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Effects of Strain and Production-Cycle on Indices of Lysine Catabolism in Turkeys

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Abstract: In typical 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 alpha-ketoglutarate (LKR) and saccharopine dehydrogenase (SDH) activities and mRNA abundance, *in vitro* lysine oxidation (LOX), L-amino acid oxidase (LAAO) 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 ($p = 0.007$), LKR mRNA ($p = 0.0004$), SDH activity ($p = 0.008$), LOX ($p < 0.0001$), lysyl oxidase activity ($p < 0.01$), LAAO ($p < 0.0001$) and CAT1 ($p = 0.005$) and 2 mRNA abundance ($p = 0.0022$) throughout the production cycle. We also found an effect of strain on SDH ($p = 0.046$) and CAT-2 mRNA abundance ($p = 0.02$); while no age or strain effects were detected for 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 LAAO 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.

Key words: Lysine, lysine alpha-ketoglutarate reductase, saccharopine dehydrogenase, lysyl oxidase, turkey, genetic strain

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 and Kerr (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 (Fig. 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 alpha-ketoglutarate forming saccharopine via lysine alpha-ketoglutarate reductase (LKR, EC 1.5.1.8). Next, saccharopine is converted to alpha-amino adipate delta-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, alpha-amino adipate delta-semialdehyde (AASS; Markovitz *et al.*, 1984; Sacksteder *et al.*, 2000). Genetic differences in LKR activity (Wang *et al.*, 1973; Wang and Nesheim, 1972) and the rate of lysine oxidation (Wang *et al.*, 1973) exist between broiler chickens selected for arginine

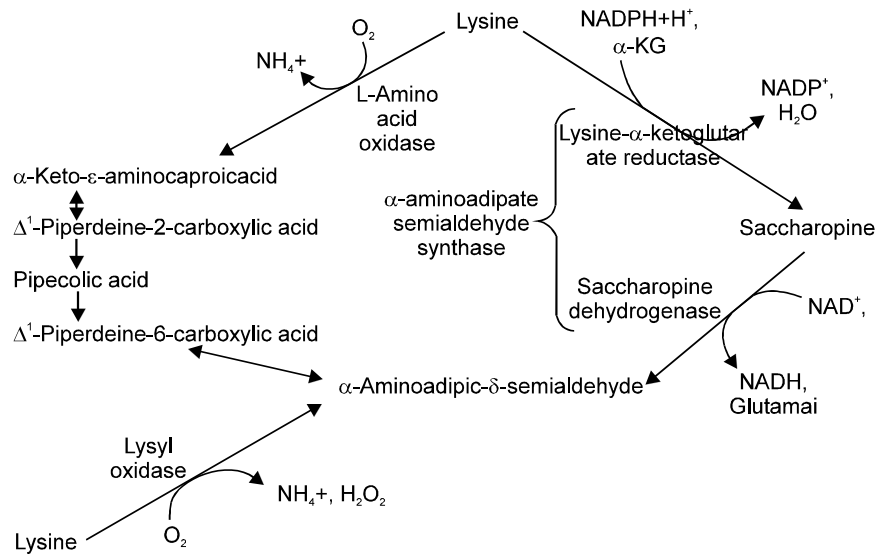


Fig. 1: Pathways of Lysine Catabolism. Pathways of lysine catabolism adapted from Wang and Nesheim (1972). The three pathways of lysine catabolism include the saccharopine-dependent, L-amino acid oxidase-dependent and the lysyl oxidase-dependent pathways

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 include lysyl oxidase (LO, EC 1.4.3.13) and L-amino acid oxidase (LAAO, EC 1.4.3.2, Fig. 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 alpha-amino adipate-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 alpha-keto-ε-aminocaproic acid (Gomez *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.*, 1973). 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 (A = Nicholas Turkeys, Aviagen Group, EW Group, Visbek, Germany; B = Hybrid Turkeys, Hendrix Genetics, Boxmeer, the Netherlands) were housed at the West Virginia University Reymann Memorial Farm Turkey Research Facility. The precise methodologies of animal housing and feeding strategies have been previously described (Evans *et al.*, 2014). 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 the experiment 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 as previously described (Evans *et al.*, 2014). 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 alpha-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 alpha-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 ($1\text{-}^{14}\text{C}$) 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 μ L of homogenate was added to the glass vial, containing 250 μ L 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 min, while oscillating at

