Guanidinoacetic acid is efficacious in improving growth performance and muscle energy homeostasis in broiler chicks fed arginine-deficient or arginine-adequate diets

A. A. DeGroot,[*] U. Braun,[†] and R. N. Dilger [*,1]

[*]*Department of Animal Sciences, University of Illinois, Urbana, IL 61801, USA; and* [†]*AlzChem Trostberg GmbH, 83308 Trostberg, Bavaria, Germany*

ABSTRACT Two studies were conducted to test the efficacy of guanidinoacetic acid (GAA) to spare Arg and serve as a precursor of creatine (Cr) by evaluating growth performance and muscle cellular energy homeostasis in broiler chicks. In both studies, 12 replicate pens of 6 chicks received dietary treatments beginning at day 2 post-hatch. At conclusion of each study, muscle biopsy samples were collected within 60 s of euthanasia for analysis of Cr-related energy metabolites. In study 1, Arg-deficient starter and grower basal diets were supplemented with 0 (negative control, NC), 0.06, 0.12, or 0.18% GAA, or supplemental Arg (positive control, PC; 0.37 and 0.32% L-Arg in starter and grower phases, respectively). Dietary GAA elicited graded improvements, with final BW, overall BW gain, and overall G:F being increased ($P < 0.05$) by 0.12% GAA compared with the NC diet with no difference to PC diet. Increases ($P < 0.001$) of phosphocreatine (PCr), total Cr (tCr), and glycogen concentrations, as well as the PCr-to-adenosine triphosphate (ATP) and glycogen:ATP ratios, were observed with supplementation of 0.12% GAA compared with the NC diet, even exceeding responses to the PC diet. In study 2, Arg-adequate starter and grower basal diets were supplemented with 0 (negative control, NC), 0.06, or 0.12% GAA, 0.12% Cr monohydrate (PC1), or salmon protein (PC2; containing total Arg concentrations equal to those of the NC diet in each phase and containing similar Cr as in PC1). Overall G:F was increased ($P < 0.05$) by PC1, but not by PC2, compared with the NC, while GAA supplementation elicited a response intermediate to NC and PC1 diets. However, GAA supplementation increased ($P < 0.01$) concentrations of tCr and glycogen, as well as the PCr:ATP and glycogen:ATP ratios, when compared with the NC (Arg-adequate) diet. Collectively, these data indicate that GAA can be used to replace Arg in practical, Arg-deficient diets and improve muscle energy homeostasis in broiler chicks receiving either Arg-deficient or Arg-adequate practical diets.

Key words: growth, broiler, arginine, guanidinoacetic acid, creatine

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INTRODUCTION

As prices of commodity crops rise due to poor weather conditions or alternative use in food or energy industry, the poultry industry is increasingly seeking alternative feed ingredients. Such feed ingredients can be highly variable, however, resulting in the possibility of lower protein and amino acid (**AA**) concentrations compared with traditional ingredients. With the increased use of alternative protein sources, along with diets formulated with lower concentrations of crude protein, there exists a need to incorporate crystalline AA to maintain optimize dietary profiles. Arginine is considered the fifth limiting AA for broiler chickens (Han et al., 1992; Fernandez et al., 1994; Waguespack et al., 2009), but is not currently available in an economically viable form for the animal feed industry. Combined with lower crude protein formulations, increased use of co- and by-product ingredients, increased growth rate of modern broilers (Havenstein et al., 2003), and a lack of de novo synthesis of Arg (Tamir and Ratner, 1963a), dietary Arg may be limiting for optimized broiler production (Han et al., 1992).

Guanidinoacetic acid (**GAA**) was studied for its Arg-sparing effects using purified diets (Edwards et al., 1958; Savage and O'Dell, 1960), and it was reported to be comparable to creatine (**Cr**) supplementation, and recent work further supports the ability for

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[1]Corresponding author: rdilger2@illinois.edu

GAA to spare Arg when included in diets based on practical ingredients (Dilger et al., 2013; DeGroot et al., 2018). As the immediate precursor of Cr, GAA appears to cause feedback inhibition of its synthesizing enzyme, L-arginine:glycine aminotransferase (Walker, 1979; Guimarães-Ferreira, 2014), thereby reducing the metabolic drain on Arg and Gly for Cr synthesis. However, only limited information exists regarding the dose-dependent ability for GAA to spare Arg in practical broiler diets, as well as effects on Cr-related metabolites in muscle (i.e., phosphagen concentrations) or glycogen. In terms of Arg-adequate diets, current research indicates that GAA improves growth performance (Michiels et al., 2012; Mousavi et al., 2013; Majdeddin et al., 2017; Cordova-Noboa et al., 2018a,b), but outcomes related to muscle energy reserves like phosphagens or glycogen, or in comparison to animal protein-containing control diets with known Cr content, are scarce (Michiels et al., 2012). Thus, we hypothesized that GAA supplementation would elicit positive effects on growth performance specifically when included in Arg-deficient, practical broiler diets, with additional benefits involving muscle phosphagen concentrations in birds receiving both Arg-deficient and Arg-adequate diets. Two studies involving dose-dependent effects of GAA were conducted to test these hypotheses using broiler chicks.

MATERIALS AND METHODS

All animal care procedures were approved by the University of Illinois Institutional Animal Care and Use Committee before initiation of the studies.

