal would act as if it were fed a protein-free diet (Said and Hegsted, 1970). This hypothesis proved true for most amino acids, however, rats fed a lysine-deficient diet lost considerably less body weight than those fed diets devoid of other essential amino acids or a protein-deficient diet (1970). This result led to the concept that lysine is conserved, allowing adaptation to lysine-deficient diets. Chicks fed a diet devoid of lysine survived twice as long, lost less weight and were stronger and more vigorous than those fed diets devoid of other essential amino acids (Ousterhout, 1960). Apparently, animals have adaptive mechanisms which conserve some amino acids when they are in short supply, and these adaptive mechanisms appear to be most pronounced for lysine (Chu and Hegsted, 1976). Explanations for prolonged survival during a lysine deficiency can be explained by drawing on peptides and protein (hemoglobin) high in lysine (Ousterhout, 1960) and/or by decreasing lysine catabolism. Decreasing lysine degradation under situations where lysine is first-limiting would allow lysine to be conserved for protein synthesis. However, the biochemical basis of lysine conservation has not been discerned. If it were possible to understand the molecular basis of lysine conservation seen under conditions of low lysine intake, it may be possible to evoke these mechanisms at higher levels of lysine intake, resulting in an increased efficiency of lysine use for protein synthesis and decreased production costs.

Pathways of lysine degradation

α -Aminoadipate δ -Semialdehyde Synthase

The degradation of lysine differs from most other amino acids, as it lacks a typical transamination reaction. The first step of lysine catabolism in the presumed predominant pathway of lysine degradation, the saccharopine-dependent pathway, is the removal of the ϵ -amino group. Lysine is converted to saccharopine in rat liver mitochondria (Higashino et al., 1965). The first step of the saccharopine-dependent pathway is catalyzed by the enzyme lysine α -ketoglutarate reductase (LKR) (E.C. 1.5.1.8); lysine is condensed with α -ketoglutarate to form saccharopine in this NADPH-dependent reaction. The requirement for α -ketoglutarate is crucial; little or no degradation occurs in the absence of α -ketoglutarate and the requirement cannot be satisfied by the addition of other tricarboxylic acid cycle intermediates (Higashino et al., 1965). Saccharopine is further metabolized to α -aminoadipic acid in rat liver mitochondria (Higashino et al., 1967). In the second step of the saccharopine-dependent pathway, saccharopine dehydrogenase (SDH) (E.C. 1.5.1.9) catalyzes the conversion of saccharopine to α -aminoadipate- γ -semialdehyde and glutamate in a NAD^+ -dependent reaction (Figure 1).

Evidence exists that both LKR and SDH activities reside on a bifunctional protein, α -aminoadipate δ -semialdehyde synthase (AASS). When LKR and SDH were copurified from bovine liver, there was a constant ratio (approximately 1.0) of both enzyme activities throughout the entire purification procedure (Markovitz et al., 1984); additionally, a single protein band was observed for LKR and SDH purified from baboon and bovine liver. Similar results were determined in human placenta (Fjellstedt and Robinson, 1975) and liver (Dancis et al., 1979). It has been hypothesized that the enzymes exist on a bifunctional enzyme that is comprised of a tetramer with identical subunits (Markovitz et al., 1984).

The saccharopine-dependent pathway is thought to be the predominant pathway of lysine degradation in animals. Lysine α -ketoglutarate reductase activity has been detected in human (Fjellstedt and Robinson, 1975), rodent (Higashino et al., 1967, Papes et al., 1999), swine (Pink et al., 2011), piscine (Higgins et al., 2006) and avian (Wang et al., 1973, Manangi et al., 2005) tissues. Initially, it was believed that lysine was oxidized almost exclusively in the liver (Miller, 1962); and LKR was first discovered in rat liver (Higashino et al., 1965). However, recent evidence suggests that extra-hepatic tissues also play a role in lysine catabolism.

Lysine degradation has also been detected in rodent (Noda and Ichiara, 1976, Maramatsu et al., 1983, Papes et al., 1999), swine (Pink et al., 2011) and avian (Manangi et al., 2005) kidney. The participation of the kidney in lysine degradation has been suggested to function in energy homeostasis, as LKR activity and LKR and SDH mRNA abundance are influenced by starvation in a similar manner as the hepatic enzymes (Papes et al., 1999). The authors also noted that the location of SDH activity in the kidney cortex may indicate that LKR and SDH play a role in the lysine reabsorption process. Other data suggests that lysine catabolism in the kidney may be related to the maintenance of cellular ion balance. Lysine degradation is affected by acid-base balance of feed; there was a 36% reduction in lysine catabolism when rats consumed a diet deficient in potassium (Forsberg and Austic, 1986).

Indices of lysine degradation have been measured in the brain of chickens (Manangi et al., 2005) and growing rats (Rao et al., 1992). In rats, LKR activity was highest during early stages of development and decreased to negligible levels at 30 days of age (Rao et al., 1992). As previously discussed, lysine degradation yields the release of glutamate, the main excitatory neurotransmitter in the mammalian central nervous system. Therefore, this may indicate that the

requirement for glutamate leads to an upregulation of LKR activity during embryonic brain development.

Initially, it was believed that the intestines did not contain the enzymes responsible for the degradation of lysine (Hutzler and Dancis, 1975, Chu and Hegsted, 1976). However, recent evidence of catabolism dominating first-pass intestinal utilization of dietary amino acids warranted further investigation (Stoll et al., 1998). In neonatal piglets, intestinal lysine oxidation accounted for one-third of whole body lysine oxidation while consuming a high protein diet (van Goudoever et al., 2000). Similarly, in 28-day-old piglets, first-pass intestinal metabolism accounted for 30% of lysine intake (Stoll et al., 1998). Although the fate of the catabolized lysine is unclear, Stoll and colleagues hypothesize it may be due to the role of lysine as an important source of energy for the small intestine (1998).

Previous research claiming that the intestine does not contain lysine catabolic enzymes used intestinal homogenates or scrapings (Hutzler and Dancis, 1975). Methods of detection may have been hindered by the gel-like properties of these samples, as a result of mucin secretion (Pink et al., 2011). Therefore, enzyme activity measurements may have been underestimated. In chickens, LKR activity and lysine oxidation were detected in whole intestinal homogenate, and represented 40% and 8% of total body LKR activity and oxidation, respectively (Manangi et al., 2005). In growing pigs, LKR activity in mitochondria of isolated intestinal epithelial cells was 50% of hepatic LKR activity (Pink et al., 2011).

Additional organs may also play minor roles in lysine catabolism. Lysine α -ketoglutarate reductase activity has been detected in pancreas of rats (Chu and Hegsted, 1976) and chickens (Manangi et al., 2005), which contributed 1.4% of total body LKR activity. Additional tissues

which play minor roles in lysine degradation include the heart, skeletal muscle, lung and spleen (Chu and Hegsted, 1976, Manangi et al., 2005, Pink et al., 2011).

Lysine degradation through the AASS pathway is under hormonal and dietary control. Injecting rats with glucagon lead to a 107 and 80% increase in LKR and SDH activity in liver mitochondria, respectively (Scislowski et al., 1994). Similar results have been shown in cultured rat hepatocytes (Shinno et al., 1980) and rat brain (Rao et al., 1992). It has been speculated that the induction of LKR expression due to glucagon may be due to an increase in intramitochondrial Ca^{2+} concentrations (Denton and McCormack, 1985), however a direct link has not been established. Conversely, insulin has also been shown to decrease LKR and SDH activity (Shinno et al., 1980). Taken together, it appears that the hormonal regulation of lysine degradation is an important physiological mediator of the metabolism of amino acids due to changes in the prandial state.

In rodents, starvation led to a 52 and 47% increase in LKR and SDH activity compared to fed animals, indicating that lysine degradation is coupled with energy balance in situations with limited carbon supply (Papes et al., 1999). Consuming diets high in protein or lysine increases LKR activity and lysine oxidation in rodent liver (Chu and Hegsted, 1976, Muramatsu et al., 1984, Blemings et al., 1998, Kiess et al., 2008) and kidney, however to a lesser extent (Muramatsu et al., 1984). Interestingly, the authors also noted that liver LKR activity increased almost linearly and reached a maximum at 7 to 10 days in rats consuming diets supplemented with various levels of lysine.

In mice consuming a high protein diet, LKR activity and AASS mRNA abundance increased, however there was no change in AASS protein abundance detected (Kiess et al., 2008). These data are consistent with post-translational modification, potentially via

phosphorylation, as the primary means of regulating LKR activity (Kiess, 2008). Similar conclusions for post-translational modifications have been observed in fish (Higgins et al., 2006) and chickens (Kiess et al., 2006). Additionally, in plants, evidence indicates that LKR is regulated by post-translational modification (Stepansky et al., 2006). In the developing tobacco seed, lysine regulates its own concentration by stimulating LKR activity. This stimulation occurs by a specific signaling cascade mediated by calcium and protein phosphorylation (Karchi et al., 1995). Similar results have been shown in soybean (Miron et al., 1997).

In vitro lysine degradation is also influenced by substrate concentrations. Pink and colleagues (2011) have determined that hepatic LKR demonstrated substrate inhibition at lysine concentrations greater than 15 mmol/L, while SDH activity demonstrated classic Michaelis-Menten kinetics. The authors also determined that LKR activity is inhibited by the lysine metabolites L-saccharopine, L-aminoadipic acid and α -ketoacid, and certain metals, including mercuric chloride and zinc chloride. Therefore, lysine degradation may be influenced by feedback regulation and dietary minerals.

Lysine degradation via the AASS pathway is also affected by genetic differences. Genetic effects on LKR activity (Wang et al., 1972, 1973) and the rate of lysine oxidation (Wang et al., 1973) exist between broiler chickens selected for arginine requirement. There is evidence of a genetic influence on the ability of broiler chicks to use lysine for protein deposition in lysine-limiting situations (Tesseraud et al., 2001).

Sequence analysis of the complete AASS protein reveals the first 30 amino acids encode for the mitochondrial localization sequence. Evidence supports the fact that lysine catabolism occurs within the mitochondria (Higashino et al., 1965). Specifically, in rat liver, LKR and SDH activities are located only within the mitochondrial matrix (Blemings et al., 1994). The location

of the initial enzymes of lysine degradation via the saccharopine-dependent pathways indicate that lysine must be transported into the matrix for its catabolism. The authors suggested that the rate of mitochondrial lysine uptake is a limiting step in lysine oxidation (Blemings et al., 1998); therefore, mitochondrial lysine transporters may be a potential target for regulating lysine catabolism. Although the specific protein(s) for mitochondrial lysine uptake have not been identified, there has been some speculation that it is similar to that of the cationic amino acid ornithine.

Ornithine is a non-essential amino acid structurally similar to lysine, only differing in one methylene group in the side-chain. Similar to lysine, the enzymes responsible for ornithine “degradation”, ornithine transcarbamylase and ornithine aminotransferase, are also found exclusively in the mitochondrial matrix. Also, a transport limitation to ornithine catabolism has been shown (Hommes et al., 1983). It has been hypothesized that ornithine and lysine are transported via similar mechanisms. When both lysine and ornithine mitochondrial uptake activities were measured simultaneously, they mutually inhibited each other, leading to the assumption that transporters for lysine uptake are the same as ornithine in rat liver mitochondria (Hommes et al., 1982). Two isoforms of ornithine transporters, ORC-1 and ORC-2 have been identified in humans (Fiermonte et al., 2003). Both isoforms are able to transport L-isomers of ornithine, lysine, arginine and citrulline by unidirectional mechanisms. The authors determined that both isoforms of the carriers are expressed in a range of human tissues, with ORC-1 being the predominant form. However, a link between ORCs and lysine degradation has not been established. Alternative mitochondrial lysine transporters have been investigated. Two mitochondrial basic amino (BAC) acid transporters in *Arabidopsis thaliana* have been examined to better understand how the amino acid arginine, also a basic amino acid, is transported into the

mitochondrion for degradation (Hoyos et al., 2003). The BAC transporters were also capable of transporting lysine into the mitochondria, albeit with decreased affinity.

Overall, lysine catabolism via the saccharopine-dependent pathway occurs in the liver; however additional tissues may play a role in whole-body lysine degradation. The enzymes of the saccharopine-dependent pathway are under dietary and hormonal control. Although the saccharopine-dependent pathway is thought to be the predominant pathway of lysine catabolism, the alternative routes, the lysyl oxidase- and L-amino acid oxidase-dependent pathways must not be overlooked.

Lysyl oxidase

The metabolite α -aminoadipic δ -semialdehyde generated by AASS in the saccharopine-dependent pathway of lysine degradation is also generated by the enzyme lysyl oxidase (Figure 1). Lysyl oxidase (LO) (E.C. 1.4.3.13) is a copper-dependent amine oxidase that initiates covalent crosslinking between and within collagen and elastin by oxidizing peptidyl lysine to peptidyl α -aminoadipic- δ -semialdehyde (Smith-Mungo and Kagan, 1998). The resulting crosslinks stabilize and precipitate collagen and elastin, making lysyl oxidase crucial in the morphogenesis and repair of connective tissues of the cardiovascular, respiratory, skeletal and other bodily systems (Kagan and Li, 2003). The preferential substrate for lysyl oxidase is protein-bound lysine, however evidence indicates that the enzyme is also able to oxidize free lysine (Trackman and Kagan, 1979).

Lysyl oxidase activity was first discovered in extracts of embryonic chick bone (Pinnell and Martin, 1968), and later in numerous mammalian (Gacheru et al., 1990, Maki and Kivirikko, 2001) and avian (Narayanan et al., 1974, Opsahl et al., 1982) tissues. In rats, LO activity was

highest in dense connective tissue, such as tendons and skin, and was also detected in aorta, kidney, lung and liver (Rucker et al., 1996).

Lysyl oxidase is under dietary and hormonal control. The enzyme contains one copper cofactor per monomer, which correlates with the maximum expression of enzyme activity (Smith-Mungo and Kagan, 1998). The consumption of a copper-deficient diet results in a significantly reduced LO activity in weanling rat cardiac muscle (Werman et al., 1995) and human skin (Werman et al., 1997). Other reports indicate a linear increase in LO activity in chick tendon when dietary copper levels increased from 0.3 to 16 ppm (Opsahl et al., 1982). Therefore, dietary copper levels may be a potentially limiting factor for enzyme activity. Follicle stimulating hormone (FSH) decreased LO mRNA expression and enzyme activity in rat granulosa cells, potentially via cAMP signaling (Harlow et al., 2003). The control of LO in the ovary may suggest coordinated regulation of ovarian extracellular matrix formation during follicular development. Also, transforming growth factor- β 1, a fibrogenic cytokine known to activate collagen synthesis, promotes LO expression in neonatal rat lung fibroblasts (Boak et al., 1994), rat vascular smooth muscle (Gacheru et al., 1997), granulosa cells (Harlow et al., 2003) and human embryos (Roy et al., 1996).

L-Amino Acid Oxidase

L-amino acid oxidase (AAOX, E.C. 1.4.3.2) is an enzyme that catalyzes the irreversible oxidation of L- α -amino acids to their corresponding α -keto acids, ammonia and hydrogen peroxide (Struck and Sizer, 1960). The enzyme is found in the microsomes of mammalian liver (Nakano et al., 1968), and brain (Murthy and Janardanasarma, 1999), avian liver (Struck and Sizer, 1960) and snake venom (Wellner and Hayes, 1968).

The affinity of different amino acids as substrates for AAOX varies. Lysine is a poor substrate for mammalian AAOX (Blanchard et al., 1944); however, the preferential substrate for AAOX in avian liver is leucine, followed by lysine (Struck and Sizer, 1960). Lysine catabolism via AAOX has been termed the pipercolic acid (PA) pathway; as the oxidation of lysine produces the intermediate pipercolic acid (Grove et al., 1968), which can eventually be converted to α -aminoadipic δ -semialdehyde, a common intermediate with the saccharopine-dependent pathway (Struck and Sizer, 1960). Although it is generally assumed that the saccharopine-dependent pathway is the major pathway of lysine degradation, participation of the pipercolic pathway is not well characterized.

Little evidence exists regarding the regulation of AAOX. Chickens consuming diets increasing in levels of dietary lysine exhibited no changes in liver AAOX activity (Wang and Neshein, 1972). However, conflicting data exists showing that chickens consuming a lysine deficient diet demonstrated a trend ($P=0.08$) for a reduction in AAOX mRNA expression compared to chickens consuming a lysine-adequate diet (Kieiss, 2006).

Lysine catabolism occurs via three pathways, the saccharopine-dependent, lysyl oxidase-dependent and L-amino acid oxidase-dependent pathways, all of which share the common intermediate α -aminoadipic- δ -semialdehyde. As with any protein or enzyme, which is a product of a gene template, enzymes involved in lysine degradation may be subject to mutations, ultimately leading to errors in lysine metabolism.

Inborn Errors of Lysine Metabolism

Mutations in genes encoding for proteins or enzymes in lysine degradation may lead to reductions in enzyme activity and errors in metabolism. Familial hyperlysinemias are autosomal

recessive disorders in the oxidative degradation of lysine. Symptoms of hyperlysinemia may include convulsions, mental retardation, delayed development, poor growth and anemia (Woody et al., 1966). Two types of hyperlysinemias have been characterized; type I refers to patients with major defects in both LKR and SDH activities, while type II refers to patients that lack SDH activity, while still retaining some measureable LKR activity (Dancis et al., 1979). In both phenotypes, plasma lysine levels are severely elevated and in some patients range from 835-1356 $\mu\text{mol/L}$ compared to normal values of 71-151 $\mu\text{mol/L}$ (reviewed by Woody, 1974). Patients with type II hyperlysinemia also exhibit elevated levels of plasma saccharopine (24 μM) compared to healthy controls (0 μM) due to the lack of SDH activity with only a reduction in LKR activity (Fellows and Carson, 1986). In cultured skin fibroblasts from these subjects, at low lysine concentrations, lysine oxidation was lower than seen in controls; but at high concentrations oxidation was close to those obtained with control cells. The authors suggest that in these cases, hyperlysinemia is due to a defect of transport of lysine into mitochondria (Oyanagi et al., 1986).

An additional symptom of hyperlysinemia is mental and motor retardation (Woody et al., 1966). However, Dancis reported mental retardation in only 1 of 3 patients analyzed, hypothesizing that no definitive statement can be made as to the impact of hyperlysinemia on mental performance other than it does not appear to be as consistently or severely damaging as it is in phenylketonuria and in maple syrup urine disease (1969). Although the cause for mental retardation due to hyperlysinemia is unknown, many hypotheses exist. Lysine degradation yields the release of glutamate, which may be decreased in individuals with hyperlysinemia; potentially leading to impaired central nervous system development (Papes et al., 2001); lysine is an important essential amino acid for protein synthesis in the central nervous system (Oyanagi et al.,

1986). Also, hyperlysinemia can lead to hyperammonemia, negatively impacting nervous system development (Gregory et al., 1989).