100 osc/min. To terminate the reactions, 100 μ L 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 alpha-keto-epsilon-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 and Trackman, 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. Samples were agitated at 4°C for 2 h 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 h 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 (beta-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 µg 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 µL were added to a 20 µL total reaction which included 10 µL 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 µM forward and reverse ARP primers or 0.625 µM of forward and reverse target gene primers. Target genes include LKR, SDH and cationic amino acid transporter (CAT) isoforms 1, 2 and 3 (Table 1). Real time PCR was performed using a BioRad iCycler IQ Detection System. The procedure began with a "hot start" at 95°C for 5 min, followed by a cycle of a 95°C denaturing step for 60 sec, an annealing step specific for each primer for 60 seconds and a 72°C extension step for 60 sec. 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 $E = 10^{(-1/\text{Slope})}$. 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) and are shown on Table 1.

SDS-PAGE was performed on a mini-gel using polyacrylamide gels (4% stacking and 12% resolving). Samples (20 µL) containing 15 µg 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).

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 CTG 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 alpha-ketoglutarate reductase (LKR), saccharopine dehydrogenase (SDH), cationic amino acid transporter (CAT), acidic ribosomal protein (ARP)

Statistical methods: 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

Measures of growth and performance have previously been reported (Evans *et al.*, 2014). In that report 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). Therefore, 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 = 0.007), but not strain or week*strain, affected LKR activity. There was also a decrease in LKR activity at week 4 compared to week 0, however activity increased to comparable levels by week 8 (Fig. 2a). By week 16, LKR activity 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 = 0.0004). There was over a 200% increase in LKR mRNA expression at week 8 compared to all other time points (Fig. 2b).

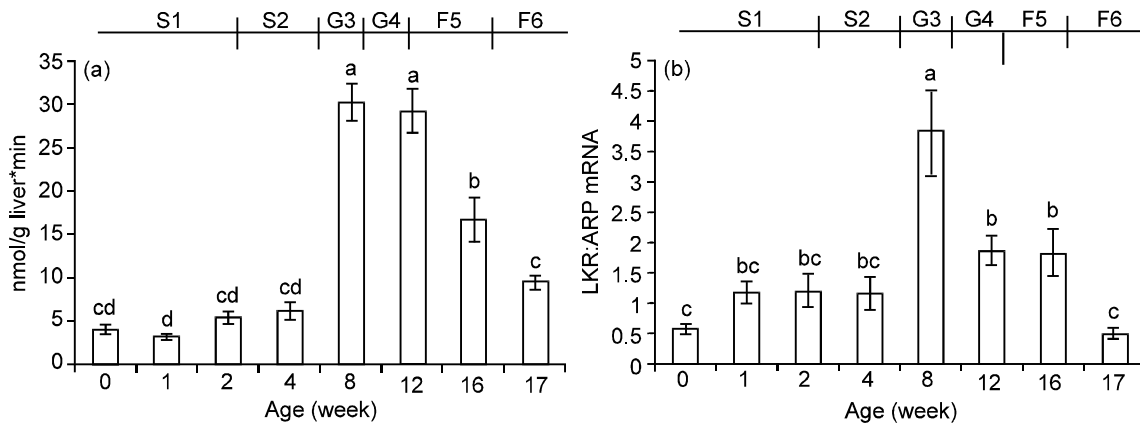


Fig. 2: LKR Activity and mRNA Abundance. Lysine alpha-ketoglutarate reductase (LKR) activity and mRNA abundance in turkey liver homogenates. Enzyme activities and mRNA abundance measured in duplicate ($n = 8$ birds/strain/week). Data are expressed as mean+SEM. Top bar represents diets consumed at each time point, including starter (S), grower (G) and finisher (F) diets. (A) 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 ($p = 0.007$) and (B) 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. Differences in superscripts indicate a significant difference ($p = 0.0004$)

There was a week ($p = 0.008$), but not strain or week*strain effect on SDH activity. SDH activity significantly increased at week 8 compared to all other weeks (Fig. 3a). Average SDH activity across strain and week was 420 nmol NADH produced/g liver*min. 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. Week ($p = 0.0015$), strain ($p < 0.046$) and a week*strain interaction ($p = 0.016$) effected SDH mRNA abundance (Fig. 3b). There was a week effect on SDH mRNA abundance in the liver of strain B birds, while abundance remained constant throughout the production cycle in the strain A birds. By week 17, SDH expression was three fold higher in strain B compared to strain A. The abundance of AASS protein was also determined via western blot (Fig. 3c) and a significant strain ($p < 0.001$), not week or week*strain effect was detected. When data were pooled across weeks, strain A birds had 60% greater AASS expression levels compared to strain B.