Animals and Husbandry

For both studies described herein, 360 male Ross 708 broiler chicks (Hoovers Hatchery, Rudd, IA) were maintained in thermostatically controlled batteries with raised-wire floors in an environmentally controlled room with continuous lighting. Water and experimental diets were provided on an ad libitum basis throughout the feeding period. Chicks were weighed, wing-banded, and assigned to treatments such that the average initial pen weights were not different among all treatments. Study diets were fed in mash form in 2 feeding phases, starter (study day 0 to 14) and grower (study day 14 to 28 in study 1; study day 14 to 27 in study 2), beginning at 2 days post-hatch. In study 1, the grower treatment diets were fed for an additional day without performance data collection to allow for temporal separation of muscle biopsy procedures as described below. On study day 14, birds were moved from starter (0.06 m^2/bird) to grower (0.09 m^2/bird) battery pens. Battery pens provided access to 13.5 and 6.7 linear cm/bird of feeder and water space, respectively, via hanging troughs in a room with adequate lighting, ventilation, and temperature control to meet agricultural standards (Federation of Animal Science Societies, 2010). Twelve replicate pens of 6 chicks received each of the 5 dietary treatments in each study. To determine body weight (**BW**) gain, feed intake, and mortality-corrected G:F (based on bird·days), chicks and feeders were weighed on study day 0, 14, and 28 of study 1, and on study day 0, 14, and 27 of study 2. In both studies, birds were selected for collection of muscle biopsy samples within 24 h after final BW measurements according to procedures described below. All birds not selected for muscle biopsy collection were humanely euthanized via CO_2 asphyxiation.

Dietary Treatments

Study 1. An Arg-deficient basal diet was formulated to meet or exceed requirements for broiler chicks (National Research Council, 1994), with the exception of Arg (Table 1). Dietary treatments included the Arg-deficient basal diet (1.08 and 0.95% total Arg in starter and grower phases, respectively) that was either unsupplemented (negative control, **NC**) or supplemented with GAA (0.06, 0.12, or 0.18%) (CreAMINO, minimum 96% GAA; AlzChem Trostberg GmbH, Germany) or L-Arg (positive control, **PC**; 0.37 and 0.32% L-Arg in starter and grower phases, respectively; Ajinomoto North America, Raleigh, NC) at the expense of inert silica sand.

Study 2. An Arg-adequate basal diet was formulated to meet or exceed requirements for broiler chicks for all nutrients (National Research Council, 1994) (Table 1). Dietary treatments included the Arg-adequate basal diet (1.42 and 1.28% total Arg in starter and grower phases, respectively) that was either unsupplemented (NC) or supplemented with GAA (0.06 or 0.12%) (CreAMINO, minimum 95% GAA; AlzChem Trostberg GmbH), 0.12% Cr monohydrate (**PC1**) (Creapure, AlzChem Trostberg GmbH) at the expense of inert silica sand. In addition, a second positive control diet (treatment 5) providing Cr levels as in PC1 but derived from a natural source (i.e., animal protein) was formulated and manufactured independently from diets 1 to 4. A screening of commercial animal proteins collected between 2012 and 2014 in the USA showed overall low concentrations of Cr in the products available, as have been reported elsewhere (Harris et al., 1997; Michiels et al., 2012; Dobenecker and Braun, 2015). Creatine levels ranged from a mean of 67 mg/kg in blood meal (n = 8), 199 mg/kg in meat and bone meal (n = 6), 885 mg/kg in meat meal (n = 20), and 1,146 mg/kg in fish meal, which would result in a maximum concentration of 0.03% Cr from the intended inclusion rate of 7%. Therefore, for the target to have a PC containing a Cr source from natural ingredients, a custom-manufactured food-grade product (salmon protein; Lipromar GmbH, Cuxhaven, Germany) was prepared and analyzed to contain 14.6 mg/kg Cr. The salmon protein-containing diet (**PC2**) was formulated

Table 1. Dietary ingredient and calculated nutrient composition of basal diets.[1]

	Study 1		Study 2			
Ingredient, %	Starter	Grower	Starter NC	Starter PC2	Grower NC	Grower PC2
Corn	53.99	55.35	54.51	58.63	59.02	62.89
Soybean meal	17.00	11.20	35.53	24.91	30.74	20.35
Corn DDGS	10.00	12.00	–	–	–	–
Corn gluten meal	8.50	10.60	–	–	–	–
Salmon protein[2]	–	–	0.00	7.00	0.00	7.00
Soy oil	3.40	4.52	4.25	3.34	5.03	4.16
Salt	0.40	0.40	0.40	0.40	0.40	0.40
Limestone	1.45	1.30	1.35	1.35	1.30	1.30
Dicalcium phosphate	2.00	1.75	2.00	2.00	1.75	1.75
Vitamin premix[3]	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix[4]	0.15	0.15	0.15	0.15	0.15	0.15
Choline chloride	0.32	0.32	0.25	0.25	0.25	0.25
L-Lysine·HCl	0.73	0.68	0.27	0.39	0.22	0.33
DL-Methionine	0.30	0.20	0.35	0.37	0.28	0.30
L-Isoleucine	0.12	0.06	0.00	0.12	0.00	0.12
L-Threonine	0.27	0.18	0.19	0.24	0.13	0.17
L-Tryptophan	0.05	0.05	–	–	–	–
L-Valine	0.12	0.04	0.05	0.15	0.03	0.13
Inert silica sand	1.00	1.00	0.50	0.50	0.50	0.50
Proximate Composition						
Crude protein, %	22.0	21.0	22.0	23.5	20.0	21.6
Ca, %	1.07	0.94	1.06	1.04	0.97	0.95
P (total), %	0.75	0.69	0.74	0.77	0.68	0.70
P (available), %	0.51	0.46	0.49	0.55	0.44	0.50
AME_N, kcal/kg	3073	3199	3050	3050	3150	3150
SID amino acids, %[5]						
Arg	0.97	0.84	1.32	1.29	1.19	1.16
Ile	0.86	0.76	0.82	0.82	0.74	0.74
Leu	2.26	2.26	1.69	1.53	1.58	1.43
Lys	1.27	1.11	1.26	1.26	1.11	1.11
Met	0.65	0.56	0.65	0.69	0.56	0.60
Met+Cys	0.95	0.85	0.95	0.95	0.84	0.84
Thr	0.89	0.77	0.89	0.89	0.76	0.76
Trp	0.21	0.18	0.23	0.22	0.21	0.20
Val	0.96	0.85	0.95	0.95	0.85	0.85

