Dietary arginine levels alter markers of arginine utilization in peripheral blood mononuclear cells and thymocytes in young broiler chicks[1]

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ABSTRACT Arginine is an essential amino acid in Aves and is also an important substrate for the immune system. Dietary Arg in avian diets must be sufficient to not only support growth but also immunity. To better understand Arg needs for immunity, 2 experiments examined markers of Arg use by the immune system in growing broiler chicks. Broiler hatchlings were fed diets containing adequate (1.2%) or high (1.35%) dietary Arg for 21 d. On d 7, the Arg importer cationic amino acid transporter-1 mRNA abundance in peripheral blood mononuclear cells was 2-fold greater in chicks fed 1.35% Arg than in chicks fed 1.2% Arg ($P < 0.05$). On d 14, chicks fed the diet containing 1.2% Arg had 2.5-fold greater mRNA abundance of the y^+L type amino acid transporter-2 exporter compared with chicks fed 1.35% Arg ($P < 0.05$). In experiment 2, broiler hatchlings were fed diets containing low (1.1%), high (1.3%), or excess (1.5%) dietary Arg for 17 d. The percentage of peripheral blood B cells at a given age tended ($P = 0.06$) to be affected by the dietary Arg level. On d 14, but not on d 10 or 17, the percentage of monocytes from chicks fed 1.5% Arg was higher than from those fed 1.1 and 1.3% Arg ($P < 0.05$). These studies indicate that the dietary Arg levels in excess of 1.2% increase the mRNA abundance of markers for Arg use by immune cells undergoing development (thymocytes) and at maintenance (peripheral blood mononuclear cells) and also increase the percentage of monocytes within peripheral blood. Understanding Arg use by the immune system will provide a better understanding of how to formulate immunosupportive diets to promote animal health.

Key words: arginine, lymphocyte, monocyte, thymocyte, transport

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INTRODUCTION

Nutrition can be used to manipulate immune responsiveness to pathogens by altering signal transduction in leukocytes, providing substrate for immune cells or pathogens, protecting against immunopathology, and influencing gut microbial populations and the hormonal environment (Klasing, 2007). Nutrients such as vitamins, trace minerals, and amino acids are substrates for immunity; however, the optimal level of supplementation of these nutrients for an animal is often unknown (Humphrey and Klasing, 2004; Klasing, 2007). Therefore, to understand the effect of nutrient supplementation on immunocompetence, studies must relate nutrient supplementation with the magnitude of leukocyte pools and the function of peripheral leukocytes (Klasing, 2007).

Arginine is an immunologic modulator due in part to its role as a substrate for the immune system (Wu et al., 2009). Arginine is an intermediate of the urea cycle and is metabolized either by NO synthase to produce NO or arginase to produce polyamines. Arginine is essential in Aves because they are uricotelic and cannot synthesize Arg de novo; however, Arg is conditionally essential in mammals due to renal synthesis (Wu and Morris, 1998). This results in avian immune cells being dependent upon acquiring dietary Arg, whereas mammalian immune cells may obtain Arg from either interorgan amino acid metabolism or the diet. Consequently, in addition to requirements for growth, dietary Arg for immunity must also be considered. Therefore, understanding Arg utilization by immune cells may help to better understand how Arg needs for immunity relate to dietary Arg levels that maximize growth.

Arginine utilization by immune cells has important implications on their function. In human peripheral blood T cells, the absence of Arg blocks in vitro proliferation by arresting them in the gap phase 0-gap phase 1 (G_0-G_1) phase of the cell cycle (Rodriguez et al., 2007). Also, the absence of Arg decreases T-cell CD3ζ chain expression in vitro in Jurkat T cells (Ro-

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driguez et al., 2002) and interferes with CD3ζ chain expression with the T-cell receptor in activated human peripheral blood T cells (Zea et al., 2004). In B cells, an Arg deficiency reduces early B-cell maturation in the bone marrow of transgenic mice at the pro- to pre-B-cell transition and decreases the proportion of B cells in the lymph nodes and the spleen but does not alter mature B cell proliferation (de Jonge et al., 2002). In avian and macrophage cell lines, production of nitrite is influenced by Arg and lipopolysaccharide concentration in a dose-dependent manner with no nitrite produced in the absence of Arg (Sung et al., 1991; Bryk et al., 2008). Therefore, functional aspects of T cells, B cells, and monocytes are reliant on mechanisms for Arg acquisition and use.

Nutrient transporters for Arg contribute to Arg retention and use in tissues. Arginine is transported by 4 transport systems (Closs et al., 2004). In nonpolarized cells, transport proteins from y^+, $b^{0,+}$, and $B^{0,+}$ systems import Arg, whereas transport proteins from the y^+L system export Arg (Closs et al., 2004). In mammalian leukocytes, Arg influx is mediated by gene products encoded for the y^+ system, known as the cationic amino acid transporters (**CAT**), whereas Arg efflux is mediated by gene products encoded for the y^+L system, known as the y^+L type amino acid transporters (**y^+LAT**) (Closs et al., 2004). For example, stimulated human peripheral blood T cells increase y^+ system activity yet only have minimal y^+L system activity in vitro (Crawford et al., 1994). Additionally, macrophage NO production is dependent upon Arg uptake via CAT-2B (Nicholson et al., 2001). Consequently, the number and type of Arg transporters expressed in tissues help to provide an understanding of Arg utilization. In chickens, the number and types of Arg transporters expressed in leukocytes in response to dietary Arg are unknown. Therefore, the objective of these experiments was to evaluate the effect of dietary Arg supplementation on Arg utilization in peripheral blood mononuclear cells (**PBMC**) and thymocytes in young, growing broiler chicks.