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 increased six-fold at week 8 compared to previous weeks, remained elevated at week 12 and further

decreased at later weeks (Fig. 4). Average LOX is 13.5 nmol CO_2 produced/g liver*min; resulting in LKR and SDH activities 18 and 31-fold higher than the predicted rate of oxidation, respectively.

Alternative routes of lysine degradation include LAO- and LO-dependent pathways. There was a significant effect of week ($p < 0.0001$), but not strain on LAO activity. L-amino acid oxidase activity increased from week 0 to week 4 and decreased with later ages (Fig. 5a). Average L-amino acid oxidase activity across weeks was 0.7 nmol alpha-keto-epsilon-aminocaproic acid produced/g liver*min. Lysyl oxidase activity was only detected in weeks 0 and 1 in the production cycle (Fig. 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. 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 (Deves *et al.*, 1998) and poultry (Humphrey *et al.*, 2004) species. CAT1 mRNA expression was detected in the liver and was affected by week ($p = 0.0053$), but not strain or week*strain. CAT1 mRNA expression was reduced 50% by week 2 compared to previous weeks and did not change thereafter (Fig. 6a).

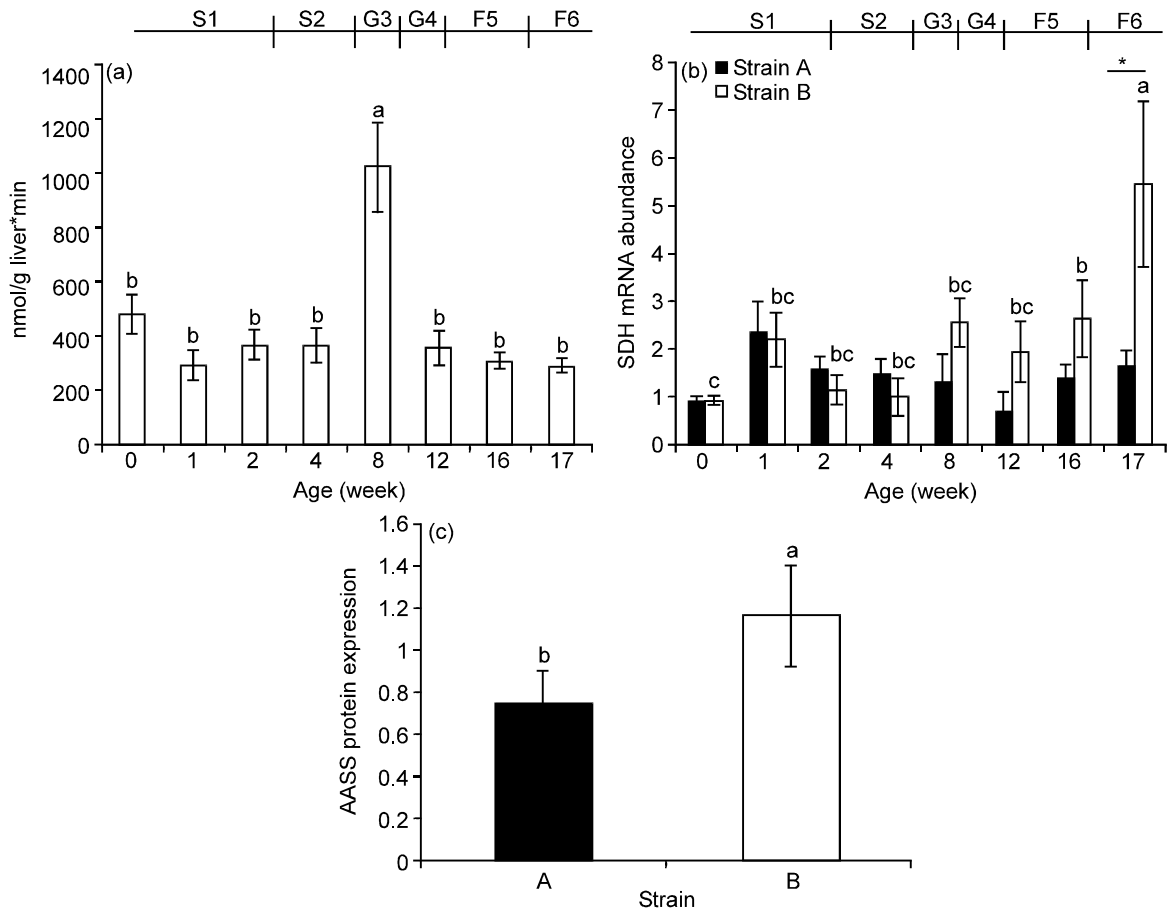


Fig. 3: SDH Activity and mRNA Abundance. Saccharopine Dehydrogenase (SDH) activity and mRNA abundance in turkey liver. Enzyme activities and mRNA abundance measured in duplicate (n = 8 birds/strain/week). Data are expressed as mean ± SEM. Top bar represents diets consumed at each time point, including starter (S), grower (G) and finisher (F) diets. (A) 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. Differences in superscripts indicates a significant difference ($p = 0.008$), (B) 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 ($p = 0.046$) and is denoted by an