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Transcriptomic analysis of genes in the nitrogen recycling pathway of meat-type chickens divergently selected for feed efficiency

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Summary

The understanding of the dynamics of ammonia detoxification and excretion in uricotelic species is lagging behind ureotelic species. The relative expression of genes involved in nitrogen recycling and feed efficiency in chickens is unknown. The objective of this study was to investigate the transcriptomics differences in key genes in the nitrogen (N) metabolism and purine biosynthesis pathway in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake at days 35 and 42 using duodenum, liver, pectoralis major (P. major) and kidney. There was a significant positive correlation between RFI and fecal N. The purine salvage pathway was activated in the LRFI compared with HRFI at days 42. The birds in the LRFI population attained greater feed efficiency by having lower FI, increasing their protein retention and producing adequate glutamine to maintain growth compared with the HRFI line. To maintain growth, excess N is deaminated mostly to generate purine nucleotides. Generating purine nucleotides primarily from the purine biosynthesis pathway is energetically costly, and to preserve energy, they preferentially generate nucleotides from the purine salvage pathway. The LRFI birds need to generate sufficient nucleotides to maintain growth despite reduced FI that then results in reduced fecal N.

Keywords glutamine, nitrogen cycling, nutrient utilization, purine biosynthesis pathway, uric acid

Introduction

The world will need 70–100% more food by 2050 (Godfray *et al.* 2010) when the global population is expected to reach 9 billion. This global demand for food will lead to competition for agricultural inputs and will create an urgent need to lower the impact of intensified food production on the environment (Royal Society of London 2009) while also being responsive to health and well-being of livestock and poultry, a concept called intensive sustainability. Research in the genetics of feed efficiency is lagging that of growth. Given that feed costs constitute about 60–70% of the total production cost and feed is a limited commodity, improvements in sustainable feed efficiency will allow for greater meat production while reducing the amount of feed required for growth, thereby reducing production costs and negative environmental impacts. The major components of feed are protein, energy, calcium and phosphorus (P). The retentions of protein and P are 50–70 and 40% respectively leading to substantial amounts of nitrogen (N) and P in poultry waste. Excreta N can be converted to ammonia and nitrates. Nitrate in water is linked to methemoglobinemia in infants, toxicity in livestock and eutrophication in both fresh and saline waters (Sharpley *et al.* 1998). Pollution of surface water by P restricts its use for fisheries, recreation and drinking due to increased growth of undesirable algae and oxygen shortages caused by their senescence and decomposition (Kotak *et al.* 1993).

Therefore, improvements in feed efficiency, especially efficiency of protein use, will improve productivity and simultaneously reduce the amount of manure N. Feeding high protein levels improves feed efficiency, but this approach is not sustainable as it also increases the amount of fecal N (Baeza *et al.* 2012). Feed efficiency is a compound trait, which makes it difficult to define the phenotype accurately. In addition, genes or genetic markers linked with feed efficiency also are limited. Consequently, transcriptomics is a critical step in understanding the mechanism that underlies the interaction of nutrition and the genome. Also, differences in gene expression associated with a

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particular gene network (e.g., nitrogen metabolism) between chickens divergently selected for RFI can provide new insight into the molecular and cellular basis of efficiency. However, there is very scant information about the molecular aspects of protein metabolism and N recycling in poultry species that excrete N in the form of uric acid. Although it was possible to use microarray gene expression to have a snapshot of the whole genome, we decided to concentrate on the expression of key genes in the N metabolism and purine biosynthesis pathways.

The objective of this study was to investigate the transcriptomics differences in key genes in the N metabolism and purine biosynthesis pathways in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake at days 35–42. In the base population used for the divergent selection, Aggrey *et al.* (2010) showed that RFI at days 28–35 is genetically correlated to body weight gain (BWG), whereas RFI at days 35–42 is not correlated with BWG. These two ages would potentially delineate the underlying mechanisms of N recycling different stages of growth in the meat-type chicken.

Table 1 Means (±SE) of feed efficiency parameters in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake.

Trait	Period[1]	LRFI ($n = 315$)	HRFI ($n = 308$)	Pr > *F*
MBW	28	171.43 ± 1.15	173.99 ± 1.27	0.1357
BWG	28–35	394.37 ± 4.90	420.89 ± 5.42	0.0003
FI	28–35	707.89 ± 8.65	803.15 ± 9.58	<0.0001
RFI	28–35	−31.86 ± 6.38	32.33 ± 7.07	<0.0001
FCR	28–35	1.80 ± 0.02	1.93 ± 0.02	<0.0001
MBW	35	222.20 ± 1.47	227.81 ± 1.62	0.0103
BWG	35–42	376.39 ± 4.72	372.09 ± 5.23	0.5406
FI (g)	35–42	776.60 ± 9.96	886.72 ± 10.91	<0.0001
RFI	35–42	−41.35 ± 8.33	54.80 ± 9.22	<0.0001
FCR	35–42	2.13 ± 0.04	2.49 ± 0.05	<0.0001
ABFW	42	28.93 ± 0.64	30.52 ± 0.69	0.0943
ABFY (%)	42	2.60 ± 0.08	2.81 ± 0.09	0.0885
NR (%)[2]	35–42	65.60 ± 1.31	57.03 ± 1.32	<0.0001

All traits measured in g except for FCR which was g/g.
MBW, metabolic body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; RFI, residual feed intake; NR, nitrogen retention; ABFW, abdominal fat weight; ABFY, abdominal fat yield.
[1]Age (d) or age range (d) that trait was measured.
[2]$n = 50$ per line.