Patients with hyperlysinemia also exhibit increased levels of plasma pipercolic acid (Dancis and Hutzler, 1986), most likely due to an increased flux through the L-amino acid oxidase pathway. Similar results were shown in patients who had increased levels of ϵ -N-acetyl-L-lysine, also involved in the AAOX pathway (Woody et al., 1967). Although lysine catabolism via this minor pathway in lysine degradation is increased in patients with hyperlysinemia, it is not able to degrade lysine at a rate comparable to the saccharopine-dependent pathway.

To determine the potential cause for hyperlysinemia, genomic DNA from a single patient previously described (Dancis et al., 1976) to have LKR and SDH activities ~10% and 3.5% of normal, respectively was sequenced (Sacksteder et al., 2000). The authors identified a homozygous 9-bp deletion in exon 15 not seen in controls. The deletion is out of frame and results in a premature stop codon in the SDH region of the protein, and is predicted to lead to a reduction in mRNA levels and ultimately reduced LKR and SDH activities. Additional research has not been conducted to examine if this deletion is present in other patients with hyperlysinemia.

Dietary treatments of hyperlysinemia include feeding diets low in lysine or protein. Effective dietary control is difficult due to the abundance of lysine in natural food, therefore synthetic diets are necessary (Dancis et al., 1983). Consuming a diet with protein levels reduced to a level required for growth lowered plasma lysine concentrations in patients with hyperlysinemia from 20 mg/dl to 12 mg/dl, or near normal levels (Dancis et al., 1983). Consuming a low lysine diet reduced plasma lysine concentrations; however the treatment was

unsuccessful at reducing plasma ammonia concentrations, leading to mild ammonia toxicity which may be a factor in the pathogenesis of hyperlysinemia (Gregory et al., 1989).

Lysine is an amino acid that has been extensively studied for decades. It is important to agriculture and human health since lysine is typically first or second limiting in grain-based diets. Lysine supplementation is an added expense to producers, therefore increasing the efficiency of lysine use for protein synthesis by decreasing lysine catabolism is a cheaper alternative. To achieve this goal, a proper understanding of lysine degradation is warranted.

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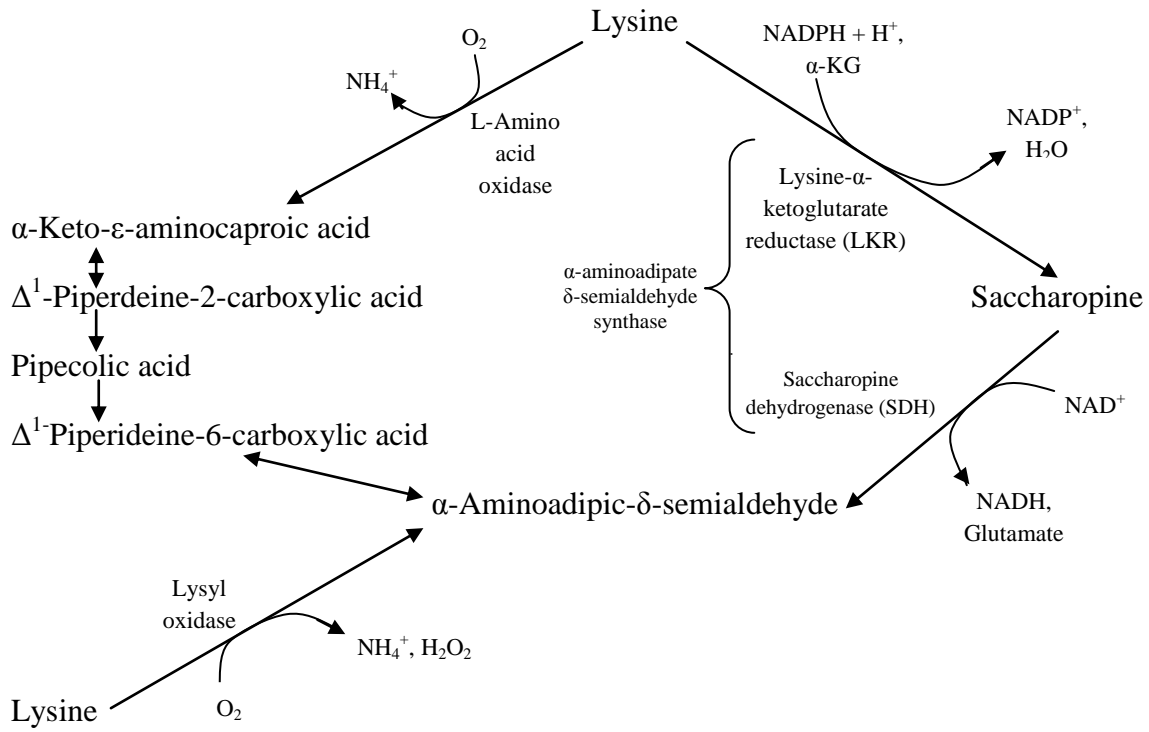
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Figure 1. Pathways of lysine degradation.



STATEMENT OF THE PROBLEM

Feed costs represent 2/3 - 3/4 of production costs, with a substantial fraction of the costs due to supplying adequate amino acids to the animal. An increase in the efficiency of amino acid use for protein synthesis would decrease production costs. Specifically targeting amino acids that are in shortest supply relative to their requirement (i.e. limiting amino acids) is an approach that will reap the greatest benefits. Lysine is an essential and limiting amino acid in most production diets; it is first-limiting in most swine and turkey diets and second-limiting in broiler diets. Thus, crystalline lysine is usually supplemented to the diets, increasing production costs. Increasing the efficiency of lysine use in protein synthesis will lower dietary lysine requirements, thus decreasing production costs. This economic benefit is most likely to be realized by a thorough understanding of lysine catabolism.

Interestingly, the efficiency of amino acid use varies with different amino acids and is not constant in all dietary situations. Animals perform better when consuming a diet devoid of lysine compared to diets devoid of protein or any other essential amino acid. The high efficiency of lysine use at low lysine intakes led to the concept that lysine is conserved, allowing adaptation to lysine-deficient diets, presumably by a decrease in lysine catabolism. Other diet-induced alterations of lysine degradation have been observed, however the mechanisms mediating these alterations are not well understood.

CHAPTER ONE

Tissue Distribution of Indices of Lysine Catabolism in Growing Swine

ABSTRACT

The primary pathway of lysine degradation in pigs presumably depends on the bifunctional protein α -amino adipate δ -semialdehyde synthase (AASS) which contains lysine α -ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH) activities. In liver, AASS is restricted to the mitochondrial matrix and lysine is presumptively transported through the plasma membrane by a cationic amino acid transporter (CAT1/2) and through the inner mitochondrial membrane by one or both mitochondrial ornithine transporters (ORC-1/ORC-2). Lysyl oxidase (LO) may represent an alternative pathway of lysine oxidation. The objective of this experiment was to analyze the distribution of indices of lysine catabolism in various pig tissues. We assessed LKR, SDH and LO activities, lysine oxidation, mRNA abundance of LKR, CAT1/2 and ORC1/2 and AASS protein abundance (via SDH antibody) in liver, heart, kidney medulla and cortex, triceps, longissimus, whole intestine, enterocytes and intestine stripped of enterocytes in growing pigs, weighing approximately 25 kilograms (n=10). LKR activity ($P<0.0001$) differed across tissues and was highest in liver, intestine and kidney samples and LKR mRNA abundance ($P<0.0001$) was highest in liver, although both were detected in all other tissues. SDH activity ($P<0.001$) and SDH mRNA abundance ($P<0.001$) was affected by tissue and were highest in liver compared to all other tissues analyzed. AASS protein abundance ($P<0.001$) was highest in whole intestine and liver. LO activity ($P<0.0001$) was highest in muscle samples. The abundance of ORC-1 ($P<0.001$) and 2 mRNA ($P<0.001$) was affected by tissues and ORC-1 was highest in liver, kidney and intestinal preparations, and ORC-2 mRNA abundance was highest in liver and intestine. Interestingly, LKR activity was correlated with ORC-1 and ORC-2 expression ($r^2=0.32$, $P<0.05$ and $r^2=0.41$, $P<0.05$, respectively). CAT-1 was

uniformly expressed in all tissues, while CAT-2 ($P < 0.01$) was highest in liver. In conclusion, these data indicate that extra-hepatic tissues contribute to lysine catabolism as do enzymes other than LKR (Support; HATCH WVA 470).

Keywords: lysine, lysine α -ketoglutarate reductase, lysyl oxidase, saccharopine dehydrogenase, swine

INTRODUCTION

Lysine catabolism by the saccharopine-dependent pathway is thought to be the predominant pathway for lysine degradation in most production species (Broquist, 1991). The enzymes of this pathway, lysine α -ketoglutarate reductase (LKR, EC 1.5.1.8) and saccharopine dehydrogenase (SDH, EC 1.5.1.9) are part of the bifunctional protein aminoadipate δ -semialdehyde synthase (AASS, Dancis et al., 1979). Initially, it was believed that lysine was oxidized almost exclusively in the liver (Miller, 1962). However, recent evidence suggests that extra-hepatic tissues play a role in lysine catabolism. Indices of lysine catabolism have been detected in kidney (Maramatsu et al., 1983, Pink et al., 2011) and brain (Rao et al., 1992, Manangi et al., 2005) in many different species, while the role of the intestines has sparked debate (Hutzler and Dancis, 1975, Stoll et al., 1998, Pink et al., 2011). However, questions remain regarding the distribution of lysine catabolism in swine tissues. Also, the role of lysyl oxidase (LO), a presumed minor pathway in lysine degradation (Smith-Mungo and Kagan, 1998) is not well characterized.

Cellular localization is an important aspect of lysine catabolism. Lysine is first transported through the plasma membrane of the cell by an isoform of cationic amino acid transporters (CAT-1 or 2). Within rat liver at least, LKR and SDH are located specifically in the mitochondrial matrix (Blemings et al., 1994), although the mechanism of transport through the inner mitochondrial membrane is unknown. However, it appears that lysine may be transported via an ornithine carrier (ORC-1 or 2, Fiermonte et al., 2003). Therefore, the objectives of this experiment were to characterize the distribution of indices of lysine catabolism in various pig tissues to obtain a better understanding of whole body lysine metabolism in growing pigs and to

determine if correlations existed among the various indices measured, especially the expression of the transport proteins.

MATERIALS AND METHODS

Sample Collection

Mixed-sex, growing pigs (~25 kg) of PIC lineage were used for tissue collection. All animals were euthanized in a humane manner via captive bolt. Immediately following euthanasia, tissue samples were removed. Jejunal sections were excised and stored in ice-cold PBS/5mM dithiothreitol (DTT) (pH 7.4). Samples of liver, kidney cortex and medulla, longissimus dorsi, triceps fermorus and heart were stored in ice cold homogenization buffer (H buffer; 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM 2-mercaptoethanol, 1 mM EGTA, 0.05% (w/v) bovine serum albumin, pH 7.4). The samples were transported to the laboratory, on ice, for further processing. Additional samples of all tissues were flash frozen in liquid nitrogen for future real-time PCR analysis. Enterocytes were prepared from the jejunum as described by Wu et al. (1994). Two replicates of 5 animals, replicate 1 and 2, were assessed over a 12 month period (n=10), whole intestinal homogenate and stripped intestine were only analyzed during replicate 2 (n=5). All protocols were approved by the West Virginia University Animal Care and Use Committee (ACUC# 09-0606)

Lysine α -Ketoglutarate Reductase and Saccharopine Dehydrogenase Enzyme Activity

Tissue LKR and SDH activities were determined on fresh tissue samples as previously described (Blemings et al., 1994). Tissues were processed using a Potter-Elvehjem homogenizer

and ice-cold H buffer to make a 25% (w/v) suspension. Lysine α -ketoglutarate reductase activity was measured as the lysine-dependent NADPH oxidation at 340 nm using a Beckman Coulter DU640 spectrophotometer. Enzyme activity was measured upon the addition of 20 μ L of tissue homogenate to a cuvette containing 1 mL of buffer (127.5 mM Hepes, 114.75 mM mannitol, 38.25 mM sucrose, 4.25 mM 2-mercaptoethanol, 0.0425% bovine serum albumin, 0.21 mM NADPH, 12.75 mM α -ketoglutarate, 0.05% (v/v) Triton-X 100 and 50 mmol L-lysine, pH 7.8). A blank without lysine was also measured to determine the disappearance of NADPH independent of lysine addition. All tissue samples were measured for LKR activity in duplicate. Saccharopine dehydrogenase activity was assessed as the saccharopine-dependent NADH appearance at 340 nm. Enzyme activity was measured upon the addition of 20 μ L of tissue homogenate to a cuvette containing 1 mL of buffer (100 mM Tris-HCl, 3 mM NAD, 5 mM 2-mercaptoethanol, 0.05% (v/v) Triton X-100 and 2 mM L-saccharopine, pH 8.7) A blank without saccharopine was also measured to determine the appearance of NADH independent of the addition of saccharopine. All tissue samples were measured for SDH activity in duplicate.

Lysine Oxidation Assay

Lysine oxidation was measured by determining the recovery of $^{14}\text{CO}_2$ from [1- ^{14}C] L-lysine on fresh tissue homogenates, as previously described in rats (Blemings et al., 1998). A base trap was constructed by the addition of 1 part ethanolamine to 2 parts methyl cellosolve. A total of 0.5 mL of a base trap was added to an Eppendorf tube, which was suspended in a 5 mL glass vial. For each reaction, 250 μ L of homogenate was added to a 5-mL vial, containing 250 μ L of buffer at 41°C (20 mM Hepes, 6mM MgCl_2 , 0.4 mM EDTA, 364 mM mannitol, 122 mM sucrose and 10 mM L-lysine (final concentration), pH 7). Additional samples were placed in

boiling water for 10 minutes to serve as a heat killed blank. All solutions were then incubated in the 41°C water bath for 30 minutes, while oscillating at 100 osc/min. To terminate the reactions, 100 µL of 1M potassium phosphate (pH 5.0) was injected via an 18 gauge needle through the serum cap covering the vial. The vials then remained oscillating in the water bath for at least 180 additional minutes, to ensure maximal recovery of the ¹⁴CO₂. After incubation, the Eppendorf tube was placed in a plastic scintillation vial along with 17 mL of Bio-Safe II liquid scintillation fluid. After the solution was vortexed, the radioactivity was measured using a Beckman LS 6500 liquid scintillation counter (Beckman Coulter Inc, Somerset, NJ). Each tissue was measured in triplicate and corrected for the heat killed blank.

Lysyl Oxidase Activity

Fresh tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until use. LO activity was measured as previously described (Palamakumura et al., 2002). Frozen samples were powdered in liquid nitrogen using a ceramic mortar and pestle. The resulting powder was homogenized with 0.01 M phosphate buffered saline (2 mL/50 mg of tissue) with a Potter-Elvehjem. Samples were agitated at 4°C for 2 hour, and then were centrifuged at 10,000xg for 30 min at 4°C. Resulting pellets were resuspended in 2 mL per initial 50 mg of tissue in 6M urea buffer, agitated for at least 18 hours at 4°C, and centrifuged at 10,000xg for 30 min at 4°C. The resulting supernatant was used for the measurement of LO activity. Supernatant (500 µL) was added to 35 µL of 0.05 M sodium borate buffer (pH 8.2). Additionally, the assay was analyzed in the presence of a 0.12 M β-aminopropionitrile (BAPN, a known LO inhibitor) in 0.05 M borate buffer. All samples were incubated in a 40°C water bath for 50 minutes, and then were added to a cuvette containing 2 mL buffer (9.7 mM lysine-HCl, 0.52 mM amplex red, 40 µg

horseradish peroxidase). Fluorescence was measured using the excitation and emission wavelengths of 563 and 587, respectively on a Varian Cary Eclipse fluorometer (Walnut Creek, Ca). Lysyl oxidase activity was calculated as a net change in fluorescence and compared to a hydrogen peroxide standard curve.

Real-time PCR Analysis

Real time RT-PCR was used to estimate the abundance of mRNA using acidic ribosomal protein (ARP) as a reference gene. The RNA was isolated and reverse transcribed with M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA (5 μ L) was added to a 20 μ L total reaction which included 10 μ L 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 μ M forward and reverse ARP primer or 0.625 μ M of forward and reverse target gene primers (Table 1). Target genes included LKR, SDH, AASS, CAT-1, CAT-2, ORC-1 and ORC-2. For LKR and SDH the primer set amplified nucleotides (nt) 212-423 and 1241-1450 of the full-length AASS gene, respectively. The AASS primer set amplified nt 944-1134, which spanned the potential linker region of LKR and SDH. The ORC-1 and ORC-2 primer set amplified nt 931-1035 and nt 656-800, respectively. The CAT-1 primer amplified nt 1072-1355 of the full CAT-1 gene, and CAT-2 primer set amplified nt 2436-2541. Real-time PCR was performed using a BioRad ICycler IQ Detection System. The procedure began with a "hot start" at 95°C for 5 minutes, followed by a cycle of a 95°C denaturing step for 30 seconds, an annealing step specific for each primer for 30 seconds, and a 72°C extension step for 30 seconds. This cycle was repeated a total for 40 times. A melt curve analysis was then performed to assess the quality of the amplification product.

Each sample was assayed in duplicate for both the target genes and ARP primers on a 96-well plate. A pooled sample was included in duplicate for both the target and housekeeping primer sets for each plate analyzed. Primer efficiencies were determined from the slope of the regression line of the log of the cDNA concentrations versus the Ct value by the equation $E=10^{(-1/\text{Slope})}$. Efficiency plates for the primer pairs were analyzed, and an acceptable efficiency for each was obtained. The efficiencies were used to calculate the relative mRNA abundance using the “efficiency corrected relative expression” equation (Pfaffl, 2001).

Protein Abundance

Western blotting was used to determine AASS abundance. SDS-PAGE was performed on a mini-gel using polyacrylamide gels (4% stacking and 12% resolving). Samples (20 μ l) containing 15 μ g of protein were loaded into each lane and electrophoresed for 2 h and 30 min at 100 volts. The primary antibody targets a 15 residue peptide fragment in the SDH-region of the mouse AASS protein raised in a rabbit (Kiess et al., 2008). The secondary antibody was goat anti-rabbit conjugated with horseradish peroxidase and was detected by incubation with Pierce SuperSignal® West Pico Chemiluminescent substrate (Pierce Biotechnology Inc, Rockford, IL) and exposure to film for 5 min. The band intensity was quantified using densitometry (FluoroChem 800, Alpha Innotech Corporation).

Statistical Analysis

Data were analyzed by ANOVA using the GLM of SAS (SAS Institute, Cary, NC). The main effect of tissue, replicate and tissue*replicate interactions was assessed. When differences

existed ($P < 0.05$), means were separated by the least significant differences procedure.