[1]Abbreviations: NC, negative control; PC2, salmon protein-containing positive control; DDGS, distiller's dried grains with solubles.
[2]Lipromar GmbH, Cuxhaven, Germany.
[3]Provided per kg of diet:retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; DL-α- tocopheryl acetate, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin, 4.41 mg; D-Ca-pantothenate, 10 mg; niacin, 22 mg; menadione sodium bisulfite, 2.33 mg.
[4]Provided as milligrams per kg of diet:Mn, 75 from MnO; Fe, 75 from $FeSO_4 \bullet 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \bullet 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; Se, 0.1 from Na_2SeO_3.
[5]Standardized ileal digestible (SID) amino acid values acquired from AMINODat 4.0 (Evonik Industries AG, Hanau-Wolfgang, Germany).

to provide total Arg concentrations equal to those of the NC diet in each feeding phase and contained 7% salmon protein.

Diet Analyses. Diets were analyzed for dry matter, crude fat, crude fiber, and ash using standardized methods (AOAC International, 2006). Total nitrogen was determined using a Leco analyzer (TruMac N, Leco Corp., St. Joseph, MI) standardized with EDTA (method 990.03, AOAC International, 2006). Dietary AA concentrations were determined by ion-exchange chromatography using postcolumn derivatization with ninhydrin. Amino acids were oxidized with performic acid, which was neutralized with Na metabisulfite (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids were liberated from the protein by hydrolysis with 6 N HCl for 24 h at 110°C and quantified with the internal standard by measuring the absorption of reaction products with ninhydrin at 570 nm. Tryptophan and tyrosine were not determined. Dietary concentrations of GAA and Cr were quantified using fully validated procedures (Dobenecker and Braun, 2015). Dietary total choline was analyzed using a validated method (AOAC International, 2006) by an analytical laboratory (Eurofins Scientific Inc., Des Moines, IA).

Muscle Biopsy Collection

Two birds per pen with BW closest to the pen average were identified for muscle collection at the final weighing event. An attempt was made to use the bird with a BW closest to the pen average for muscle sampling. If that bird displayed more than minimal movement prior to muscle sampling (as assessed by a single observer), an alternate bird with a BW next closest to the pen average was chosen. In total, a muscle sample from 1 bird per pen was collected and analyzed. Apart from the time birds were being monitored for movement immediately before sampling (i.e., 15-min period), selected birds had ad libitum access to dietary

treatments, so muscle sampling was conducted in what was assumed to be a fed state.

To begin the procedure, the selected bird was injected intramuscularly (on the contralateral side of the pectoralis major where biopsy was to occur) with 2 mg/kg xylazine and 10 mg/kg ketamine (Maiti et al., 2006) to reduce the incidence of flapping prior to muscle collection. Birds remained calm (i.e., no handling or disruption) in this anesthetized state for 8 to 12 min before muscle biopsies were collected to ensure that muscle phosphagen status was normalized prior to sampling; times required for anesthetization are based on preliminary data from our lab (data not shown). Constant monitoring was provided by a single observer for all birds during both studies, and included time of anesthetic administration and bird condition (e.g., sedated, alert, relaxed, or flapping/moving) recorded every 2 min during the anesthetic period. No birds died as a result of anesthesia prior to euthanasia, and alternate birds were selected only if the initial bird exhibited one or more bouts of movement during the anesthesia period.

Following the anesthetic period, birds were euthanized via an intracardiac injection of sodium pentobarbital (390 mg/mL) at 0.2 mL of injected solution/kg BW to facilitate rapid collection of breast muscle tissue. As quickly as possible, a muscle sample (1 to 5 g of wet tissue) was collected within 30 s of euthanasia and immediately immersed directly in liquid nitrogen until gas elaboration ceased (up to 45 s of emersion). Time from euthanasia to flash-freezing of the muscle biopsy was no more than 60 s for any individual bird. Snap-frozen muscle biopsy samples were then shattered using blunt force, with frozen muscle aliquots randomly dispensed into pre-cooled cryovials and placed back in liquid nitrogen until transferred to storage at –80°C. At no point were flash-frozen muscle biopsy samples allowed to thaw.

Analysis of Cr-related Metabolites in Muscle

Muscle phosphocreatine (**PCr**), free Cr, adenosine triphosphate (**ATP**), and glycogen were measured simultaneously by an external laboratory (Harlan Laboratories, Itingen, Switzerland) using fully validated procedures as described previously (DeGroot et. al, 2018). In brief, muscle biopsy samples were freeze-dried, powdered, and dissected, and following extraction of the powder with perchloric acid, an aliquot of 25 μL of neutralized extract (corresponding to 250 μg of dried muscle) was used for the simultaneous determination of ATP and PCr. For determination of Cr, the neutralized extract was diluted 1:5 with assay buffer, and in a single run, undiluted extract was used for the analysis of Cr. The analytical method was based on enzymatic determinations, which ultimately resulted in either reduction of NADP to NADPH (for ATP, PCr) or oxidation of NADH to NAD (for Cr), measured spectrophotometrically at 340 nm in perchloric acid-extracted, dried tissue samples. Linearity, accuracy, precision, and selectivity of these assays were tested and achieved prior to analysis of muscle samples from birds enrolled in each study. Final data included the absolute concentration of each Cr-related metabolite (ATP, PCr, and Cr), along with calculation of absolute concentration of total Cr (**tCr**; PCr plus free Cr) and relative proportions (PCr:ATP, free Cr:ATP, tCr:ATP, and PCr:tCr). As muscle ATP is an indicator for the muscle tissue content in the sample, the PCr:ATP ratio is more appropriate than absolute PCr values since it cancels out variance due to differences in biopsy samples in their contents of blood and connective tissue (Harris et al., 1992).

