EFFICACY OF DIETARY GUANIDINOACETIC ACID IN BROILER CHICKS

BY

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THESIS

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ABSTRACT

Three studies were conducted to determine the arginine (Arg) sparing effect of guanidinoacetic acid (GAA) and the efficacy of GAA to support growth performance and muscle phosphagen homeostasis in chicks when supplemented in either Arg-deficient or Arg-adequate diets. Study 1 established that 0.12% GAA supplementation increased ($P < 0.05$) gain:feed (G:F) by 7% on average compared with the 0.0% GAA negative controls. Bodyweight gain (BWG) increased ($P < 0.05$) and G:F ($P < 0.05$) for d 15 to 22 and d 8 to 22 with increasing Arg and GAA supplementation, proving that diets were successfully Arg deficient, and Arg and GAA supplementation alleviated the deficiency. Muscle metabolites, including phosphocreatine (PCr) and PCr:ATP, were increased ($P < 0.05$) by an average of 101% and 103%, respectively, with 0.12% GAA compared with the 0.0% GAA negative controls. Total Cre (tCre) was increased (Arg and GAA interaction, $P < 0.05$) by 41 and 51% with 0.12% GAA when included in diets containing 0.0 or 0.16% added Arg, respectively. Hematological and clinical chemistry outcomes were unaffected by GAA treatment, and plasma concentrations of essential amino acids appeared to be reflective of improvements in growth performance. Study 2 confirmed that 0.12% GAA supplementation increased ($P < 0.05$) BWG and G:F ($P < 0.05$) from d 0 to 28 by 6 and 8%, respectively, when compared with the negative control diet. Muscle concentrations of both PCr and tCre were increased ($P < 0.05$) with addition of 0.12% GAA by 66 and 49%, respectively, compared with the negative control (Arg-deficient). In Study 3, 0.12% GAA supplementation increased ($P < 0.05$) G:F d 14 to 27 by 3% when compared with the negative control (Arg-adequate) diet. Muscle PCr and tCre concentrations were also increased ($P < 0.05$) in chicks receiving 0.12% supplemental GAA by 26 and 18%, respectively, as compared with the negative

control (Arg adequate). Improvements in growth performance and muscle phosphagen concentrations in broiler chicks consuming practical diets supplemented with GAA indicates that this feed additive is a suitable replacement for Arg.

For Matt Nothnagel

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LIST OF ABBREVIATIONS

AA	Amino acid(s)
ADG	Average daily gain
ADP	Adenosine diphosphate
AGAT	Arginine:glycine amidinotransferase
AL	Argininosuccinate lysase
Arg	Arginine
AS	Argininosuccinate synthase
ATP	Adenosine triphosphate
BW	Body weight
BWG	Body weight gain
Cit	Citrulline
CK	Creatine kinase
CK-B	Creatine kinase brain
CK-M	Creatine kinase cytosol of muscle
CK-mit	Creatine kinase mitochondria of muscle
Cre	Creatine
Crn	Creatinine
CPS-I	Carbamylphosphate synthetase I
DDGS	Distillers dried grains with solubles
GAA	Guanidinoacetic acid
Gly	Glycine
GS	Glutamine synthetase
Met	Methionine
NADH	Nicotinamide adenine dinucleotide
NC	Negative control
NRC	National Research Council
OAT	Ornithine aminotransferase
Orn	Ornithine
OTC	Ornithine transcarbamoylase
P-5-C	Pyrroline-5-carboxylate
PC	Positive control
PCr	Phosphocreatine
PDG	Phosphate-dependent glutaminase
RCBD	Randomized complete block design
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
tCre	Total Creatine
XDH	Xanthine dehydrogenase