Correlations were assessed using Pearson's Correlation test.

RESULTS AND DISCUSSION

Lysine is frequently the first-limiting amino acid in typical swine rations. Therefore, a complete understanding of the fate of ingested lysine will help enable animal scientists to increase the efficiency of lysine use for protein synthesis. The tissue specific contribution of the different pathways of lysine degradation is not well discerned. Moreover, knowledge of the expression of genes involved in lysine transport, especially into mitochondria, is lacking. To enhance our knowledge of these aspects of lysine degradation, we examined indices of lysine catabolism in various pig tissues, as well as any correlations to known amino acid transporters.

Lysine α -ketoglutarate reductase activity was first described in rat liver (Higashino et al., 1965), and activity was thought to be predominately hepatic. Recently, LKR activity and lysine oxidation (LOX) have been detected in extra-hepatic tissues in chickens (Manangi et al., 2005) and swine (Pink et al., 2011), demonstrating that tissues other than liver contribute to whole-body lysine catabolism. However, a more detailed picture of the distribution of lysine catabolism is needed in production species, especially swine, to potentially improve production efficiency. In agreement with others (Hutzler and Dancis, 1975, Pink et al., 2011), LKR activity differed across tissues ($P < 0.0001$) and was highest in liver (384.5 nmol NADPH consumed/g liver*min) and whole intestines (Figure 1a). There was also an effect of replication ($P < 0.01$), which was most evident in the longissimus samples, where LKR activity was higher in replicate 2 compared to 1. There was no interaction between replication and tissue. The average LKR

activity across all tissues was 115.6 nmol of NADPH consumed/g tissue*min. Activity was detected in all tissues analyzed, including all intestinal preparations.

Initially, it was believed that the intestines did not contain the enzymes responsible for the degradation of lysine (Hutzler and Dancis, 1975, Chu and Hegsted, 1976, Wu, 1998). Previous findings indicated negligible and undetectable levels of LKR and SDH activities, respectively in enterocytes of piglets age 0 to 21 days (Chen et al., 2009). However, evidence of catabolism dominating first-pass intestinal utilization of dietary amino acids warranted further investigation (Stoll et al., 1998). In neonatal piglets, intestinal lysine oxidation accounted for one-third of whole body lysine while consuming a high protein diet (van Goudoever et al., 2000). In the present data, LKR activity was first detected in isolated enterocytes in replicate 1. Similar results have been shown where mitochondria isolated from intestinal epithelial cells exhibited LKR activity equal to 50% of that found in liver (Pink et al., 2011). In replicate 2, we were also interested in measuring indices of lysine catabolism in whole intestinal homogenate and intestines stripped of enterocytes. Interestingly, the average LKR activity of whole intestine, stripped intestine and isolated enterocytes was 445.5, 168, and 266 nmol of NADPH consumed/g tissue*min, respectively. Therefore, stripped intestine contributed 38%, and enterocytes 60% to whole intestinal homogenate LKR activity. Although the fate of the catabolized lysine in the intestine is unclear, Stoll and colleagues (1998) hypothesized that lysine is an important energy source for the small intestine.

The expression of LKR mRNA also differed across tissues ($P < 0.0001$); LKR mRNA abundance was highest in liver (Figure 1b). The abundance of LKR mRNA was also affected by replicate ($P < 0.05$) where replicate 1 had increased abundance compared to replicate 2; however, the pattern of distribution remained similar over the two time replicates and there was no

interaction between tissue and replication. Interestingly, LKR mRNA abundance was extremely low in intestinal preparations.

Saccharopine dehydrogenase activity also differed across tissues ($P < 0.0001$, Figure 2a), and exhibited no effects of replication or a replication by tissue interaction. Activity was highest in liver (378.2 nmol NADH produced/g tissue*min), and was detected in all tissues measured, with the exception of whole intestine and stripped intestine. Saccharopine dehydrogenase activity was detected in isolated enterocytes, measuring about 20% of the activity found in liver, differing from previous research which reported no SDH activity in mitochondria isolated from pig intestinal epithelial cells (Pink et al., 2011). Average SDH activity across tissues was 133.1 nmol NADH produced/g tissue*min. The expression of SDH mRNA differed across tissues ($P < 0.0001$), however it was highest in liver and kidney preparations (Figure 2b). Lysine degradation has also been detected in rodent (Noda and Ichiara, 1976, Maramatsu et al., 1983, Papes et al., 1999), swine (Pink et al., 2011) and avian (Manangi et al., 2005) kidney. The participation of the kidney in lysine degradation has been suggested to function in energy homeostasis, as LKR activity and LKR and SDH mRNA abundance are influenced by starvation in a similar manner as the hepatic enzymes (Papes et al., 1999). The authors also noted that the location of SDH activity in the kidney cortex may indicate that LKR and SDH play a role in the lysine reabsorption process.

Lysine α -ketoglutarate reductase and saccharopine dehydrogenase activities are thought to exist on a single bifunctional protein, α -amino adipate δ -semialdehyde synthase (AASS; Marcovitz et al., 1984). The expression of AASS mRNA differed across tissues ($P < 0.001$) and was highest in liver (Figure 2c). There was also an effect of replication on AASS mRNA abundance ($P < 0.05$), with replicate 2 having increased abundance compared to replicate 1.

Similar to LKR activity, AASS protein abundance differed across tissues ($P < 0.001$) and was highest in liver and whole intestine (Figure 2d). While associations between LKR activity and AASS protein exist regarding tissue distribution, LKR mRNA abundance, activity and AASS protein abundance are not well correlated in dietary studies. Mice consuming a high protein diet had increased LKR activity and AASS mRNA abundance, however there was no change in AASS protein abundance (Kiess et al., 2008). These data are consistent with post-translational modification, potentially via phosphorylation, as in plants (Karchi et al., 1995), as the primary means of regulating LKR activity.

In vitro lysine oxidation was measured as proxy for lysine transport into the mitochondrial matrix (Benevenga and Blemings, 2007). Oxidation differed across tissues ($P < 0.05$) and was numerically highest in liver and kidney tissues (Figure 3a). Average lysine oxidation across tissues was 9.23 nmol of CO_2 produced/ g tissue*min, representing only about 7.5% of LKR and SDH enzyme activities. Similar results have been found in poultry, where LKR and SDH activities were 18-31-times higher than the rate of lysine oxidation in turkey liver (Gatrell et al., 2011).

An alternative pathway for lysine degradation includes lysyl oxidase, a copper-dependent enzyme responsible for cross-linking in proteins such as collagen and elastin (Pinnell and Martin, 1968). It was originally thought that lysyl oxidase only acted on peptide-bound lysine; however, evidence shows that free lysine is also a substrate (Trackman and Kagan, 1979). The role of LO in lysine catabolism, in swine especially, has not been characterized. In the present study, LO activity differed across tissues ($P < 0.0001$) and was highest in striated muscle (longissimus, triceps and heart; Figure 3b). A similar result was detected in rats, where LO was highest in dense connective tissue, such as tendons and skin, and was also detected in aorta, kidney, lung

and liver (Rucker et al., 1996), however in this experiment, lysine was not used as the substrate. In the present experiment, the average LO activity across tissues was 0.61 nmol H₂O₂ produced/g tissue*min, representing only 0.5% of the activity of enzymes involved in the saccharopine-dependent pathway.

It is important to note that LKR and SDH activities were measured very near V_{max} conditions, while LO activity was measured at less than V_{max} conditions. Enzyme velocities at physiological concentrations of lysine (1 mM) were calculated using the average value of each enzyme activity, regardless of any effect of tissue or replication (Table 2). The average velocities for LKR were 35.1, 5.2, 28.9, 13.2 and 32.6 nmol/g*min for liver, heart, kidney, muscle and small intestine, respectively. The average velocities for LO were 0.02, 0.31, 0.05, 0.67 and 0.08 nmol/g*min for liver, heart, kidney, muscle and small intestine, respectively, indicating that even at physiological conditions, the saccharopine-dependent pathway is the predominant pathway of lysine degradation in pig tissues.

It is clear that on a per gram basis, lysine degradation occurs predominantly in the liver, intestine and kidney for the saccharopine-dependent pathway, and in striated muscle for lysyl oxidase. However, when the mass of each tissue is considered, total LKR activity in the muscle was almost 12-fold that in the liver. Also, whole tissue contributions to LO activity was much higher in the muscle, at ~8.4 mmol/min, compared to all other tissues (3-30 nmol/min). These data indicate that extra-hepatic tissues do play a key role in whole body lysine catabolism.

In rat liver (Blemings et al., 1994), and presumably in pig liver, LKR is located exclusively in the mitochondrial matrix. Therefore lysine needs to be transported through the inner mitochondrial membrane for its catabolism. It has been hypothesized that lysine transport into the matrix limits its oxidation (Blemings et al., 1998). Yet, there has been very little

research into the mechanism of lysine transport into the mitochondrion. There has been some speculation that the mechanism of transport for lysine is similar to that of the cationic amino acid ornithine (Hommes et al., 1982). There is also evidence that lysine and ornithine mutually inhibit each other's uptake into the mitochondrial matrix (Hommes et al., 1983). Two isoforms of ornithine transporters, ORC-1 and ORC-2 have been identified (Fiermonte et al., 2003). Both isoforms of the carrier are expressed in a range of human tissues, with ORC-1 being the predominant form (2003); however it is unclear if these carriers are associated with lysine transport. The mRNA expression of ORC-1 and 2 in various pig tissues was analyzed to obtain a greater understanding of their potential roles in lysine transport and catabolism. The ORC-1 ($P < 0.001$) and ORC-2 mRNA ($P < 0.001$) differed across tissues; ORC-1 mRNA was highest in liver, kidney and intestinal preparations (Figure 4a) and ORC-2 mRNA was highest in liver, followed by the whole intestines and stripped intestines (Figure 4b). The mRNA expression of the plasma membrane transporters CAT-1 and 2 were measured in various tissues. The CAT transport system is the primary system to concentrate the amino acids lysine, arginine and ornithine into cellular amino acid pools for use in metabolism (Broer, 2002). There are 3 main CAT isoforms, CAT-1, CAT-2 and CAT-3, however only CAT-1 and CAT-2 were analyzed in this experiment because we could not obtain acceptable amplification with any CAT-3 primer sets. The CAT-1 mRNA was uniformly expressed throughout all tissues ($P > 0.05$) and average CAT-1:ARP ratio was 2.79. CAT-2 expression differed across tissues ($P < 0.001$) and was expressed predominantly in the liver (Figure 4c). Similar distribution patterns have been reported in other mammals (Deves and Boyd, 1998) and poultry (Humphrey et al., 2004).

Correlation analysis was performed to assess the relationships among the indices of lysine catabolism measured (Appendix 1). Lysine α -ketoglutarate reductase activity was

positively correlated with SDH activity ($P < 0.0001$, $R = 0.58$), LKR mRNA ($P < 0.01$, $R = 0.35$), SDH mRNA ($P < 0.01$, $R = 0.33$), AASS mRNA abundance ($P < 0.01$, $R = 0.33$) and AASS protein ($P < 0.001$, $R = 0.41$). SDH activity was also positively correlated with LKR mRNA ($P < 0.01$, $R = 0.38$), SDH mRNA ($P < 0.01$, $R = 0.38$), AASS mRNA ($P = 0.10$, $R = 0.23$) and AASS protein ($P < 0.001$, $R = 0.47$). These data are in agreement with LKR and SDH activities existing on a single protein AASS (Dancis et al., 1979). LKR and SDH activities were also negatively correlated with lysyl oxidase ($P < 0.05$, $R = -0.26$) and ($P < 0.01$, $R = -0.38$), respectively. There was a positive correlation between CAT-2 mRNA abundance and LKR ($P < 0.001$, $R = 0.38$) and SDH activity ($P < 0.0001$, $R = 0.50$), indicating that the transport of lysine through the plasma membrane via CAT-2 may be an important step in lysine degradation. Interestingly, LKR activity was also positively correlated with ORC-1 mRNA abundance ($P < 0.05$, $R = 0.25$) and ORC-2 mRNA abundance ($P < 0.01$, $R = 0.32$) and SDH activity correlated with ORC-1 ($P < 0.01$, $R = 0.38$) and ORC-2 ($P < 0.01$, $R = 0.41$). The tissue distribution of ORC mRNA is correlated with the initial enzymes of the primary route of lysine degradation, suggesting that these transporters deliver lysine to its site of catabolism.

The present data have shown that extra-hepatic tissues play a role in lysine catabolism. Notably, muscle, kidney and intestinal lysine catabolism may be important contributors to whole-body lysine degradation via the saccharopine-dependent pathway. Since dietary modulation of enzyme activities involved in lysine catabolism occur in liver (Kiess et al., 2008), it would be beneficial to investigate the effect in other tissues that degrade lysine. Also, ORC transporters may play a role in the transport of lysine into the mitochondrial matrix for its catabolism. Further investigation is warranted, since it has been hypothesized that the rate of transport of lysine into the matrix is a limiting step in lysine catabolism.

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Table 1. Primer sequences for LKR, SDH, AASS, CAT, ORC and ARP mRNA¹.

Gene	Primer Sequence	Primer Efficiency
LKR	5 [′] - GTC AAA GCT GGT GGC ATT CT 3 [′] - TGC CAC TAC CCG TAC TCC TC	2.0
SDH	5 [′] -TGG CCG GCA CAG CTC CCA AT 3 [′] -GCA CGT TCC CTG CTC TCC CG	2.10
AASS	5 [′] -TGA GCG CTA CAC AAGTCG TT 3 [′] -TTG CCA CAA GTT TGT GTG GT	2.10
ORC-1	5 [′] -CGG GGA GAT CAA AAG ATG AA 3 [′] -TTT GAT ACA ATC CAC GGG GT	2.00
ORC-2	5 [′] -TGG AGC ACT CTG CTT CAA GA 3 [′] -AAT TCC AGC AAT TCC ACC AC	2.08
CAT-1	5 [′] -GCC ACG AAG GTG GGG AAG CC 3 [′] - ACG TGC TTG AAG GCG TCG GG	2.10
CAT-2	5 [′] -ATG TGC CGG GCA TGG TTG GG 3 [′] -AAC GGG TAG GAC CCC AGG CA	1.98
ARP	5 [′] -GCT AAG GTG CTC GGT TCT TC 3 [′] -GTG CGG ACC AAT GCT AGG	2.00

¹Abbreviations: lysine α -ketoglutarate reductase (LKR), saccharopine dehydrogenase (SDH), amino adipate δ -semialdehyde synthase (AASS), cationic amino acid transporter (CAT), ornithine carrier (ORC), acidic ribosomal protein (ARP)

Table 2. Whole Tissue Lysine α -Ketoglutarate Reductase and Lysyl Oxidase Activities.

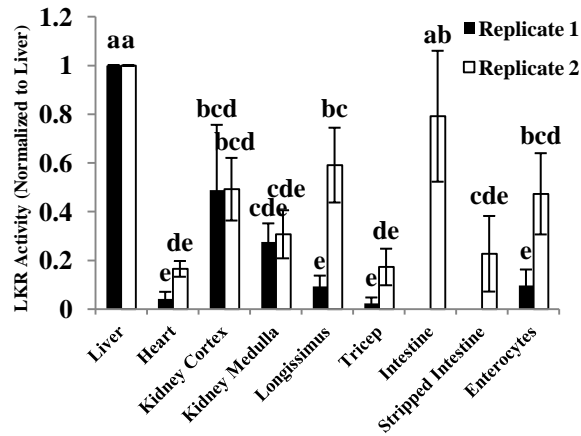
	Average Velocity ¹	grams of tissue ²	Total Tissue Activity
Liver			
LKR	35.1 (nmol/g*min)	375	13.16 mmol /min
LO	0.02 (nmol/g*min)	375	7.5 nmol /min
Heart			
LKR	5.2 (nmol/g*min)	75	0.39 mmol/min
LO	0.31 (nmol/g*min)	75	23.25 nmol/min
Kidney			
LKR	28.9 (nmol/g*min)	75	2.17 mmol/min
LO	0.05 (nmol/g*min)	75	3.75 nmol/min
Muscle			
LKR	13.2 (nmol/g*min)	12500	164.7 mmol/min
LO	0.67 (nmol/g*min)	12500	8.38 mmol/min
Small Intestine			
LKR	32.6 (nmol/g*min)	375	12.25 mmol/min
LO	0.08 (nmol/g*min)	375	30.0 nmol/min

¹LKR and LO activities were scaled to a lysine concentration of 1 mmol/L by the use of the Michaelis-Menten equation using 9.7 and 5.2 as the Km for LKR and LO, respectively.

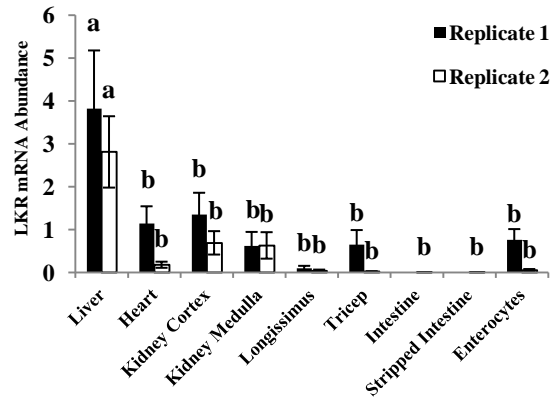
²Tissue weights (in grams) were based on estimates from the 25 kg pigs based on work performed by Anugwa et al., 1989.

Figure 1. LKR Activity and mRNA Abundance.

Lysine α -ketoglutarate reductase (LKR) activity and mRNA abundance in swine tissue homogenates. Enzyme activities and mRNA abundance measured in duplicate (n=10 for all tissues except whole and stripped intestine, n=5). Data are expressed as mean \pm SEM. LKR activity and mRNA abundance werenot determined (nd) in replicate 1 intestine or stripped intestine samples.



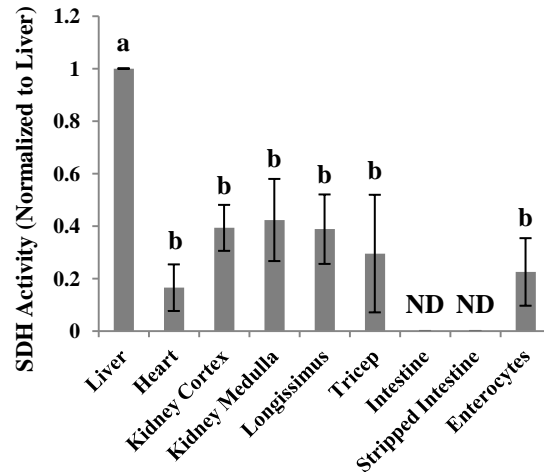
Panel A LKR activity in swine tissues. Data are expressed as a ratio to the liver sample. There was an effect of tissue ($P < 0.0001$) and replicate ($P < 0.05$). Differences in superscripts indicate a significant difference determined by the LSD test ($P < 0.05$).