Materials and Methods

Experimental population

We used duodenum, liver, P. major (major breast muscle) and kidney tissues from a chicken population (Arkansas Random bred, ARB) that is divergently selected for residual feed intake (RFI). The ARB was established in 1996 from poultry industry elite breeding lines (seven males and six females) and propagated yearly as a random mating population. The selection program was based on low or high days 35–42 RFI breeding values for three generations. At days 35 and 42, four male chickens from each line were selected. There were 16 samples per tissue representing two lines and two ages. Tissues were taken from birds based on days 28–35 and 35–42 RFI. The RFI values of the birds used for the current study at days 28–35 were –108 and 486 for LRFI and HRFI respectively and that for days 35–42 were –202 and 324 for LRFI and HRFI respectively. Chicks were sexed at hatch, placed in pens with litter and fed a ration containing 225 g/kg protein, 52.8 g/kg fat, 25.3 g/kg fiber, 12.90 MJ ME/kg, 9.5 g/kg calcium (Ca) and 7.2 g/kg total P (4.5 g/kg available P) until day 18. Thereafter, they were fed 205 g/kg protein, 57.6 g/kg fat, 25.0 g/kg fiber, 13.20 MJ ME/kg, 9.0 g/kg Ca and 6.7 g/kg total P (4.1 g/kg available P). At day 28, birds were fasted for 12 hours and transferred to individual metabolism cages until day 42. Birds were kept on a 14L:10D light regimen. The feed intake, RFI and BWG were taken for days 28–35 and 35–42 and are presented in Table 1. We randomly selected 50 birds each from the HRFI and LRFI lines at days 35–42 and determined the fecal nitrogen (FN) using the method described by AOAC (1995) and also N retention (NR). We estimated the correlation coefficient between RFI and FN, and between FN and nitrogen intake (NI) using the PROC CORR procedure in SAS (SAS 2000). The NI was estimated from the N concentration in the feed and the amount of feed consumed. All animal protocols were approved by the Animal Care and Use committee of the University of Georgia. Birds were killed by cervical dislocation.

RNA extraction and quantitative real-time PCR analysis

Total RNA were extracted from duodenum, breast, liver and kidney tissues using Trizol reagent (Invitrogen Corp.) according to manufacturer's instructions. The concentration of the extracted RNA was assessed with UV absorbance, and the $OD_{260/280}$ ratios for all samples were >1.9. RNA integrity was verified with a Bioanalyzer (Agilent Technologies). All RNA samples displayed an RNA integrity number (RIN) of ≥9. For cDNA synthesis, 2 μg of total RNA was reversed transcribed with high capacity cDNA reversed transcription kits according to manufacturer's protocol (Applied Biosystems). Real-time PCR reactions were performed using the StepOnePlus (Applied Biosysems). 0.5 μl of cDNA served as a template in a 20-μl PCR mixture containing 0.3 μl each of forward and reverse primers from 10 μM stocks and 2X Fast SYBR Green Master Mix (Applied Biosystems). The PCR conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. In addition, at the end of each reaction, a melting temperature curve of every PCR reaction was determined. RT-PCR for each sample was carried out in duplicate. Data were analyzed according to $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) and were normalized by β-actin expression in each

sample. We first tested the efficacy of using *glyceraldehyde 3 phosphate dehydrogenase* (*GAPDH*), *hypoxanthine phosphoribosyltransferase 1* (*HPRT1*) and *β*-actin as internal standards and ascertained that *β*-actin was more appropriate than were *GAPDH* and *HPRT1* for the current study. Differential gene expression was expressed as LRFI/HRFI. Statistical difference in gene expression between lines was determined using PROC GLM in SAS (SAS 2000), and $P \leq 0.05$ was considered as significant. The NCBI accession numbers, forward and reverse primers and annealing temperatures of genes used in this study are provided in Table S1.

Results

The feed efficiency parameters of the LRFI and HRFI lines are provided in Table 1. The divergent selection was based on days 35–42 RFI. The LRFI line improved feed efficiency by reducing FI compared with the feed inefficient HRFI line at days 35–42. However, for the correlated response at days 28–35, the LRFI line improved feed efficiency by reducing both FI and BWG. Nitrogen retention was higher ($P \leq 0.001$) in LRFI compared with HRFI. The correlation between RFI and FN was 0.45 ($P \leq 0.001$) and that between NI and FN was 0.33 ($P \leq 0.001$). Abdominal fat yields also were different between LRFI and HRFI but not statistically significant ($P = 0.09$). LRFI had a lower abdominal fat yield compared with HRFI.