Statistical Analysis

All data were analyzed as a randomized complete block design by a 1-way ANOVA using the mixed procedure of SAS (SAS Inst., Cary, NC). Diet and replicate were independent variables in this model, and pen of birds served as the experimental unit for all response variables. Treatments were compared using least squares means separation. Overall treatment effects with a probability of $P < 0.05$ were accepted as statistically significant, and trends were defined as $0.05 < P < 0.10$.

RESULTS

Overall, formulation objectives were achieved in terms of creating both Arg-deficient and Arg-adequate basal diets, and graded supplementation of Arg and GAA was achieved in the experimental dietary treatments (Table 2). As such, formulated and analyzed concentrations of target nutrients (Arg, GAA, and Cr) were similar (Table 3).

Study 1

Growth Performance. The PC diet increased ($P < 0.05$) final BW by 10% and daily BW gain day 14 to 28 and day 0 to 28 by 11 and 10%, respectively, compared with the NC diet (Table 4). Supplementation with 0.06 or 0.12% GAA elicited graded improvements in FCR and BWG. Additionally, 0.12% GAA increased ($P < 0.05$) both final BW and daily BW gain (day 0 to 28) by 6% compared with the NC diet. In each case, responses to 0.12% GAA supplementation were statistically equivalent to the PC diet. Supplementation with 0.06 or 0.18% GAA improved BW numerically, with no significant differences evident compared with either the NC or PC diets. There were no significant effects of dietary supplementation on feed intake during any period in this study.

Mortality-corrected G:F was increased ($P < 0.05$) by 10% on average in birds fed the PC diet, compared

Table 2. Analyzed composition of dietary control treatments (%, as-is basis).[1]

	Study 1[2]				Study 2[3]					
	NC		PC		NC		PC1		PC2[4]	
Nutrient	Starter	Grower	Starter	Grower	Starter	Grower	Starter	Grower	Starter	Grower
Dry matter	89.3	89.3	89.7	89.8	89.4	89.2	89.3	88.9	89.1	89.1
Crude protein	22.6	23.6	23.6	21.3	22.6	19.9	23.2	20.6	24.1	21.8
Crude fat	6.9	6.8	6.8	8.1	7.0	7.1	6.7	7.8	6.3	7.6
Crude fiber	2.8	2.7	2.7	2.9	2.9	2.3	2.7	2.3	1.9	1.9
Ash	6.5	6.1	6.1	5.8	6.4	6.0	6.0	6.2	6.5	6.2
Choline	0.19	0.19	0.20	0.19	0.20	0.20	0.20	0.21	0.20	0.19
Amino acids[5]										
Essential										
Arg	1.05	0.98	1.47	1.25	1.54	1.31	1.45	1.36	1.47	1.31
His	0.51	0.48	0.52	0.47	0.58	0.53	0.54	0.54	0.49	0.47
Ile	0.95	0.87	0.92	0.85	0.97	0.85	0.89	0.87	0.86	0.81
Leu	2.36	2.47	2.30	2.49	2.01	1.79	1.89	1.83	1.70	1.56
Lys	1.35	1.21	1.43	1.20	1.42	1.23	1.38	1.27	1.36	1.20
Met	0.68	0.56	0.66	0.58	0.61	0.59	0.66	0.61	0.68	0.62
Phe	1.09	1.08	1.08	1.08	1.17	1.01	1.10	1.03	0.97	0.85
Thr	0.98	0.93	0.99	0.87	0.97	0.84	0.98	0.87	0.96	0.84
Val	1.05	0.98	1.07	0.96	1.13	0.97	1.06	0.99	1.04	0.95
Non-essential										
Ala	1.34	1.37	1.31	1.37	1.12	1.03	1.06	1.05	1.29	1.24
Asp	1.69	1.58	1.72	1.54	2.38	2.04	2.23	2.12	2.00	1.82
Cys	0.36	0.35	0.36	0.35	0.39	0.35	0.36	0.36	0.31	0.28
Glu	3.88	3.86	3.84	3.83	4.19	3.64	3.95	3.75	3.63	3.27
Gly	0.74	0.70	0.75	0.68	0.94	0.81	0.89	0.83	1.69	1.61
Pro	1.49	1.59	1.47	1.57	1.36	1.25	1.27	1.28	1.50	1.45
Ser	1.01	0.99	1.00	0.99	1.11	1.00	1.06	1.03	1.02	0.95

[1]Abbreviations: NC, negative control; PC, Arg-adequate positive control (study 1); PC1, creatine monohydrate-containing positive control (study 2); PC2, salmon protein-containing positive control (study 2).
[2]Basal diet formulated to contain 1.08% total Arg (0.97% digestible Arg) and 0.95% total Arg (0.84% digestible Arg) in the starter and grower phases, respectively.
[3]Basal diet formulated to contain 1.42% total Arg (1.32% digestible Arg) and 1.28% total Arg (1.19% digestible Arg) in the starter and grower phases, respectively.
[4]Basal diet formulated to contain 1.42% total Arg (1.29% digestible Arg) and 1.28% total Arg (1.16% digestible Arg) in the starter and grower phases, respectively.
[5]Analyzed total amino acid content values standardized to a dry matter content of 88%.

with the NC diet, during each feeding phase and over the entire 28-D feeding period. Moreover, G:F increased ($P < 0.05$) with graded supplemental concentrations of 0.06, 0.12, and 0.18% GAA during both the starter (6, 11, and 8%, respectively) and finisher (3, 6, and 7%, respectively) periods, respectively, with equivalent effects noted when comparing the 0.12% GAA-supplemented diet with the PC diet during each feeding period. Diets supplemented with GAA at 0.12 or 0.18% improved ($P < 0.05$) G:F compared with the NC diet during the entire 28-D feeding period.