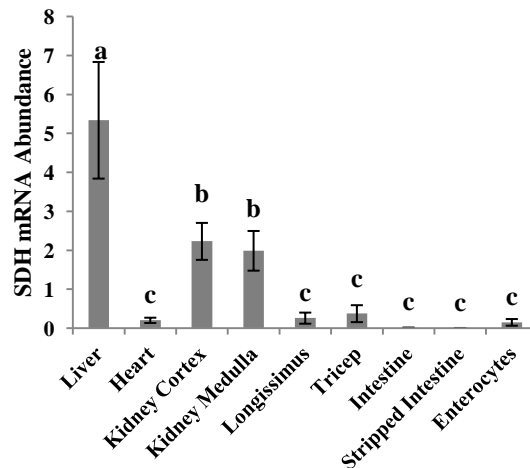
Panel B LKR mRNA abundance in swine tissues. LKR mRNA is expressed as a ratio to the housekeeping gene Acidic Ribosomal Protein (ARP). There was an effect of tissue ($P < 0.0001$) and an effect of replicate ($P < 0.05$). Differences in superscripts indicate a significant difference determined by the LSD test ($P < 0.05$).

Figure 2. SDH Activity and mRNA Abundance.

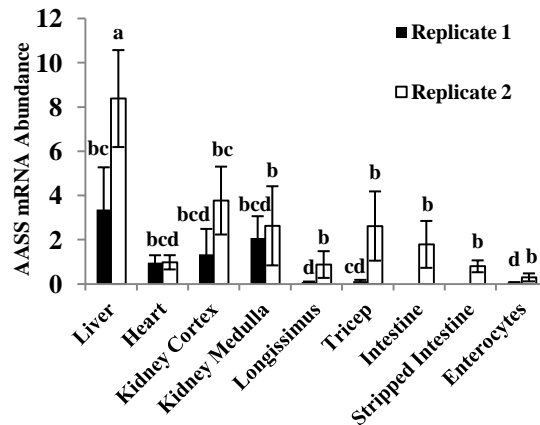
Saccharopine Dehydrogenase (SDH) activity and mRNA abundance in swine tissues. Enzyme activities and mRNA abundance measured in duplicate (n=10 for all tissues except whole and stripped intestine, n=5). Data are expressed as mean \pm SEM.



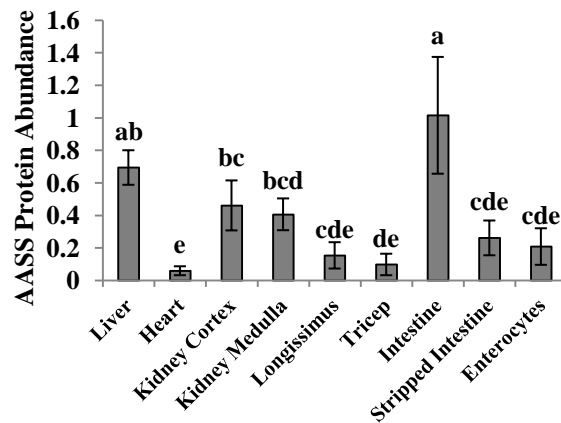
Panel A SDH Activity in swine tissues. Data are expressed as a ratio to the liver sample. No differences between replicate or tissue*replicate interactions were detected ($P>0.05$), therefore data were analyzed only for tissue effect ($P<0.0001$). Differences in superscripts indicates a significant difference determined by the LSD test ($P<0.05$). SDH activity was not determined in replicate 1, and not detected in replicate 2 for the intestine and stripped intestine samples.



Panel B SDH mRNA abundance in swine tissues. SDH mRNA is expressed as a ratio to the housekeeping gene acidic ribosomal protein (ARP). No differences between replicate or tissue*replicate interactions were detected ($P>0.05$), therefore data were analyzed only for tissue effect ($P<0.0001$). Differences in superscripts indicates a significant difference determined by the LSD test ($P<0.05$).

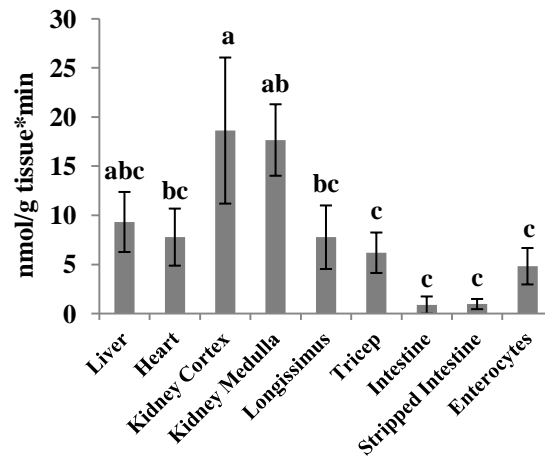


Panel C AASS mRNA abundance in swine tissues. AASS mRNA is expressed as a ratio to the housekeeping gene Acidic Ribosomal Protein (ARP). There was an effect of tissue ($P < 0.001$) and an effect of replicate ($P < 0.05$). Differences in superscripts indicate a significant difference determined by the LSD test ($P < 0.05$).

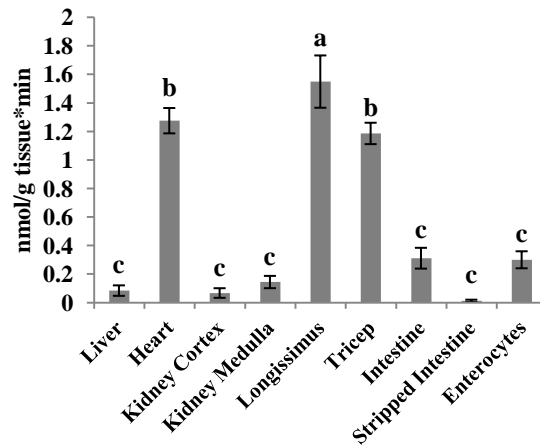


Panel D α -Amino adipate Semialdehyde Synthase (AASS) protein abundance in swine tissue. The abundance of AASS protein was analyzed via western blot with a probe targeting the SDH region of the protein. Data are expressed as a ratio to a pooled sample. A tissue, but not replicate or tissue*replicate effect was detected, so data were pooled across tissue ($P < 0.001$). Differences in superscripts indicate a significant difference determined by the LSD test ($P < 0.05$).

Figure 3. Lysine Oxidation and Lysyl Oxidase Activity.



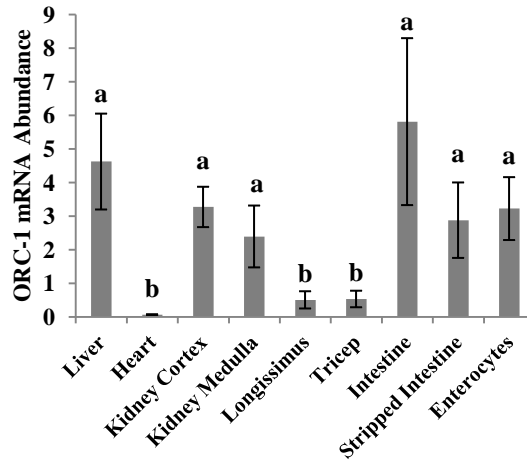
Panel A Lysine oxidation. n=10 for all tissues except whole and stripped intestine, n=5. No effect of replicate or tissue*replicate interactions were detected ($P>0.05$), therefore data were analyzed only for tissue effect ($P<0.05$). Differences in superscripts indicate a significant difference determined by the LSD test ($P<0.05$).



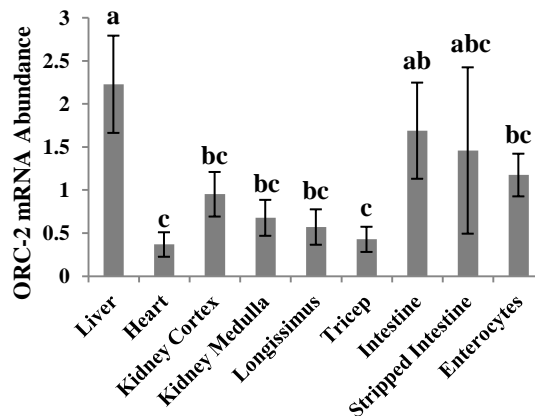
Panel B Lysyl oxidase activity. Data are expressed as nmol H_2O_2 produced/g liver*min. No effect of replicate or tissue*replicate interactions were detected ($P>0.05$), therefore data were analyzed only for tissue effect ($P<0.0001$). Differences in superscripts indicate a significant difference determined by the LSD test ($P<0.05$).

Figure 4. Abundance of Ornithine Carrier (ORC) mRNA and Cationic Amino Acid Transporter (CAT) mRNA.

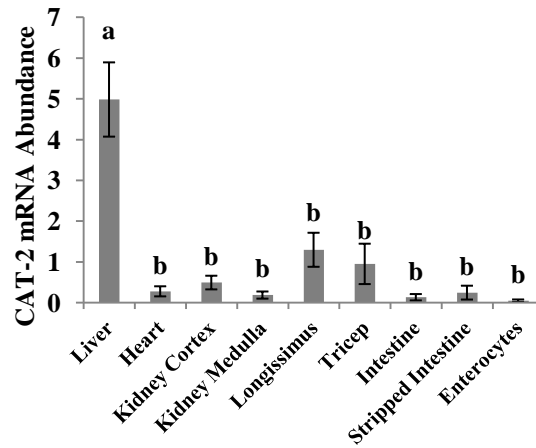
ORC and CAT mRNA is expressed as a ratio to the housekeeping gene Acidic Ribosomal Protein (ARP). Data are expressed as mean \pm SEM (n=10 for all tissues except whole and stripped intestine, n=5).



Panel A ORC-1 mRNA abundance. No effect of replicate or tissue*replicate interactions were detected ($P>0.05$), therefore data were analyzed only for tissue effect ($P<0.001$). Differences in superscripts indicate a significant difference determined by the LSD test ($P<0.05$).



Panel B ORC-2 mRNA abundance. No effect of replicate or tissue*replicate interactions were detected ($P>0.05$), therefore data were analyzed only for tissue effect ($P<0.001$). Differences in superscripts indicates a significant difference determined by the LSD test ($P<0.05$).



Panel C CAT-2 mRNA abundance. No effect of replicate or tissue*replicate interactions were detected ($P > 0.05$), therefore data were analyzed only for tissue effect ($P < 0.01$). Differences in superscripts indicate a significant difference determined by the LSD test ($P < 0.05$).

Appendix 1. Correlation Analysis.

	SDH Act	LOX	Lysyl Ox	LKR mRNA	SDH mRNA	AASS mRNA	AASS Protein	CAT-1 mRNA	CAT-2 mRNA	ORC-1 mRNA	ORC-2 mRNA
LKR Act	R= 0.58 P<0.0001	R=0.08 P>0.05	R=-0.26 P<0.05	R=0.35 P<0.01	R=0.33 P<0.01	R=0.33 P<0.01	R=0.41 P<0.001	R=0.09 P>0.05	R=0.38 P<0.001	R=0.25 P<0.05	R=0.32 P<0.01
SDH Act		R=-0.05 P>0.05	R=-0.38 P< 0.01	R=0.38 P<0.01	R=0.38 P<0.01	R=0.23 P<0.1	R=0.47 P<0.001	R=-0.06 P>0.05	R=0.50 P<0.0001	R=0.38 P<0.01	R=0.41 P<0.01
LOX			R=-0.17 P>0.05	R=16 P>0.05	R=0.16 P>0.05	R=0.19 P>0.05	R=-0.09 P>0.05	R=-0.11 P>0.05	R=0.04 P>0.05	R=0.08 P>0.05	R=-0.1 P>0.05
Lysyl Ox				R=-0.28 P<0.05	R=-0.33 P<0.01	R=-0.29 P<0.05	R=-0.39 P<0.001	R=-0.31 P<0.05	R=-0.10 P>0.05	R=-0.49 P<0.0001	R=-0.38 P<0.001
LKR mRNA					R=0.58 P<0.001	R=0.37 P<0.01	R=0.17 P>0.05	R=-0.08 P>0.05	R=0.33 P<0.01	R=0.21 P<0.10	R=0.12 P>0.05
SDH mRNA						R=0.71 P<0.0001	R=0.18 P>0.05	R=0.05 P>0.05	R=0.34 P<0.01	R=0.36 P<0.01	R=0.05 P>0.05
AASS mRNA							R=0.24 P<0.05	R=-0.13 P>0.05	R=0.41 P<0.001	R=0.23 P>0.10	R=-0.04 P>0.05
AASS Protein								R=0.02 P>0.05	R=0.21 P<0.10	R=0.09 P>0.05	R=0.29 P<0.01
CAT-1 mRNA									R=-0.09 P>0.05	R=0.05 P>0.05	R=-0.001 P>0.05
CAT-2 mRNA										R=0.05 P>0.05	R=0.46 P<0.0001
ORC-1 mRNA											R=0.38 P<0.001

Correlation analysis performed via Pearson's correlation analysis across all tissues

CHAPTER TWO

Diet-induced modifications of lysine catabolism occur in pig liver, but not kidney or heart

ABSTRACT

The molecular mechanisms responsible for alterations in lysine degradation via the saccharopine-dependent pathway, the primary pathway of lysine catabolism in mammalian species, are unknown. This pathway presumably depends on the bifunctional protein α -amino adipate δ -semialdehyde synthase (AASS), which contains both lysine α -ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH) activities. In the liver, LKR and SDH are restricted to the mitochondrial matrix and lysine is presumably transported through the plasma membrane via a cationic amino acid transporter (CAT-1/2) and through the inner mitochondrial membrane potentially by one or both isoforms of mitochondrial ornithine transporters (ORC-1/2). The aim of this experiment was to discern the mechanisms responsible for alterations of lysine catabolism in pigs fed diets either in excess or deficient in either protein or lysine. Weanling pigs (n=35) were fed either a control (C, 18% CP, 0.95% lysine), high protein (HP, 23% CP, 0.95% lysine), low protein (LP, 13% CP, 0.95% lysine), high lysine (HL, 18% CP, 1.25% lysine) or low lysine (LL, 18% CP, 0.65% lysine) diet for 10 days. No differences in weight gain or feed intake were detected. Liver LKR activity ($P < 0.05$) and AASS protein expression ($P < 0.01$) were reduced in pigs consuming the LL diet compared to C. Liver SDH mRNA expression was reduced ($P < 0.08$) with the consumption of the LL diet compared to C, and AASS mRNA was reduced ($P < 0.05$) with the consumption of the LL diet compared to the HP and HL diets. No significant dietary alterations in lysine catabolism were detected in heart or kidney. There was a significant increase in liver ORC-1 mRNA expression with the consumption of the HL diet ($P < 0.05$); ORC-2 mRNA expression was not affected by any dietary treatments. In conclusion, these data indicate that diet induced modifications occur in pig liver, but not heart or kidney (Support; HATCH WVA 470).

INTRODUCTION

Lysine is an essential and limiting amino acid in most cereal-based human diets (Bobwell et al., 1981) and frequently the first limiting amino acid in typical corn/soybean meal-based swine diets. Increasing the efficiency of use of lysine for protein synthesis may improve human health and potentially lower the dietary requirement, thus decreasing the need for lysine supplementation and thereby decreasing production costs. Interestingly, animals perform better when consuming a diet devoid of lysine compared to diets devoid of any other essential amino acid (Said and Hegsted, 1970). The relatively high efficiency of lysine use in animals fed diets devoid of lysine led to the concept that lysine is conserved, allowing adaptation to lysine-deficient diets. Lysine conservation has been observed in rodents (Said and Hegsted, 1970, Gahl et al. 1991), poultry (Ousterhout, 1960) and suggested in swine (Mnilk et al. 1996). Lysine conservation could be explained by drawing on body stores of peptides and protein containing lysine (Ousterhout, 1960) and/or by decreasing lysine catabolism. The potential benefits of increasing the efficiency of lysine use for protein synthesis in production species offers incentives to understanding the regulation of lysine catabolism.

Lysine catabolism via the saccharopine-dependent pathway is the predominant route of lysine degradation in most production species (Broquist 1991). Lysine α -ketoglutarate reductase (LKR, E.C. 1.5.1.8) condenses lysine with α -ketoglutarate to form saccharopine in a NADPH-dependent reaction. Next, saccharopine dehydrogenase (SDH, E.C. 1.5.1.9) catalyzes the conversion of saccharopine to α -aminoadipate- γ -semialdehyde and glutamate in a NAD⁺-dependent reaction. The enzymes LKR and SDH are part of the bifunctional protein α -aminoadipate δ -semialdehyde synthase (AASS, Markovitz et al., 1984).

Initially, it was believed that lysine was oxidized almost exclusively in the liver (Miller, 1962). However, recent evidence suggests that extra-hepatic tissues also play a role in lysine catabolism in humans (Hutzler and Dancis, 1975), poultry (Manangi et al. 2005) and swine (Pink et al. 2011). Several reports have also suggested that, at least in rodents, lysine catabolism via the saccharopine-dependent pathway is under dietary control. Consuming diets high in protein or lysine increases LKR activity and lysine oxidation in rodent liver (Chu and Hegsted 1976, Muramatsu et al. 1984, Blemings et al. 1998, Kiess et al. 2008) and kidney, however to a lesser extent (Muramatsu et al. 1984). While several reports have investigated diet-induced alterations on whole body lysine oxidation in swine, studies on diet-induced alterations on LKR activity are lacking.

With respect to cellular localization, lysine is transported through the plasma membrane of the cell by an isoform of cationic amino acid transporters (CAT-1, 2 or 3). Next, specifically in rat liver, LKR and SDH activities are located exclusively within the mitochondrial matrix (Blemings et al. 1994). The location of the initial enzymes of lysine degradation via the saccharopine-dependent pathway indicates that lysine must be transported into the matrix for its catabolism. It has been suggested that mitochondrial lysine uptake is a limiting step in lysine oxidation (Blemings et al. 1998); therefore, the protein(s) responsible for mitochondrial lysine uptake may be a potential target for regulating lysine catabolism. Although specific proteins responsible for lysine uptake in pig liver mitochondria are unknown, we speculate that they are similar to proteins responsible for ornithine uptake. Two isoforms of ornithine transporters, ORC-1 and ORC-2 have been identified in human liver mitochondria (Fiermonte et al. 2003), however; a link between ORCs and lysine degradation has not been established. Therefore, the objectives of this study were to 1) determine the effect of high and low protein and high and low

lysine diets on indices of lysine degradation in liver, kidney and heart 2) investigate molecular mechanisms responsible for potential lysine conservation and 3) correlate mRNA expression of known ORC transporters to indices of lysine catabolism.

