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Lysine mediation of neuroendocrine food regulation in guinea fowl

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ABSTRACT In poultry, obesity is partly influenced by food intake, and is increasingly becoming a nationwide problem. Hypothalamic food intake mechanisms are involved metabolically and neurologically via two peptide hormones, leptin and ghrelin, and the amino acid glutamate, which is enzymatically derived from lysine metabolism. We hypothesize that lysine homeostasis mediates regulation of feed intake and performance characteristics via the brain–liver axis through glutamate sensing. The objective was to examine the effects of lysine homeostasis in avian food regulation and performance through neuroendocrine signaling. One-day-old male French Guinea fowl (GF) keets (n = 270) were weighed and randomly assigned to 5 dietary treatments (0.80%, 0.86%, 0.92%, 1.10% control, and 1.22% lysine) in 3 replicates. At 4 and 8 wk of age 20% of experimental birds were randomly selected, weighed and euthanatized. The liver, pancreas, and hypothalamus were excised, snap frozen in liquid nitrogen and stored at –80°C until use. Tissue mRNA was extracted and cDNA synthesized for qPCR assays. Lysine at 0.80 and 0.86% hindered growth, development of digestive organs, expression of brain and liver glutamate and leptin receptors, and caused high mortality in GF. The fold change for metabotropic glutamate receptor I was lower ($P < 0.05$) in liver and higher in brain at 0.86 and 0.92% than the control (1.10%) and 1.22% lysine. The 1.22% lysine exhibited highest expression of ionotropic glutamate receptor, while brain ghrelin receptor expression was highest at 0.86 and 0.92% lysine. Therefore, dietary lysine concentration may influence signaling pathways regulating food intake in brain-liver axis via glutamate synthesis.

Key words: lysine food regulation, glutamate, leptin and ghrelin, guinea fowl, obesity

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INTRODUCTION

Obesity is a nationwide and global health problem that is projected to affect 2.3 billion adults by 2015 according to the World Health organization (Suzuki et al., 2010). According to a 2009 report on health policies, obesity accounts for 20 to 30% of the healthcare costs in the United States. On the other hand, obesity has been shown to correlate with cardiovascular complication, stroke, cancer, type 2 diabetes, and neurological and sleep disorders (Suzuki et al., 2010). Consumption of animal products, such as poultry, containing excess amounts of fat may be, in part, a contributing factor to the increasing incidence of human obesity and other associated health risks. Poultry is a major source of human diet and therefore minimizing the excess fat deposition will minimize consumption of such fat in diets.

Feed intake is influenced by behavioral, genetic, and physiological states (Berthoud, 2002; Kaiya et al., 2009; Richards et al., 2010; Fang et al., 2014; Mostafa et al., 2014). Extensive research has focused on all three factors that may have an impact on human health, but the major emphasis has been placed on the regulatory functions of the hypothalamus. Appetite monitoring gut and peptide hormones such as leptin and ghrelin, respectively, have been shown to be involved in the regulation of food intake. Although leptin hormone production in chicken has not been confirmed, avian genomes contain a highly conserved leptin receptor (Adachi et al., 2008; Prokop et al., 2014) which is capable of activating the Janus kinase (JAK)-signal transducers and activation of transcription (STAT) signal transduction and other pathways that may be associated with nutrient sensing and metabolism (Ohkubo et al., 2007; Hen et al., 2008; Ohkubo and Adachi, 2008). Adachi et al. (2008) reported that leptin receptor expressed in chicken tissues may be capable of binding endogenous ligand as well as exogenous mammalian leptin. The leptin receptor gene may also be responsive to divergent extremes of nutrient supply.

In poultry, the balance of sustenance from the gastrointestinal tract and energy expenditure is maintained by the balance of endocrine factors such as the anorexigenic leptin and the orexigenic ghrelin (Richards and Weglarz, 2007). Furthermore, proteins and amino acids have been shown to control food intake in rats

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(Tome, 2004). Tome, in his paper entitled "Protein, amino acids and the control of food intake," discusses that the influence of internal proteins and amino acids in food intake is based on three known conditions: 1) an antagonistic response to diets depleted or bereft of protein or lacking in slightly one essential amino acid, 2) adjustments in the body to limited amino acid or protein consumption by increasing protein dietary intake, and 3) satiety provided by protein, which lowers the need for a high-protein diet (Tome, 2004). Tome's report showed that rats have been used to investigate the role of nutritional imbalance from a high fat diet and its effects on amino acids. One amino acid in particular is lysine, which is a dietary supplement in mammalian and avian diets and is involved in the synthesis of connective tissue, and carnitine. Also, lysine plays a role in fatty acid metabolism and one of its metabolites is glutamate. Glutamate arises from the metabolism of lysine to saccharopine and is catalyzed by glutamate dehydrogenase. Recently, it has been shown that glutamate plays a role in energy homeostasis and food intake (Delgado, 2013).

Most of the research on glutamate and energy homeostasis has been done on the mouse (Bradbury et al., 2005; Jinap and Hajeb, 2010) whereas several studies on chickens have also been reported (Wang and Nesheim, 1972; Kobayashi et al., 2011). Genetic variations among species are usually associated with differences in endocrine-hypothalamic mediated feed intake and energy metabolism and are an important resource in elucidating the mechanisms surrounding feed intake and energy metabolism in poultry (Swennen et al., 2007; Huang et al., 2008). As an alternative animal protein source to chickens, the guinea fowl is an avian species that are reared in various parts of the world including Africa, Europe and the United States (Nahashon et al., 2006). Previous reports indicate that genetic similarity between the Pearl Grey guinea fowl and the Single Comb White Leghorn chickens is as low as 31.3% (Nahashon et al., 2010). Earlier, Kilonzo-Nthenge et al. (2008) reported that the guinea fowl exhibited significantly higher ($P < 0.05$) resistance to pathogenic microbial infection than chickens. These differences among species provide various dimensions into the mechanisms of feed intake in food animals and especially poultry. Therefore, the broad hypothesis in this study is that in the guinea fowl, lysine homeostasis mediates regulation of feed intake and performance characteristics via the brain-liver axis through glutamate sensing.