The relative gene expression levels in LRFI compared with HRFI of *aldehyde dehydrogenase 4 family, member A1* (*ALDH4A1*), *asparagine synthetase (glutamine-hydrolyzing)* (*ASNS*), *cysteine conjugate-beta lyase 1, cytoplasmic* (*CCBL1*), *glycine amidinotransferase (L-arginine:glycine amidinotransferase)* (*GATM*), *glutaminase* (*GLS*), *glutamate dehydrogenase 1* (*GLUD1*), *glutamic-oxaloacetate transaminases, soluble* (*GOT1*), *glutamic-oxaloacetate transaminases, mitochondrial* (*GOT2*), *glutamate-ammonia ligase* (*GLUL*) and *phosphoribosyl pyrophosphate amidotransferase* (*PPAT*) in the duodenum, breast, liver and kidney tissues at days 35 and 42 are presented in Tables 2 and 3 respectively. There were relative differences in gene expression between tissues and also between ages. *ALDH4A1* was down-regulated in the duodenum, muscle and kidney at days 35 but up-regulated in the muscle, liver and kidney at day 42. *GLUL* was down-regulated in the duodenum at day 35 but up-regulated at day 42, and *GLUL* was up-regulated in the muscle, liver and kidney. The expression of *GLS* was opposite between days 35 and 42 for each of the four tissues investigated. The *PPAT* gene was down-regulated in the duodenum and kidney at day 35; however, at day 42, the gene was down-regulated in the muscle and up-regulated in the liver and kidney tissues. Interestingly, both *GOT1* and *GOT2* were down-regulated in all tissues at day 35, but at day 42, *GOT1* was up-regulated only in the liver and kidney, whereas *GOT2* was down-regulated in the duodenum and up-regulated in the muscle, liver and kidney. The *GATM* gene was down-regulated in the duodenum, but up-regulated in the muscle of LRFI at day 35; however, at day 42, *GATM* was up-regulated in the duodenum, muscle and liver in LRFI compared with HRFI. *Adenylosuccinate lyase* (*ADSL*) was down-regulated in the duodenum and muscle at day 35 but was up-regulated in the duodenum, muscle, liver and kidney at day 42; however, *adenylosuccinate synthetase 1 like*

Table 2 Differential expression of genes (±SE) at day 35 in the nitrogen recycling pathway in chickens divergently selected for low (LRFI) or high (HRFI) residual feed intake.

		Relative expression							
Symbol	Gene name	Duodenum		Muscle		Liver		Kidney	
ALDH4A1	*Aldehyde dehydrogenase 4 family, member A1*	–1.34**	(0.05)	–1.34***	(0.03)	–1.01	(0.01)	–2.06***	(0.03)
ASNS	*Asparagine synthetase (glutamine-hydrolyzing)*	1.58**	(0.15)	1.32***	(0.06)	1.80*	(0.26)	–1.44***	(0.02)
CCBL1	*Cysteine conjugate-beta lyase 1, cytoplasmic*	–1.21**	(0.04)	–1.16	(0.09)	1.39**	(0.08)	–1.10	(0.06)
GATM	*Glycine amidinotransferase (L-arginine:glycine amidinotransferase)*	–1.28	(0.11)	1.04	(0.14)	2.18	(0.64)	1.14	(0.13)
GLS	*Glutaminase*	–1.78***	(0.04)	1.26	(0.14)	1.38**	(0.08)	–1.10*	(0.03)
GLUD1	*Glutamate dehydrogenase 1*	–1.63***	(0.02)	–1.01	(0.08)	–1.05	(0.04)	–1.29**	(0.06)
GOT1	*Glutamic-oxaloacetate transaminase 1, soluble*	–1.81***	(0.04)	–1.17	(0.08)	–1.20	(0.09)	–1.36***	(0.04)
GOT2	*Glutamic-oxaloacetate transaminase 2, mitochondrial*	–1.45***	(0.04)	–5.03***	(0.01)	–1.12	(0.06)	–1.19	(0.08)
GLUL	*Glutamate-ammonia ligase*	–1.29	(0.11)	–1.14	(0.06)	1.85*	(0.34)	1.05	(0.15)
PPAT	*Phosphoribosyl pyrophosphate amidotransferase*	–1.38***	(0.04)	1.02	(0.04)	1.09	(0.22)	–1.29***	(0.05)
ADSL	*Adenylosuccinate lyase*	–1.44***	(0.03)	–1.47***	(0.04)	1.57*	(0.23)	–1.05	(0.05)
ADSSL1	*Adenylosuccinate synthetase like 1*	1.02	(0.08)	1.29*	(0.08)	2.40**	(0.45)	1.21**	(0.05)
GMPR	*Guanosine monophosphate reductase*	–1.03	(0.11)	–1.12	(0.09)	1.12	(0.05)	1.00	(0.07)
GMPS	*Guanosine monophosphate synthetase*	–1.10	(0.11)	–1.29***	(0.03)	–1.06	(0.06)	–1.19***	(0.03)
HPRT1	*Hypoxanthine phosphoribosyltransferase 1*	–1.41	(0.06)	–1.38***	(0.05)	–1.27*	(0.07)	–1.11	(0.06)
IMPDH2	*IMP (inosine 5′-monophosphate) dehydrogenase 2*	1.19	(0.19)	–1.14	(0.04)	1.11	(0.09)	–1.07	(0.04)
XDH	*Xanthine dehydrogenase*	1.02	(0.12)	–1.12	(0.09)	1.71*	(0.26)	–1.21**	(0.06)

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; negative values represent those that were down-regulated in LRFI, and positive values represent those that were up-regulated in LRFI in comparison with HRFI (data based on four individuals per line).