Chapter 1

INTRODUCTION

Feed constitutes the greatest proportion of costs associated with poultry production, with most of that cost related to the high price of protein-furnishing ingredients. As commodity crop prices rise due to poor weather conditions, increased demand for products, and alternative land-usage, poultry producers increasingly look for alternative feed ingredients to use as substitutes. These alternative feed ingredients can be categorized as co-products, by-products, and non-traditional feed ingredients. However, these alternatives may have lower quality and higher variability of protein and amino acid concentrations compared with the original sources. This variability can be addressed with supplementation of commercially-available, crystalline amino acids. Unfortunately, not all amino acids are commercially available; an example that is not readily available is arginine (Arg). Arginine is the fifth limiting essential AA in most poultry diets and is needed for mainly bodily functions concerning maintenance (i.e., creatine production) and growth (i.e., protein synthesis) (Klose et al., 1938; Waguespack et al., 2009; Wu and Morris Jr., 1998). Due to the increased growth rate of modern broilers (Havenstein et al., 2003), lack of Arg *de novo* synthesis (Tamir and Ratner, 1963), and bodily need for Arg-based metabolites (i.e., Cre) (Walker, 1979), dietary Arg supplementation may be required to support optimal growth and maintenance (Han et al., 1992). Without an economically-feasible and commercially-available source of Arg, the increased use of lower crude protein formulations and alternative ingredients, including distillers dried grains with solubles (DDGS), necessitates the inclusion of crystalline AA beyond those which are currently available. Therefore, the poultry

industry may soon have a need to supplement Arg, and one way to relieve the formulation pressure may be providing a feed additive that can spare the use of Arg for Cre synthesis. Guanidinoacetic acid (GAA) is a compound formed from Arg and Gly, and is produced via chemical synthesis from glycine cyanamide commercially as CreAMINO®. Guanidinoacetic acid is able to spare Arg that would otherwise be used for Cre synthesis (Savage and O'Dell, 1960) because it is converted into Cre in both the avian liver and kidney (Van Pilsum et al., 1972). Creatine is used to make the high-energy phosphagen PCr and assists in maintaining energy homeostasis in muscles (Guimarães-Ferreira, 2014). If supplemented as an Arg replacement (Dilger et al., 2013), GAA has been reported to both spare Arg in Arg-deficient diets, but also to improve growth performance in Arg-adequate diets (Michiels et al., 2012). If the cost of GAA is less expensive than, or equal to, commercially available Arg, then GAA supplementation would be more advantageous to supplement because of the improvement observed in Arg-adequate diets. Therefore, the poultry industry may choose to use GAA as an Arg replacement, relieving the necessity for Arg supplementation in current poultry diets.

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Chapter 2

LITERATURE REVIEW

Arginine (Arg) is needed for maintenance and growth in vertebrates and is required for the synthesis of nitric oxide, creatine (Cre), the non-essential amino acids proline and glutamate, and for protein synthesis (Wu and Morris Jr., 1998). Synthesis of muscle protein and production of Cre are particularly important for young, growing animals due to the high rate of muscle and nerve growth that occurs (Walker, 1979). Because the bodily need for Arg is so high during this phase of increased growth, Arg is considered semi-essential in mammals and essential in poultry and reptiles (Klose et al., 1938). Commercial poultry, in particular broiler chickens, have been bred for increased rates of growth and muscle development, reaching production size between 6 and 8 weeks of age. Because of the increased rate of growth for current broiler chickens, which increases growth and maintenance requirement for Arg, and due to the limitations of chickens to synthesize Arg *de novo* (Klose et al., 1938; Tamir and Ratner, 1963a), supplemental dietary Arg may be required to support optimal growth and maintenance (Han et al., 1992).

Arginine Metabolism

Due to the insufficient capacity for *de novo* Arg production, chickens require twice as much Arg as pigs as proportion of the diet. During the swine grower phase, the National Research Council (NRC) Arg requirement is 0.50% to 0.32% of the diet, while during the same period in broiler chickens, the NRC Arg requirement is 1.1% of the diet (NRC, 2012; NRC, 1994). This difference in requirement is because pigs, like other mammals, are capable of producing a sizeable proportion of their required Arg *de novo*.