MATERIALS AND METHODS

Birds and Dietary Treatments

All animal studies adhered to the institutional animal care and use committee's (IACUC) guidelines and were approved by IACUC. Two hundred and seventy male 1-day-old guinea keets of the pearl gray variety were obtained from Ideal Poultry Breeding Farms (Cameron, Texas). These birds were randomly assigned to four dietary treatments in a completely randomized design. The dietary treatments comprised lysine levels of 0.80%, 0.86%, 0.92%, 1.10%, and 1.22% (Table 1). The control diet contained the 1.10% lysine, which was estimated from lysine requirement of Single Comb White Leghorn chickens (NRC, 1994). The starter diets were isocaloric (3,100 Kcal/kg ME) and isonitrogenous (23% CP) and were fed from hatch to 4 wk of age (WOA). During the growing period (5 to 8 WOA), the lysine concentrations were maintained the same in the four dietary treatments; however, the grower rations contained 3,150 Kcal/kg ME and 21% CP (Table 2). Bell and Weaver (2002) suggested that in order for birds to attain optimum growth and at economical scale, key nutrients such as energy and protein must be phased to justify requirement and to produce the bird at the lowest cost possible. The dietary concentrations of ME and CP fed to the Pearl Grey guinea fowl in this study were previously reported by Nahashon et al. (2006). Each dietary treatment was replicated 3 times with 18 birds per replicate. Feed was provided in mash form and both feed and water were provided at free choice throughout the study period. Mortality was recorded and monitored daily as it occurred.

Table 1. Composition of Experimental Rations (%) fed at 0–4 Wk of Age.

Lysine %	1.10	0.80	0.86	0.92	1.22
Feed Ingredient					
Corn (8% CP)	48.95	50.67	50.67	50.1	49.21
Soybean meal (48% CP)	29.4	14.1	17.02	19.8	30.81
Corn Gluten meal (60% CP)	7.12	17.6	15.8	13.7	6
Wheat middlings	5.14	10.36	8.86	8.27	4.4
Alfalfa meal (17% CP)	1	1	1	1	1
Poult. Blend. Fat	4.5	2.3	2.7	3.1	4.62
Dical. Phosphate	1.74	1.74	1.75	1.75	1.73
Limestone flour	1.5	1.45	1.40	1.3	1.45
D, L-Methionine (98%)[2]	0.1	0.03	0.05 0.0	0.06	0.12
L-Arg	0	0	0.2	0.23	0
L-Thr	0	0	0	0.12	0
L-lle	0	0	0	0	0
L-Val	0	0	0	0.02	0
L-Lys HCl	0	0	0	0	0.11
Salt	0.3	0.3	0.3	0.3	0.3
Vitamin-Mineral premix[1]	0.25	0.25	0.25	0.25	0.25
Sand	0	0	0	0	0
Calculated analyses					
Crude Protein, %	23	23	23 23 23	23	23
ME (Kcal/kg diet)	3,100	3100	3,100	3,100	3,100
Calcium, %	1.00	1.04	1.00	1.00	1.00
Total Phosphorus, %	0.72	0.72	0.72	0.72	0.72
Methionine, %	0.50	0.42	0.50	0.50	0.50
Methionine + cysteine, %	0.90	0.92	0.90	0.90	0.90
Lysine, %	1.10	0.80	0.86	0.92	1.22

[1]Provided per kg of diet: retinyl acetate, 3,500 IU; cholecalciferol, 1,000 ICU; DL-α-tocopheryl acetate, 4.5 IU; menadione sodium bisulfite complex, 2.8 mg; vitamin B_{12}, 5.0 mg; riboflavin, 2.5 mg; pantothenic acid, 4.0 mg; niacin, 15.0 mg; choline, 172 mg; folic acid, 230 mg; ethoxyquin, 56.7 mg; manganese, 65 mg; iodine, 1 mg; iron, 54.8 mg; copper, 6 mg; zinc, 55 mg; selenium, 0.3 mg.

[2]Degussa Corporation, Kennesaw, GA.

Table 2. Composition of experimental diets (%) fed at 5 to 8 wk of age.

Lysine %	1.10	0.80	0.86	0.92	1.22
Feed Ingredient					
Corn (8% CP)	56.73	64.11	57.78	61.14	55.85
Soybean meal (48% CP)	31	16.4	17.1	22.2	34.28
Corn Gluten meal (60% CP)	2.5	13	12	8.9	0.13
Wheat middlings	0.91	0.98	6	1	0.2
Alfalfa meal (17% CP)	1	1	1	1	1
Poult. Blend. Fat	4.55	1.1	2.7	2.45	5.2
Dical. Phosphate	1.3	1.35	1.35	1.33	1.27
Limestone flour	1.35	1.4	1.4	1.37	1.33
D, L-Methionine (98%)[2]	0.11	0.03	0.04	0.06	0.12
L-Arg	0	0	0.04	0	0
L-Thr	0	0	0	0	0
L-lle	0	0	0	0	0
L-Val	0	0	0	0	0
L-Lys HCl	0	0	0.04	0	0.07
Salt	0.3	0.3	0.3	0.3	0.3
Vitamin-Mineral premix[1]	0.25	0.25	0.25	0.25	0.25
Sand	0	0	0	0	0
Calculated analyses					
Crude Protein, %	21	21	21 21	21	21
ME (Kcal/kg diet)	3,150	3150	3,150	3,150	3,150
Calcium, %	0.90	0.90	0.90	0.90	0.90
Total Phosphorus, %	0.60	0.60	0.60	0.60	0.60
Methionine, %	0.45	0.45	0.45	0.45	0.45
Methionine + cystine, %	0.83	0.83	0.83	0.83	0.83
Lysine, %	1.10	0.80	0.86	0.92	1.22

[1]Provided per kg of diet: retinyl acetate, 3,500 IU; cholecalciferol, 1,000 ICU; DL-α-tocopheryl acetate, 4.5 IU; menadione sodium bisulfite complex, 2.8 mg; vitamin B_{12}, 5.0 mg; riboflavin, 2.5 mg; pantothenic acid, 4.0 mg; niacin, 15.0 mg; choline, 172 mg; folic acid, 230 mg; ethoxyquin, 56.7 mg; manganese, 65 mg; iodine, 1 mg; iron, 54.8 mg; copper, 6 mg; zinc, 55 mg; selenium, 0.3 mg.

[2]Degussa Corporation, Kennesaw, GA.

Management of Experimental Birds

At 1 d of age, experimental birds were weighed individually, wing banded and randomly assigned to electrically heated, temperature controlled Petersime^{TM} battery brooders (Petersime brooders model 2SD12, Petersime Incubator Co., Gettysburg, OH) equipped with raised wire floors for the first 4 WOA. The battery cages measure 99 × 66 × 25 cm and each housed 18 birds. The temperature was kept at 32°C for the first wk and was gradually reduced by 2.8°C to a steady temperature of 23.9°C, and at this point on no artificial heat was provided to the experimental birds. At five WOA the guinea keets were transferred into growing batteries, which were not supplied with supplemental heating. However constant room temperature was maintained at 21°C. The growing cages measured 163 × 69 × 33 cm and each housed 7 to 8 birds from 5 to 8 WOA. Birds were provided a 23-h light regimen from hatch to 8 WOA. Ventilation within the brooders and growing cages were maintained by thermostatically controlled exhaust fans.