(*ADSS1*) was up-regulated in all four tissues in the two age periods in LRFI compared with HRFI. *Hypoxanthine phosphoribosyltransferase 1* (*HPRT1*) was down-regulated in LRFI in the liver and kidney at day 35, but was up-regulated in the kidney at day 42. *Xanthine dehydrogenase* (*XDH*) was up-regulated in the liver and down-regulated in the kidney at day 35, but this gene was up-regulated in all tissues at day 42 in LRFI compared with HRFI. The relative expression of genes in the kidney for the purine biosynthesis and salvage pathways is shown in Fig. 1.

Discussion

Dietary protein is metabolized to provide amino acids for growth and various physiological processes in the body. The byproduct of amino acid catabolism is ammonia, which is toxic and, in avian species, eliminated as uric acid (Milroy 1903). Ammonia also is produced endogenously mainly in skeletal muscles, kidney and the brain (Spanaki & Plaitakis 2012), and efficient detoxification of ammonia requires coordinated effort between tissues to maintain ammonia homeostasis. The primary reaction disposing of excess ammonia and controlling the concentration of ammonia in chicken liver is the synthesis of glutamine (Mapes & Krebs 1978), which is a precursor in purine biosynthesis (Krebs 1978).

In the LRFI line (determined between days 28–35), there was down-regulation of *GLS*, *GLUD1* and *GLUL* in the duodenum, suggesting a possible lower interconversion of glutamine, glutamine and keto-acids. Most catabolized amino acids are transaminated to form glutamate (Campbell 1995), and glutamate is metabolized by GLUD, GLS and GLUL (Hudson & Daniel 1993). GLUD catalyzes the interconversion of glutamate to α-ketoglutarate and NH_4^+ (Labow *et al.* 2001). GLUL catalyzes the conversion of glutamate and ammonia into glutamine (Newsholme *et al.* 2003), and the reverse reaction is catalyzed by GLS (Curthoys & Watford 1995). Glutamine therefore serves and functions as a major transport of ammonia in the body. The intestine may not be the prominent tissue for generating N for excretion in LRFI compared with HRFI. Mammalian skeletal muscles express high GLUL and low GLS (Wu *et al.* 1991), and this was also found in the chicken where high level of glutamine corresponded with increased muscle protein synthesis (Watford & Wu 2005). Further, increase in chicken liver GLUL on high protein diet has been found to be in concordance with *GLUL* mRNA level (Campbell 1995). The regulation of GLS and GLUL suggests a decline in protein synthesis at day 35 in LRFI and that may be in concordance with its BWG, which is reduced compared with HRFI. *GLUD1* was down-regulated in the kidney in LRFI. It appears that at day 35 the physiology of LRFI supports reduced levels of glutamine entering the purine biosynthesis pathway compared with HRFI. There was a down-regulation of *GLS* and up-regulation of *GLUL* in the breast muscle, suggesting an increase in conversion of glutamate to glutamine in LRFI compared with HRFI at day 42. From the study of Wu *et al.* (1991), if mRNA levels of *GLUL* and *GLS* in the muscle correlated with activity levels, then this may suggest increased protein synthesis in the breast muscle of LRFI, even with reduced FI. However, it should be pointed out that N retention was significantly

Table 3 Differential gene expression of genes (±SE) at day 42 in the nitrogen recycling pathway in chickens divergently selected for low (LRFI) or high (HRFI) residual feed intake.

		Relative expression							
Symbol	Gene name	Duodenum		Muscle		Liver		Kidney	
ALDH4A1	*Aldehyde dehydrogenase 4 family, member A1*	1.13	(0.06)	1.36**	(0.11)	1.83***	(0.13)	1.97*	(0.35)
ASNS	*Asparagine synthetase (glutamine-hydrolyzing)*	1.16	(0.14)	1.19	(0.12)	1.72***	(0.15)	1.65*	(0.27)
CCBL1	*Cysteine conjugate-beta lyase 1, cytoplasmic*	1.42*	(0.19)	1.15	(0.10)	–1.08	(0.15)	–1.02	(0.06)
GATM	*Glycine amidinotransferase (L-arginine:glycine amidinotransferase)*	2.19*	(0.48)	1.55	(0.40)	1.32	(0.49)	–1.13*	(0.04)
GLS	*Glutaminase*	1.33	(0.17)	–1.26*	(0.09)	–1.11	(0.17)	1.47**	(0.10)
GLUD1	*Glutamate dehydrogenase 1*	1.12*	(0.02)	1.13	(0.12)	1.43*	(0.18)	1.23*	(0.08)
GOT1	*Glutamic-oxaloacetate transaminase 1, soluble*	–1.03	(0.06)	1.19*	(0.09)	1.20**	(0.04)	1.34**	(0.09)
GOT2	*Glutamic-oxaloacetate transaminase 2, mitochondrial*	–1.30***	(0.04)	1.22	(0.12)	1.36***	(0.04)	1.22**	(0.05)
GLUL	*Glutamate-ammonia ligase*	–1.02	(0.04)	3.11*	(0.79)	2.25***	(0.40)	1.62**	(0.18)
PPAT	*Phosphoribosyl pyrophosphate amidotransferase*	1.14	(0.08)	–1.31***	(0.05)	1.73***	(0.08)	1.45**	(0.05)
ADSL	*Adenylosuccinate lyase*	1.33*	(0.13)	1.48*	(0.20)	2.29***	(0.19)	2.09**	(0.24)
ADSSL1	*Adenylosuccinate synthetase like 1*	1.67*	(0.22)	1.56*	(0.16)	1.54***	(0.09)	1.49***	(0.12)
GMPR	*Guanosine monophosphate reductase*	–1.17*	(0.06)	–1.07	(0.09)	1.11	(0.04)	1.04	(0.07)
GMPS	*Guanosine monophosphate synthetase*	1.01	(1.06)	–1.04	(0.06)	–1.01	(0.07)	1.00	(0.11)
HPRT1	*Hypoxanthine phosphoribosyltransferase 1*	1.18	(0.21)	1.16	(0.10)	1.11	(0.08)	1.52***	(0.09)
IMPDH2	*IMP (inosine 5′-monophosphate) dehydrogenase 2*	1.11	(0.09)	1.37*	(0.11)	1.08	(0.07)	1.15	(0.08)
XDH	*Xanthine dehydrogenase*	1.13	(0.18)	1.52***	(0.08)	3.02*	(0.77)	1.51*	(0.20)