MATERIALS AND METHODS

Animals and diets. Litters of piglets from PIC lineage were weaned at 28 days of age. Five pigs from each litter were separated into individual pens and fed a standard weanling diet for 7 days. In total, 32 of the 35 animals were barrows, the other 3 were gilts. Animals were exposed to a 12-h light:dark period and had constant access to food and water. After the 7d acclimation period, body weights were taken and animals were randomly allotted to receive one of five diets: control (C), low protein (LP), high protein (HP), low lysine (LL) or high lysine (HL, Table 1). Feed intake was recorded daily and body weights were taken after 10 days of dietary treatment. Animals were then euthanized in a humane manner via captive bolt. Immediately following euthanasia, samples of liver, kidney and heart were taken, weighed and one portion was stored in ice cold homogenization buffer (H buffer; 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM 2-mercaptoethanol, 1 mM EGTA, 0.05% (w/v) bovine serum albumin, pH 7.4). The samples were transferred to the laboratory, on ice, for further processing. The other portion of all tissues was flash frozen in liquid nitrogen for mRNA analysis. This experimental procedure was replicated 7 times (n=7 pigs/treatment). Animal care and conduct of experiments were approved by the West Virginia University Animal Care and Use Committee (ACUC#-09-0606).

LKR (EC 1.5.1.8) and SDH (EC 1.5.1.9) activities. Tissue LKR and SDH activities were determined on fresh tissue samples as previously described (Blemings et al. 1994). Each sample was analyzed in duplicate using a 25% (w/v) homogenate.

Lysine oxidation in tissue homogenates. Lysine oxidation (LOX) was measured by determining the recovery of $^{14}\text{CO}_2$ from [1- ^{14}C] L-lysine on fresh tissue homogenates, as previously described in rats (Blemings et al. 1998). A total of 0.5 mL of a base trap (1 part ethanolamine to 2 parts methyl cellosolve) was added to an Eppendorf tube, which was suspended in a 5-mL glass vial. A 25% (w/v) tissue homogenate was made with fresh tissue and H buffer. Homogenate (250 μL) was added to the glass vial, containing 250 μL of buffer at 41°C (20 mM Hepes, 6 mM MgCl_2 , 0.4 mM EDTA, 364 mM mannitol, 122 mM sucrose and 10 mM L-lysine (final concentration), pH 7), for each sample. Additional sets of each sample were placed in boiling water for 10 minutes to obtain a heat-killed sample. All solutions were then incubated in the 41°C water bath for 30 minutes, while oscillating at 100 osc/min. To terminate the reactions, 100 μL of 1M potassium phosphate buffer (pH 5.0) was injected via an 18 gauge needle through the serum cap covering the vial. The vial then remained in the water bath for at least 180 additional minutes while oscillating. The Eppendorf tube was placed in a plastic scintillation vial along with 17 mL of Bio-Safe II liquid scintillation fluid. The radioactivity was measured using a Beckman LS 6500 liquid scintillation counter (Beckman Coulter Inc, Somerset, NJ). Each tissue was measured in triplicate and corrected for the heat-killed sample.

Real-time PCR analysis. Real time RT-PCR was used to estimate the abundance of mRNA of various target genes using acidic ribosomal protein (ARP) as a reference gene. The RNA was isolated and reverse transcribed using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The resulting cDNA (2 μL) was added to a 20 μL total

reaction which included 10 μ L 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 μ M forward and reverse ARP primer or 0.625 μ M of forward and reverse target gene primers. Target genes included LKR, SDH, AASS, CAT-1, CAT-2, ORC-1 and ORC-2 (Table 2). For LKR, the primer set was designed to amplify nucleotides (nt) 212-423 of the full-length AASS gene. The SDH primer set amplified nt 1241-1450 of the full-length gene, and AASS amplified nt 944-1134, which spanned the potential linker region of LKR and SDH. The ORC-1 primer set amplified nt 931-1035, and ORC-2 was designed for nt 656-800. The CAT-1 primer set was designed to amplify nt 1072-1355 of the full CAT-1 gene, and CAT-2 primer set amplified nt 2436-2541. Real time PCR was performed using a BioRad ICycler IQ Detection System. The procedure began with a “hot start” at 95°C for 5 minutes, followed by a 95°C denaturing step for 30 seconds, an annealing step specific for each primer for 30 seconds, and a 72°C extension step for 30 seconds. This cycle was repeated a total for 40 times. A melt curve analysis was then performed to assess the quality of the amplification product.

Each sample was analyzed in duplicate for both the target genes and ARP primers on a 96-well plate. A pooled sample was included in duplicate for both the gene of interest and the housekeeping gene on each plate analyzed. Primer efficiencies (Table 2) were determined from the slope of the regression line of the log of the cDNA concentrations versus the Ct value by the equation $E=10^{(-1/Slope)}$. Efficiency plates for the primer pairs were analyzed, and an acceptable efficiency for each was obtained. The efficiencies were used to calculate the relative mRNA abundance using the “efficiency corrected relative expression” equation (Pfaffl, 2001).

AASS protein abundance. Western blotting was used to determine AASS protein abundance. SDS-PAGE was performed on a mini-gel using polyacrylamide gels (4% stacking and 12% resolving). Samples (20 μ l) containing 20 μ g of protein were loaded into each lane and

electrophoresed for 2 h and 30 min at 100 volts. The primary antibody targets a 15 residue peptide fragment in the SDH-region of the mouse AASS protein raised in a rabbit (Kiess et al. 2008). The secondary antibody was goat anti-rabbit conjugated with horseradish peroxidase and was detected by incubation with Pierce SuperSignal® West Pico Chemiluminescent substrate (Pierce Biotechnology Inc, Rockford, IL) and exposure to film for 5 min. The band intensity was quantified using densitometry (FluoroChem 800, Alpha Innotech Corporation).

Statistical analysis. Data were analyzed by ANOVA using the GLM of SAS (SAS Institute, Cary, NC). The main effect of diet and replication were assessed. When differences existed ($P < 0.05$) means were separated by the least significant differences procedure. Also, the effect of tissue was analyzed and when differences existed, separated by the least squares means procedure. The effect of sex was also assessed by comparing the means for each variable with and without the inclusion of the females. Means for all data were not affected by the females, so data were pooled across sex. Correlation analysis was also assessed using Pearson's Correlation test.

RESULTS

Food intake, average daily gain and organ weights. Average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency were not affected by dietary treatment (**Table 3**). However, there was a numerical reduction in feed efficiency in the pigs consuming the LP and LL diet compared to the control. There was, also an effect of replication on ADG ($P < 0.05$), ADFI ($P < 0.001$) and feed efficiency ($P < 0.01$), potentially due to the differences in starting weights between replications due to differences in litter sizes. Relative liver weight was highest

in the pigs consuming the HP diet ($P < 0.05$) compared to all other dietary treatment except LL (**Table 3**). Relative kidney weight was also highest in the pigs consuming the HP ($P < 0.05$) diet compared to those consuming the LP or LL diets (**Table 3**). There was also a replication effect on both relative liver ($P < 0.01$) and kidney weight ($P < 0.001$). There was no dietary effects on relative heart weights ($P > 0.05$).

Diet-induced modifications on indices of lysine catabolism. The effect of diet on liver enzyme activities and protein expression are shown in **Figure 1**. There was an effect of replicate on the absolute LKR activity ($P < 0.05$), but no replication effect was detected when the activity was scaled to the control. Liver LKR activity, expressed as a ratio to C, was significantly affected by dietary treatment ($P < 0.05$); activity was reduced in pigs consuming the LP (25%) and the LL diet (30%) compared to those consuming the C diet (**Figure 1a**). The control liver LKR activity averaged 629.0 ± 96 nmol NADPH/g liver*min over all seven replicates. Liver SDH activity, expressed as a ratio to the C, was reduced ($P < 0.05$) in pigs consuming the LL diet compared to those consuming the C or HP diet (**Figure 1b**). Liver SDH activity was also reduced in the pigs consuming the LP diet compared to the HP diet. The control liver SDH activity averaged 715.7 ± 130 nmol NADH/g liver*min over all seven replicates. Liver AASS protein abundance, expressed as a ratio to a pooled sample, was reduced in pigs consuming the LL diet compared to pigs consuming all other dietary treatments (**Figure 1c**, $P < 0.01$).

The dietary modifications on liver LOX and tissue mRNA expression are shown in **Table 4**. Liver LOX, expressed as nmol CO₂ produced/g tissue*min, was not affected by dietary treatment or replication ($P > 0.05$). All mRNA expression is expressed as a ratio to the housekeeping gene ARP. There was no dietary or replication effects ($P > 0.05$) on liver LKR mRNA expression (**Table 4**). There was a trend ($P < 0.08$) for a reduction in SDH mRNA

expression in the livers of pigs consuming the LL diet compared to those consuming the C, HP or HL diets (**Table 4**). There was a 76% reduction in SDH mRNA expression in the LL diet compared to the C. Liver AASS mRNA abundance was reduced 60 and 68% ($P<0.05$) in pigs consuming the LL diet compared to the HP and HL diets, respectively (**Table 4**). There was no effect of replication on SDH or AASS mRNA expression ($P>0.05$).

ORC mRNA expression was analyzed as we hypothesize that it is responsible for transport of lysine across the inner mitochondrial membrane. There was an increase in the expression of liver ORC-1 mRNA ($P<0.05$) with the consumption of the HL diet compared to all diets, with a ~170% increase compared to the C diet (**Figure 1d**). There was also a ~50% numerical decrease in ORC-1 mRNA expression in the livers of pigs consuming the LL compared to C diets. The CAT mRNA expression was also analyzed to determine any dietary modifications of the expression of known transporters of lysine across the plasma membrane. The CAT-1 mRNA expression was increased ($P<0.01$) in the livers of pigs consuming the HL compared to all other diets, with a ~330% increase compared to the C diet (**Table 4**). The CAT-2 expression was not affected by dietary treatment (**Table 4**). There was no effect of replication on ORC or CAT mRNA expression.

There was no effect of replication or dietary treatment on LKR and SDH activity or AASS protein abundance in the kidney (data not shown). There was, however, a trend for a dietary effect on kidney SDH expression ($P<0.10$). There was an increase in SDH mRNA expression in the kidneys of pigs consuming the HP diet compared to those consuming the C, HL or LL diets (**Table 4**), with a 100% increase compared to the C diet. There was a trend for diet effect on kidney ORC-1 mRNA expression ($P<0.10$). The ORC-1 mRNA was increased with the consumption of the HP diet compared to the C and LP diets, with a ~270% increase

compared to the C diet (**Table 4**). There was also a replication effect on kidney SDH and ORC-1 mRNA expression ($P < 0.05$). There were no dietary or replication effects on any indices of lysine catabolism in the heart, including enzyme activity, protein abundance or mRNA expression for any gene studied ($P > 0.05$).

Tissue distribution of indices of lysine catabolism. To analyze the effect of tissue on indices of lysine catabolism, the data from the pigs consuming the C diet were analyzed, and the results are shown in **Table 5**. Liver LKR activity was 300 and 140% greater ($P < 0.0001$) than heart and kidney, respectively. SDH activity was higher in the liver than kidney and was not detected in the heart ($P < 0.0001$). The expression of AASS protein was highest in the liver ($P < 0.0001$) compared to heart or kidney; however there was no effect of tissue on LOX. The expression of LKR and AASS mRNA was not different across tissues. The expression of SDH ($P < 0.10$), ORC-1 ($P < 0.05$), ORC-2 ($P < 0.10$) and CAT-2 ($P < 0.10$) mRNA was highest in the liver, followed by kidney and then heart. No tissue differences in CAT-1 were detected.

Correlation analysis. Correlation analysis was performed to examine the correlation of indices of lysine catabolism to potential lysine transporters. ORC-1 mRNA abundance was positively correlated with AASS ($R = 0.59$, $P < 0.001$), ORC-2 ($R = 0.62$, $P < 0.001$), CAT-1 ($R = 0.51$, $P < 0.01$) and CAT-2 mRNA abundance ($R = 0.36$, $P < 0.08$) and AASS Protein Abundance ($R = 0.36$, $P < 0.05$). ORC-2 mRNA abundance was positively correlated with LKR ($R = 0.33$, $P < 0.09$), SDH ($R = 0.39$, $P < 0.05$), AASS ($R = 0.50$, $P < 0.01$) and CAT-2 mRNA abundance ($R = 0.35$, $P < 0.08$).

DISCUSSION

This is the first study, to our knowledge, examining the diet-induced modifications in indices of lysine catabolism in swine. The present results clearly demonstrated that LKR activity in pig liver is decreased with the consumption of a LL and LP diet compared to the C. Similar findings in rodent liver have been reported. Chu and Hegsted (1976) showed that hepatic LKR activity decreased 70% when adult rats consumed a lysine-free diet compared to those consuming a complete amino acid diet. The increased efficiency of lysine use when lysine is limiting indicate that pigs have adaptive mechanisms which conserve lysine when it is in short supply. However, the consumption of the LL diet did not result in a decrease in LOX. Moehn and others (2004) demonstrated that at moderate levels of lysine intake restrictions (10-30% below the requirement for maximum protein deposition), lysine oxidation in growing pigs appeared to be independent of lysine intake as a result of inevitable lysine catabolism.

Present findings demonstrated no increase in LOX, LKR or SDH activities with the consumption of the HL or HP diets, compared to the control. These data are in disagreement with previous reports which showed an increase in LKR activity and LOX with excess protein and lysine in rodent liver (Chu and Hegsted, 1976, Muramatsu et al. 1984, Blemings et al. 1998, Kiess et al. 2008). The reason for the discrepancy between this experiment and the latter may be due to the degree of excess of both lysine and protein. In the present experiment, there is a ~30% increase in protein and lysine in the HP and HL diets with respect to the dietary requirement. The previous experiments use a 100-200% increase of protein and 100% increase in lysine above the requirement. Perhaps the HP and HL diets used in the current experiment

were not enough to induce these changes. Also, the differences between the responses of rodent and swine to lysine deficiency indicate that responses to dietary amino acid deficiencies may potentially be species specific.

The appreciation of the contribution of liver to lysine catabolism has been noted for decades (Higashino et al. 1965). However, recent evidence suggests that extra-hepatic tissues also play a role in lysine catabolism. Various measures of lysine degradation have been detected in rodent (Noda and Ichiara, 1976, Maramatsu et al. 1983, Papes et al. 1999), swine (Pink et al. 2011) and avian (Manangi et al. 2005) kidney. The participation of the kidney in lysine degradation has been suggested to function in energy homeostasis, as LKR activity and LKR and SDH mRNA abundance are influenced by starvation in a similar manner as the hepatic enzymes (Papes et al. 1999). Indices of lysine catabolism in pig kidney were detected in the present study, with LKR activity representing ~50% of that found in the liver. Our present data also indicate that indices of lysine degradation in the saccharopine-dependent pathway do not respond to changes in dietary lysine and protein in the kidney. In contrast, Muramatsu and others (1984) stated that the kidney responds to increasing levels of lysine and protein, though the response is lower than that of the liver. The discrepancy between these data may also be due to the level of excess of the dietary protein and lysine, or it may be a species-specific effect.

To understand these diet-induced alterations in lysine catabolism mechanistically, the abundance of AASS protein and mRNA abundance of LKR, SDH and AASS were measured. AASS protein abundance was significantly decreased with the consumption of the LL diet. This is the first report of changes in AASS protein expression due to diet-induced alterations in mammals. A significant increase in AASS mRNA expression with the consumption of the HL diet, with no resulting increase in AASS protein abundance was also detected. These data are

consistent with posttranslational modifications, potentially via phosphorylation, as a means of regulating LKR activity, at least in situations of excess lysine. Similar conclusions for post-translational modifications have been observed in rodents (Kiess et al. 2008), fish (Higgins et al. 2006), chickens (Kiess et al. 2006) and plants (Miron et al. 1997, Stepansky et al. 2006). In the developing tobacco seed, lysine regulates its own concentration by stimulating LKR activity. This stimulation occurs by a specific signaling cascade mediated by calcium and protein phosphorylation (Karchi et al. 1995).

Diet induced alterations of mRNA expression of CAT isoforms 1 and 2 were measured to try and understand how cellular uptake could influence lysine metabolism. The CAT transport system is the primary system to concentrate cationic amino acids into cellular amino acid pools for use in metabolism (Broer, 2002). In liver, CAT-1 mRNA expression was increased with the consumption of the HL diet. The expression of CAT-1 is subject to amino-acid sensitive regulation at a number of posttranslational steps (reviewed by Hyde et al. 2003). Such adaptive responses may be to prevent excessive amounts of amino acids in the body, and presumably to increase the likelihood of lysine catabolism via LKR/SDH.

Liver LKR and SDH are housed exclusively within the mitochondrial matrix (Blemings et al. 1994), therefore lysine must be transported into mitochondria for LKR-dependent catabolism. It has been hypothesized that the uptake of lysine into the mitochondrion limits the rate at which it is oxidized in rat liver (Blemings et al. 1998). Exactly how lysine is transported into mitochondria is unknown, although we speculate that its transport is similar to that of the cationic amino acid ornithine. Similar to lysine, the degrading enzymes involved in ornithine catabolism are restricted to the mitochondrial matrix (Ip et al. 1974) and transport seems to limit ornithine degradation (Cohen et al. 1987, Hommes et al. 1982). When lysine and ornithine

mitochondrial uptake activities were measured, they mutually inhibited each other, leading to the assumption that transporters for lysine uptake are the same as ornithine in rat liver mitochondria (Hommes et al. 1982). Two isoforms of ornithine transporters, ORC-1 and ORC-2 have been identified in humans (Fiermonte et al. 2003). Both isoforms are able to transport L-isomers of ornithine, lysine, arginine and citrulline by unidirectional mechanisms. The authors also determined that both isoforms of the carriers are expressed in a range of human tissues, with ORC-1 being the form predominantly expressed.

Connections between ORC mRNA expression and lysine degradation have not been previously assessed. In the present experiment, consuming a HL diet led to a ~170% increase in liver ORC-1 mRNA abundance, while consuming a LL diet decreased liver ORC-1 mRNA abundance 50% compared to control diets. There was, however, no effect of high or low protein feeding on ORC-1 mRNA expression. Also, there was a 76% numerical decrease in ORC-2 mRNA abundance with the consumption of the LL diet compared to the C. The reduction of both LKR activity and ORC-1 and ORC-2 mRNA abundance as a result of the consumption of the LL diet may suggest that this transporter delivers lysine to its site of catabolism. Moreover, the response of the ORC mRNA to low lysine diets implicates these transporters as the molecular basis of lysine conservation.