Tissue Analysis. Muscle tCr and PCr were reduced in the NC diet as compared with the PC diet ($P < 0.05$), while glycogen concentrations were unaffected. Differences in muscle total Cr and PCr were 66 and 58% for NC and PC treatments, respectively, providing evidence that cellular energy supply was depleted due to ingestion of Arg-deficient diets. Supplementation of 0.06, 0.12, or 0.18% GAA gradually elevated ($P < 0.05$) PCr concentrations by 18, 66, and 105%, respectively, when compared with the NC diet. Supplementation of 0.18% GAA was needed to increase glycogen concentrations above those elicited by the NC diet. The PCr:ATP and glycogen:ATP ratios were also increased due to graded GAA supplementation, with the most prominent effects observed when supplementing GAA at 0.12 and 0.18% (Table 5). Total Cr increased ($P < 0.05$) by 16, 49, and 76% with 0.06, 0.12, or 0.18% GAA supplementation, respectively, compared with the NC diet. Supplementation of GAA at 0.12% elicited a tCr concentration that was equal to the PC diet, and addition of 0.18% GAA caused tCr to be 11% greater ($P < 0.05$) than tCr concentrations elicited by the PC diet. There were no differences between treatments for absolute concentrations of ATP or relative proportions of PCr:tCr.

Study 2

Growth Performance. BW gain of chicks receiving Arg-adequate diets did not respond to the supplementation of GAA or Cr during study day 0 to 14; results from day 0 to 27 suggested a trend for higher ($P = 0.06$) ending BW due to GAA supplementation (Table 6). During day 14 to 27, BW gain in PC2 was reduced when compared with the NC diet, but BW was greater ($P < 0.05$) in chicks fed diets supplemented with either GAA (11% higher) or Cr (PC1; 8% higher) compared with the salmon protein-containing diet (PC2). There

Table 3. Formulated and analyzed concentrations of targeted nutrients in experimental dietary treatments (as-is basis).[1]

Study	Feeding Phase	Treatment	Arginine, %		GAA, mg/kg		Creatine[2], mg/kg	
			Formulated	Analyzed	Formulated	Analyzed	Formulated	Analyzed
1	Starter	NC	1.08	1.05	0	ND	0	–
		0.06% GAA	1.08	1.10	600	513	0	–
		0.12% GAA	1.08	1.10	1,200	1,098	0	–
		0.18% GAA	1.08	1.11	1,800	1,835	0	–
		PC	1.45	1.47	0	ND	0	–
	Grower	NC	0.95	0.98	0	ND	0	–
		0.06% GAA	0.95	0.98	600	590	0	–
		0.12% GAA	0.95	0.97	1,200	1,181	0	–
		0.18% GAA	0.95	0.96	1,800	1,771	0	–
		PC	1.27	1.25	0	ND	0	–
2	Starter	NC	1.42	1.54	0	ND	0	ND
		0.06% GAA	1.42	1.51	600	702	0	ND
		0.12% GAA	1.42	1.48	1,200	1,281	0	ND
		PC1	1.42	1.45	0	ND	1,200	1,031
		PC2	1.42	1.47	0	ND	1,200	1,069
	Grower	NC	1.28	1.31	0	ND	0	ND
		0.06% GAA	1.28	1.32	600	650	0	ND
		0.12% GAA	1.28	1.28	1,200	1,248	0	ND
		PC1	1.28	1.36	0	ND	1,200	1,158
		PC2	1.28	1.31	0	ND	1,200	1,095

[1]Abbreviations: GAA, guanidinoacetic acid; NC, negative control; ND, not detectable; PC, Arg-adequate positive control (study 1); PC1, creatine monohydrate-containing positive control (study 2); PC2, salmon protein-containing positive control (study 2).
[2]Creatine concentrations were only quantified in study 2 and were assumed to be under the quantifiable detection limit in study 1.

Table 4. Growth performance of chicks fed Arg-deficient diets (study 1).[1]

Variable	Dietary treatment[2]					Pooled SEM	Overall *P*-value
	NC	0.06% GAA	0.12% GAA	0.18% GAA	PC		
Body weight, g							
Day 0	33.2	33.2	33.2	33.2	33.2	0.01	0.143
Day 14	259.5	269.2	273.1	262.9	273.7	5.13	0.222
Day 28	950.8^{a}	987.9^{a-c}	1010.4^{b,c}	982.3^{a,b}	1042.4^{c}	19.83	0.030
Daily BW gain, g/chick							
Day 0 to 14	16.2	16.9	17.1	16.4	17.2	0.37	0.222
Day 14 to 28	49.4^{a}	51.0^{a}	52.6^{a,b}	51.2^{a}	54.8^{b}	1.18	0.028
Day 0 to 28	32.8^{a}	34.1^{a-c}	34.9^{b,c}	33.9^{a,b}	36.0^{c}	0.71	0.030
Daily feed intake, g/chick							
Day 0 to 14	23.8	23.5	22.5	22.4	22.9	0.51	0.235
Day 14 to 28	76.0	75.6	76.1	72.5	77.6	1.27	0.091
Day 0 to 28	49.4	48.2	48.5	47.0	49.5	0.80	0.180
Gain:feed, g/kg							
Day 0 to 14	675.3^{a}	715.5^{b}	749.5^{c}	730.2^{b,c}	749.7^{c}	10.03	<0.001
Day 14 to 28	643.3^{a}	661.0^{a}	680.8^{b}	685.8^{b}	706.0^{c}	7.14	<0.001
Day 0 to 28	646.0^{a}	663.7^{a}	696.6^{b,c}	685.3^{a,b}	712.6^{c}	8.79	<0.001
Mortality, no. of chicks							
Day 0 to 14	3	3	5	2	5	–	–
Day 14 to 28	2	5	1	5	1	–	–
Day 0 to 28	5	8	6	7	6	–	–

[a–c]Means within a row lacking a common superscript letter differ ($P < 0.05$).
[1]Values are means of 12 replicate pens of 6 chicks during the feeding period 2 to 30 D post-hatch. Abbreviations: GAA, guanidinoacetic acid; NC, negative control; PC = positive control; SEM, standard error of the mean.
[2]Basal diet formulated to contain 1.08% total Arg (0.97% digestible Arg) and 0.95% total Arg (0.84% digestible Arg) in the starter and grower phases, respectively.

were no effects of dietary supplementation on feed intake during any feeding period in this study.