Arginine Synthesis

In mammals, Arg synthesis occurs via the culmination of multiple metabolic pathways. In mature animals, the major precursor for Arg synthesis is citrulline (Cit) produced in the small intestine (De Jonge et al., 1998; Herzfeld and Raper, 1976; Hurwitz and Kretchmer, 1986; Wu, 1998), with glutamine and glutamate serving as the major Cit precursors (Matthews et al., 1993; Stoll et al., 1998; Windmueller and Spaeth, 1975; Wu, 1998). Citrulline is transported from the small intestine to the kidney where argininosuccinate synthase (AS) and argininosuccinate lysase (AL) convert Cit to Arg (**Figure 2.1**), accounting for nearly 60% of net Arg synthesis in mammals (Dhanakoti et al., 1990; Funahashi et al., 1981; Levillain et al., 1992; Wu and Morris Jr., 1998).

Although, Arg is synthesized as part of the urea cycle in mammals, it is immediately converted to ornithine (Orn) and urea, thereby continuing the cycle with no net synthesis of Arg (Morris Jr., 1992; Wu and Morris Jr., 1998). It was theorized, however, that net Arg synthesis could occur if it was maintained through the introduction of intermediates, like Orn from other sources (Wu and Morris Jr., 1998), such as the production of guanidinoacetic acid (GAA) (Brosnan and Brosnan, 2010). The production of GAA would continue the urea cycle by converting Arg and glycine (Gly) to Orn and GAA via the enzyme arginine:glycine amidinotransferase (AGAT), with Orn returning to the cycle to be converted to Arg again (**Figure 2.2**). In rats, McGuire et al. (1986) were able to detect AGAT protein not only in the kidney of the rat, but also in the hepatocytes, thus prompting further study of Cre synthesis in the kidney. Incubating rat hepatocytes with isotope-labeled ammonia, along with Gly, methionine (Met), and pyruvate, Brosnan and Brosnan (2010) reported no detectable levels of Cre, though it was previously demonstrated that rat hepatocytes could convert labeled ammonia to labeled urea,

and GAA to Cre (da Silva et al., 2009). Because there was no detectable production of Cre in rat hepatocytes when incubated with GAA precursors and stimulation of the urea cycle, it was concluded that AGAT was not producing GAA with Arg from the urea cycle.

Unlike mammals, birds and other uricotelic animals excrete nitrogen as uric acid via the "urate cycle" (Campbell, 1995). Development of uric acid excretion, as well as formation of the bird's cloaca, is theorized to have begun due to an increasingly arid environment during the late Triassic period (Benton, 1983). The arid environment pushed for the conservation of water as a means of survival, and thus the evolution of the avian kidney has allowed for the excretion of nitrogenous waste as a semi-solid paste resulting in little water excretion via the production of uric acid (Laverty and Skadhauge, 2008; Schmidt-Nielsen, 1988). Uricogenesis is the excretion of nitrogen, through the breakdown of purines, as uric acid or urate. This process uses fifteen enzymatic steps to recycle ribose-5-phosphate and synthesize urate (Campbell, 1995) (**Figure 2.3**). The major enzymes that are important in uric acid production include glutamine synthetase (GS) and xanthine dehydrogenase (XDH). Glutamine synthetase is used to fix ammonia; it is the uricotelic equivalent of mammalian carbamylphosphate synthetase I (CPS-I) (Campbell, 1995). Xanthine dehydrogenase is the final enzyme in the production of uric acid; it is a source of reduced nicotinamide adenine dinucleotide (NADH) for uricogenesis (Campbell, 1995).