From these data it can be concluded that diet-induced alterations in lysine catabolism do occur in pig liver. Also, ORC transporters may play a role in the transport of lysine into the mitochondrial matrix for its catabolism. Present data also support the model of post-translational modifications suggested by others (Karchi et al. 1995, Cleveland et al. 2008, Kiess et al. 2008), especially in situations with excess lysine. Future studies aimed at understanding the role of the ORC-1 transporter and LKR phosphorylation in regulating lysine oxidation seem likely to be

fruitful in terms of decreasing lysine degradation and ultimately reducing lysine requirements. If these modifications can be determined in mammalian species, it may be possible to manipulate lysine catabolism to decrease its degradation, ultimately reducing the dietary lysine requirement. Previous work from our laboratory has already shown that decreasing lysine catabolism decreases the lysine requirement (Cleveland et al. 2008).

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Table 1. Diet formulations.

Ingredient	Control	High Protein	Low Protein	High Lysine	Low Lysine
Corn	73.07	60.74	83.65	73.61	72.15
Soybean Meal (48%)	12.19	23.73	7.67	10.79	12.23
Corn gluten meal (60%)	7.81	9.84	1.58	8.34	8.24
Corn DDGS	2.41	0.58	2.29	2.28	2.29
Wheat middlings	0.50	0.50	0.50	0.50	1.48
Limestone	1.29	1.26	1.30	1.30	1.30
Soybean oil	1.00	2.06	1.00	1.00	1.00
Tricalcium Phosphate	1.00	0.94	1.04	1.01	0.99
NB3000 (vit/min premix)	0.25	0.25	0.25	0.25	0.25
Salt	0.05	0.08	0.05	0.05	0.05
BMD	0.03	.03	0.03	0.03	0.03
DL - Methionine	--	--	0.03	--	--
HCl Lysine	0.40	--	0.62	0.84	--
Calculated nutrients					
ME (kcal/lb)	1474	1474	1474	1474	1474
CP %	18	23	13	18	18
Lysine %	0.95	0.95	0.95	1.25	0.65
Calcium %	0.70	0.70	0.70	0.70	0.70
Available Phosphorus %	0.32	0.32	0.32	0.32	0.32
Sodium	0.10	0.10	0.10	0.10	0.10
Analyzed nutrients					
CP (%)	17.2	20.4	14.1	17.0	16.1
Lysine (%)	0.96	0.90	0.88	1.14	0.64

Diets meet the NRC requirements for all nutrients except for protein and lysine where noted.

Table 2. Primer sequences for LKR, SDH, AASS, CAT, ORC and ARP mRNA¹.

Gene	Primer Sequence	Primer Efficiency
LKR	5'- GTC AAA GCT GGT GGC ATT CT 3'- TGC CAC TAC CCG TAC TCC TC	2.0
SDH	5'-TGG CCG GCA CAG CTC CCA AT 3'-GCA CGT TCC CTG CTC TCC CG	2.10
AASS	5' -TGA GCG CTA CAC AAGTCG TT 3' -TTG CCA CAA GTT TGT GTG GT	2.10
ORC-1	5'-CGG GGA GAT CAA AAG ATG AA 3'-TTT GAT ACA ATC CAC GGG GT	2.00
ORC-2	5'-TGG AGC ACT CTG CTT CAA GA 3'-AAT TCC AGC AAT TCC ACC AC	2.08
CAT-1	5'-GCC ACG AAG GTG GGG AAG CC 3'- ACG TGC TTG AAG GCG TCG GG	2.10
CAT-2	5'-ATG TGC CGG GCA TGG TTG GG 3'-AAC GGG TAG GAC CCC AGG CA	1.98
ARP	5'-GCT AAG GTG CTC GGT TCT TC 3'-GTG CGG ACC AAT GCT AGG	2.00

¹Abbreviations: lysine α -ketoglutarate reductase (LKR), saccharopine dehydrogenase (SDH), amino adipate δ -semialdehyde synthase (AASS), ornithine carrier (ORC), cationic amino acid transporter (CAT), acidic ribosomal protein (ARP)

Table 3. Effect of dietary treatment on performance.

	Control	High Protein	Low Protein	High Lysine	Low Lysine
ADG (g)	73.9±19.2	139.6±25.9	99.1±43.1	134.4±29.0	75.1±15.1
ADFI (g)	372.6±68	390.3±56	380.4±57	385.2±61	393±62
Feed Efficiency	0.51±0.17	0.57±0.13	0.41±0.11	0.62±0.17	0.36±0.14
Relative Liver Wt (%)	2.8±0.13 ^b	3.2±0.12 ^a	2.7±0.15 ^b	2.89±0.17 ^b	2.93±0.32 ^{ab}
Relative Kidney Wt (%)	0.64±0.02 ^{ab}	0.68±0.03 ^a	0.54±0.03 ^b	0.61±0.04 ^{ab}	0.54±0.07 ^b
Relative Heart Wt (%)	0.64±0.02	0.65±0.03	0.61±0.04	0.65±0.02	0.72±0.10

¹Abbreviations: average daily gain (ADG), average daily feed intake (ADFI)

²Measurements are averaged over all replicates within dietary treatment. Data are expressed as mean ± SEM (n=7). Within a row, values that do not share a common superscript are different (P<0.05).

Table 4. Effect of dietary treatment on indices of lysine catabolism¹.

	Control	High Protein	Low Protein	High Lysine	Low Lysine
Liver LOX (nmol/g*min)	7.66±2.63	12.30±2.63	9.33±1.65	10.66±2.19	7.37±2.34
Liver LKR mRNA [#]	1.20±0.48	1.80±0.66	1.08±0.55	1.20±0.39	0.51±0.12
Liver SDH mRNA ²	2.28±0.70 ^a	2.48±0.68 ^a	0.98±0.54 ^{ab}	2.37±0.49 ^a	0.55±0.15 ^b
Liver AASS mRNA	2.53±0.55 ^{bc}	4.55±0.39 ^{ab}	2.01±0.90 ^{bc}	5.78±1.96 ^a	1.84±0.48 ^c
Liver ORC-2 mRNA	0.81±0.19	0.79±0.22	1.25±0.64	1.53±0.68	0.19±0.06
Liver CAT-1 mRNA	0.69±0.20 ^b	1.21±0.33 ^b	0.48±0.19 ^b	2.99±0.84 ^a	0.61±0.23 ^b
Liver CAT-2 mRNA	0.80±0.18	1.58±0.30	1.22±0.52	4.25±2.68	3.98±1.29
Kidney SDH mRNA ³	0.91±0.35 ^b	1.82±0.52 ^a	1.22±0.51 ^{ab}	0.61±0.26 ^b	0.69±0.19 ^b
Kidney ORC-1 mRNA ³	1.15±0.27 ^b	4.30±1.95 ^a	1.67±0.67 ^b	2.21±0.86 ^{ab}	2.06±0.63 ^{ab}

¹Measurements are averaged over all replicates within dietary treatment. Data are expressed as mean ± SEM (n=7). Within a row, values that do not share a common superscript are different (P<0.05), ²P<0.08, ³P<0.10

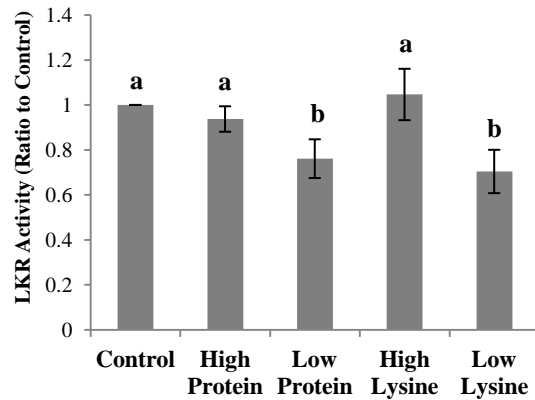
[#]All mRNA are represented as a ratio to liver ARP

Table 5. Tissue distribution of indices of lysine catabolism.

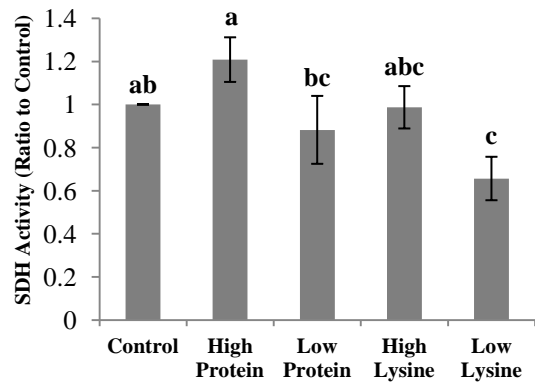
	Liver	Heart	Kidney
LKR Activity (nmol/g*min)**	628.6±96.0 ^a	152.0±55.5 ^b	258.7±67.7 ^b
SDH Activity (nmol/g*min)**	715.7±129.9 ^a	ND	148.6±41.0 ^b
AASS Protein (Ratio to liver pool)**	0.82±0.09 ^a	0.03±0.01 ^b	0.10±0.03 ^b
LOX (nmol/g*min)	7.66±2.62	7.35±2.42	8.18±2.30
LKR mRNA (Ratio to ARP)	1.20±0.48	0.11±0.07	0.71±0.36
SDH mRNA (Ratio to APR) [#]	2.28±0.68 ^a	0.54±0.30 ^b	0.91±0.35 ^{ab}
AASS mRNA (Ratio to ARP)	2.28±0.56	1.70±0.85	2.45±1.35
ORC-1 mRNA (Ratio to ARP) [*]	2.08±0.59 ^a	0.24±0.12 ^b	1.15±0.27 ^{ab}
ORC-2 mRNA (Ratio to ARP) [#]	0.81±0.19 ^a	0.22±0.12 ^b	0.55±0.12 ^{ab}
CAT-1 mRNA (Ratio to ARP)	0.69±0.20	0.51±0.30	0.86±0.34
CAT-2 mRNA (Ratio to ARP) [#]	0.80±0.18 ^a	0.24±0.16 ^b	0.44±0.15 ^{ab}

¹Measurements are averaged over all replicates for control diet only. Data are expressed as mean ± SEM (n=7). ND is not detected. Values within a row that do not share a common superscript are different, *P<0.05, **P<0.0001, #P<0.10

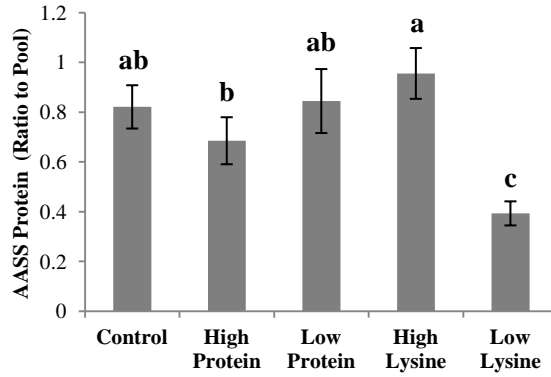
Figure 1 Dietary effect on indices of liver lysine catabolism.



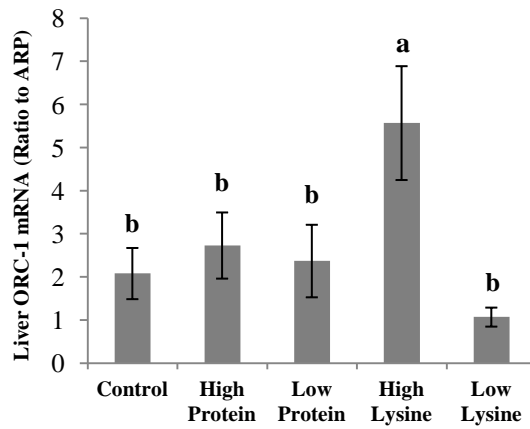
Panel A Dietary effects on liver LKR activity. Enzyme activity was measured in duplicate (n=7). Data are expressed as mean \pm SEM. Data are expressed as a ratio to the control. Values that do not share a common superscript are different (P<0.05).



Panel B Dietary effects on liver SDH activity. Enzyme activity was measured in duplicate (n=7). Data are expressed as mean + SEM. Data are expressed as a ratio to the control. Values that do not share a common superscript are different (P<0.05).



Panel C Dietary effects on liver AASS protein expression. Data are expressed as a ratio to a pooled liver sample as mean + SEM. Values that do not share a common superscript are different ($P < 0.01$).



Panel D Dietary effects on liver ORC-1 mRNA abundance. Data are expressed as a ratio to the expression of the housekeeping gene ARP as mean + SEM. Values that do not share a common superscript are different ($P < 0.05$).

CHAPTER THREE

Effects of strain and production-cycle on indices of lysine catabolism in turkeys

ABSTRACT

In typical commercial turkey production diets, lysine is frequently the second limiting amino acid. Understanding its catabolism could provide opportunities to increase the efficiency of lysine use for protein synthesis. We hypothesize that indices of lysine catabolism in turkey liver vary throughout the production cycle. Two commercial strains of turkey, Strain A and B, were analyzed 8 times over a period of 17 weeks (n=8 birds/sampling time/strain) for lysine α -ketoglutarate (LKR) and saccharopine dehydrogenase (SDH) activities and mRNA abundance, *in vitro* lysine oxidation (LOX), amino acid oxidase (AAOX) activity, lysyl oxidase (LO) activity and mRNA expression of cationic amino acid transporters (CAT) 1, 2 and 3. We found differences in hepatic LKR activity (P<0.05), LKR mRNA (P<0.01), SDH activity (P<0.05), LOX (P<0.0001), AAOX activity (P<0.05), lysyl oxidase activity (P<0.01) L-amino acid oxidase (P<0.0001), and CAT1 mRNA abundance (P<0.05) throughout the production cycle. We also found an effect of strain on SDH mRNA abundance; while no age or strain effects were detected for CAT2 and CAT3 mRNA abundance. Interestingly, the average LKR and SDH activities across strains and weeks was 240 and 420 nmol per minute per gram of liver, respectively, as opposed to the average AAOX activity, lysyl oxidase activity and LOX which were 0.70, 0.10 and 13.5 nmol per minute per gram liver, respectively. These data indicate that the saccharopine-dependent pathway is the predominant pathway of lysine degradation in turkey liver and that indices of hepatic lysine catabolism vary throughout the production cycle. (Support; HATCH WVA 470).

Keywords: Lysine, lysine α -ketoglutarate reductase, saccharopine dehydrogenase, lysyl oxidase, turkey

INTRODUCTION

In the poultry industry, increasing production efficiency is important for reducing production costs and environmental impact. Genetic selection to optimize efficiency has been used as selection criteria for decades. Genetic differences in body weight gain, feed conversion ratio and breast yield have been reported in poultry (Smith et al., 1998, Sterling et al., 2006). Also, in growing turkeys, genetic strain influenced growth rate, carcass components and aspects of meat quality characteristics early in the production-cycle (Roberson et al., 2003). It is for this reason, that genetic strain is an important consideration in maximizing production efficiency.

Carcass components are also widely affected by dietary amino acids; particularly lysine which is essential for muscle growth and typically second limiting in poultry production diets. Lysine deficiency in poultry leads to a reduction in body weight, growth rate, and muscle weight and an increase in feed conversion ratio (**FCR**) (Tesseraud et al., 1999). Kidd et al. (1998) established that increasing lysine content above its requirement in the starter and grower-finisher diets of broiler chickens increased performance and breast yield.

Relationships between genetics, growth and lysine requirements have been reported. Tesseraud et al. (1999) demonstrated that a line of broilers selected for improved carcass quality had increased body and muscle weights compared to a control line while consuming a lysine-deficient diet. Sterling et al. (2006) showed a three-way interaction between dietary protein, lysine levels and genotype for weight gain and FCR. Acar et al. (1991) demonstrated interactions between strain and dietary lysine for abdominal fat, breast fillet and tender yield. A potential explanation for the interaction between strain and lysine requirement could be due to differences in lysine catabolism.

In poultry, lysine is thought to be degraded predominantly via the saccharopine-dependent pathway (Figure 1). This pathway takes place in a variety of organs, most notably the liver (Manangi et al., 2005). The saccharopine-dependent pathway is initiated by the condensation of lysine and α -ketoglutarate forming saccharopine via lysine α -ketoglutarate reductase (**LKR**, EC 1.5.1.8). Next, saccharopine is converted to α -aminoadipate δ -semialdehyde and glutamate by saccharopine dehydrogenase (**SDH**, EC 1.5.1.9). There is evidence in mammals that both LKR and SDH activities are found on a bifunctional protein, α -aminoadipate δ -semialdehyde (**AASS**; Markovitz et al., 1984, Sacksteder et al., 2006). Genetic differences in LKR activity (Wang et al., 1972, 1973) and the rate of lysine oxidation (Wang et al., 1973) exist between broiler chickens selected for arginine requirement. Selecting for both growth and a decrease in lysine catabolism could lead to increased production and feed efficiency, ultimately decreasing feed costs.

Other pathways of lysine degradation depend on lysyl oxidase (**LO**, EC 1.4.3.13) or L-amino acid oxidase (**AAOX**, EC 1.4.3.2, Figure 1). Lysyl oxidase is a copper-dependent enzyme that is important to the formation of connective tissue by cross-linking collagen and elastin (Pinnell and Martin, 1968); lysyl oxidase catalyzes the oxidation of peptidyl-lysine residues to α -aminoadipate- δ -semialdehyde. It was originally thought that lysyl oxidase was only active towards peptide-bound lysine; however, evidence shows that free lysine is also a substrate (Trackman and Kagan, 1979). L-amino acid oxidase converts L-lysine to α -keto- ϵ -aminocaproic acid (Gómez et al., 2006). L-amino acid oxidase is inactive towards L-lysine in mammalian tissues (Blanchard et al., 1944); however activity has been measured in liver of chicks (Wang et al., 1972). The extent of these alternative pathways in lysine degradation in poultry liver is not well understood.

Cellular localization is an important aspect of lysine catabolism. Lysine must first be transported into the cell, through the plasma membrane. One mechanism of transport for lysine is a cationic amino acid transport (CAT) system (Deves and Boyd, 1998). The CAT system is the primary amino acid transport system to concentrate lysine, arginine and ornithine into cellular amino acid pools (Broer, 2002). Four main CAT isoforms, CAT1, CAT2A, CAT2B and CAT3 exist, all differing in tissue localization. Isoform CAT2A is predominantly expressed in the liver of mammals (Deves and Boyd, 1998) and aves (Humphrey et al., 2004).

The objectives of the present study are to investigate the effect of strain on lysine catabolism in commercial turkey and also to characterize changes in indices of lysine catabolism throughout the typical production cycle. Understanding these changes throughout the production cycle may aid in the reduction of lysine catabolism, increasing its use for protein synthesis. We also measured the three pathways of lysine degradation to obtain a better understanding of the roles of each pathway in total liver lysine catabolism. This is the first report in which all three pathways of lysine catabolism have been simultaneously measured.