Mortality-corrected G:F was increased ($P < 0.05$) by addition of 0.12% GAA as compared with the NC diet from study day 0 to 14 and day 14 to 27. Supplementation of Cr increased ($P < 0.05$) G:F by 6, 4, and 4%, respectively, in the starter, grower, and overall periods as compared with the NC diet. While the salmon protein-containing diet (PC2) elicited superior G:F relative to all other treatments during day 0 to 14, it also produced the lowest G:F for day 14 to 27 and day 0 to 27.

Tissue Analysis. A main effect of dietary treatment was observed for the concentration of muscle ATP, with the NC diet eliciting the highest ($P < 0.05$) muscle ATP concentration and graded addition of GAA reducing ($P < 0.05$) the ATP concentration in a dose-dependent manner (Table 7). Therefore, the appropriate way to compare PCr and Cr values is by normalization with ATP, which inherently corrects for

Table 5. Muscle analyses of creatine-related metabolites in chicks fed Arg-deficient diets (study 1).[1]

Variable	Dietary treatment[2]					Pooled SEM	Overall *P*-value
	NC	0.06% GAA	0.12% GAA	0.18% GAA	PC		
ATP, mmol/kg DW	35.93	36.90	34.40	35.84	33.73	0.90	0.107
PCr, mmol/kg DW	52.81^{a}	62.42^{a}	87.69^{b}	108.17^{c}	87.60^{b}	4.25	<0.001
PCr:ATP ratio	1.45^{a}	1.72^{a}	2.56^{b}	3.02^{c}	2.62^{b}	0.12	<0.001
Free Cr, mmol/kg DW	35.64^{a}	39.88^{a}	43.85^{a,b}	47.11^{b}	51.73^{b}	3.39	0.017
Free Cr:ATP ratio	1.01^{a}	1.09^{a,b}	1.12^{a,b,c}	1.32^{b,c}	1.55^{c}	0.11	0.011
Total Cr, mmol/kg DW[3]	88.45^{a}	102.29^{b}	131.54^{c}	155.28^{d}	139.33^{c}	4.33	<0.001
Total Cr:ATP ratio	2.45^{a}	2.81^{a}	3.85^{b}	4.34^{c}	4.17^{b,c}	0.14	<0.001
PCr:total Cr, %	58.93	61.37	66.80	69.68	62.69	2.96	0.094
Glycogen, mmol/kg DW	191.50^{a,b}	180.67^{a}	212.50^{a,b}	265.42^{c}	225.67^{b}	12.70	0.0002
Glycogen:ATP ratio	5.64^{a,b}	5.15^{a}	6.30^{a,b}	7.81^{c}	6.75^{b}	0.43	0.0008

$^{a–d}$Means within a row lacking a common superscript letter differ ($P < 0.05$).

[1]Values are means of 12 replicate chicks (i.e., 1 chick per pen) with muscle samples collected on day 30 post-hatch. Abbreviations: ATP, adenosine triphosphate; Cr, creatine; DW, dry weight; GAA, guanidinoacetic acid; PCr, phosphocreatine; NC, negative control; PC, Arg-adequate positive control; SEM, standard error of the mean.

[2]Basal diet formulated to contain 1.08% total Arg (0.97% digestible Arg) and 0.95% total Arg (0.84% digestible Arg) in the starter and grower phases, respectively.

[3]Calculated as PCr plus free Cr.

Table 6. Growth performance of chicks fed Arg-adequate diets (study 2).[1]

Variable	Dietary treatment[2]					Pooled SEM	Overall *P*-value
	NC	0.06% GAA	0.12% GAA	PC1[3]	PC2[4]		
Body weight, g							
Day 0	34.3	34.3	34.3	34.3	34.3	0.24	0.672
Day 14	431.1	438.7	449.3	440.5	448.4	9.42	0.642
Day 27	1418.1	1440.4	1490.2	1451.3	1378.3	26.81	0.066
Daily BW gain, g/chick							
Day 0 to 14	28.4	28.9	29.6	29.0	29.6	0.67	0.644
Day 14 to 27	75.6^{a,b}	76.9^{b}	79.2^{b}	77.7^{b}	71.5^{a}	1.51	0.011
Day 0 to 27	51.3	52.1	53.9	52.5	51.4	1.21	0.538
Daily feed intake, g/chick							
Day 0 to 14	36.7	36.0	37.3	35.2	33.7	0.87	0.053
Day 14 to 27	97.1	96.0	100.2	95.5	94.1	1.76	0.168
Day 0 to 27	66.6	64.5	67.8	65.0	62.8	1.32	0.092
Gain:feed, g/kg							
Day 0 to 14	772.6^{a}	785.4^{a,b}	801.3^{b,c}	818.4^{c}	851.3^{d}	9.55	<0.001
Day 14 to 27	719.5^{a}	727.9^{a,b}	738.7^{b,c}	746.0^{c}	703.7^{a}	6.10	<0.001
Day 0 to 27	729.8^{a,b}	727.3^{a}	748.5^{b,c}	759.9^{c}	740.2^{a-c}	7.01	0.013
Mortality, no. of chicks							
Day 0 to 14	1	7	4	1	7	–	–
Day 14 to 27	2	5	3	2	1	–	–
Day 0 to 27	3	12	7	3	8	–	–

$^{a–d}$Means within a row lacking a common superscript letter differ ($P < 0.05$).

[1]Values are means of 12 replicate pens of 6 chicks during the feeding period 2 to 29 D post-hatch. Abbreviations: GAA, guanidinoacetic acid; NC, negative control; PC1, creatine monohydrate-containing positive control; PC2, salmon protein-containing positive control; SEM, standard error of the mean.

[2]Basal diet formulated to contain 1.42% total Arg (1.32% digestible Arg) and 1.28% total Arg (1.19% digestible Arg) in the starter and grower phases, respectively.