Performance Analysis

Growth performance was measured by average daily gain (ADG) and feed: gain (F:G) ratio. Body weight of each guinea fowl from which average daily gain was determined, and the amount of feed intake (FI) by each individual bird and replicate were measured weekly. The ADG and F:G ratios were calculated weekly. The amount of feed given to each replicate in the treatment groups was recorded and at the end of each wk the remainder of the feed was deducted from the aggregated amount of feed appropriated throughout the wk. The F:G ratios were calculated by dividing the daily FI by the ADG.

Tissue Collection, Analyses, and Sample Preparation

At 4 and 8 WOA, 20% of the experimental birds were randomly selected from each replicate. The birds were euthanatized by cervical dislocation and immediately whole heads, liver, and pancreas were excised, weighed and flash-frozen in liquid nitrogen. All tissue samples (2 g) were stored at –80°C until assayed for gene expression. The hypothalami and surrounding tissue were excised by micro-dissection from the frozen heads, snap frozen in liquid nitrogen, and then transferred into –80°C freezer until assayed for gene expression.

Total RNA Extraction and cDNA Synthesis

The total RNA was extracted from tissues of 60 randomly selected experimental birds (28 and 32 at 4 and 8 WOA, respectively). Briefly, 10 mg of each liver and pancreas tissues, and the hypothalami and its surrounding tissue were added to 200 μL of Qiazol within a single Eppendorf tube and homogenized using a Fisher tissue homogenizer® (Fisher Scientific, Pittsburgh, PA). mRNA was extracted using the Bio-Rad Total RNA Extraction Kit® (Bio-Rad, Hercules, CA) and the Rneasy® RNA extraction kits (Qiagen Corporation, Valencia, CA). RNA was reverse transcribed into cDNA using the Qiagen Quantitect Reverse Transcription kit®. RNA and cDNA were tested for quantity (concentration) and quality (contamination) using the Nanodrop^{TM} Spectrophotometer and agarose gel electrophoresis.

Gene Expression Assays

A. Primer Design Sequences for the guinea fowl leptin receptor, ionotropic receptor, and metabotropic glutamate I receptor were extracted from a contiguous database and compiled after de novo assembly of the guinea fowl pancreas transcriptome using Trinity software (GenBank id: 3052, 105945 and 174385, respectively, and BLASTX ID: XM_420582.4, NM_001113186.1, and XM_004941942.1, respectively). These were the only receptors whose sequences for the guinea fowl were available from a contiguous database compiled after de novo assembly of the guinea fowl transcriptome by our laboratory. Oligonucleotide primer for

the ghrelin receptor Growth hormone secretagogue receptor 1A (GHSR1A) was designed using National Center for Biotechnology Information (NCBI's) Primer3-Blast software. Since the Guinea fowl sequence for this locus was unavailable in GenBank, Clustal Omega alignment was used to identify the most highly conserved regions of the locus among chicken, human, mouse, guinea pig and cattle. Highly conserved sequences were used as inputs to design primers. Each primer was designed to have a minimum guanosine-cytosine (GC) content of 45 to 60%.

B. Quantitative Real-time Polymerase Chain Reaction (QT-PCR) Two-step qPCR reactions were prepared using QiaGen's QuantiTect® SYBR® Green PCR Kit (Qiagen Corporation, Valencia, CA) in a final volume of 20 μL. PCR primers were designed using Primer Express 2.0 (Applied Biosystems, Carlsbad, CA) and optimized using the Eppendorf 5531 Master Cycler Gradient Thermo Cycler® (102 Motor Parkway, Hauppauge, NY) to a final concentration of 0.8μM. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as the internal control. The primers were validated using melting curve analysis of each qPCR reaction. Sequences of the primers are available in Supplementary Table S1. The cDNA was diluted in TE buffer to 10 ng/μL and confirmed after dilution with a NanoDrop Spectrophotometer. Amplification was carried out using 40 ng cDNA in Applied Biosystems 7000® Prism Sequence Detection System (Applied Biosystems, Carlsbad, CA) with the following cycling parameters: initial denaturation at 95°C for 15 minutes; followed by 40 cycles of denaturation at 94°C for 15 seconds, primer annealing at 58°C for 30 seconds; and primer extension at 72°C for 30 seconds. Data was presented as threshold cycle (Ct) relative to the internal control Glyceraldehyde- 3- phosphate dehydrogenase (GAPDH). Fold change equals $2^{-\Delta\Delta C_T}$, where $-\Delta\Delta C_T$ = [C_T gene of interest - C_T internal control Sample A] – [C_T gene of interest- C_T internal control sample B]. Fold change was calculated using the comparative C_T method discussed by Livakand Schmittgen (2001; Schmittgen and Livak, 2008). qPCR products were visualized via 2% agarose gel electrophoresis.

Statistical Analysis

Percent data was transformed into Log form prior to analysis. The data was back transformed for tabulation and discussion. All variables were analyzed as repeated measurements. Growth performance and gene expression data were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) of the SAS® software version 9.3 (SAS Institute, 2011) as completely randomized design with dietary treatments as main effects. The statistical model used was: $Y_{ijk} = \mu + L_i + R_{ij} + \gamma_{ijk,}$ where Y_{ijk} = response variables from each individual replication, μ = the overall mean; L_i = the effect of dietary Lysine; R_{ij} = the inter-experimental unit (replications) error term; and γ_{ijk} = the intra-experimental unit error term. Differences in mortality among dietary treatments were analyzed using the chi-square method. Least significant difference and the *t*-test comparisons were made between treatment means for main effects when there was a significant F-value ($P < 0.05$; DF = 53, 6 and 7 for growth performance, and tissue weight and gene expression at 4 and 8 wk of age, respectively). Correlations were determined using the correlation procedure (Proc. Corr.) of SAS (SAS® software version 9.3, SAS Institute, 2011).