MATERIALS AND METHODS

Birds and Diets

Mixed sex Cobb 500 broilers were obtained from a commercial hatchery (Cedar Hatchery, Fresno, CA) and were raised in Petersime brooder batteries (Petersime Incubator Co., Gettysburg, OH) contained in an environmentally controlled room (27°C; 24 h of light). In all experiments, chicks were provided ad libitum access to water and feed. In experiment 1, a wheat-soybean meal diet (Table 1) was formulated according to the NRC (1994) recommendations for a young, growing broiler chick, except for Arg. Diets were formulated to contain an adequate or high dietary Arg level as compared with the NRC requirement for Arg (1.25%). Diets were analyzed for amino acid content at a commercial laboratory (University of Missouri Agricultural Experiment Station Chemical Laboratory, Columbia, MO; method 982.30, AOAC, 2006) and the analyzed values were 1.2% Arg for the adequate-Arg diet and 1.35% Arg for the high-Arg diet. In experiment 2, a corn-soybean meal diet (Table 1) was formulated according to NRC (1994) recommendations for a young, growing broiler chick, except for Arg. Diets were formulated to contain a low, high, or excess dietary Arg level as compared with the NRC requirement for Arg (1.25%). Experimental diets were analyzed for amino acid content at a commercial laboratory (University of Missouri Agricultural Experiment Station Chemical Laboratory; method 982.30, AOAC, 2006) and contained either 1.1% (low), 1.3% (high), or 1.5% (excess) Arg. All experiments and procedures were approved by the California Polytechnic State University Animal Care and Use Committee.

Experimental Design

In experiment 1, mixed sex hatchlings (d 1) were selected for uniform BW from a larger population and 12 chicks were placed into 1 of 6 replicate pens per dietary treatment. On d 3, 7, 14, and 21 posthatch, pen weights were measured and 3 chicks per pen were killed by CO_2 overdose for whole blood and organ collection. Whole blood from 1 chick per pen was collected via cardiac puncture into a syringe containing a 2% EDTA solution (2854, Sigma-Aldrich, St. Louis, MO) and 4 thymic lobes from the chick's left side were removed aseptically and stored in RPMI-1640 (1260354, MP Biomedicals LLC, Solon, OH) on ice. From whole blood and thymic lobes, respectively, PBMC and thymocytes were isolated to determine Arg transporter mRNA abundance. From the remaining 2 chicks per pen, 4 thymic lobes from the chick's left side were removed and weighed. Spleen and pectoralis major and minor were excised from 3 chicks per pen and weighed.

In experiment 2, mixed sex hatchlings (d 1) were selected for uniform BW from a larger population and 9 chicks were placed into 1 of 8 replicate pens per dietary treatment. On d 10, 14, and 17 posthatch, pen weights were measured and 2 chicks per pen were killed by CO_2 overdose for whole blood and organ collection. Whole blood from 1 chick per pen was collected into a heparinized (0409-1402-31, Hospira Inc., Lake Forest, IL) syringe and 6 thymic lobes from the chick's left side were removed aseptically and stored in RPMI-1640 on ice. From whole blood, PBMC were isolated for phenotyping. From thymic lobes, thymocytes were cultured for a proliferation assay.

PBMC Isolation

Peripheral blood mononuclear cells were isolated from approximately 3 mL of whole blood. Whole blood was overlaid onto Histopaque-1077 (10771, Sigma-Aldrich) and centrifuged at 350 × g for 15 min at 25°C. In experiment 1 only, the buffy coat was collected and over-

Table 1. Composition of diets fed to growing broiler chicks in experiments 1 and 2

	Experiment 1		Experiment 2		
Item	Adequate Arg	High Arg	Low Arg	Adequate Arg	High Arg
Ingredient (g/kg)					
Wheat	626.8	626.8	—	—	—
Corn	—	—	483.7	483.7	483.7
Soybean meal	158.6	158.6	139.5	139.5	139.5
Corn gluten meal	—	—	113.2	113.2	113.2
Alfalfa meal	—	—	112.4	112.4	112.4
Safflower meal	90.0	90.0	—	—	—
Vegetable oil	66.1	66.1	45.0	45.0	45.0
Wheat millrun	—	—	58.1	58.1	58.1
Dicalcium phosphate	18.3	18.3	16.6	16.6	16.6
Calcium carbonate	16.2	16.2	—	—	—
Limestone	—	—	12.6	12.6	12.6
L-Lys-HCl	5.5	5.5	6.5	6.5	6.5
Salt	3.4	3.4	4.1	4.1	4.1
DL-Met	3.4	3.4	1.4	1.4	1.4
Choline chloride	—	—	4.8	4.8	4.8
Vitamin-mineral premix[1]	2.5	2.5	1.5	1.5	1.5
L-Leu	2.0	2.0	—	—	—
L-Arg-HCl	—	2.0	—	2.5	5.0
L-Thr	1.8	1.8	0.6	0.6	0.6
L-Cys	1.6	1.6	—	—	—
Rice hulls	1.3	1.3	—	—	—
L-Val	1.2	1.2	—	—	—
Copper sulfate	1.0	1.0	—	—	—
L-Ile	0.3	0.3	—	—	—
L-Trp	0.01	0.01	—	—	—
Calculated composition					
ME (kcal/kg)	3,058	3,058	3,080	3,080	3,080
Ca (%)	1.0	1.0	0.97	0.97	0.97
Available P (%)	0.5	0.5	0.45	0.45	0.45
Analyzed composition (%)					
CP	19.1	19.1	22.24	22.96	23.68
Crude fat	7.95	7.95	6.01	6.01	6.01
Arg	1.21	1.34	1.12	1.31	1.53
Lys	1.21	1.22	1.35	1.35	1.34
Met	0.61	0.58	0.50	0.51	0.51