asterisk. There was also an effect of production cycle week on SDH activity in the strain A birds and differences in superscripts indicate a significant difference ($p = 0.0015$). There was also an interaction between week and strain ($p = 0.016$) and (C) Panel C alpha-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, but not week or week*strain effect was detected, so data were pooled across weeks ($p < 0.001$)

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 ($p = 0.002$), strain effect ($p < 0.02$) and a week by strain interaction ($p < 0.014$) on CAT2 mRNA abundance (Fig. 6b). The most pronounced strain effect is at week 12, where strain B had a 430% increased relative expression of CAT2

compared to strain A. 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

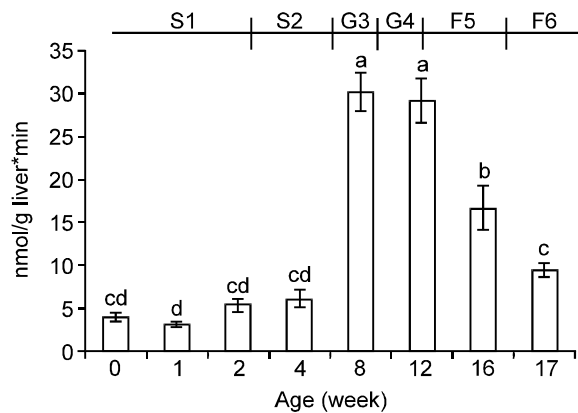


Fig. 4: Lysine Oxidation. Lysine oxidation was measured as $^{14}\text{CO}_2$ produced from $[1\text{-}^{14}\text{C}]$ L-lysine and data are expressed as nmol CO_2 produced/g liver*min ($n = 8$ birds/strain/week). Top bar represents diets consumed at each time point, including starter (S), grower (G) and finisher (F) diets. No effect of strain or week*strain interactions were detected ($p > 0.05$), therefore data were analyzed only for week effect. Differences in superscripts indicates a significant difference ($p < 0.001$)

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 LAOA ($p < 0.01$, $r = -0.314$) and CAT1 mRNA abundance ($p < 0.01$, $r = -0.27$). There was a trend for LAOA to be negatively correlated with SDH mRNA abundance ($p = 0.059$, $r = -0.22$). The negative correlation between the saccharopine-dependent pathway and LAOA 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 (Evans *et al.*, 2014) by week. 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, LAOA 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