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; negative values represent those that were down-regulated in LRFI, and positive values represent those that were up-regulated in LRFI in comparison with HRFI (data based on four individuals per line).

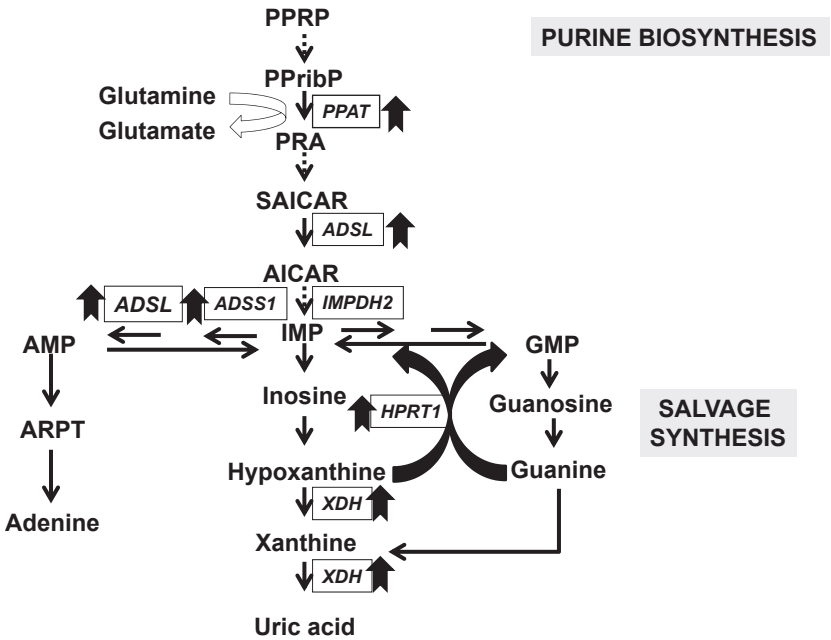

Figure 1 Relative gene expressions at day 42 in kidneys of chickens selected for low (LRFI) or high (HRFI) residual feed intake. Full arrow = direct step; dashed arrow = several steps; open arrow = up-regulation in LRFI/HRFI. PPRP, phosphoribosylpyrophosphate; PPribP, phosphoribosyl phosphate; PRA, phosphoribosylamine; SAICAR, succinylaminoimidazole-carboxamide ribose-5′-phosphate; AICAR, aminoimidazole-4-carboxamide ribonucleotide; AMP, adenine monophosphate; IMP, inosine monosphosphate; GMP, guanine monophosphate; APRT, adenine phosphoribosyltransferase; PPAT, phosphoribosyl pyrophosphate amidotransferase; ADSL, adenylosuccinate lyase; ADSSL1, adenylosuccinate synthetase 1; IMPDH2, IMP (inosine 5′-monophosphate) dehydrogenase 2; HPRT1, hypoxanthine-guanine phosphoribosyltransferase 1; GMPS, guanine monophosphate synthetase; and XDH, xanthine dehydrogenase.

higher in LRFI than in HRFI. The *GLUL*, *GLUD1* and *GLS* expression differences between LRFI and HRFI at day 42 together with their corresponding FI and BWG suggest that there is a higher production of glutamine from breast muscle of LRFI than of HRFI which is transported to the liver and kidney, in addition to the glutamine produced in both the liver and kidney.

Glutamic-oxaloacetic transaminase is a pyridoxal phosphate-dependent enzyme that exists in cytoplasmic and inner membrane mitochondrial forms, GOT1 and GOT2 respectively. GOT plays a role in amino acid metabolism and in urea and tricarboxylic acid cycles (Mavrides & Christen 1978). GOT also catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate. During aspartate catabolism, the glutamate formed undergoes deamination to form NH_4^+, which is excreted (Kirsch *et al.* 1984). Asparagine synthetase catalyzes the amidation of aspartate and glutamine in the presence of Mg^{2+} to asparagines. At day 35, *ASNS* was up-regulated in the duodenum, muscle and liver and down-regulated in the kidney of LRFI, whereas both *GOT1* and *GOT2* were down-regulated in all four tissues. The dynamics of *ASNS*, *GOT1* and *GOT2* point to increased glutamate formation and reduced conversion of glutamine to asparagine in the duodenum, muscle and liver; but in the kidney of LRFI, both *ASNS* and *GOT1* were down-regulated, supporting reduced formation of glutamate. However, at day 42, besides *GOT2*, which was down-regulated in the duodenum, *ASNS*, *GOT1* and *GOT2* were all up-regulated in the three tissues in LRFI compared with HRFI.