[1]Vitamins and trace minerals were provided as described in the NRC Standard Reference Diet for Chicks (NRC, 1994). Supplied per kilogram of diet: choline chloride, 0.3 g; folic acid, 0.0004 g; niacin, 0.033 g; pantothenic acid, 0.011 g; riboflavin, 0.0044 g; thiamine, 1.1 mg; pyridoxine, 2.2 mg; menadione, 1 mg; vitamin B_{12}, 9 μg; Cu, 5.1 mg; I, 2.5 mg; Fe, 45 mg; Mn, 66 mg; Se, 0.2 mg; Zn, 60 mg; Ca, 375 mg; P, 0.3 mg; Mg, 3.3 mg; K, 2.6 mg; S, 26 mg; Cl, 1.5 mg; vitamin A, 7,200 IU (retinyl acetate); vitamin D_3, 3,320 IU; and vitamin E, 10 IU (DL-α-tocopheryl acetate).

laid on isovolumetric Histopaque-1077 a second time to remove excessive red blood cell contamination. In both experiments, the buffy coat was reconstituted in RPMI-1640 and centrifuged at 500 × *g* for 5 min at 25°C. The pelleted cells were resuspended in 1 mL of RPMI-1640 and cells were enumerated with a hemocytometer (experiment 1) or ViaCount Reagent (experiment 2; 4000, Millipore, Billerica, MA) per the manufacturer's instructions. Peripheral blood mononuclear cells from experiment 1 were aliquoted (4.5×10^6 cells) and centrifuged at 500 × *g* for 5 min at 25°C, snap-frozen in liquid N, and stored at −80°C until further analysis.

PBMC Phenotyping

In experiment 2, PBMC phenotype was determined via flow cytometry using the EasyCyte Plus System (Millipore) with a 488-nm argon laser. Fluorescein isothiocyanate (**FITC**)-conjugated mouse anti-chicken CD4, phycoerythrin (**PE**)-conjugated mouse anti-chicken CD8α, FITC-conjugated mouse anti-chicken Bu-1, and PE-conjugated mouse anti-chicken KUL01 (8210-02, 8220-09, 8395-02, and 8420-09, SouthernBiotech, Birmingham, AL) were used as markers for $CD4^+$, $CD8\alpha^+$, B cells, and monocytes, respectively. Approximately 10^6 cells were incubated with 0.2 μg (PE) or 1 μg (FITC) of antibody for 30 min at 4°C. Samples were washed by centrifugation with wash buffer (250 μL of PBS, 2% BSA, 0.1% NaN_3) at 4°C for 5 min at 500 × *g*. The cells were then resuspended in wash buffer and diluted 1:10 for analysis. Lymphocyte and monocyte subpopulations were gated by forward and side-scatter characteristics and 5,000 gated events were analyzed for FITC or PE fluorescence. The proportions of $CD4^+$, $CD8^+$, Bu-1^+, and KUL01^+ cell populations were expressed as a percentage of gated lymphocytes or gated monocytes, as appropriate.

Thymocyte Isolation

In experiments 1 and 2, thymic lobes were removed aseptically and placed into sterile 60-mm tissue culture plates (353002, Becton, Dickinson and Company, Franklin Lakes, NJ) containing 1.5 mL of RPMI-1640 as

described previously (Rudrappa and Humphrey, 2007). Briefly, thymic lobes were minced gently with forceps to release thymocyte populations into the media. The media from each sample was filtered through a sterile 70-μm nylon cell strainer (352350, BD Falcon, BD Biosciences, Bedford, MA) and thymocyte concentration was determined with a hemocytometer (experiment 1) or ViaCount Reagent per the manufacturer's protocol (experiment 2). Thymocytes from experiment 1 were aliquoted (2×10^7 cells) and pelleted at $500 \times g$ for 5 min at 25°C, snap-frozen in liquid N, and stored at −80°C until further analysis.

Thymocyte Proliferation

Thymocytes (5×10^5 cells) were cultured in complete medium (RPMI-1640, 5% fetal bovine serum, 1% penicillin-streptomycin) in each well of 2 sterile 96-well flat-bottom plates (353072, BD Falcon, BD Biosciences) with either 0 (control) or 30 μg/mL of phytohemagglutinin-P (L1668, Sigma-Aldrich) as described previously (Humphrey and Klasing, 2005). Thymocyte proliferation was determined after 48 h using Cell Proliferation ELISA, BrdU (colorimetric) (11647229001, Roche Diagnostics, Indianapolis, IN) per the manufacturer's instructions. Data were expressed as absorbance at 370 nm.

Total RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from PBMC and thymocyte samples using the NucleoSpin RNA II Kit (740955.50, Macherny-Nagel, Bethlehem, PA) per the manufacturer's instructions. Samples were homogenized with a Tissuemiser homogenizer (Fisher Scientific, Pittsburgh, PA). Optical density at 260 nm was used to quantify RNA concentrations. Peripheral blood mononuclear cells and thymocyte total RNA (250 ng) was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (170-8891, BioRad, Hercules, CA) per the manufacturer's protocol. Quantitative real-time PCR analysis of target gene mRNA abundance was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions used Fast SYBR Green Master Mix (4385610, Applied Biosystems), 1 μL of reverse transcription product, and 10 μmol/L of each primer (Table 2). Thermal cycling parameters were 1 cycle of enzyme activation at 95°C for 20 s and 40 cycles of denaturing at 95°C for 3 s and annealing and extending at 60°C for 30 s. Melting curve analysis was performed after 40 cycles to confirm product specificity such that melt curve analyses containing a specific product peak and primer dimer peak were excluded from analysis and considered to be below the limit for accurate detection. Melting curve analysis was performed at 95°C for 15 s, 60°C for 60 s, followed by a linear temperature increase of 0.5°C/s to 95°C while continuously monitoring fluorescence. The change in sample mRNA abundance was calculated using the Δ-Δ equation with modifications as described previously (Rudrappa and Humphrey 2007). Sample PCR amplification efficiencies were determined in the log-linear phase with the LinRegPCR program (Ramakers et al., 2003). Target gene mRNA abundance was normalized by geometric averaging of raw nonnormalized values

Table 2. Primer sequences for quantitative real-time PCR[1]