Although chickens and other uricotelic animals do not excrete nitrogen via the urea cycle, enzymes integral to this cycle are present in various organs in the chicken. While only low levels of arginase activity are observed in chicken liver, both AS and AL are present in the chicken kidney (Tamir and Ratner, 1963a). The activity of renal AS and AL in chickens was determined to be similar to that observed in rats, 10 μg $AS \cdot h^{-1} \cdot g^{-1}$ kidney tissue wet weight and 11 μg $AL \cdot h^{-1} \cdot g^{-1}$ kidney tissue wet weight vs. 22 μg $AS \cdot h^{-1} \cdot g^{-1}$ kidney tissue wet weight and 16 μg $AL \cdot h^{-1} \cdot g^{-1}$

kidney tissue wet weight (Tamir and Ratner, 1963a). Because both of these enzymes are present in the kidney, Cit is able to replace Arg in chicks (Klose et al., 1938; Tamir and Ratner, 1963b). Tamir and Ratner (1963b) conducted a study to prove the conversion of Cit to Arg in the kidney by feeding labeled Cit to chicks fed a low Arg diet. Labeled Arg was observed in organs, blood, and muscle tissues, proving the conversion of Cit to Arg was possible with approximately 30% efficiency. Although Cit can be converted to Arg in birds, similar to mammals, Cit cannot be synthesized *de novo*, as in mammals. Tamir and Ratner (1963b) conducted an additional study to determine if Cit could be synthesized in the chicken, not just converted to Arg. Chickens fed a low Arg diet were supplemented with unlabeled Cit and ^{14}C labeled sodium carbonate. These authors theorized that if ornithine transcarbamoylase (OTC) was present in the chicken, any carbamoyl phosphate containing the ^{14}C label would be detectable as labeled Cit. Although labeled ^{14}C was observed in glutamic acid and aspartic acid pools, no labeled Arg was measured, indicating that Cit could not be formed by the action of OTC in the chicken.

Wu and Flynn (1995) also conducted a study to determine if Cit was synthesized in the intestines of the chick. After incubating chick enterocytes in either glutamine or Orn, these authors reported that neither Orn, Cit, nor Arg were converted from glutamine, and neither Cit nor Arg were synthesized from Orn. To confirm their conclusions, the activities of pyrroline-5-carboxylate (P-5-C) synthase and OTC were measured, along with many other enzymes connected with Cit, Orn, and Arg synthesis. Compared with levels observed in pig enterocytes, AS, AL, and arginase levels were lower in chickens, 0.15 ± 0.02, 0.22 ± 0.03, 0.34 ± 0.06 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein, respectively vs. 1.72 ± 0.24, 3.01 ± 0.29, 2.28 ± 0.35 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein, respectively (Wu et al., 1995). When comparing CPS-I, levels were similar in both chickens (1.27 ± 0.06 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein) and pigs (Wu et al., 1994). However, phosphate-

dependent glutaminase (PDG) and ornithine aminotransferase (OAT) levels, 1.95 ± 0.37 and 1.42 ± 0.39, nmol·min^{-1}·mg^{-1} protein respectively, were lower in chickens as compared with pigs, 17% and 3% activity, respectively (Wu et al., 1994). The lower levels of PDG and OAT in chickens indicate that glutamine could not be used to synthesize Orn. No activity of either P-C-5 synthase or OTC were detected in the enterocytes of chickens, confirming observations from the *in vitro* studies.

Guanidinoacetic Acid Production

In all vertebrates, one of the main uses for Arg, from either dietary or *de novo* origins, is the production of GAA, which is the precursor to Cre. Guanidinoacetic acid is synthesized through the transfer of an amidino group from Arg to Gly via AGAT, forming GAA and Orn (**Figure 2.4**). This *de novo* synthesis of GAA accounts for 40-60% of the total Cre in rats (Goldman and Moss, 1959), indicating that a large amount of Arg is used for the production of GAA in the body.