Aldehyde dehydrogenase 4 family, member A1 is a mitochondrial matrix NAD-dependent dehydrogenase that catalyzes the conversion of pyrroline-5-carboxylate to glutamate in the proline degradation pathway (Valle *et al.* 1979). ALDH4 has been identified as a target of p53, and expression of *ALDH4* mRNA was induced in response to DNA damage (Yoon *et al.* 2004). At day 35, *ALDH4A1* was down-regulated in all four tissues but was up-regulated in the muscle, liver and kidney at day 42 in LHRFI. N intake in LRFI was significantly lower at both age periods, but at day 35 BWG declined, whereas at day 42 BWG was similar compared with HRFI. It is likely that proline may be used as a N source by degradation into glutamate, especially at day 42 to maintain growth in LRFI.

Glutamine phosphoribosyl pyrophosphate amidotransferase is an enzyme encoded by the *PPAT* gene, and GPAT is the first limiting enzyme in the purine biosynthesis pathway (Moat & Friedman 1960; Koenigsknecht *et al.* 2007). It catalyzes the conversion of 5-phosphoribosylpyrophosphate (PRPP) to 5-phosphoribosylamine (PRA) (Koenigsknecht *et al.* 2007) by deamination of glutamine. PRA, through several steps, yields inosine 5′-phosphate (IMP). IMP can be converted to adenosine 5′-nucleotidase (AMP) and GMP to synthesize the nucleic acids adenine and guanine. In uricotelic species, including chickens, IMP is also converted to inosine and subsequently to uric acid. The final enzyme in the uric acid formation is XDH (Remy & Westerfeld 1951; Richert & Westerfeld 1951), and in chickens, Chou (1972) showed that the kidney is relatively more important in the synthesis of uric acid than is liver. At day 35, *PPAT* was down-regulated in the duodenum and kidney of LRFI, suggesting a possible relatively lower deamination of glutamine compared to HRFI. A lower rate of deamination could lead to less N entering the purine biosynthesis cycle and could be the basis of less FN in LFRI. At day 42, there was a down-regulation of *PPAT* in breast muscle of LRFI. It

could be that glutamine is needed to be maintained for protein synthesis rather than entering the purine biosynthesis pathway for deamination. *PPAT* was up-regulated in the liver and kidney LRFI at day 42. In non-uricotelic species, the purine pathway functions mainly to supply purine nucleotides, and synthesis is regulated through feedback inhibition of *PPAT* by adenine and guanine nucleotides (Wyngaarden & Ashton 1959; Zhou *et al.* 1993). However, uricotelic species require additional regulatory mechanisms, as uric acid synthesis from excess N must proceed even when cells have their full complement of nucleotides. According to Caskey *et al.* (1964), the glutamine concentration, the primary product of ammonia detoxification, is a major controlling factor as it reverses the inhibition of PPAT by nucleotides in uricotelic species. The correlation between RFI and FN was positive, indicating lower concentration of fecal N in LRFI compared with HFRI. Taken together, the up-regulation of *PPAT* in LRFI at day 42 could be in response to the need to synthesize more purine nucleotides than to reduce ammonia burden, as fecal N was lower in LRFI then HRFI.

Biosynthesis of purine nucleotides increases the growth rate mainly through ATP production and G(1)/S transition (Kondo *et al.* 2000). We can therefore hypothesize that chickens in the LRFI line, despite significantly reducing their FI (protein intake), maintain their BW by retaining more N, excreting less N and putatively synthesizing more purines to maintain growth compared with the HRFI chickens. However, because production of nucleotides through the purine biosynthesis pathway is energy costly (Krebs 1978), a large proportion of purine nucleotides may be generated via the purine salvage pathway, which is energetically less costly.

Xanthine dehydrogenase, also known as xanthine oxidoreductase (XOR), is the key enzyme in the catabolism of purines, oxidizing hypoxanthine (Hx) into xanthine and then xanthine to uric acid. The synthesis of the XDH enzyme has been shown to be proportional to its mRNA level (Thompson *et al.* 1979). *Xanthine dehydrogenase* was up-regulated in the liver and kidney at day 42 in LRFI compared with HRFI. XDH is a substrate-inducible enzyme (Fisher *et al.* 1974) that increases in response to increased amounts of Hx and xanthine. Given that *HPRT1*, *ADSL* and *ADSS1* were all up-regulated in the LRFI compared with HRFI, the LRFI birds are preferentially using the purine salvage pathway to generate nucleotides. The LRFI birds have low excreta N compared with the HRFI (Table 1). Excreta N contains N from other sources in addition to uric acid; however, that quantity may not be sufficient to significantly alter the scale of excreta N. The lack of concordance between the up-regulation of *XDH* and low excreta N in LRFI could be explained by the preferential need of those birds to generate sufficient nucleotides through the purine salvage pathway after which the remaining Hx is converted to uric acid. Purine nucleotides are synthesized preferentially by the salvage pathway as long as hypoxanthine is available for energy conservation (Kondo *et al.* 2000). Hypoxanthine is the most essential substrate, and HPRT is the most essential enzyme for the nucleotide salvage pathway (Fig. 1). HPRT converts Hx and guanine to IMP and GMP respectively (Yamaoka *et al.* 1997). There are two ways by which Hx from IMP in the avian liver can occur: the reversible reaction of IMP to Hx or the conversion of Hx from IMP through inosine. The increased activity of the salvage pathway in LRFI points to the reversible reaction of IMP to Hx rather than conversion of Hx from IMP through inosine, which is more energy costly (Mapes & Krebs 1978). The *ADSL* gene is involved in both *de novo* synthesis of purines and formation of AMP from IMP (Kmoch *et al.* 2000), and the *ADSS1* gene encodes the enzyme that catalyzes the GTP-dependent conversion of IMP and aspartic acid to AMP (Sun *et al.* 2005).