RESULTS

Average Daily Gains and Feed to Gain Ratios

Average daily gain (ADG) of pearl grey guinea keets fed diets varying in lysine concentrations at 1 to 4 WOA are presented in Figure 1A. At the beginning of this experiment (wk 1), differences in mean ADG among replicates and dietary lysine concentrations (0.80, 0.86, 0.92, 1.10, and 1.22%) were not significant for all five dietary treatments. The diets with lower lysine concentrations contained 0.80, 0.86 and 0.92% lysine. The highest amount of lysine was 1.22% while the control diet contained 1.10% lysine. Birds fed the 0.80% lysine diet exhibited ADG that were significantly lower ($P < 0.05$) that those of other dietary treatments at 1 to 4 WOA. Although the ADG of birds fed diets containing 0.86 and 0.92% lysine were not statistically different ($P > 0.05$) at 1 to 3 WOA, birds on the 0.92% lysine had higher ADG ($P < 0.05$) than those fed diets containing 0.86% lysine. The highest ADG were observed in birds fed the control (1.10% lysine) and 1.22% lysine diets. These ADG were statistically higher ($P < 0.05$) than those of other dietary lysine concentrations. Birds fed the 1.22% lysine exhibited higher ADG ($P < 0.05$) than those fed the 1.10% lysine diets, the control.

The poor performance of birds fed diets containing the 0.80% lysine when compared to other dietary treatments was also associated with significantly high mortality ($P < 0.05$) which was about 95% and significantly higher than that of other dietary treatments (Figure 2). There were significant differences ($P < 0.05$) in mortality of birds fed the 0.92 and 1.10% lysine when compared to those fed 0.86% lysine diets. At 3 to 4 wk of age birds consuming diets containing 0.80% Lysine exhibited impaired physical characteristics such as malnutrition, impaired growth and muscle development, poor feather development, significant reduction in ADG and ultimately high mortality. Due to high mortality in birds fed the 0.80% lysine diets at 1 to 4 wk of age, this treatment was excluded from the study during wk 5 to 8.

Figure 1B shows the ADG of guinea fowls fed the various dietary lysine concentrations at 5 to 8 wk of age. At wk 5 birds fed diets containing either the 0.86

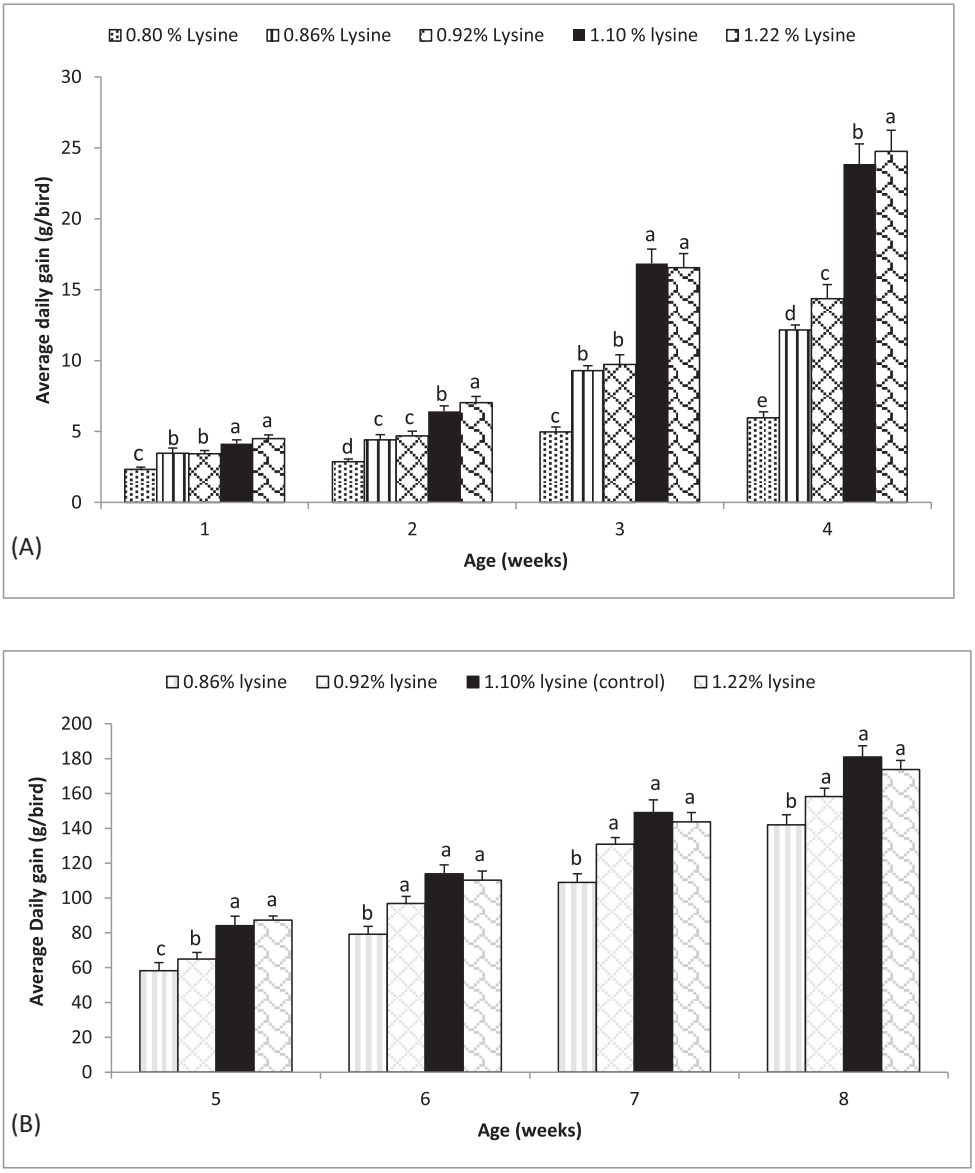

Figure 1. Average daily gain of French guinea fowl broilers fed diets varying in lysine concentrations at 1–4 wk of age (A) and 5–8 wk of age (B). [a,b]Within individual charts, bars representing average daily gain with no common superscript differ significantly ($P < 0.05$).