MATERIALS AND METHODS

Two strains of turkey, Strain A and B were housed at the West Virginia University Reymann Memorial Farm Turkey Research Facility. The precise methodologies of animal housing and feeding strategies are described in Part I by Swiger and others (2011). In short, birds were provided feed and water *ad libitum*. Phase feeding was utilized to mimic production settings with a total of 6 diets; starter 1 (w 0-2), starter 2 (w 2-5), grower 3 (w 5-8), grower 4 (w 8-11), finisher 5 (w 11-16), and finisher 6 (w 16-18). One objective of Part I was to examine the effect of phosphorous level in the finisher diets on performance. Finisher 5 and 6 diets had either

a high or low phosphorous content. All diets were manufactured at a commercial feed mill (Virginia Poultry Growers Cooperative, Broadway, VA) and contained corn, soybean meal, poultry by-product meal, wheat middlings and animal/vegetable blended fat. Diet formulations were proprietary; however, proximate analysis was performed and is reported in Part I (West et al., 2011). Eight birds per strain were euthanized in a humane manner by cervical dislocation at week 0, 1, 2, 4, 8, 12, 16 and 17 of life. After euthanasia, liver samples were collected and homogenized into a 25% (w/v) solution using a Potter-Elvehjem device in ice-cold H buffer (220 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM 2-Mercaptoethanol, 1 mM EGTA, 0.05% (w/v) bovine serum albumin, pH 7.4) and transported back to West Virginia University on ice. Animal care and conduct of experiments were approved by the West Virginia University Animal Care and Use Committee (ACUC#-11-0703).

Hepatic LKR and SDH activities were determined on fresh liver as previously described (Blemings et al., 1994). Lysine α -ketoglutarate reductase activity was measured by the lysine-dependent NADPH oxidation at 340 nm using a Beckman Coulter DU 640 spectrophotometer. Enzyme activity was measured by the addition of 25 μ L of homogenate to a cuvette containing 1 mL of buffer (127.5 mM Hepes, 114.75 mM mannitol, 38.25 mM sucrose, 4.25 mM 2-mercaptoethanol, 0.0425% (w/v) bovine serum albumin, 0.21 mM NADPH, 12.75 mM α -ketoglutarate, 0.05% (v/v) Triton-X 100 and 50 mM L-lysine, pH 7.8). A blank without lysine was also measured to assess the disappearance of NADPH, independent of lysine addition. All liver samples were measured for LKR activity in duplicate and the reactions started with the addition homogenate. Saccharopine dehydrogenase activity was measured by the saccharopine-dependent NADH appearance at 340 nm. Enzyme activity was measured by the addition of 25 μ L of homogenate to a cuvette containing 1 mL of buffer (100 mM Tris-HCl, 3 mM NAD, 5

mM 2-mercaptoethanol, 0.5% (v/v) Triton X-100 and 2 mM L-saccharopine, pH 8.7). A blank without saccharopine was also measured to assess the appearance of NADH independent of saccharopine addition. All liver samples were measured for SDH activity in duplicate and the reactions started with the addition of homogenate.

Lysine oxidation (**LOX**) was measured in fresh liver samples by determining the recovery of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ L-lysine, as previously described (Blemings et al., 1998). A base trap was constructed by the addition of 1 part ethanolamine to 2 parts methyl cellosolve. A total of 0.5 mL of a base trap was added to an Eppendorf tube, which was suspended in a 4-mL glass vial. A 25% (w/v) tissue homogenate was made with fresh liver and H buffer. For each reaction, 250 μL of homogenate was added to the glass vial, containing 250 μL of buffer at 41°C (10 mM Hepes, 3mM MgCl_2 , 0.2 mM EDTA, 182 mM mannitol, 61 mM sucrose and 10 mM L-lysine (final concentrations), pH 7). The solution was then incubated in the 41°C water bath for 30 minutes, while oscillating at 100 osc/min. To terminate the reactions, 100 μL of 100 mM potassium phosphate buffer (pH 5) was injected via an 18 gauge needle through the serum cap covering the vial. The vial then remained in the water bath for at least 180 additional minutes, to ensure maximal recovery of the $^{14}\text{CO}_2$. After incubation, the Eppendorf tube was placed in a plastic scintillation vial along with 17 mL of Bio-Safe II liquid scintillation fluid. After the solution was vortexed, the radioactivity was measured using a Beckman LS 6500 liquid scintillation counter (Beckman Coulter Inc, Somerset, NJ). Each tissue was measured in triplicate.

L-amino acid oxidase (LAAO) was measured as the formation of α -keto- ϵ -aminocaproate semibarbazone according to Danson et al. (2002). A total of 5 mL of the 25% liver homogenate was centrifuged at 10,000 X g for 10 minutes. The resulting supernatant was centrifuged for an

additional hour at 100,000 X g to yield a microsomal pellet. The pellet was resuspended in 1 mL of H Buffer. Enzyme activity was measured by the addition of 25 μ L of microsomal suspension to 975 μ L of buffer (100 mM potassium phosphate, 250 mM semicarbazide, 25,000 units/L catalase, 0.1% (v/v) triton-X and 10 mM L-lysine HCl). A blank without lysine was also assayed to measure the semibarbazone formation, independent of lysine addition. All samples were measured in duplicate. The reaction was initiated by the addition of lysine and incubated for 90 min with gentle shaking at 40°C. The reaction was terminated by the addition of 100 μ L of 6N HCl. Samples were centrifuged for 2 min at 2,000 X g, and 200 μ L of the supernatant was loaded onto a 96 well plate. Absorbance at 248 nm was determined using the SPECTRAmax®PLUS³⁸⁴.

Fresh liver samples were frozen in liquid nitrogen and stored at -80°C until use. Lysyl Oxidase (LO) was assessed as previously described (Palamakumbura et al., 2002). Frozen samples were powdered in liquid nitrogen using a ceramic mortar and pestle. After powdering, 2 mL of 0.05M phosphate buffered saline per 50 mg of tissue was used to homogenize the samples in a Potter-Elvehjem device. Samples were agitated at 4°C for 2 hour, and then were centrifuged at 10,000 X g for 30 min at 4°C. The resulting supernatants were discarded and the pellets were resuspended in 2 mL of a 6M urea solution per initial 50 mg of tissue. Samples were then agitated for at least 18 hours at 4°C and centrifuged at 10,000 X g for 30 min at 4°C. The resulting supernatant was used for the LO assay. Assays were performed by adding 500 μ L extract (supernatant) to 35 μ L 0.05M sodium borate buffer (pH 8.2), all samples were in duplicate. Assays were also performed in the presence of a LO inhibitor (β -aminopropionitrile-BAPN), in which 500 μ L of the supernatant was added to 35 μ L of a 0.12 M BAPN solution in 0.05M sodium borate buffer, in duplicate. All samples were incubated in a 40°C water bath for

50 min. After incubation, samples were added to a cuvette containing 2 mL buffer (9.7 mM lysine-HCl, 0.52 mM Amplex red, 40 ug of horseradish peroxidase). Fluorescence was measured using excitation and emission wavelengths of 563 and 587 nm, respectively on a Varian Cary Eclipse fluorometer (Walnut Creek, Ca). Lysyl oxidase activity was calculated as the net change in fluorescence and compared to a hydrogen peroxide standard curve.

Real time RT-PCR was used to estimate the abundance of mRNA using acidic ribosomal protein (**ARP**) as a reference gene. The RNA was isolated and reverse transcribed using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was diluted 1:2 with nuclease-free water and 2 μ L were added to a 20 μ L total reaction which included 10 μ L 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 μ M forward and reverse ARP primers or 0.625 μ M of forward and reverse target gene primers. Target genes include LKR, SDH, and cationic amino acid transporter (CAT) isoforms 1, 2 and 3 (Table 2). Real time PCR was performed using a BioRad iCycler IQ Detection System. The procedure began with a "hot start" at 95°C for 5 minutes, followed by a cycle of a 95°C denaturing step for 60 seconds, an annealing step specific for each primer for 60 seconds, and a 72°C extension step for 60 seconds. This cycle was repeated a total for 40 times. A melt curve analysis was then performed to assess the quality of the amplification product.

Each sample was assayed in duplicate for both the target genes and ARP primers on a 96-well plate. A pooled sample was included in duplicate for both primers for each plate analyzed. Primer efficiencies were determined from the slope of the regression line of the log of the cDNA concentrations versus the threshold cycle (Ct) value by the equation $E=10^{(-1/Slope)}$. Efficiency plates for the primer pairs were analyzed, and an acceptable efficiency for each was obtained.

The efficiencies were used to calculate the relative mRNA abundance using the “efficiency corrected relative expression” equation (Pfaffl, 2001), and are shown on Table 1.

SDS-PAGE was performed on a mini-gel using polyacrylamide gels (4% stacking and 12% resolving). Samples (20 μ L) containing 15 μ g of protein were loaded into each lane and electrophoresed for 2 h and 30 min at 100 volts. Western blotting was used to determine AASS abundance. The primary antibody targets a 15 residue peptide fragment in the SDH-region of the mouse AASS protein raised in a rabbit (Kiess et al., 2008). The secondary antibody was goat anti-rabbit conjugated with horseradish peroxidase and was detected by incubation with Pierce SuperSignal® West Pico Chemiluminescent substrate (Pierce Biotechnology Inc, Rockford, IL) and exposure to film for 5 min. The band intensity was quantified using densitometry (FluoroChem800, Alpha Innotech Corporation).

Statistical Analyses

Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC). The main effects of strain and week were assessed, as well as strain by week interactions (n=8 birds/strain/week). When differences existed ($P < 0.05$), means were separated by the least significant differences procedure. If there were no strain effects, data from both strains were pooled and analyzed for week effect. Again, when differences existed ($P < 0.05$), means were separated by the least squares means procedure. The effect of phosphorous level in the finisher diets was also examined and no effect was detected on any of the indices measured. Therefore, data were pooled and analyzed only for week and strain effects. Correlation analysis was also assessed using Pearsons Correlation test.

RESULTS AND DISCUSSION

Measures of growth and performance are reported in Part 1 by West et al. (2011). In part 1, the effect of phosphorous levels on growth was assessed; therefore finisher diets 5 and 6 were constructed to contain either normal or low phosphorous levels. There was no effect of finisher diet phosphorous level on any indices of lysine catabolism or cationic amino acid transporter abundance ($P>0.05$). All data for finisher diets were pooled to examine the effect of strain and week only.

Lysine degradation via the saccharopine-dependent pathway was measured via LKR and SDH activity. Here, week ($P<0.05$), but not strain or week*strain, affected LKR activity. The lack of a strain effect on LKR and SDH activities was not surprising as these two strains were selected for growth; however this is the first known report to compare lysine catabolism in two commercially-available strains of turkey. There was also a decrease in LKR activity at week 4 compared to week 0, however activity increased to comparable levels by week 8 (Figure 2a). By week 16, LKR activity significantly decreased compared to all previous time points. Average LKR activity across weeks was 240 nmol NADPH consumed/g liver*min. LKR mRNA abundance, expressed as a ratio to ARP, was also affected only by week ($P<0.0001$). There was over a 200% increase in LKR mRNA expression at week 8 compared to all other time points (Figure 2b). The lack of correlation between LKR activity and mRNA abundance is in agreement with the hypothesis of post-translational modification of LKR (Karchi et al., 1995) as the primary mode of regulation. Keiss et al. (2008) demonstrated changes in LKR activity with no effect on AASS mRNA abundance or protein expression in livers from mice consuming a high protein diet.

There was a week ($P < 0.0001$), but not strain or week*strain effect on SDH activity. SDH activity significantly increased at week 8 compared to all other weeks (Figure 3a). Average SDH activity across strain and week was 420 nmol NADH produced/g liver*min. Both week and strain had an effect on SDH mRNA abundance (Figure 3b). There was a significant week effect on SDH mRNA abundance in the liver of strain A birds, while abundance remained constant throughout the production cycle in the strain B birds. By week 17, SDH expression was three fold higher in strain A compared to strain B. The abundance of AASS protein was also determined via western blot (Figure 3c) and a significant strain ($P < 0.001$), not week or week*strain effect was detected. When data were pooled across weeks, strain B birds had 60% greater AASS expression levels compared to strain A.

The rate of lysine oxidation, a measurement of flux through the saccharopine-dependent pathway and a proxy for lysine transport into mitochondria (Blemings and Benevenga, 2007), was affected by week ($P < 0.0001$), regardless of strain. Lysine oxidation significantly increased six-fold at week 8 compared to previous weeks, remained elevated at week 12, and further decreased at later weeks (Figure 4). Average LOX is 13.5 nmol CO₂ produced/g liver*min; resulting in LKR and SDH activities 18 and 31-fold higher than the rate of oxidation, respectively. Blemings et al. (1998) demonstrated similar results where LKR and SDH activity ranged from 6 to 107-times that of LOX in livers of rats fed varying levels of protein. Previous reports have shown that LKR and SDH are located in the mitochondria matrix (Blemings et al., 1994), indicating the need for transport of lysine into the matrix for catabolism to occur, suggesting that lysine transport into the mitochondrial matrix limits the rate of catabolism. The mode of transport is unknown and needs to be further investigated as it seems an important control point in lysine catabolism.

The 100-600% increase in LKR mRNA abundance, SDH activity and lysine oxidation at week 8 in production, corresponding to the grower 3 diet, indicates an increase in lysine catabolism. The increase in indices of lysine catabolism may be a result of hormonal and/or dietary control. Glucagon (Scislawski et al., 1994) and diets high in protein (Blemings et al., 1998, Keiss et al., 2008) and lysine (Foster et al., 1993) increased liver LKR activity in rodents. Also, the week effects demonstrated in this experiment may be a result of a “diet effect” due to the phase-feeding schedule implemented during the production cycle. These data indicate that presumably the efficiency of lysine use for protein synthesis is lower at these time points and modifying the diets may increase the efficiency of use. Alternatively, the increase at week 8 may also be a result of the relatively low protein level of the starter 2 diet.

Alternative routes of lysine degradation include LAAO- and LO-dependent pathways. There was a significant effect of week ($P < 0.0001$), but not strain on LAAO activity. L-amino acid oxidase activity increased from week 0 to week 4 and decreased with later ages (Figure 5a). Average L-amino acid oxidase activity across weeks was 0.7 nmol α -keto- ϵ -aminocaproic acid produced/g liver*min, which is only 0.21% of the activity of the enzymes in the saccharopine-dependent pathway. Our data are in agreement with Wang et al. (1972) who also showed that LAAO was present in the liver of poultry and is active towards free lysine. Lysyl oxidase activity was only detected in weeks 0 and 1 in the production cycle (Figure 5b). Activity was effected by week ($P < 0.05$) and not strain, and average activity was 0.1 nmol H_2O_2 produced/g liver*min, which is only about 0.03% of the activity of enzymes involved in the saccharopine-dependent pathway. These data are the only known report to analyze the three pathways of lysine degradation. However, it is important to note that LKR and SDH activities were measured at V_{max} conditions, whereas LO and LAAO activities were measured approaching V_{max}

conditions. Enzyme velocities at physiological concentrations of lysine were calculated using the average value of each enzyme activity, regardless of any strain or week effects. The average velocities of LKR, SDH, LAAO and LO are 22, 297, 0.1 and 0.025 nmol/g liver*min, respectively, indicating that even at physiological conditions, the saccharopine-dependent pathway is the predominant pathway of lysine degradation in turkey liver.

Cationic amino acid transporter (CAT) mRNA expression was also measured as a potential indicator of lysine transport activity through the plasma membrane and into the cell. Cationic amino acids are transported across the plasma membrane via four main CAT isoforms, CAT1, CAT2A, CAT2B and CAT3, all differing in tissue localization. CAT2A is predominantly expressed in the liver of mammalian (Devés et al., 1998) and poultry (Humphrey et al., 2004) species. CAT1 mRNA expression was detected in the liver and was affected by week ($P<0.001$), but not strain or week*strain. CAT1 mRNA expression was reduced 50% by week 2 compared to previous weeks, and did not change thereafter (Figure 6a). These data are in agreement with Humphrey et al. (2004) who also detected a reduction in liver CAT1 expression at 14-days post-hatch compared to 7-days post-hatch in chickens. Primers for CAT2 were designed in the shared region of the CAT2A and 2B splice variants, as per Humphrey and others (2004), therefore overall CAT2 mRNA was detected. There was a week effect ($P<0.01$), strain effect ($P<0.05$) and a week by strain interaction ($P<0.05$) on CAT2 mRNA abundance (Figure 6b). The most pronounced strain effect is at week 12, where strain A had a 430% increased relative expression of CAT2 compared to strain B. For CAT3, no interaction or main effects were detected and the average CAT3:ARP ratio was 0.62.

Correlation analysis was also performed on indices of lysine catabolism using Pearson's Correlation test. SDH activity was positively correlated with LKR mRNA abundance ($P<0.001$,

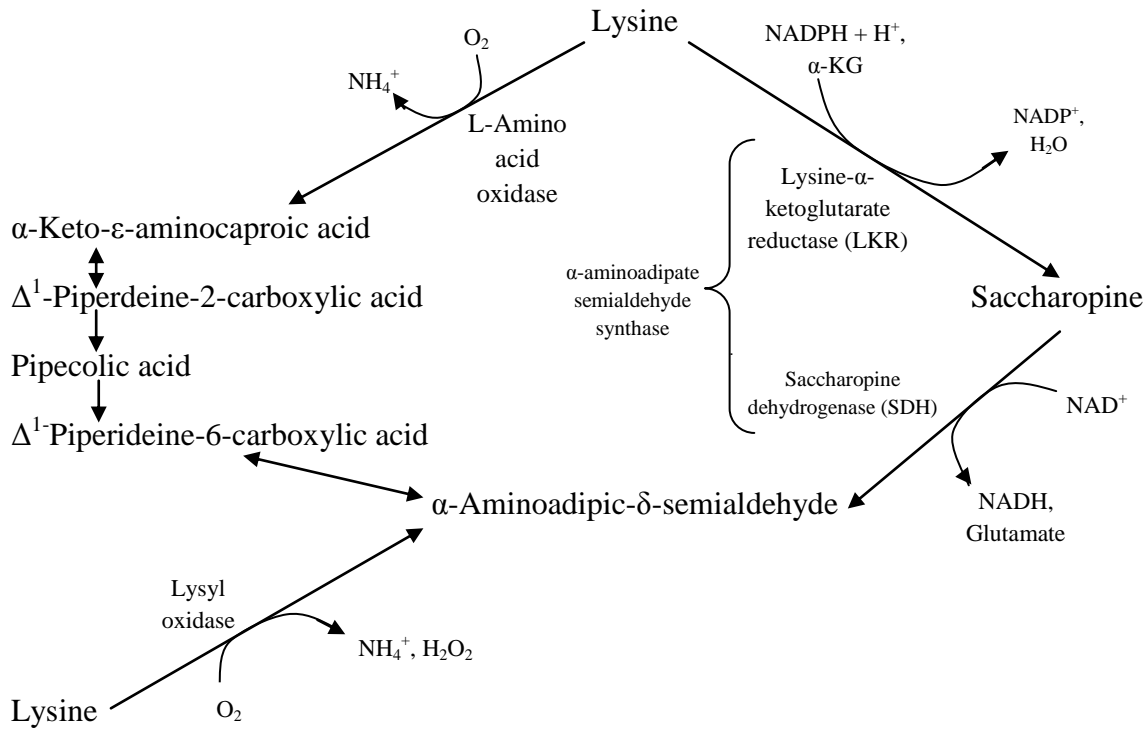
R=0.31), LOX ($P<0.001$, $R=0.323$) and had a trend for correlation with AASS protein abundance ($P=0.09$, $R=0.185$). The correlation between SDH activity, LKR mRNA and AASS mRNA supports the fact that activities of LKR and SDH exist as the bifunctional protein AASS (Markovitz et al., 1984). Lysine oxidation is a measurement of the flux through the saccharopine-dependent pathway, and is positively correlated with LKR mRNA abundance ($P<0.001$, $R=0.41$). There was a negative correlation between LOX and LAAO ($P<0.01$, $R= -0.314$) and CAT1 mRNA abundance ($P<0.01$, $R= -0.27$). There was a trend for LAAO to be negatively correlated with SDH mRNA abundance ($P=0.059$, $R= -0.22$). The negative correlation between the saccharopine-dependent pathway and LAAO is unclear. CAT1 mRNA abundance was positively correlated with CAT2 mRNA abundance ($P<0.01$, $R=0.24$), indicating that age and/or diet effects are consistent for these 2 isoforms.