[3]Arg-adequate diet supplemented with 0.12% creatine monohydrate.

[4]Basal diet formulated to contain 1.42% total Arg (1.29% digestible Arg) and 1.28% total Arg (1.16% digestible Arg) in the starter and grower phases, respectively. This Arg-adequate diet was supplemented with 7.0% salmon protein to achieve 0.12% digestible creatine to match PC1 diet.

differences in biopsy sample quality (e.g., relative composition of muscle, blood, and connective tissue). As such, the PCr:ATP ratio was improved ($P < 0.05$) in chicks receiving either PC diet. Graded addition of GAA numerically increased ($P = 0.07$) the absolute muscle PCr concentration, while the PCr:ATP ratio was increased ($P < 0.05$) by 26% when the NC diet was supplemented with 0.12% GAA. Moreover, 0.12% GAA supplementation elicited a PCr:ATP ratio that was not different from either the PC1 or PC2 diets. Neither absolute nor relative concentrations of free Cr were affected by GAA supplementation of Arg-adequate diets. For tCr, when expressed as the tCr:ATP ratio, both GAA-supplemented diets elicited responses that were higher ($P < 0.05$) than the NC diet and equivalent in magnitude to both the PC1 and PC2 diets. No dietary effects were noted for the PCr:tCr ratio in this study. The absolute muscle glycogen concentration was lower ($P < 0.05$) in the NC diet compared with either PC diets, and only 0.12% GAA supplementation

Table 7. Muscle analyses of creatine-related metabolites in chicks fed Arg-adequate diets (study 2).[1]

Variable	Dietary treatment[2]					Pooled	Overall
	NC	0.06% GAA	0.12% GAA	PC1[3]	PC2[4]	SEM	*P*-value
ATP, mmol/kg DW	33.04^{c}	28.34^{a,b}	27.39^{a}	30.90^{b,c}	30.85^{b,c}	0.95	0.001
PCr, mmol/kg DW	78.89	73.78	82.90	90.45	85.66	4.13	0.066
PCr:ATP ratio	2.40^{a}	2.61^{a,b}	3.03^{c}	2.94^{c}	2.77^{b,c}	0.12	0.003
Free Cr, mmol/kg DW	71.09	74.75	72.19	74.05	78.39	4.47	0.812
Free Cr:ATP ratio	2.21	2.69	2.66	2.41	2.60	0.19	0.356
Total Cr, mmol/kg DW[5]	149.99^{a}	148.53^{a}	155.09^{a,b}	164.50^{c}	164.05^{b,c}	3.24	0.001
Total Cr:ATP ratio	4.61^{a}	5.30^{b}	5.69^{b}	5.35^{b}	5.36^{b}	0.16	<0.001
PCr:total Cr, %	52.70	49.60	53.62	54.98	51.99	2.59	0.664
Glycogen, mmol/kg DW	179.17^{a}	178.50^{a}	209.50^{a,b}	223.33^{b}	264.92^{c}	11.31	<0.001
Glycogen:ATP ratio	5.53^{a}	6.39^{a,b}	7.72^{c,d}	7.30^{b,c}	8.57^{d}	0.40	<0.001

$^{a–d}$Means within a row lacking a common superscript letter differ ($P < 0.05$).

[1]Values are means of 12 replicate chicks (i.e., 1 chick per pen) with muscle samples collected on day 30 post-hatch. Abbreviations: ATP, adenosine triphosphate; Cr, creatine; DW, dry weight; GAA, guanidinoacetic acid; PCr, phosphocreatine; NC, negative control; PC1, creatine monohydrate-containing positive control; PC2, salmon protein-containing positive control; SEM, standard error of the mean.

[2]Basal diet formulated to contain 1.42% total Arg (1.32% digestible Arg) and 1.28% total Arg (1.19% digestible Arg) in the starter and grower phases, respectively.

[3]Arg-adequate diet supplemented with 0.12% creatine monohydrate.

[4]Basal diet formulated to contain 1.42% total Arg (1.29% digestible Arg) and 1.28% total Arg (1.16% digestible Arg) in the starter and grower phases, respectively. This Arg-adequate diet was supplemented with 7.0% salmon protein to achieve 0.12% digestible creatine to match PC1 diet.

[5]Calculated as PCr plus free Cr.

elicited a numerical increase in the muscle glycogen concentration above that observed for the NC diet. The glycogen:ATP ratio observed for the NC diet was lower ($P < 0.05$) than for either PC diet, and GAA supplementation elicited graded increases such that addition of 0.12% GAA produced a ratio that was not different from the PC diets.

DISCUSSION

The present study was designed to test the efficacy of GAA for supporting growth and muscle phosphagen status in fast-growing broiler chicks fed either Arg-deficient or Arg-adequate diets based on practical ingredients. When GAA was supplemented in Arg-deficient diets, G:F increased markedly, such that diets containing 0.12% GAA elicited growth performance responses that were overall equivalent to the Arg-adequate PC diet in study 1. Moreover, responses due to GAA were evident in muscle phosphagen concentrations as GAA-supplemented diets caused PCr and tCr concentrations to meet or exceed those of the Arg-adequate PC diet. These results were specifically notable as more L-Arg was added to NC to formulate PC (0.37 and 0.32% Arg in starter and grower resulting in 21 and 18 mmol, respectively) compared to gradual additions of GAA (0.06, 0.12, and 0.18% in starter and grower phases resulting in 5, 10, and 15 mmol, respectively). In Arg-adequate diets (study 2), GAA supplementation also markedly increased G:F, such that diets containing 0.12% GAA elicited growth performance responses that were overall equivalent to the Cr-supplemented and salmon protein-supplemented PC diets. Moreover, responses due to GAA were evident in muscle phosphagen concentrations as even 0.06% GAA supplementation caused the PCr and tCr:ATP ratios to meet or exceed those of the Cr-supplemented and salmon protein-supplemented PC diets. These observations provide clear and direct evidence of GAA efficacy in terms of growth performance and muscle phosphagen status in broiler chicks fed practical Arg-deficient and Arg-adequate diets.