In mammals, the production of GAA occurs mainly in the kidneys, where AGAT activity is highest, although small amounts of AGAT have been detected in the liver (Walker, 1979). The rat, for example, has AGAT activity of 22.2 μmol GAA·h^{-1}·g^{-1} kidney tissue, while humans have an AGAT activity of 10.4 μmol GAA·h^{-1}·g^{-1} kidney tissue (Van Pilsum et al., 1972). In the liver however, the AGAT activity in rats is <0.01 μmol GAA·h^{-1}·g^{-1} liver, while in humans it is 1.0 μmol GAA·h^{-1}·g^{-1} liver, indicating that very little GAA is produced in liver compared with kidney in mammals. Chickens, on the other hand, are able to synthesize GAA in both the kidney and liver, with AGAT activity of 2.5 μmol GAA·h^{-1}·g^{-1} kidney and 3.5 μmol GAA·h^{-1}·g^{-1} liver (Van Pilsum et al., 1972). Comparatively, enzyme activity of AGAT in the chicken is about 25%

of that observed in the human, but the enzyme is more distributed in the chicken than the human. The enzyme activity of AGAT is very important for the production of Cre, with AGAT being both the limiting reaction for Cre synthesis and the most regulated step (Walker, 1979; Wyss and Kaddurah-Daouk, 2000).

The rate-limiting nature of AGAT may be related to availability of GAA precursor compounds. In rats, although the K_m values of AGAT for Gly and Arg are relatively similar (2.4-2.8 mM and 3.0-3.1 mM, respectively), the renal concentrations of these amino acids are extremely different (Brosnan et al., 1983), leading to concentrations that are about 2.5 mM for Gly and 0.32 mM for Arg (Edison et al., 2007). These concentration differences indicate that Arg is the limiting substrate in production of GAA, which could be problematic if animals were fed an Arg-deficient diet. In mammals this is not a concern, as there is ample *de novo* synthesis of Arg. In birds and other uricotelic animals, however, many metabolic consequences could arise, such as decreased growth, impaired metabolic functions, and impaired muscle development (Wietlake et al., 1954).

Creatine Metabolism

Creatine Synthesis

Creatine synthesis is the only known metabolic function of GAA (Walker, 1979), and is synthesized when GAA receives a methyl moiety from S-adenosylmethionine (SAM) via guanidinoacetate methyltransferase (GAMT); S-adenosylhomocysteine (SAH) is released as a byproduct of this reaction. Creatine helps to maintain the energy balance in cells and tissues by accepting high energy phosphate groups from adenosine triphosphate (ATP) to create

phosphocreatine (PCr), and then releasing the high energy phosphate group to form ATP when energy demand is high (Guimarães-Ferreira, 2014; Wyss and Kaddurah-Daouk, 2000).

In mammals, Cre is synthesized primarily in the liver, where GAMT activity is highest, although small amounts of GAMT are detectable in the kidney (Walker, 1979). The rat, for example has GAMT activity of 0.77 μmol Cre·h^{-1}·g^{-1} liver, while humans have GAMT activity of 0.85 μmol Cre·h^{-1}·g^{-1} liver (Van Pilsum et al., 1972). In the kidney, however, the GAMT activity in rats is 0.02 μmol Cre·h^{-1}·g^{-1}, while in humans it is 0.07 μmol Cre·h^{-1}·g^{-1}, indicating that little Cre is produced in kidney as compared with liver in mammals. Chickens, on the other hand, are able to synthesize Cre in both the kidney and liver, with GAMT activity at 1.21 μmol Cre·h^{-1}·g^{-1} liver and 1.06 μmol Cre·h^{-1}·g^{-1} kidney (Van Pilsum et al., 1972).