Correlation analysis was also performed between indices of lysine catabolism and bird performance by week, reported by West and others (2011). At week 1 of production, there was a trend for a positive correlation between LOX and FCR ($P=0.07$, $R=0.47$) and a negative correlation between LOX and bird weight ($P<0.01$, $R= -0.58$). At week 12 of production, there was a negative correlation between bird weight and LKR activity ($P<0.07$, $R= -0.52$) and SDH activity ($P<0.05$, $R= -0.53$). At week 17 there was a negative correlation between bird weight and LKR mRNA abundance ($P<0.01$, $R= -0.68$). At week 8, AAOX was positively correlated with FCR ($P<0.0001$, $R=0.97$) and negatively with bird weight ($P<0.0001$, $R= -0.97$). Taken together, these results indicate that at various points in the production cycle, an increase in lysine degradation resulted in a reduced body weight, and increased FCR. There was a negative correlation between FCR and CAT1 mRNA abundance at week 2 ($P<0.05$, $R= -0.60$) and week

16 ($P < 0.06$, $R = -0.59$) and between FCR and CAT2 mRNA abundance at week 12 ($P < 0.05$, $R = -0.61$).

In conclusion, differences in indices of lysine catabolism due to strain in these 2 commercial lines of turkey were not detected. However, there are numerous effects related to the stage of the production cycle; including an increase in catabolism at week 8 of the cycle, potentially indicating that lysine or protein levels are too high for efficient use for protein synthesis. Overall, decreasing lysine catabolism would improve production efficiency. Present data also support the model of post-translational modification of enzymes in the saccharopine-dependent pathway and is supported by others (Karchi et al., 1995, Cleveland et al., 2008, Keiss et al., 2008). Currently, work is being conducted to construct a polyclonal antibody towards the full-length mouse AASS protein. The generation of these antibodies will allow further research to understand the regulation of lysine catabolism.

Figure 1. Pathways of lysine catabolism.



Pathways of lysine catabolism adapted from Wang and Nesheim (1972). The three pathways of lysine catabolism include the saccharopine-dependent, L-amino acid oxidase-dependent and the lysyl oxidase-dependent pathways.

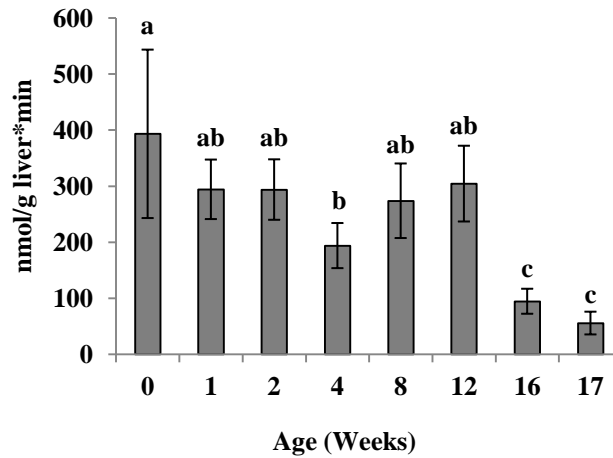
Table 1. Primer sequences for LKR, SDH, CAT1, CAT2, CAT3 and ARP mRNA¹.

Gene	Primer Sequence	Primer Efficiency
LKR	5 [′] - GGT GTG CAT TCC GCC CTG CT 3 [′] - AGG CGA CCG TGG GTT CGT AC	2.10
SDH	5 [′] - GGT GGC CTG CCA GCT CCA GA 3 [′] - CGC CCT CCT CGC AAC GAA TC	2.01
CAT-1	5 [′] - GGG CTG TGG CAC GGG TGA AC 3 [′] - CAA CCG CTC GAC ACC CGG AA	2.01
CAT-2	5 [′] - GTT TCC TTC CTC ATT GCT GC 3 [′] - CCA CTC CAG GCT CTT GCT AC	2.01
CAT-3	5 [′] - CCA AGA CTG GCT CTG CCT AC 3 [′] - TAG TAG CCG TTG GTT TAG AG	1.97
ARP	5 [′] - GCA GCT GAT TAA GAC CGG AG 3 [′] - TGG GAC TTC ACG ACC TGT AG	1.90

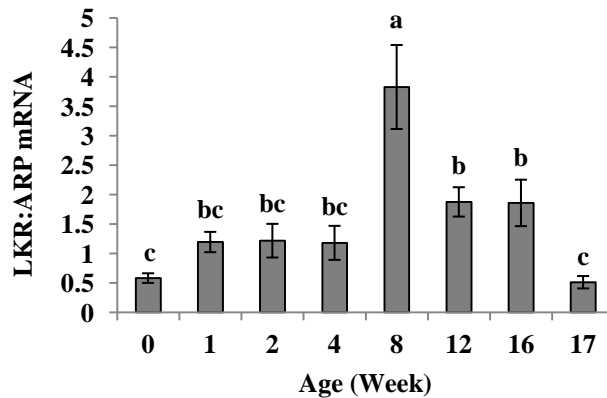
¹Abbreviations: lysine α -ketoglutarate reductase (LKR), saccharopine dehydrogenase (SDH), cationic amino acid transporter (CAT), acidic ribosomal protein (ARP)

Figure 2. LKR Activity and mRNA Abundance.

Lysine α -ketoglutarate reductase (LKR) activity and mRNA abundance in turkey liver homogenates. Enzyme activities and mRNA abundance measured in duplicate (n=8 birds/strain/week). Data are expressed as mean \pm SEM.



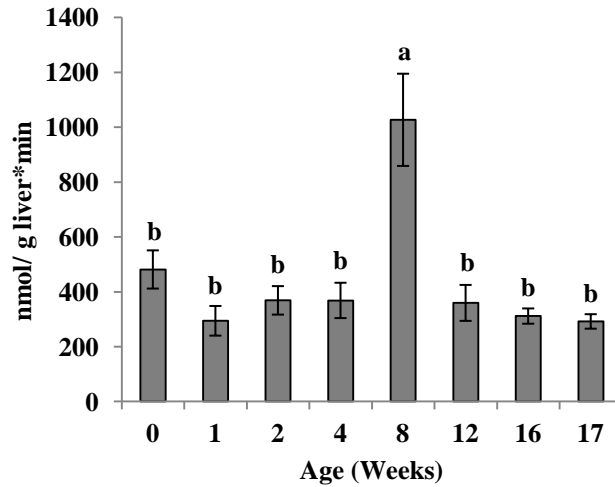
Panel A LKR Activity across weeks of the production cycle. LKR activity was measured as the lysine-dependent disappearance of NADPH in liver homogenates, and data are expressed as nmol NADPH consumed/g liver*min. No differences between strains or week*strain interactions were detected ($P > 0.05$), therefore strains were pooled and analyzed only for week effect. Differences in superscripts indicates a significant difference as detected by LSD ($P < 0.05$).



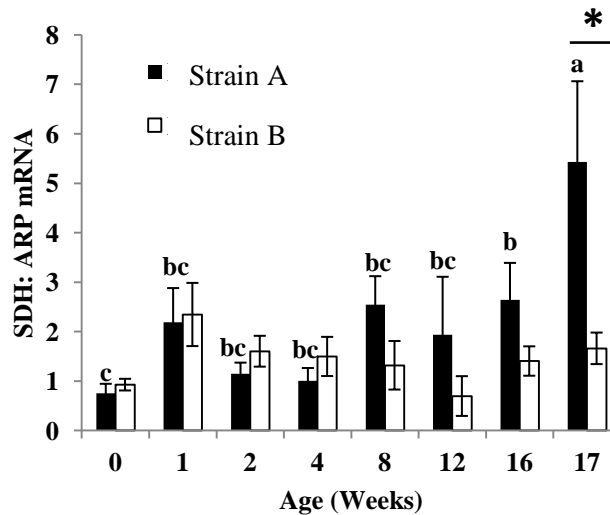
Panel B LKR mRNA abundance across the production cycle. LKR mRNA is expressed as a ratio to the housekeeping gene Acidic Ribosomal Protein (ARP). No differences between strains or week*strain interactions were detected ($P > 0.05$), therefore strains were pooled and analyzed only for week effect ($P < 0.001$). Differences in superscripts indicate a significant difference as detected by LSD ($P < 0.05$).

Figure 3. SDH Activity and mRNA Abundance.

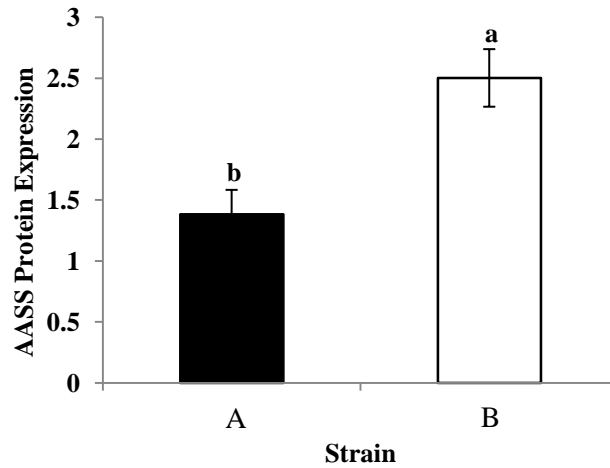
Saccharopine Dehydrogenase (SDH) activity and mRNA abundance in turkey liver. Enzyme activities and mRNA abundance measured in duplicate (n=8 birds/strain/week). Data are expressed as mean \pm SEM.



Panel A SDH Activity across weeks of the production cycle. SDH activity was measured as the saccharopine-dependent appearance of NADH in prepared liver homogenates, and data are expressed as nmol NADH produced/g liver*min. No differences between strains or week*strain interactions were detected ($P > 0.05$), therefore data were analyzed only for week effect ($P < 0.001$). Differences in superscripts indicates a significant difference as detected by LSD ($P < 0.05$).

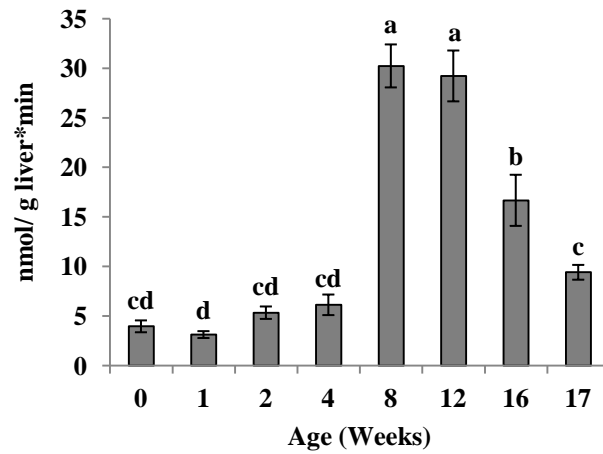


Panel B SDH mRNA abundance across the production cycle. SDH mRNA is expressed as a ratio to the housekeeping gene Acidic Ribosomal Protein (ARP). An effect of strain was detected ($P < 0.01$) and is denoted by an asterisk. There was also an effect of production cycle week on SDH activity ($P < 0.001$) in the strain A. Differences in superscripts indicates a significant difference as detected by LSD ($P < 0.05$). There was no interaction between week and strain.



Panel C α -Amino adipate Semialdehyde Synthase (AASS) protein abundance in liver homogenates. The abundance of AASS protein was analyzed via western blot with a probe targeting the SDH region of the protein. Data are expressed as a ratio to a pooled sample. A strain ($P < 0.001$), but not week or week*strain effect was detected, so data were pooled across weeks. Differences in superscripts indicates a significant difference as detected by LSD ($P < 0.05$).

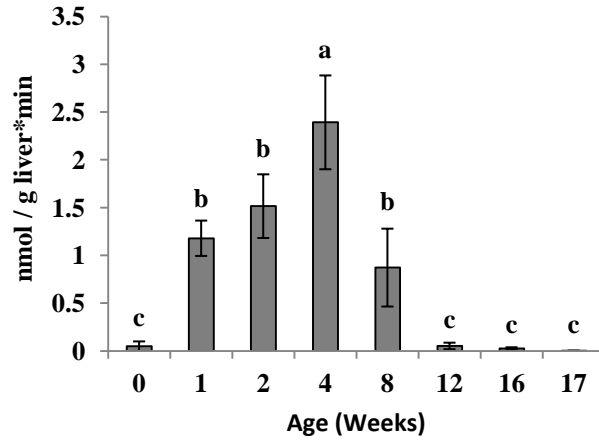
Figure 4. Lysine Oxidation.



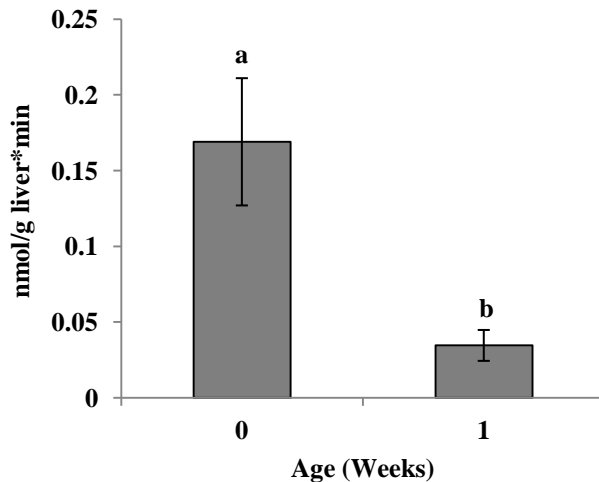
Lysine oxidation was measured as $^{14}\text{CO}_2$ produced from $[1\text{-C}^{14}]$ L-lysine, and data are expressed as nmol CO_2 produced/g liver*min (n=8 birds/strain/week). No effect of strain or week*strain interactions were detected ($P>0.05$), therefore data were analyzed only for week effect ($P<0.001$). Differences in superscripts indicates a significant difference as detected by LSD ($P<0.05$).

Figure 5. Alternative Pathways of Lysine Degradation.

Alternative pathways in lysine degradation were assessed. Data are expressed as mean \pm SEM (n=8 birds/strain/week).



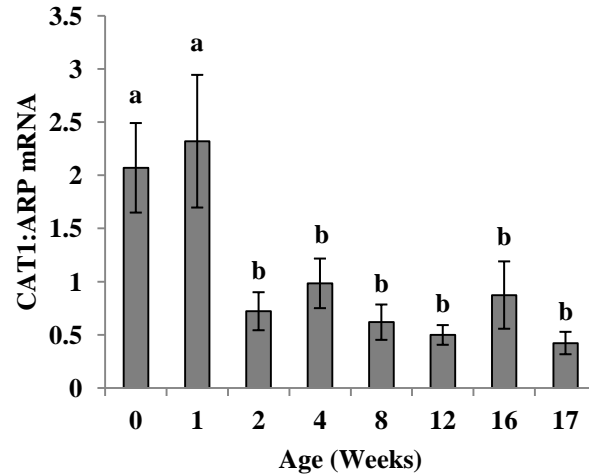
Panel A L-Amino acid oxidase activity was measured as the lysine-dependent formation of α -keto- ϵ -aminocaproate semibarbazone, data are expressed as nmol α -keto- ϵ -aminocaproate semibarbazone produced/g liver*min. No effect of strain or week*strain interactions were detected ($P>0.05$), therefore data were analyzed only for week effect ($P<0.001$). Differences in superscripts indicates a significant difference as detected by LSD ($P<0.05$).



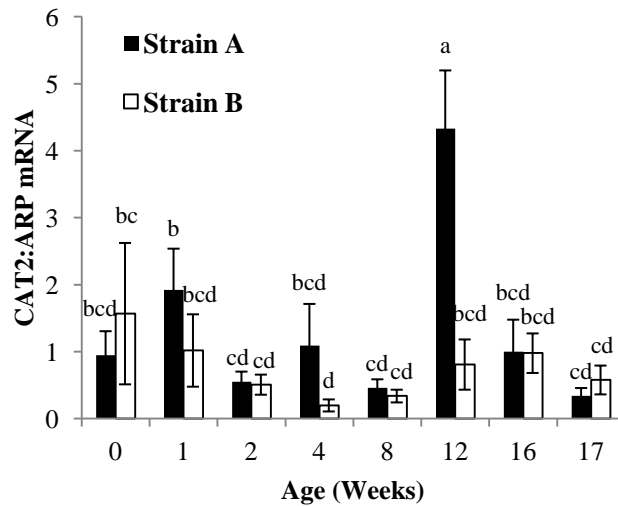
Panel B Lysyl oxidase activity was determined fluorometrically as the rate of BAPN-inhabitable H_2O_2 production, via a reaction coupled with Horseradish Peroxidase, data are expressed as nmol H_2O_2 produced/g liver*min. Activity was only detected as weeks 0 and 1 of the production cycle, and no differences in strain were observed. There was a significant week effect ($P<0.05$).

Figure 6. Abundance of Cationic Amino Acid Transporter (CAT) mRNA.

CAT mRNA is expressed as a ratio to the housekeeping gene Acidic Ribosomal Protein (ARP). Data are expressed as mean \pm SEM (n=8 birds/strain/week).



Panel A CAT1 mRNA abundance. No differences between strains were detected ($P>0.05$), therefore data were analyzed for week effect ($P<0.001$). Differences in superscripts indicates a significant difference as detected by LSD ($P<0.05$).



Panel B CAT2 mRNA abundance. There was an effect of week ($P<0.01$), strain ($P<0.05$) and a week by strain interaction ($P<0.05$).

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