Gene	Primer sequence	Product size (bp)	Accession number
y^+LAT-1			
Sense	5′-GTTGGAGCCAGAGAAGGACATC-3′	165	XM_418326
Antisense	5′-AAGCCAGTAGTTGAAGCAGTAGTAG-3′		
y^+LAT-2			
Sense	5′-TTGTTCTCTTATTCTGGTTGGGATAC-3′	100	XM_001231336
Antisense	5′-TTGGCATAGACACAGCAATAGC-3′		
CAT-1			
Sense	5′-ACCTGCCATCGTCATCTCCTTC-3′	252	EU360441
Antisense	5′-AAGTCTTCAATGTGCCCACCTATG-3′		
CAT-2A			
Sense	5′-TGCTTTGTCTACAAGTCTTCTCG-3′	165	EU360448
Antisense	5′-AATGCCATAATACCAGAGATGACC-3′		
CAT-3			
Sense	5′-CCACGGGCACCAAACAGAAG-3′	150	XM_420204
Antisense	5′-CAGTCAGCACCACGCAGATG-3′		
β-Actin			
Sense	5′-ACCCCTGTGATGAAACAAAACCC-3′	265	NM_205518
Antisense	5′-GCGAGTAACTTCCTGTAACAATGC-3′		
HPRT-1			
Sense	5′-GCCAGACTTTGTTGGATTTGAAG-3′	213	NM_204848
Antisense	5′-AGAGTTGAAGCCTGTGAGAGATAG-3′		
TBP			
Sense	5′-TTTAGCCCGATGATGCCGTATG-3′	196	NM_205103
Antisense	5′-CTGTGGTAAGAGTCTGTGAGTGG-3′		

[1]CAT = cationic amino acid transporter; HPRT-1 = hypoxanthine phosphoribosyltransferase-1; TBP = TATA box binding protein; y^+LAT = y^+L type amino acid transporter.

of β-actin, hypoxanthine phosphoribosyltransferase-1, and TATA box binding protein using geNorm software (Vandesompele et al., 2002). Data are presented as the normalized fold change in mRNA abundance relative to 1.2% Arg on d 7 for PBMC or d 3 for thymocytes.

Statistical Analysis

Dependent variables were analyzed by GLM (JMP Software, SAS Institute Inc., Cary, NC) using a 2-way ANOVA. When main effects or their interactions were significant ($P < 0.05$), means were compared by Student's *t*-test pairwise comparisons. Peripheral blood mononuclear cell phenotyping data were transformed using arcsine to meet conditions for ANOVA. Data are reported as nontransformed means and pooled SE.

RESULTS

Experiment 1

Performance and Tissue Weights. Chicks fed 1.35% dietary Arg tended to have greater ADG ($P = 0.09$) and BW ($P = 0.06$) compared with chicks fed 1.2% Arg. Average daily feed intake and feed conversion were similar between chicks fed either 1.2% Arg (33.7 ± 0.8 and 1.08 ± 0.03, respectively) or 1.35% Arg (33.9 ± 0.8 and 1.03 ± 0.03, respectively) over the 3-wk experimental period. When organ weights were corrected for individual BW (relative tissue weights) from d 3 to 21, thymus, spleen, and pectoralis relative weights increased 84, 49, and 451%, respectively (Table 3; $P < 0.05$).

PBMC and Thymocyte Concentrations. Peripheral blood mononuclear cell concentration increased approximately 145% from 1.41×10^7 cells/mL to 3.46×10^7 cells/mL from d 3 to 7 ($P < 0.05$) and remained constant through d 21. From d 3 to 21 posthatch, thymocyte concentration was dependent upon the dietary Arg level ($P < 0.05$; Figure 1). On d 21, thymocyte concentration from chicks fed 1.2% Arg was 25% higher than that from chicks fed 1.35% dietary Arg ($P < 0.05$).

Arg Transporter mRNA Abundance. In PBMC, the mRNA of several Arg transporters including CAT-1, CAT-3, y^+LAT-1, and y^+LAT-2 were detected, whereas the mRNA of CAT-2A and CAT-2B was not detected. When data were expressed relative to CAT-1 mRNA abundance on d 7 from chicks fed 1.2% Arg, both 1.2 and 1.35% dietary Arg decreased CAT-1 mRNA abundance between d 7 and 14; however, on d 7, PBMC from chicks fed 1.35% Arg had 2-fold higher CAT-1 mRNA abundance compared with PBMC from chicks fed 1.2% Arg ($P < 0.05$; Table 4). In PBMC, CAT-1 and y^+LAT-2 mRNA had the highest abundance, whereas y^+LAT-1 mRNA had the lowest, regardless of dietary Arg level or age.

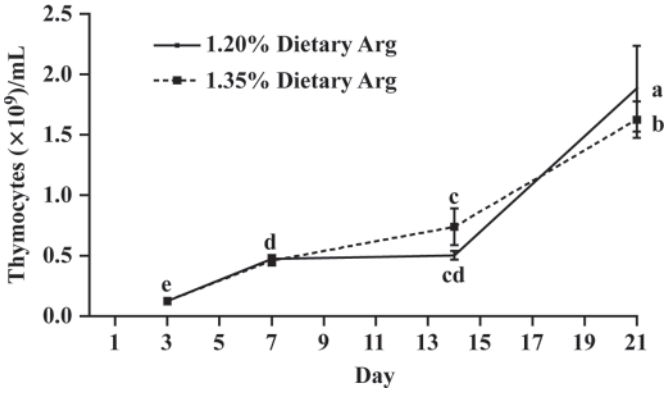

Figure 1. Thymocyte concentration on d 3, 7, 14, and 21 posthatch from experiment 1 broiler chicks fed 1.2 or 1.35% dietary Arg. Values represent means ± SEM (n = 6 replicates of 1 bird per replicate pen). Means not sharing a common letter differ ($P < 0.05$).