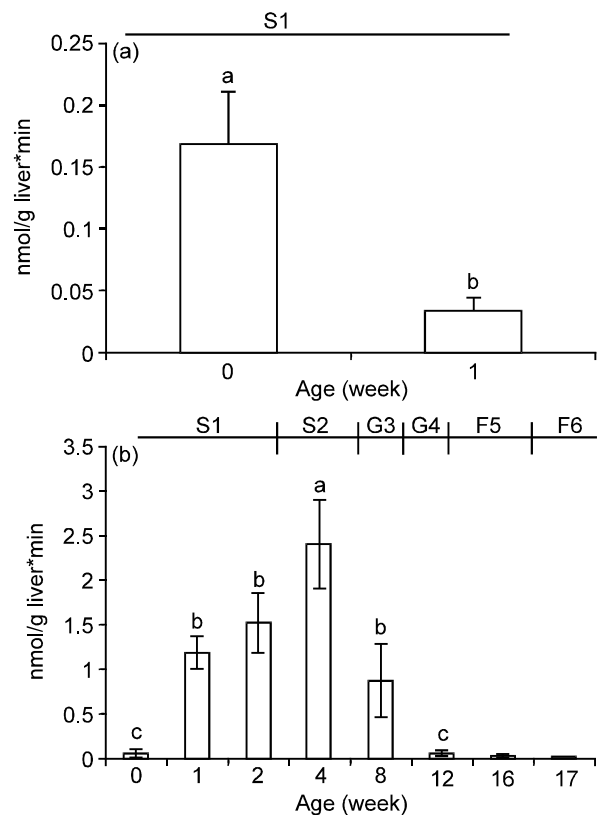


Fig. 5: Alternative Pathways of Lysine Degradation. Alternative pathways in lysine degradation were assessed. Data are expressed as mean+SEM ($n = 8$ birds/strain/week). Top bar represents diets consumed at each time point, including starter (S), grower (G) and finisher (F) diets. (A) Panel A L-Amino acid oxidase activity was measured as the lysine-dependent formation of alpha-keto-epsilon-aminocaproate semi-barbazone, data are expressed as nmol alpha-keto-epsilon-aminocaproate semi-barbazone 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. Differences in superscripts indicates a significant difference ($p < 0.001$) and (B) 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 ($p < 0.05$)

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

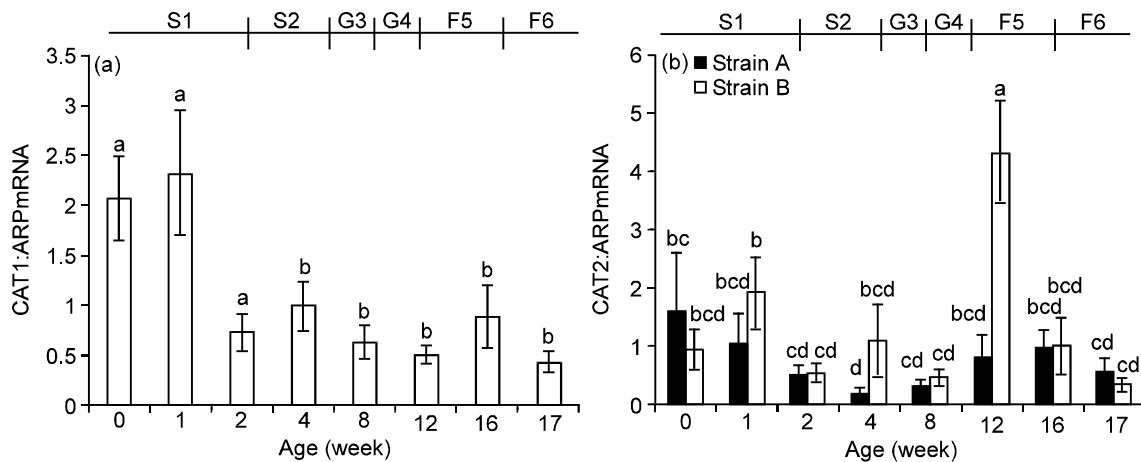


Fig. 6: Abundance of Cationic Amino Acid Transporter (CAT) mRNA. CAT mRNA is expressed as a ratio to the housekeeping gene Acidic Ribosomal Protein (ARP). Data are expressed as mean+SEM (n = 8 birds/strain/week). Top bar represents diets consumed at each time point, including starter (S), grower (G) and finisher (F) diets. (A) Panel A CAT1 mRNA abundance. No differences between strains were detected ($p > 0.05$), therefore data were analyzed for week effect. Differences in superscripts indicate a significant difference ($p = 0.0053$) and (B) Panel B CAT2 mRNA abundance. There was an effect of week ($p = 0.0022$), strain ($p = 0.02$) and a week by strain interaction ($p = 0.014$)

($p < 0.05$, $r = -0.60$) and week 16 ($p < 0.06$, $r = -0.59$) and between FCR and CAT2 mRNA abundance at week 12 ($p < 0.05$, $r = -0.61$).

DISCUSSION

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 an important mode of regulation. Kiess *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; consistent with post-translational modification.

The LKR and SDH activity findings are similar to Blemings *et al.* (1998) 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 may also be an important control point in lysine 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 dietary control. Glucagon (Scislowski *et al.*, 1994) and diets high in protein (Blemings *et al.*, 1998; Kiess *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.

Our data are in agreement with Wang *et al.* (1973) who also showed that LAAO was present in the liver of poultry and is active towards free lysine. However, LAAO activity was only 0.21% of the activity of the enzymes in the saccharopine-dependent pathway. Relative to the LO pathway, the average 0.1 nmol H_2O_2 produced/g liver*min 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.

Conclusion: 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; Kiess *et al.*, 2008).

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