Phosphocreatine Synthesis

Phosphocreatine is formed when an energy rich phosphate group is removed from ATP and attached to Cre by creatine kinase (CK) in a reversible reaction (Figure 2.2). The phosphorylation cycle of Cre and PCr is incredibly important to energy distribution and for the maintenance of energy in cells (Guimarães-Ferreira, 2014). Creatine kinase facilitates the exchange of energy rich phosphate groups between Cre and PCr, thereby using ATP and adenosine diphosphate (ADP) as metabolic intermediates (**Figure 2.5**) (Brosnan and Brosnan, 2010; Guimarães-Ferreira, 2014; Wyss and Kaddurah-Daouk, 2000). Creatine kinase is composed of four isoforms observed in the cytosol of muscles (CK-M) and brain (CK-B), and in the mitochondria of muscles and all other tissues (Cit-mit) (Guimarães-Ferreira, 2014; Wyss and Kaddurah-Daouk, 2000). It is through the compartmentalization of CK that the PCr "transport" or "shuttle" theory is possible (Guimarães-Ferreira, 2014; Wyss and Kaddurah-Daouk, 2000).

The “shuttle” theory is the cyclical movement of ATP and ADP through the use of PCr and Cre. Beginning with the formation of ATP in the mitochondria, CK-mit cleaves the phosphate from ATP forming ADP and PCr. Phosphocreatine, due primarily to size and ease of diffusion (Minajeva et al., 1996; Yoshizaki et al., 1990), moves from the mitochondria to the cytoplasm where isomers of CK-M or CK-B remove the phosphate group from PCr, forming Cre, and regenerating ATP from ADP. The regenerated ATP is converted back into ADP by an ATPase in muscle, brain, or other tissues, where the phosphate group is used for metabolic work (e.g., muscle contraction). Creatine diffuses back into the mitochondria to be used again in the cycle (Guimarães-Ferreira, 2014; Wyss and Kaddurah-Daouk, 2000).

Without a functioning PCr “shuttle” system, muscles would be unable to contract. Using frog muscles, Cain and Davies (1962) showed that when CK is inhibited, isolated muscle could only contract about 10% of that of control muscles (3 muscle contractions vs. > 30 muscle contractions), with no measureable difference in PCr before or after contractions. If the “shuttle” system were not able to function properly in poultry, then there would be little to no muscle growth. Thus, if Cre and PCr concentrations were suboptimal, e.g., due to Arg deficiency, muscle growth could be impaired not only because of less Arg for protein accretion, but also due to impaired ATP distribution in muscles.

Creatinine Synthesis

While converting between Cre and PCr in the PCr “shuttle”, some molecules of both Cre and PCr are converted into and excreted as creatinine (Crn) through a non-enzymatic and spontaneous reaction (Wyss and Kaddurah-Daouk, 2000). Under *in vitro* conditions, the reversible conversion of Cre to Crn is dependent on both pH and temperature. Low pH and high

temperature favors the conversion to Crn, while high pH and low temperature favors the conversion to Cre (Kreider et al., 1996). However, this metabolic regulation has not been confirmed *in vivo*, mainly because conversion from Cre to Crn appears to be irreversible (Bloch and Schoenheimer, 1939; Walker, 1979). Originally it was theorized that Crn could be converted to Cre due to an increase in muscle Cre when Crn was supplemented (Almquist et al., 1941). Using labeled Cre and Crn, subsequent studies proved the conversion was irreversible (Bloch and Schoenheimer, 1939; Wyss and Kaddurah-Daouk, 2000), with ingestion of labeled Cre being quantified as muscle Cre and urinary Crn. When labeled Crn was supplemented, however, the labeled isotope was observed mainly in the urine with no significant levels of isotope observed in body Cre (Bloch and Schoenheimer, 1939; Wyss and Kaddurah-Daouk, 2000). When studied *in vivo*, it was concluded that 1.1% of endogenous Cre and 2.6% of endogenous PCr were irreversibly converted into Crn each day in humans, with an average of 1.7% of total Cre (i.e., Cre + PCr) turned over daily (Walker, 1979; Wyss and Kaddurah-Daouk, 2000).

Regulation of Creatine Production

Due to the important roles of Cre and PCr in energy maintenance of brain and muscle tissues, the production of Cre is highly regulated (Wyss and Kaddurah-Daouk, 2000), and all regulation of this metabolic pathway is due to the AGAT enzyme.