In thymocytes, the mRNA of Arg transporters including CAT-1, CAT-3, y^+LAT-1, and y^+LAT-2 was detected, whereas the mRNA of CAT-2A and CAT-2B was not detected. In thymocytes, y^+LAT-2 had the highest mRNA abundance, whereas y^+LAT-1 had the lowest mRNA abundance, regardless of dietary Arg level or age (Table 5). Dietary Arg did not change the mRNA abundance of CAT-1 and CAT-3 in thymocytes ($P > 0.05$); however, y^+LAT-1 mRNA abundance tended ($P = 0.07$) to be lower in thymocytes from chicks fed 1.2% Arg. Thymocyte CAT-1, CAT-3, and y^+LAT-1 mRNA abundance decreased 47, 51, and 68%, respectively, from d 3 to 21 ($P < 0.05$). Thymocyte y^+LAT-2 mRNA abundance was dependent upon the dietary Arg level ($P < 0.05$). On d 14, thymocytes from chicks fed the diet containing 1.2% Arg had 2.5-fold higher

Table 3. Tissue weights from experiment 1 broiler chicks fed 1.2 or 1.35% dietary Arg from d 1 to 21 posthatch[1]

	1.2% Arg				1.35% Arg					*P*-value		
Item	d 3	d 7	d 14	d 21	d 3	d 7	d 14	d 21	SEM	Arg	Day	Arg × day
Thymus (g)	0.077	0.184	0.546	1.091	0.066	0.208	0.497	1.213	0.062	0.654	<0.0001	0.565
Thymus (% BW)	0.088	0.113	0.133	0.146	0.079	0.125	0.120	0.160	0.010	0.902	<0.0001	0.390
Spleen (g)	0.067	0.166	0.378	0.811	0.062	0.181	0.396	0.866	0.033	0.377	<0.0001	0.837
Spleen (% BW)	0.078	0.099	0.094	0.109	0.071	0.113	0.094	0.111	0.008	0.646	0.0001	0.656
Pectoralis (g)	1.78	11.74	37.06	85.77	1.87	11.97	36.71	89.73	1.73	0.442	<0.0001	0.585
Pectoralis (% BW)	2.03	6.99	9.06	11.58	2.16	7.16	8.86	11.50	0.20	0.947	<0.0001	0.753

[1]Values are means ± pooled SEM (n = 6 replicates of 3 birds per replicate pen for spleen and pectoralis weights and 2 birds per pen for thymus weight).

Table 4. Mean mRNA abundance of Arg transporters relative to CAT-1 in peripheral blood mononuclear cells from experiment 1 broiler chicks fed 1.2 or 1.35% dietary Arg[1,2]

	1.2% Arg			1.35% Arg				*P*-value		
Item	d 7	d 14	d 21	d 7	d 14	d 21	SEM	Arg	Day	Arg × day
CAT-1	1.000[b]	0.230[c]	0.297[c]	2.020[a]	0.515[c]	0.264[c]	0.146	<0.01	<0.01	<0.01
CAT-3	1.192	0.171	0.210	1.379	0.366	0.129	0.228	0.594	<0.01	0.788
y^+LAT-1	1.179	0.023	0.018	0.922	0.010	0.017	0.289	0.688	<0.01	0.881
y^+LAT-2	1.114	1.388	1.763	1.024	1.897	1.239	0.260	0.868	0.101	0.164

[a–c]Means within a row not sharing a common superscript differ ($P < 0.05$).

[1]Values are means ± pooled SEM (n = 6 replicates of 1 bird per replicate pen). Data are expressed relative to CAT-1 mRNA abundance on d 7 from chicks fed 1.2% Arg.

[2]CAT = cationic amino acid transporter; y^+LAT = y^+L type amino acid transporter.

y^+LAT-2 mRNA abundance compared with thymocytes from chicks fed 1.35% Arg ($P < 0.05$).

Experiment 2

Performance. An Arg × diet interaction demonstrates that chicks fed 1.3 and 1.5% Arg had greater BW compared with chicks fed 1.1% Arg, but only on d 14 and 17 ($P < 0.05$; Table 6). Over the entire experimental period, chicks fed 1.3% Arg consumed more feed and had greater ADG than chicks fed 1.1% Arg ($P < 0.05$), whereas chicks fed 1.5% Arg were intermediate to both for each dependent variable. Chicks fed 1.3 and 1.5% Arg had lower feed conversion over the entire experiment compared with chicks fed 1.1% Arg ($P < 0.05$).

PBMC Concentration and Phenotyping. Peripheral blood mononuclear cell concentration did not change in response to dietary Arg; however, the concentration of viable PBMC increased approximately 35% from d 10 to maximum levels reached on d 14 ($P < 0.05$; Table 7). There was no significant effect of dietary Arg or age on percentage of $CD4^+$ PBMC ($P > 0.05$). The ratio of $CD4^+$ to $CD8^+$ cells in peripheral blood was not affected by dietary Arg ($P > 0.05$). The percentage of $CD8^+$ PBMC was highest on d 17, increasing 42% from levels on d 14 ($P < 0.05$). There tended ($P = 0.06$) to be an age × Arg interaction for the percentage of peripheral blood B cells. On d 17, the percentage of B cells was numerically greater from chicks fed 1.5% Arg compared with those fed 1.1 and 1.3% Arg. An age × dietary Arg interaction demonstrates that chicks fed 1.5% Arg had the highest percentage of monocytes in peripheral blood, but only on d 14 ($P < 0.05$; Figure 2).

Thymocyte Concentration. The concentration of thymocytes did not depend on dietary Arg levels ($P > 0.05$) but did differ by day posthatch ($P < 0.05$; Table 8). From d 10 to 17, the concentration of viable and dead thymocytes increased 180 and 312%, respectively, whereas the concentration of apoptotic thymocytes increased 125% from d 14 to 17 ($P < 0.05$), but not from d 10 to 14 ($P > 0.05$).