There are limitations in the current study, and the down-regulation or up-regulation between tissues and/or age should be considered as the relative gene expression between divergent lines. However, with all the data taken together, we hypothesize that the birds in the LRFI population attain feed efficiency by reducing FI, increasing their protein retention and producing adequate glutamine to maintain growth compared with the HRFI. To maintain growth, excess N is deaminated mostly to generate purine nucleotides. Generating purine nucleotides primarily from the purine biosynthesis pathway is energy costly, and to preserve energy, they preferentially generate nucleotides from the purine salvage pathway. The need to generate sufficient nucleotides to maintain growth despite reduced FI then results in reduced fecal N.

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References

Aggrey S.E., Karnuah A.B., Sebastian B. & Anthony N.B. (2010) Genetic properties of feed efficiency parameters in meat-type chickens. *Genetics Selection and Evolution* **42**, 25.

Association of Official Analytical Chemists (1995) Animal Feed (Chapter ed W.R Windham) AOAC Official Method 968.06, Protein (crude) in Animal Feed. In: *Official Methods of Analysis of the Association of Analytical Chemists* (Ed. by P Cunniff), 16th edn, Vol. 1. pp. 13–5. Association of Analytical Chemists, Washington, DC.

Baeza E., Bernadet M.D. & Lessire M. (2012) Protein requirements for growth, feed efficiency and meat production in growing mule ducks. *Journal of Applied Poultry Research* **21**, 21–31.

Campbell J.W. (1995) Excretory nitrogen metabolism in reptiles and birds. In: *Nitrogen Metabolism and Excretion*. (Ed. by P.J. Walsh & P. Wright), pp. 147–78. CRC Press, Inc., Boca Raton, FL.

Caskey C.T., Ashton D.M. & Wyngaarden J.B. (1964) The enzymology of feedback inhibition of glutamine phosphoribosylpyrophosphate amidotransferase by purine ribonucleotides. *Journal of Biological Chemistry* **239**, 2570–9.

Chou S.T. (1972) Relative importance of liver and kidney in synthesis of uric acid in chickens. *Canadian Journal of Physiology and Pharmacology* **50**, 936–9.

Curthoys N.P. & Watford M. (1995) Regulation of glutaminase activity and glutamine metabolism. *Annual Review of Nutrition* **15**, 133–59.

Fisher J.R., Woodard W.D., Lee D.C. & Wu J. (1974) Regulation of xanthine dehydrogenase and purine nucleoside phosphorylase levels in chick liver. *Advances in Experimental Medicine and Biology* **41A**, 65–74.

Godfray H.C.J., Beddington J.R., Crute I.R., Haddad L., Lawrence D., Muir J.F., Pretty J., Robinson S., Thomas S.M. & Toulmin C. (2010) Food security: the challenge of feeding 9 billion people. *Science* **327**, 812–27.

Hudson R.C. & Daniel R.M. (1993) L-glutamate dehydrogenase: distribution, properties and mechanism. *Comparative Biochemistry and Physiology B* **106**, 767–92.

Kirsch J.F., Eirchele G., Ford G.C., Vincent M.G. & Jansonius J.N. (1984) Mechanism of action of aspartate aminotransferase proposed on the basis of its spatial structure. *Journal of Molecular Biology* **174**, 497–525.

Kmoch S., Hartmannová H., Stibůrková B., Krijt J., Zikánová M. & Sebesta I. (2000) Human adenylocuccinate lyase (ADSL), cloning and characterization of full-length cDNA and its isoform, gene structure and molecular basis for ADSL deficiency in six patients. *Human Molecular Genetics* **9**, 1501–13.

Koenigsknecht M.J., Ramos I. & Downs D.M. (2007) Glutamine phosphoribosylpyrophosphate amidotransferase-independent phosphoribosyl amine synthesis from ribose 5-phosphate and glutamine or asparagines. *Journal of Biological Chemistry* **282**, 28379–84.

Kondo M.T., Yamaoka T., Honda S., Miwa Y., Katashima R., Moritani M., Yoshimoto K., Hayashi Y. & Itakura M. (2000) The rate of cell growth is regulated by purine biosynthesis via ATP production and G(1) to S phase transition. *Journal of Biochemistry* **28**, 57–64.