Arginine:Glycine Amindinotransferase

The formation of GAA by AGAT is the rate-limiting, and most highly regulated, step in Cre production (Wu, 1998; Wyss and Kaddurah-Daouk, 2000). The primary regulation of AGAT is feedback repression by Cre, which causes a decrease in mRNA expression, AGAT concentration, and AGAT activity, indicating that Cre regulates AGAT at both transcriptional

and translational levels (Guthmiller et al., 1994; Walker, 1979). This down-regulation of AGAT can be absolute, although total repression is uncommon. Arginine and GAA are also purported regulatory molecules, as both need to be converted to Cre to decrease AGAT activity (Magri et al., 1975; Walker and Wang, 1964; Wyss and Kaddurah-Daouk, 2000).

The expression of AGAT is also controlled via hormonal influence. Using rats without thyroid or pituitary glands, injection of thyroid hormone and growth hormone caused AGAT activity to increase until levels were normal (Guthmiller et al., 1994; Van Pilsum et al., 1992). Thyroid and growth hormone administration caused an increase in the expression of AGAT level and activity, indicating that the hormonal regulation was similar to Cre regulation by influencing the level of gene expression (McGuire et al., 1980). However, when growth hormone was injected into hypophysectomized rats consuming Cre-supplemented diets, there was no change in AGAT activity compared with non-supplemented, hypophysectomized rats. This indicates that Cre and growth hormone are antagonistic in regard to AGAT activity (McGuire et al., 1980; Van Pilsum et al., 1992).

In addition to the regulatory effects of Cre on AGAT activity, complete cessation of nutrients (i.e., fasting) also influences AGAT (Van Pilsum et al., 1992). Walker (1960) discovered that chicks fed a diet with 3% supplemental Cre had a decrease in AGAT activity after 3 days of feeding (7.3 μmol GAA$\cdot h^{-1} \cdot g^{-1}$liver wet weight in control birds compared with 0.5 μmol GAA$\cdot h^{-1} \cdot g^{-1}$ liver wet weight in treated birds). Fasting also causes decreased AGAT levels due to the increase in blood Cre levels that occurs when an animal is fasted (Kim et al., 1983). Although determined first in mice (Kim et al., 1983), Walker (1960) reported similar trends in chicks. As was discussed above, chicks fed a diet with supplemental Cre had decreased AGAT activity. After receiving a diet supplemented with 2% Cre for 74 h, AGAT levels in chicks

decreased to approximately 10% of control levels (17 μmol GAA·h^{-1}·g^{-1} liver weight wet). At that point, the investigators divided the birds into two groups and they were either returned to the control diet or given no food (i.e., fasted). After 48 h off of Cre supplementation, chicks returned to the control diet had AGAT activity that was about 70% of control levels, indicating a transient response to dietary Cre supplementation. However, fasted birds had virtually no AGAT activity when compared with birds remaining on the Cre-supplemented diet. Whereas no explanation was given in the original manuscript, it is now understood that fasting also increased the circulating Cre in the body to inhibit AGAT activity.

Guanidinoacetic Acid Methyltransferase

Unlike AGAT, GAMT expression and activity are not regulated by Cre (da Silva et al., 2009; Wyss and Kaddurah-Daouk, 2000). Guanidinoacetic acid methyltransferase is consistently inhibited by SAH, as are virtually all methyltransferases (Clarke and Banfield, 2001; Fujioka et al., 1988). da Silva et al. (2009) reported that GAMT activity in hepatocytes was not affected in rats fed either Cre-free or Cre-supplemented diets. More importantly, these investigators reported that the rate of conversion of GAA to Cre was not altered with either dietary treatment when hepatic cells were incubated with GAA. Therefore, if GAA is supplemented to cells, Cre production continues without inhibition.