Thymocyte Proliferation. Thymocyte proliferation was dependent upon dietary Arg at d 10 posthatch (Table 9). On d 10, chicks fed 1.5% Arg reduced thymocyte proliferation compared with chicks fed 1.1 and 1.3% Arg ($P < 0.05$). Phytohemagglutinin-P addition increased thymocyte proliferation 132, 45, and 19% on d 10, 14, and 17, respectively, compared with controls ($P < 0.05$).

DISCUSSION

Formulation of immunosupportive diets requires an understanding of the nutrient needs of the immune system. In the case of Arg, diets were formulated to contain multiple levels of Arg ranging from 1.1 to 1.5% to measure markers of Arg use by the immune system.

Table 5. Mean mRNA abundance of Arg transporters relative to CAT-1 in thymocytes from experiment 1 broiler chicks fed 1.2 or 1.35% dietary Arg[1,2]

	1.2% Arg				1.35% Arg					*P*-value		
Item	d 3	d 7	d 14	d 21	d 3	d 7	d 14	d 21	SEM	Arg	Day	Arg × day
CAT-1	1.000	0.761	0.698	0.544	0.858	0.718	0.430	0.442	0.131	0.1310	<0.01	0.851
CAT-3	0.992	0.588	0.618	0.457	0.820	0.450	0.592	0.425	0.111	0.2363	<0.001	0.883
y^+LAT-1	0.999	0.437	0.307	0.520	1.913	0.686	0.544	0.424	0.250	0.0767	<0.001	0.247
y^+LAT-2	2.352[c]	3.317[bc]	6.054[a]	3.129[bc]	3.858[bc]	4.494[ab]	3.152[bc]	2.660[bc]	0.722	0.7384	0.080	<0.05

[a–c]Means within a row not sharing a common superscript differ ($P < 0.05$).

[1]Values are means ± pooled SEM (n = 6 replicates of 1 bird per replicate pen). Data are expressed relative to CAT-1 mRNA abundance on d 3 from chicks fed 1.2% Arg.

[2]CAT = cationic amino acid transporter; y^+LAT = y^+L type amino acid transporter.

Table 6. Experiment 2 broiler performance in response to 1.1, 1.3, or 1.5% dietary Arg fed from d 1 to 17 posthatch[1]

					P-value		
Item	1.1% Arg	1.3% Arg	1.5% Arg	SEM	Arg	Day	Arg × day
BW (g)							
d 1	42.6[f]	42.6[f]	42.6[f]	7.2	<0.0001	<0.0001	<0.05
d 10	260.6[e]	278.4[e]	276.6[e]	7.2			
d 14	392.9[d]	430.3[c]	433.5[c]	7.2			
d 17	524.9[b]	567.0[a]	560.7[a]	7.2			
ADFI (g)							
d 1 to 10	27.8	29.1	28.3	0.8	<0.05	<0.0001	0.683
d 10 to 14	40.9	41.9	40.2	0.8			
d 14 to 17	44.9	45.8	43.3	0.8			
ADG (g)							
d 1 to 10	21.8	23.6	23.4	1.2	<0.05	<0.0001	0.759
d 10 to 14	34.4	38.3	38.1	1.2			
d 14 to 17	42.3	45.6	43.5	1.2			
Feed conversion (g/g)							
d 1 to 10	1.27	1.23	1.21	0.03	<0.05	<0.0001	0.272
d 10 to 14	1.19	1.09	1.07	0.03			
d 14 to 17	1.07	0.94	1.02	0.03			

[a–f]Means within a row not sharing a common superscript are significantly different ($P < 0.05$).

[1]Values are means ± pooled SEM (n = 8 replicates of 2 birds per replicate pen).

These diets were fed to mixed sex chicks, so potential sex differences on markers of Arg use by the immune system cannot be inferred from these studies. To assess Arg use by the immune system, the mRNA abundance of genes coordinating Arg uptake (CAT-1, CAT-2, and CAT-3) and Arg release (y^+LAT-1 and y^+LAT-2) were measured in quiescent (PBMC) and developing immune cells (thymocytes). In addition, leukocyte numbers, function, and type were also measured to determine the effect of dietary Arg level on the magnitude and type of leukocyte pools. Taken together, the results from these studies indicate that the effect of dietary Arg on markers of Arg use by the immune system is both age- and leukocyte-dependent.

Primary and secondary lymphoid organs are often weighed as a measure of immunity given their critical role in the development and function of the immune system (Kwak et al., 1999). Thymic weight is assumed to relate to the magnitude of developing T cells, whereas spleen weight is assumed to relate to the proliferation of immune cells within this secondary lymphoid tissue during periods of infection (Elmore, 2006; Pozo et al., 2009). In our studies, thymus and spleen weights increased with age, though at a much slower rate than pectoralis, but their growth was not altered by dietary Arg levels. Kidd et al. (2001) reported that feeding Arg at 1.48 and 1.68% also did not alter thymus or spleen weights relative to BW. Taken together, these results indicate that at the tissue level, dietary Arg level ranging from 1.2 to 1.68% may be in excess of the requirement for maximizing lymphoid mass. In the present studies, despite no change in thymus mass, 1.2% dietary Arg resulted in the greatest thymocyte concentration on d 21, whereas 1.1% Arg resulted in the greatest thymocyte proliferation on d 10. These data indicate that dietary Arg levels below those needed for maximum growth may be sufficient for developing T cells. Nonetheless, these data indicate that changes in tissue mass do not always translate to their cellular contents and perhaps a more detailed examination of lymphoid organs is needed when assessing the immune system's need for nutrients or energy.