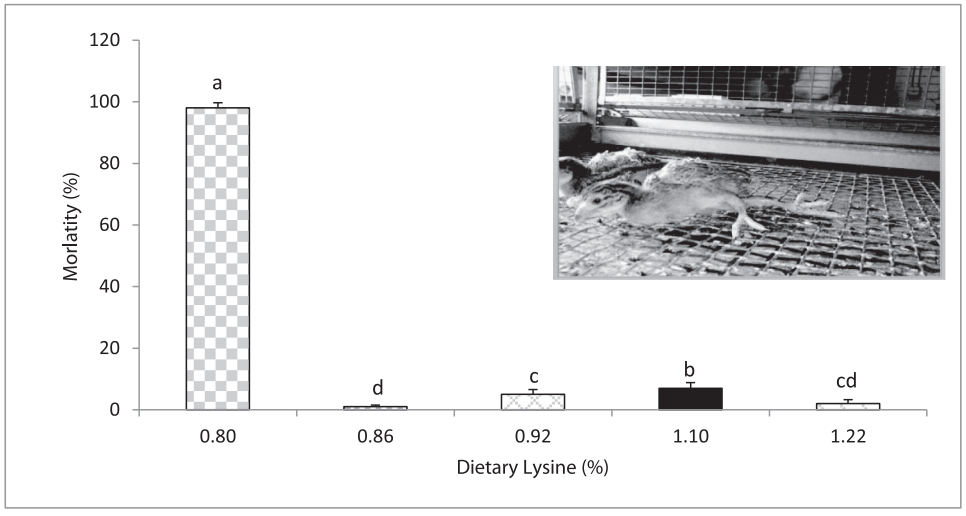

Figure 2. Cumulative percent mortality of French guinea fowl broilers fed varying concentrations of dietary lysine from hatch to 8 wk of age. [a,b]Within individual charts, bars representing mean percent mortality with no common superscript differ significantly ($P < 0.05$).

Table 3. Feed: Gain of French guinea fowl broilers fed diets varying in Lysine concentrations.

	Wk of age								
Average Lysine-(g feed intake/g daily gain)	1	2	3	4	5	6	7	8	
0.80	2.18^{a}	2.67^{a,b}	3.15^{a}	2.78^{a}	3.20^{a}	ND	ND	ND	ND
0.86	1.97^{a,b}	2.88^{a}	2.65^{b}	2.36^{b}	3.07^{a}	3.18^{a}	2.84^{a}	2.80^{b}	2.71^{a}
0.92	1.92^{b}	2.47^{b}	2.41^{b,c}	2.02^{c}	2.30^{b}	2.54^{b}	2.47^{b}	3.59^{a}	2.46^{b}
1.10	1.44^{c}	2.22^{c}	1.98^{c}	1.85^{d}	2.17^{c}	2.69^{b}	2.65^{a,b}	3.50^{a}	2.31^{c}
1.22	1.55^{c}	2.16^{c}	2.05^{c}	1.71^{d}	2.37^{b}	3.11^{a}	2.51^{b}	2.99^{b}	2.30^{c}
SEM	0.07	0.11	0.10	0.07	0.09	0.12	0.20	0.13	0.11

ND = not determined due to high mortality

$^{a–d}$Means within columns with no common superscript differ significantly ($P < 0.05$).

SEM = standard error of mean

or 0.92% lysine concentration showed significantly lower ADG ($P < 0.05$) when compared with the other dietary treatments. During wk 6 to 8, ADG of the birds fed the 0.86% lysine remained significantly lower than all other dietary treatments, however differences in ADG of birds fed the 0.92, 1.10, and 1.22% lysine concentrations were not significant ($P < 0.05$).

Mean feed to gain (F:G) of the French guinea fowl fed diets containing varying lysine concentrations are presented in Table 3. The F:G ratios of birds fed the 1.10 and 1.22% lysine diets were significantly lower ($P < 0.05$) than those of other dietary treatments. Highest F:G ratios were exhibited by birds fed the 0.80 and 0.86% lysine diets. While differences in F:G ratios of birds fed the 1.10 and 1.22% lysine diets were not significant in most part, they were significantly lower than those of birds fed the 0.92% lysine diets.

Organ Weights

Organ weight of French guinea fowl fed diets varying in lysine concentrations are presented in Figure 3. During the fourth wk of age, the weight of guinea fowl liver was significantly lower ($P < 0.05$) at the lowest concentration of dietary lysine (0.86%) when compared to the control (1.10%), 0.92, and 1.22% lysine concentrations Figure 3(I). The mean weight of the liver for the 0.92% lysine diets was similar to the control and the excess lysine (1.22%). During the fourth wk of age, the weight of the pancreas of birds fed the lower dietary lysine concentrations (0.86 and 0.92%) was slightly lower in than that of birds fed higher concentrations of lysine (1.10% or control and 1.22%); however, these differences were not significant ($P > 0.05$). At eight wk of age change in dietary lysine concentration did not significantly alter liver weight (Figure 3III). The fourth-wk pancre-

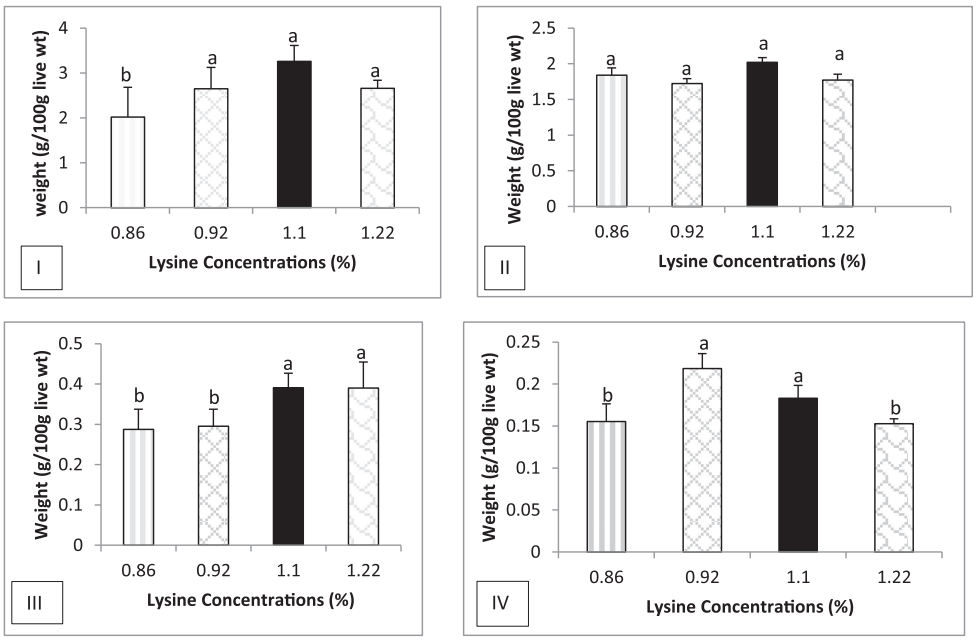

Figure 3. Relative weight of liver and pancreas of French guinea fowl broilers fed diets containing varying concentrations of lysine from hatch to 8 wks of age (WOA). The liver and pancreas were weighed at 4 WOA (I and III, respectively) and 8 WOA (II and IV, respectively). ^{a,b}Within individual charts, bars representing mean organ weights with no common superscripts differ significantly ($P < 0.05$).