Improvements in growth performance when GAA is added to Arg-deficient diets are in agreement with data from Savage and O'Dell (1960), Dilger et al. (2013), and DeGroot et al., (2018). Unlike previous observations, however, supplementation of 0.12% GAA elicited both final BW and overall BW gain improvements that were greater than the NC diet and equal to the PC diet. Moreover, G:F was improved as has been consistently observed (Dilger et al., 2013; DeGroot et al., 2018). Our data strongly indicate an Arg-sparing effect (Almquist et al., 1941), which would allow Arg to be used for functions other than Cr synthesis, most notably muscle growth (Edwards et al., 1958). In general, GAA restored growth performance of birds receiving Arg-deficient diets containing practical ingredients common to the U.S. poultry industry.

Supplementation of GAA increased concentrations of Cr-related metabolites in chicken breast muscle, with PCr and tCr concentrations increasing 18 and 15%, respectively, with 0.06% GAA supplementation and 66 and 49%, respectively, for 0.12% GAA supplementation, when compared with the NC diet in study 1. When compared with the PC diet, PCr and tCr concentrations due to 0.12% GAA supplementation were comparable, with 0.18% GAA increasing concentrations of these metabolites even further. These muscle phosphagen outcomes concur with observations from Michiels et al. (2012), who reported increases in breast muscle tCr concentrations with GAA supplementation. Because of the tandem increases in muscle PCr and tCr concentrations, we can assume that dietary GAA is successfully absorbed and metabolized to Cr in the broiler

chicken (Wyss and Kaddurah-Daouk, 2000), and when combined with the increase in growth performance, these observations indicate that GAA supplementation spares Arg from Cr synthesis. Possibly, growth might have been affected more directly by elevated muscle Cr and PCr as it has been reported that Cr and PCr interact with phospholipids and hence will stabilize cell membranes (Tokarska-Schlattner, 2012). Furthermore, an increase in muscle glycogen from dietary GAA, as observed most prominent in the Arg-deficient diets at a supplementation level of 0.18% addition, may provide additional energy for growth.

The increase in muscle metabolites, especially the PCr:ATP ratio, indicates that potential for PCr to regenerate ATP is increased, and thus, there appears to be an increased potential for energy expenditure in chickens consuming supplemental GAA. This likely occurs because GAA supplementation circumvents downregulation of the L-arginine:glycine aminotransferase enzyme caused by Cr synthesis (Wyss and Kaddurah-Daouk, 2000), and because the production of Cr from GAA is unregulated and continues until all GAA is consumed or excreted (da Silva et al., 2009). Thus, supplemental GAA essentially bypasses enzymatic regulation and permits increased concentrations of Cr-related metabolites in muscle, which is a unique outcome for this nutritional additive. In this way, tCr and PCr concentrations increase the overall potential for muscle energy homeostasis (Guimarães-Ferreira, 2014), thereby allowing for ATP to be consumed at a higher rate in support of improved metabolic function.

When 0.12% GAA was supplemented to the Arg-adequate diet, G:F increased 3% relative to the NC diet. This is in agreement with results of research conducted by Michiels et al. (2012) and Mousavi et al. (2013), who reported that feed efficiency was improved by 2 and 5%, respectively, when added to Arg-adequate diets. As all diets in study 2 were Arg-adequate, there was theoretically no Arg to be spared by GAA for protein accretion. Because GAA is converted to Cr via a simple methyltransferase reaction (Wyss and Kaddurah-Daouk, 2000), and both muscle PCr and tCr concentrations increased due to GAA supplementation, it is assumed that GAA was synthesized to Cr with high efficacy, thereby producing results very similar to direct Cr supplementation. The advantage of using GAA, however, is that this chemically synthesized metabolite is more stable when incorporated into mixed diets compared with Cr monohydrate. Muscle energy metabolites, namely Cr and PCr, may be elevated from natural sources of Cr; however, products with relevant concentrations of Cr are not readily available on the market.

Both of our studies resulted in improvements in BW gain, G:F, and PCr:ATP ratios with 0.12% GAA supplementation. In an Arg-deficient diet, GAA is likely alleviating the Arg deficiency by sparing Arg for use in lean tissue accretion and thereby increasing BW gain (Dilger et al., 2013; DeGroot et al., 2018). In the Arg-adequate diet, however, the rate of muscle protein synthesis should be equivalent because all diets contained adequate and equal quantities of Arg, yet muscle concentrations of Cr-related metabolites continued to increase due to GAA supplementation. While this did not manifest as an improvement in BW gain in our study, this phenomenon has been previously reported to increase muscle growth (Ingwall, 1976; Ingwall and Wildenthal, 1976). Whereas 0.06 and 0.12% GAA supplementation elicited increases in the PCr:ATP and tCr:ATP ratios in both Arg-deficient and Arg-adequate diets, improvements in the Arg-deficient scenario were 2 to 3 times greater than when diets were Arg-adequate. With either dietary Arg status, GAA supplementation increased the concentration of Cr observed in the muscle, and this effect was predominantly through alteration of PCr concentrations.

Overall, we conclude that GAA is capable of sparing dietary Arg when broiler chicks are fed either Arg-deficient or Arg-adequate diets based on practical ingredients. From these studies, we conclude that 0.12% GAA supplementation is the most effective level of supplementation, such that growth performance and Cr-related metabolites were superior to those elicited by the NC diets and equivalent to the PC diets. Future studies should focus on dose-dependent changes in L-arginine:glycine aminotransferase enzyme activity to better quantify the Arg-sparing effects of GAA. Moreover, investigating the effects of dietary GAA on muscle glycogen concentrations and whether GAA supplementation affects muscle protein synthesis in vivo is warranted.

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