Peripheral blood mononuclear cells include T cells, B cells, and monocytes and their measurement allows for assessment of both adaptive (T and B cells) and innate (monocyte) immunity. In contrast to developing T cells, the metabolic activity of PBMC is low because these cells are in the gap phase 0 (G_0) phase of the cell cycle and are not proliferating or synthesizing effector molecules (Ardawi and Newsholme, 1985). Leukocytes require energy and nutrients to support basal metabolism (Buttgereit et al., 2000), and alterations in their

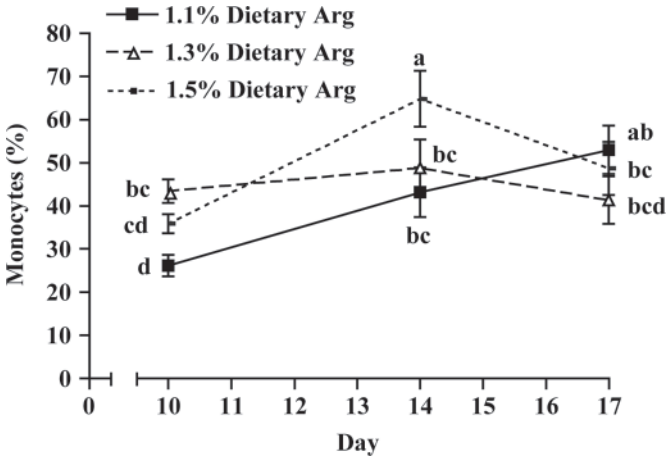

Figure 2. Peripheral blood mononuclear cell phenotyping of monocytes isolated from experiment 2 broiler chicks fed 1.1, 1.3, or 1.5% dietary Arg on d 10, 14, and 17 posthatch. Values represent means ± SEM (n = 8 replicates of 1 bird per replicate pen). Means not sharing a common letter differ ($P < 0.05$).

Table 7. Peripheral blood mononuclear cell (PBMC) phenotyping of cells isolated from experiment 2 broiler chicks fed 1.1, 1.3, or 1.5% dietary Arg on d 10, 14, and 17 posthatch[1]

	1.1% Arg			1.3% Arg			1.5% Arg				*P*-value		
Item	d 10	d 14	d 17	d 10	d 14	d 17	d 10	d 14	d 17	SEM	Arg	Day	Arg × day
PBMC (10^7/mL)	8.23	9.31	11.62	8.14	11.09	12.99	8.37	12.88	12.30	0.85	0.111	<0.0001	0.258
$CD4^+$ (%)	10.2	8.39	8.77	10.51	9.71	9.66	8.81	9.74	10.21	1.20	0.756	0.8375	0.755
$CD8^+$ (%)	4.78	3.74	4.84	3.98	3.91	5.54	3.42	3.27	5.13	0.69	0.599	<0.05	0.675
CD4:CD8	2.26	2.60	2.15	3.32	2.74	1.77	2.68	3.10	2.17	0.42	0.6123	<0.05	0.465
B cells (%)	2.21	5.40	2.74	4.15	2.26	2.78	2.33	5.09	6.33	1.22	0.562	0.2026	0.060

[1]Values represent means ± pooled SEM (n = 8 replicates of 1 bird per replicate pen). Peripheral blood mononuclear cell concentration and phenotype were determined via flow cytometry. Peripheral blood mononuclear cell concentration was determined using ViaCount (Millipore, Billerica, MA) according to the manufacturer's instructions and represents total viable cells. Lymphocyte and monocyte subpopulations were gated by forward and side-scatter characteristics and 5,000 gated events were analyzed for fluorescein isothiocyanate fluorescence. The proportions of $CD4^+$, $CD8^+$, and Bu-1^+ cell populations were expressed as a percentage of gated lymphocytes.

nutrient supply or energy homeostasis can lead to alterations in PBMC concentration (Field et al., 1991). In the present studies, PBMC concentration was measured to determine responsiveness to dietary Arg levels. Peripheral blood mononuclear cell concentrations were not responsive to any dietary Arg level examined and this may be due to low rates of protein synthesis for "housekeeping" functions in these cell types (Buttgereit et al., 2000; Schmid et al., 2000; Frauwirth and Thompson, 2004). Therefore, the present studies indicate that dietary Arg levels as low as 1.1% may be adequate for maintaining PBMC concentrations in young, growing broiler chicks.

Arginine transporters CAT-1, CAT-3, y^+LAT-1, and y^+LAT-2 were expressed in PBMC and thymocytes, but CAT-2A and CAT-2B were below the limit of detection. The Arg transporters CAT-1 and y^+LAT-2 were highest in both of these cell types. In mammalian leukocytes, Arg utilization by monocytes-macrophages is rate-limiting for NO production. In both monocyte-macrophage and endothelial cells, Arg utilization for NO production is dependent upon the induction of CAT-2B, with minimal contributions from CAT-1 or CAT-3 (Nicholson et al., 2001; Yeramian et al., 2006). The birds used in the present studies were not challenged with an infectious agent to induce an immune response, and total NO production by monocytes in the absence of an immune response is negligible (Bowen et al., 2009). Additionally, total plasma NO levels were measured in our studies and found to be below the limit of detection (B. D. Humphrey, unpublished data). Consequently, genes other than CAT-1 may be involved in Arg uptake by PBMC during an immune response, but this cannot be inferred from these studies.

In both thymocytes and PBMC, y^+LAT-2 had the highest mRNA abundance of all Arg transporters examined. In nonpolarized cells, the y^+LAT-2 transporter is an amino acid exchanger and exports intracellular Arg in exchange for the import of extracellular neutral amino acids or glutamine (Wagner et al., 2001). Glutamine is of particular importance to leukocytes given its metabolic role as an energy source and precursor for protein, fatty acid, and nucleic acid biosynthesis (Ardawi and Newsholme, 1985; Brand, 1985). Furthermore, glutamine supplementation improves indices of immunity in growing broilers, particularly lymphocyte function (Bartell and Batal, 2007). Therefore, perhaps the high mRNA abundance of y^+LAT-2 is due in part to a need for glutamine. Additionally, y^+LAT-2 forms a heterodimer with the heavy chain 4F2hc to localize properly within the cell (Verrey et al., 2000). In addition to amino acid transport, the 4F2hc protein, also known as the cluster of differentiation 98hc, also participates in cell signaling and coordinates cell adhesion and proliferation (Devés and Boyd 2000). In mammalian lymphocytes, cluster of differentiation 98hc is essential for proper lymphocyte responses (Cantor et al., 2009). Consequently, the y^+LAT-2 heterodimer is associated with several important functions other than Arg use and this may contribute to the high mRNA abundance of this transporter measured in both thymocytes and PBMC.