Kotak B.G., Kenefick S.L., Fritz D.L., Rousseaux C.G., Prepas E.E. & Hrudey S.E. (1993) Occurrence and toxicological evaluation of cyanobacterial toxins in Alberta lakes and farm dugouts. *Water Research* **27**, 495–506.

Krebs H.A. (1978) Regulatory mechanisms in purine biosynthesis. *Advances in Enzyme Regulation* **16**, 409–22.

Labow B.I., Souba W.W. & Abcouwer S.F. (2001) Mechanism governing the expression of the enzymes of glutamine metabolism – glutaminase and glutamine synthase. *Journal of Nutrition* **131**, 2467S–74S.

Livak K.J. & Schmittgen T. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–8.

Mapes J.P. & Krebs H.A. (1978) Rate-limiting factors in urate synthesis and gluconeogenesis in avian liver. *Biochemical Journal* **172**, 193–203.

Mavrides C. & Christen P. (1978) Mitochondrial and cytosolic aspartate aminotransferase from chicken: activity towards amino acids. *Biochemical and Biophysical Research Communications* **85**, 769–73.

Milroy T.H. (1903) The formation of uric acid in birds. *Journal of Physiology* **30**, 47–60.

Moat A.G. & Friedman H. (1960) The biosynthesis and interconversion of purines and their derivatives. *Bacteriological Review* **24**, 309–39.

Newsholme P., Procopio J., Lima M.M.R., Pithon-Curi T.C. & Curi R. (2003) Glutamine and glutamate – their central role in cell metabolism. *Cell Biochemistry and Function* **21**, 1–9.

Remy C. & Westerfeld W.W. (1951) The effect of diet on xanthine dehyrogenase in chicken tissues. *Journal of Biological Chemistry* **193**, 659–67.

Richert D.A. & Westerfeld W.W. (1951) Xanthine oxidases in different species. *Proceedings of the Society for Experimental Biology and Medicine* **76**, 252–4.

Royal Society of London (2009) *Reaping the Benefits: Science and Sustainable Intensification of Global Agriculture*. Royal Society, London.

SAS Institute (2000) *SAS/STAT User's Guide*. Version 8 Ed. SAS Inst. Inc., Cary, NC.

Sharpley A., Meisinger J.J., Breeuwsma A., Sims J.T., Daniel T.C. & Schepers J.S. (1998) Impact of animal manure management on ground and surface water quality. In: *Animal Waste Utilization: Effective Use of Manure as a Soil Resource* (Ed. by J.L. Hatfield & B.A. Stewart), pp. 173–242. Lewis Publishers, Boca Raton, FL.

Spanaki C. & Plaitakis A. (2012) The role of glutamate dehydrogenase in mammalian ammonia metabolism. *Neurotoxicity Research* **21**, 117–27.

Sun H., Li N., Wang X., Chen T., Shi L., Zhang L., Wang J., Wan T. & Cao X. (2005) Molecular cloning and characterization of novel muscle adenylocuccinate synthetase Adssl1, from human bone marrow stromal cells. *Molecular and Cellular Biochemistry* **269**, 85–94.

Thompson J.M., Nickels J.S. & Fisher J.R. (1979) Synthesis and degradation of xanthine dehydrogenase in chick liver. *In vivo* and *in vitro* studies. *Biochimica et Biophysica Acta* **568**, 157–76.

Valle D., Goodman S.I., Harris S.C. & Phang J.M. (1979) Genetic evidence of common enzyme catalyzing the second step in the degradation of proline and hydroxyproline. *Journal of Clinical Investigation* **64**, 1365–70.

Watford M. & Wu G. (2005) Glutamine metabolism in uricotelic species: variation in skeletal muscle glutamine synthase, glutaminase, glutamine levels and rate of protein synthesis. *Comparative Biochemistry and Physiology Part B* **140**, 607–14.

Wu G., Thompson J.R. & Baracos V.E. (1991) Glutamine metabolism in skeletal muscles from the broiler chick (*Gallus domesticus*) and laboratory rat (*Rattus norvegicus*). *Biochemistry Journal* **274**, 769–74.

Wyngaarden J.B. & Ashton D.M. (1959) The regulation of activity of phosphoribosyl-pyrophosphate amidotransferase by purine ribonucleotides: a potential feedback control of purine biosynthesis. *Journal of Biological Chemistry* **234**, 1492–6.

Yamaoka T., Kondo M., Honda S., Iwahana H., Moritani M., Ii S., Yoshimoto K. & Itakura M. (1997) Amidophosphoribosyltransferase limits the rate of cell growth-linked *de novo* purine synthesis in the presence of constant capacity of

salvage purine synthesis. *Journal of Biological Chemistry* **272**, 17719–25.

Yoon K.A., Nakamura Y. & Arakawa H. (2004) Identification of *ALDH4* as a p53-inducible gene and its protective role in cellular stresses. *Journal of Human Genetics* **49**, 134–40.

Zhou G., Charbonneau H., Colman R.F. & Zalkin H. (1993) Identification of site for feedback regulation of glutamine 5-phosphoribosylpyrophosphate amidotransferase by nucleotides and relationship to residues important for catalysis. *Journal of Biological Chemistry* **268**, 10471–81.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer pairs used to analyzed gene expression by quantitative RT-PCR, and size of product.