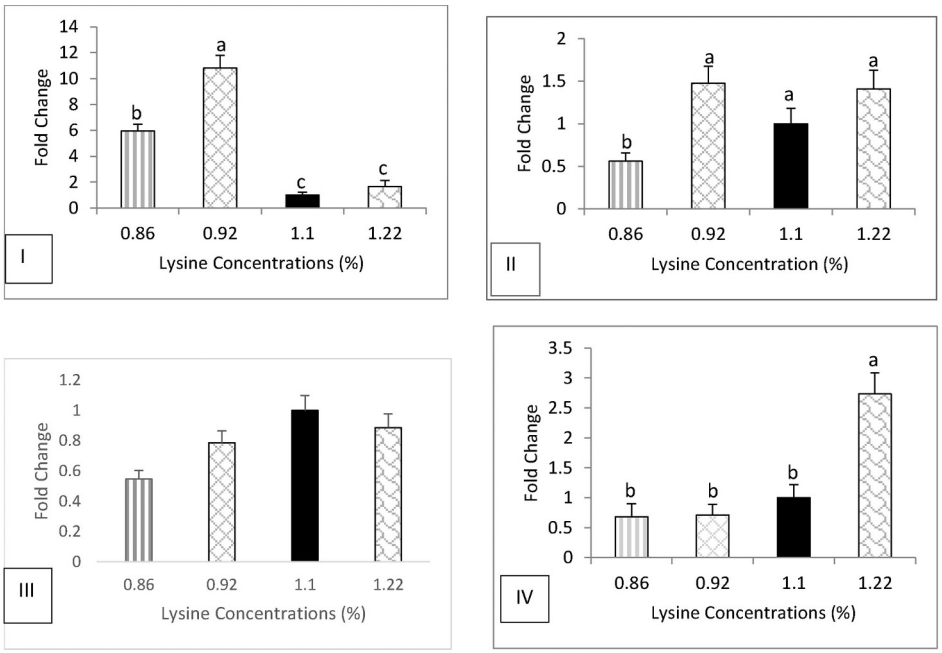

Figure 4. Expression of brain metabotropic glutamate receptor I (I), brain ionotropic glutamate receptor (II), liver metabotropic glutamate receptor I (III) and liver ionotropic glutamate receptor (IV) of French guinea fowl broilers fed diets with varying concentrations of lysine. [a,b]Within individual charts, bars representing mean gene expression with no common superscripts differ significantly ($P < 0.05$).

atic weight of the guinea fowl were significantly higher ($P < 0.05$) for birds fed the 1.10 and 1.22% dietary lysine than those fed the 0.86 and 0.92% lysine. However, differences in mean pancreatic weights of guinea fowl fed diets containing the 0.86% and 0.92% lysine were not significant ($P < 0.05$). A similar trend was observed between birds fed the 1.10 and 1.22% lysine. On the other hand, at 8 WOA, pancreatic weight was significantly lower ($P < 0.05$) in birds fed diets containing 0.86% lysine and 1.22% lysine than those fed the 0.92 and 1.10 (control) diets. However, the 0.92 lysine concentrations was not different from the control; likewise, mean pancreatic weights of birds fed the 0.86% lysine diets were not statistically different from those of birds fed the 1.22% lysine diets (Figure 3IV).

Gene Expression Assays

Gene expression was assayed by two-step real-time quantitative PCR using 4 specific primers: metabotropic glutamate receptor I, ionotropic glutamate receptor, leptin receptor, and ghrelin receptor (Supplementary Table S1). The expression of brain and liver metabotropic and ionotropic glutamate receptors is presented in Figure 4. Varying dietary lysine concentrations alter the expression of these receptor genes in French guinea fowl broilers. Birds that received the diet with lowest amount of lysine (0.86%) showed a significant reduction (4-fold difference) in expression of brain metabotropic glutamate receptor I (BmGluR1) when compared with birds fed the 0.92% lysine diets (Figure 4 [I]). Also the expression of BmGluR1 was significantly higher ($P < 0.05$) in the 0.92% lysine diets than the control (1.10% lysine) and the 1.22% lysine diets (12.5 and 13.5-fold difference, respectively). As shown in Figure 4 (III) the expression of liver metabotropic glutamate receptor I (LmGluR1) gene was not altered by changes in dietary lysine concentrations. When compared to the other treatments the LmGluR1expression of the 0.86% lysine treatment was similar to the control (1.10%) and the excess (1.22% lysine) diet. Figure 4 (II) demonstrated that varying dietary lysine concentrations alter the expression of these receptor genes in French guinea fowl broilers. While fold-change differences in birds fed the 0.92% lysine diets and those fed the control (1.10% lysine) and the 1.22% lysine diets were not significant ($P > 0.05$), they were statistically higher ($P < 0.05$) than those of birds fed the 0.86% lysine diets (2 to 3-fold difference). On the other hand, the liver ionotropic glutamate receptor (LiGluR) displayed a higher gene expression at 1.22% lysine than all other lysine concentrations (2.75 to 3.90-fold difference) as evidenced in Figure 4 (IV). Differences in expression of the LiGluR gene of birds fed diets containing the 0.86, 0.92 and 1.10% lysine were not significant.

The expression of the brain leptin receptor (BLepR) gene of birds fed diets containing the 0.92% lysine and the control were not different (Figure 5 [I]). However, the 0.86% lysine diets had 2.5-fold reduction in BLepR expression when compared to the control and the excess dietary lysine (1.22%). Also the GF fed the 1.22% lysine showed similar gene expression profile when compared to those fed the 0.86% lysine diets. It was also evident that birds fed the control and the 0.92% lysine diets exhibited a 10-fold increase ($P < 0.05$) in expression of the BLepR when compared to birds fed the 1.22%