Table 8. Mean cell concentrations of thymocytes isolated from experiment 2 broiler chicks fed 1.1, 1.3, or 1.5% dietary Arg on d 10, 14, and 17 posthatch[1]

	1.1% Arg			1.3% Arg			1.5% Arg				*P*-value		
Thymocytes (10^9/mL)	d 10	d 14	d 17	d 10	d 14	d 17	d 10	d 14	d 17	SEM	Arg	Day	Arg × day
Viable	0.581	0.969	1.763	0.743	1.224	1.725	0.670	1.237	2.080	0.126	0.108	<0.0001	0.466
Apoptotic	0.010	0.008	0.028	0.009	0.011	0.023	0.008	0.012	0.019	0.002	0.553	<0.0001	0.132
Dead	0.052	0.113	0.245	0.072	0.141	0.221	0.051	0.137	0.254	0.018	0.766	<0.0001	0.481

[1]Values represent means ± pooled SEM (n = 8 replicates of 1 bird per replicate pen).

Table 9. Mean absorbance of thymocyte proliferation of cells isolated from experiment 2 broiler chicks fed 1.1, 1.3, or 1.5% dietary Arg on d 10, 14, and 17 posthatch[1,2]

	1.1% Arg		1.3% Arg		1.5% Arg			*P*-value		
Day	−	+	−	+	−	+	SEM	Arg	PHA-P	Arg × PHA-P
10	0.273	0.662	0.269	0.633	0.221	0.472	0.035	<0.01	<0.0001	0.109
14	0.234	0.363	0.244	0.384	0.266	0.337	0.019	0.684	<0.0001	0.139
17	0.231	0.290	0.242	0.297	0.240	0.263	0.016	0.502	<0.001	0.460

[1]Values represent means ± pooled SEM (n = 8 replicates of 1 bird per replicate pen). + = PHA-P included in media; − = PHA-P not included in media.

[2]PHA-P = phytohemagglutinin-P.

Dietary Arg levels in excess of the growth requirement increased the mRNA abundance of an Arg importer (CAT-1) in PBMC on d 7 posthatch and decreased the mRNA abundance of an Arg exporter (y^+LAT-2) in thymocytes on d 14. These changes in Arg importers and exporters may have implications on Arg use by PBMC and thymocytes, but this cannot be inferred from these studies because only mRNA abundance and not transport was measured. In comparison, the effect of dietary Arg on Arg transporter expression in mammalian leukocytes is not well understood. Mammalian leukocytes express CAT and y^+LAT genes (Yeramian et al., 2006), but the effect of dietary Arg on the mRNA abundance or activities of these systems has not been reported to date. This may be due to potential confounding effects of de novo Arg synthesis and the potential for macrophages to recycle citrulline, a product of the NO synthase pathway, to Arg for continued NO production (Bryk et al., 2008). However, in vitro studies have shown mammalian CAT-1 translation to be responsive to amino acid availability via an amino acid sensing mechanism (Fernandez et al., 2002; Lopez et al., 2007). Identification of Arg-responsive genes in chicken leukocytes (CAT-1 and y^+LAT-2) may help to assess the Arg needs for the immune system by serving as markers in young broiler chicks.

Differences in the phenotype of leukocytes in peripheral blood in response to dietary nutrients may promote specific types of immune responses. The percentages of monocytes (d 14) and B cells (d 17) were both increased by 1.5% Arg. Although these measurements are a proportion within a cell population, the total number of PBMC increased to maximum numbers by 2 wk posthatch (Table 7). Therefore, the increased percentage of monocytes and B cells in response to dietary Arg levels resulted in increased concentrations of these leukocytes in peripheral blood and may be due to alterations in hematopoiesis as shown for B cells in mammals (de Jonge et al., 2002). In broiler chicks, high dietary Arg levels increased the percentage of B cells in peripheral blood, but this was in almost 7-wk-old birds and measured 19 d after vaccination with a commercial vaccine (Abdukalykova et al., 2008). The changes in peripheral blood monocytes in response to dietary Arg in broilers have not been previously reported, but the dramatic changes in their abundance within peripheral blood during a lipopolysaccharide challenge (Bowen et al., 2009) warrant further investigation into how dietary Arg may alter this dynamic response.

It has been found that T cells help to coordinate immune responses. Nutrient supplementation that bolsters a specific T-cell population in peripheral blood may influence this function; however, when supplemented with dietary Arg in healthy chicks, the percentage of $CD4^+$ T cells, $CD8^+$ T cells, and the CD4:CD8 in peripheral blood were similar. The percentage of $CD4^+$ T cells in PBMC in vaccinated broiler chicks also did not change; however, $CD8^+$ T cells were increased in birds supplemented with 2.2% Arg compared with 1.2% Arg (Abdukalykova et al., 2008). Taken together, this indicates that peripheral blood $CD4^+$ and $CD8^+$ T-cell populations in healthy chicks are not responsive to Arg.

In summary, these experiments have identified markers of Arg utilization in different leukocyte populations that are responsive to dietary Arg levels. Changes in the mRNA abundance of these Arg utilization markers were most evident when dietary Arg levels were above 1.2% Arg. Future studies are needed to better understand how the changes in these markers relate to Arg use (i.e., Arg transport) as well as any potential modulating effect on the immune response.

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