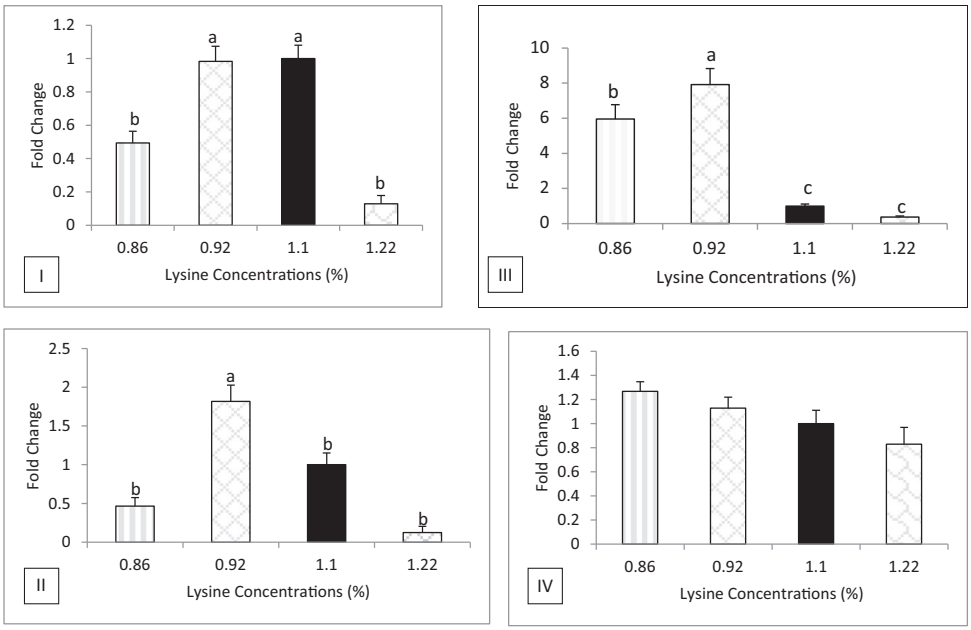

Figure 5. Expression of brain leptin and ghrelin receptors (I and II, respectively) and liver ghrelin and leptin receptors (III and IV, respectively) of French guinea fowl broilers fed diets with varying concentrations of lysine. [a,b]Within individual charts, bars representing mean gene expression with no common superscripts differ significantly ($P < 0.05$).

lysine diets. On the other hand, the expression of the liver leptin receptor (LLepR) of birds fed the 0.92% lysine was significantly higher ($P < 0.05$) than that of birds on the 0.86, 1.10, and 1.22% lysine (Figure 5 [II]). The fold change difference between the 0.92% lysine and the 0.86, 1.10, and 1.22% lysine was about 2, 1.5, and 2.5, respectively. However, the fold change differences among the 0.86, 1.10 and 1.22% lysine concentrations were not significant ($P > 0.05$). The 0.92% lysine concentration increased the expression of the brain ghrelin receptor (BGhrR) gene by about an 8-fold difference [Figure 5 (III)]. A similar trend was observed with the 0.86% lysine exhibiting a 6-fold change in the expression of the BGhrR gene when compared with the control. It was also noted that the expression of the BGhrR gene of the 0.86% lysine diets was lower ($P < 0.05$) than that of birds fed the 0.92% lysine diets. The control and excess lysine concentrations displayed lowest mean gene expression profiles in the BGhrR gene, which were not significantly different from each other ($P > 0.05$). The expression of the liver ghrelin receptor (LGhrR) gene of French guinea fowl broilers is presented in Figure 5 (IV). Differences in the mean expression of the LGhrR gene among all lysine concentrations were not significant ($P < 0.05$).

Correlations

Table 4 presents correlation coefficients between dietary lysine concentrations, performance characteristics, and the expression of glutamate, ghrelin and leptin receptors of the French guinea fowl broiler. Positive correlations were observed between lysine concentrations and average daily gain, pancreatic weight and expression levels of LiGluR. Negative correlations were also observed between lysine concentrations and the expression levels of LmGluR and BmGluR. Correlations between 1 to 4 wk average daily gain and both LLepR and iGluRs were positive and significant. Pancreatic weight was also positively correlated with the level of expression of BGhrR, LepR, mGluR, and iGluR. Also, the expression of BLepR and LLepR were positively correlated with the expression of both pancreatic and BmGluR and iGluRs. On the other hand, correlations between LLepR expression and LiLepR expression were also positive and significant. However, correlations between the expression of BmGluR and iGluR and expression of LiGluR were negative and significant ($P < 0.01$). Also, LGhrR and LepR expression were negatively and highly correlated with BiGluR expression ($P < 0.01$).

DISCUSSION

Lysine as a limiting amino acid in the chicken was shown to have significant effects on performance of the French guinea fowl broiler. A low level (0.80%) of dietary lysine caused significantly high mortality (Figure 2). Previous research has shown that reduced dietary lysine levels have contributed to a reduction in appetite and low metabolic activity in the early stages of avian growth and development (Kidd and Fancher, 2001). Previous research has also shown that a depletion of a single amino acid can cause metabolic homeostatic problems (Tome, 2004). Averous et al. (2003) explains that regulation of amino acid homeostasis is required in mammals for two reasons: 1) Higher level organisms cannot synthesize all amino acids, and 2) nonessential amino acids cannot be stored (in contrast

Table 4. Correlation coefficients among performance traits and expression of genes associated with feed intake and lysine metabolism.

	BW 0–4	BW 5–8	Liver weight	Pancreas weight	Brain GhrR	Liver GhrR	Brain LepR	Liver LepR	Brain mGluR	Liver mGluR	Brain iGluR	Liver iGluR
Lysine	0.565*	0.606**	0.268*	0.766**	−0.819**	−0.352	−0.272	0.068	−0.743**	−0.552*	−0.044	0.619**
BW 0–4		0.185	−0.377	−0.384	−0.156	0.338	−0.282	0.715**	−0.385	0.005	0.162	0.484*
BW 5–8			0.124	0.113	−0.439*	0.008	−0.106	0.181	−0.491*	−0.184	−0.207	0.334*
Liver weight				0.127	0.371	0.159	−0.081	−0.567*	−0.434*	−0.026	−0.042	−0.238
Pancreas weight					0.471*	0.281	0.585*	−0.068	0.683**	0.420*	0.745**	−0.185
Brain GhrR						0.708**	0.482*	−0.009	0.811**	0.617**	0.590*	−0.598*
Liver GhrR							−0.287	0.875**	0.165	−0.089	−0.981*	−0.188
Brain LepR								−0.551*	0.221	0.704**	0.252	−0.205
Liver LepR									0.215	0.013	−0.998*	0.871**
Brain mGluR										0.037	0.328	−0.903*
Liver mGluR											0.429*	0.009
Brain iGluR												−0.832*

LepR = Leptin Receptor; iGluR = ionotropic glutamate receptor; GhrR = Growth Secretagouge Receptor (Ghrelin receptor); mGluR1 = Glutamate metabotrophic receptor 1;
** $P < 0.01$; * $P < 0.05$.

to lipids, and sugars). Using the guinea fowl as a model due to its leanness, we were able to observe a physiological and anatomical change due to varied levels of administered lysine values (Figure 1A and B). Observation of the lowest allocation at 0.80% lysine showed early developmental problems (Figure 1A). The detected effects were hindered growth rate, withered feathers, and reduced muscle development. This was further evidence to confirm the role of lysine in the early development of avian and mammalian organisms (Benevenga and Blemings, 2007). Evaluation of the organ weights confirm that alterations in lysine can contribute to overexertion of the liver and pancreas (Figure 1A and B). The positive correlations between lysine concentrations and average daily gain and organ weight (Table 4) further supports the premise that lysine had direct influence in growth and tissue development in the guinea fowl. Lysine deficiency contributed to the decreased size of the 4-week-old guinea keet liver and pancreas. Also evaluating the effect of dietary lysine on pancreatic weight [Figure 3 (II) and (IV)] showed the various effects caused by the neuropeptic hormones leptin and ghrelin. The alterations of lysine may have contributed to other digestive functions as seen by the similar reduction of weights of the pancreas and liver in the lowest level of lysine. Dietary lysine concentration of 1.10% or higher contributed to optimum performance characteristics of the French guinea fowl. Another point was that muscle homeostasis was affected, which led to the idea that through hindered lysine levels the glutamate metabolite suppressed proper muscle formation. Also, it has been shown through our research that a single change in the lysine metabolic pathway may cause a transformation in the final product of acetyl coenzyme A (Acetyl CoA). Papes et al. (2001) showed how the saccharopine pathway (which is utilized by higher celled organisms) is the precursor to glutamate and also its association with the enzymatic reactions that lead to Acetyl CoA and the metabolite glutamate. Our investigation into the link between lysine and glutamate in regulation of food intake, showed that lysine in low levels causes varied modifications in glutamate metabolism. Jinap and Hajeb (2010) focused on the role of dietary glutamate to elicit distinct flavor and the umami taste, which cause a more desired necessity to devour these foods. Also, Uneyama (2011) used the same reasoning to explain the glutamate appetite regulation. Figure 4 (III) and (IV) confirm the fact that lysine, the precursor to metabolic glutamate, can mediate food intake by way of a neuronal and glutamate sensing mechanism via the liver (Jensen et al., 2013; Torii et al., 2013). The negative correlations between lysine concentrations and both LmGluR and BmGluR (Table 4) suggest a negative feedback mechanism where deficiency of lysine stimulates the expression of the glutamate receptors in attempt to increase lysine uptake. Jensen et al. (2013) argued that the liver serves an amino acid sensing function and it can act through its own innervated system to communicate to the brain. Negative and highly

significant correlations were observed between LiGluR and BiGluR expression (Table 4). Our research showed that glutamate metabolism acting via the liver can cause a state of satiation as observed in Figure 4 (III) and (IV). Gene expression analysis shown in Figure 4 (I) and (III) demonstrated that varying lysine levels influenced specifically the expression of metabotropic glutamate receptor I. Bradbury et al. (2005) discussed the role of metabotropic glutamate receptor 5 (mGluR5) regulating feed intake via a dopamine-mediated reward system and its role in modulating glutamate sensory conduction. Our findings indicated that mGluR5 may have the same function as that of mGluRI (Pieper et al., 2011: Bradbury et al., 2005). The role of anorexigenic and orexigenic hormones in controlling food absorption has been thoroughly researched and in two specific hormones leptin and ghrelin (Kojima and Kangawa, 2005; Gao and Horvath, 2007; Nirmala et al., 2009). Our supposition was that an amino acid could affect the activities of these hormones and cause a change in food intake. As illustrated in Figure 5 (I) and (IV), lysine levels did affect leptin receptor expression in both the liver and brain. In earlier reports, Xu et al. (2013) argues that glutamate acts on the same leptin/insulin signaling cascade thus regulating food intake. We proposed the hypothesis that glutamate and lysine share a relationship that involves regulating food intake through the leptin signaling pathway. Figure 4 (I & III) and 5 (I and IV) substantiate the gene expression analysis of the glutamate metabotropic receptor I displayed comparable levels to leptin at low lysine concentrations in the brain and liver. These findings led us to conclude that the lowest amounts of lysine contribute to a suppression of eating and drinking, leading to starvation. The exploration into glutamate led us to study the ionotropic glutamate receptor (iGluR) as well. The iGluR has not been thoroughly studied due to its unique structure (McFeeters and Oswald, 2004). Figure 4 (II) and (IV) show that in the LiGluR high levels of leptin receptor expression at 1.22% lysine led to the idea that this is caused by increased ligand binding. The correlation table further demonstrates that the iGluR and LepR acting through varied lysine modifications change pancreas activity leading to early and late stage body transformations. The evaluation of the BiGluR as viewed in Figure 4 (II) displayed lower expression in the brain at low lysine levels. These results further confirm that lower lysine levels may cause decreased ligand binding or ionic suppression. Further investigation as to how changes in glutamate affect the ionotropic receptors has yet to be done. Our lab hypothesized that lysine acting through ghrelin signaling would increase food intake. Parello and Zigman (2012) discusses ghrelin's actions by way of a reward-based system. The study results of Figure 5 (II) and (III) revealed that ghrelin displayed no significant variation in the liver. This could be due to the combined actions of glutamate and lysine to suppress ghrelin's function. However, when viewing Figure 5 (II), in the brain, ghrelin expression was reduced in the control and higher in the lower ranges of lysine. The correlation table shows that the GhrRs interacted with LepR and mGluRs and contributed to changes in the gut–liver axis as showcased in Table 4. There may be some other effect in the lysine metabolism that may cause a negation of ghrelin, which was observed in the lowered levels of lysine. Based on all of the findings, the final conjecture was that glutamate metabolism through the lysine saccharopine pathway can interact with leptin to decrease food intake or ghrelin to increase feed intake via glutamate sensing in the liver. Exploration of the signaling pathways that integrate the brain-liver axis could give a better understanding of the mechanisms that control the cognitive sensations, or impulses that drive hunger. This will in turn guide management and breeding practices to minimize excessive fat deposition in poultry and consumer susceptibility to the diseases associated with consumption of fatty foods.

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SUPPLEMENTARY DATA

Table S1. Oligonucleotide Primers used to amplify leptin, glutamate, Ghrelin and metabotropic glutamate receptors of the Guinea Fowl fed diets containing varying concentrations of lysine.

Supplementary data is available at PSA *Journal* online.

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