Arginine-Sparing Effects

With adequate Cre supplementation, AGAT enzyme concentration and activity are reduced such that *de novo* Cre synthesis is decreased or completely repressed (Walker, 1960; Wyss and Kaddurah-Daouk, 2000). Because there is no need for GAA formation when Cre is supplemented to a diet, the precursors of GAA production and Cre formation are able to be

spared for use elsewhere in the body. The sparing of components is only possible because AGAT is regulated by the end-product of Cre. In regard to GAA production, both Arg and Gly can be used for other functions in the body, such as protein accretion, nitric oxide production, or *de novo* amino acid synthesis. Moreover, in regard to Cre production, methionine and SAM can be spared for use in other parts of the body, such as protein synthesis and methylating other compounds, respectively.

Creatine

Almquist et al. (1941) reported that supplementing Cre, GAA, or Crn stimulated growth of chicks fed Arg-deficient diets, indicating that all compounds exhibited Arg-sparing effects. Wietlake et al. (1954) used Cre, Arg, and Gly to determine how best to alleviate Arg deficiency in casein-based diets fed to chicks. It was observed that Cre supplementation did improve growth rates in chicks fed an Arg-deficient diet, but had little to no effect when supplemented in an Arg-adequate diet. Fisher et al. (1956), contrary to previous research, reported that Cre supplementation elicited very little growth improvement, compared with other diets supplemented with Arg, Gly, Met, or their combinations. Savage and O'Dell (1960) reported that Cre supplementation improved growth performance of chicks fed Arg-deficient diets, and spared about 0.42% Arg. Whereas Cre supplementation can clearly spare Arg, use of Cre in poultry diets is impractical due to issues with product stability and cost (Baker (2009).

Guanidinoacetic Acid

Guanidinoacetic acid may serve as a better feed additive because it is more stable and cost effective than Cre. Almquist et al. (1941) theorized that GAA could spare Arg, along with Cre and Crn. This theory was later supported by Edwards Jr. et al. (1958), providing clear

evidence that GAA was converted to Cre in chicks. These studies also indicated that Arg was spared and was used for muscle protein accretion (i.e., body weight gain). Other studies have also shown that supplementation of GAA is comparable to Cre supplementation in chicks fed Arg deficient diets (Savage and O'Dell, 1960). Michiels et al. (2012) reported that GAA supplementation of 0.12% increased the gain:feed ratio when compared with a negative control diet, and both 0.06% and 0.12% GAA supplementation improved gain:feed by an average of 2.3% compared with the negative control. Michiels, et al. (2012) also reported improved beast meat yield with GAA supplementation that was comparable to the positive control diet. Mousavi et al. (2013) reported that addition of 0.06% GAA improved feed conversion ratio over periods d 23 to 40 and d 0 to 40 by 4% and 3%, respectively, when compared with a practical Arg-adequate diet without added GAA. Mousavi et al. (2013) also reported that feed conversion ratio was improved with 0.06% GAA supplementation from d 0 to 40 to a nutritionally-complete diet. Dilger et al. (2013) reported that there was an improvement in gain:feed ratio when feeding 0.12% GAA in practical Arg-deficient diets, which was comparable to 0.15% Cre and 0.25% Arg supplementation. Murakami, et al.(2014) reported improved weight gain and feed conversion in offspring of GAA supplemented breeder quail, indicating that GAA supplementation may be advantageous beyond growth. Collectively, results of these reports indicate GAA may serve as a commercially-viable compound to improve growth performance of broiler chickens by sparing Arg for use in protein synthesis, and GAA supplementation should theoretically also positively influence muscle phosphagen concentrations.

Conclusion

With the increasing use of non-traditional feed ingredients and lower protein poultry diets, there is risk of experiencing amino acid deficiencies when using practical diets. Currently, crystalline Arg is too expensive to be included in practical diets, yet Arg is the fifth limiting amino acid in most poultry diets (Waguespack et al., 2009). Therefore, the following studies were conducted to determine the effects of GAA supplementation on growth performance and muscle energy metabolites in practical diets for broiler chickens.

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Figures

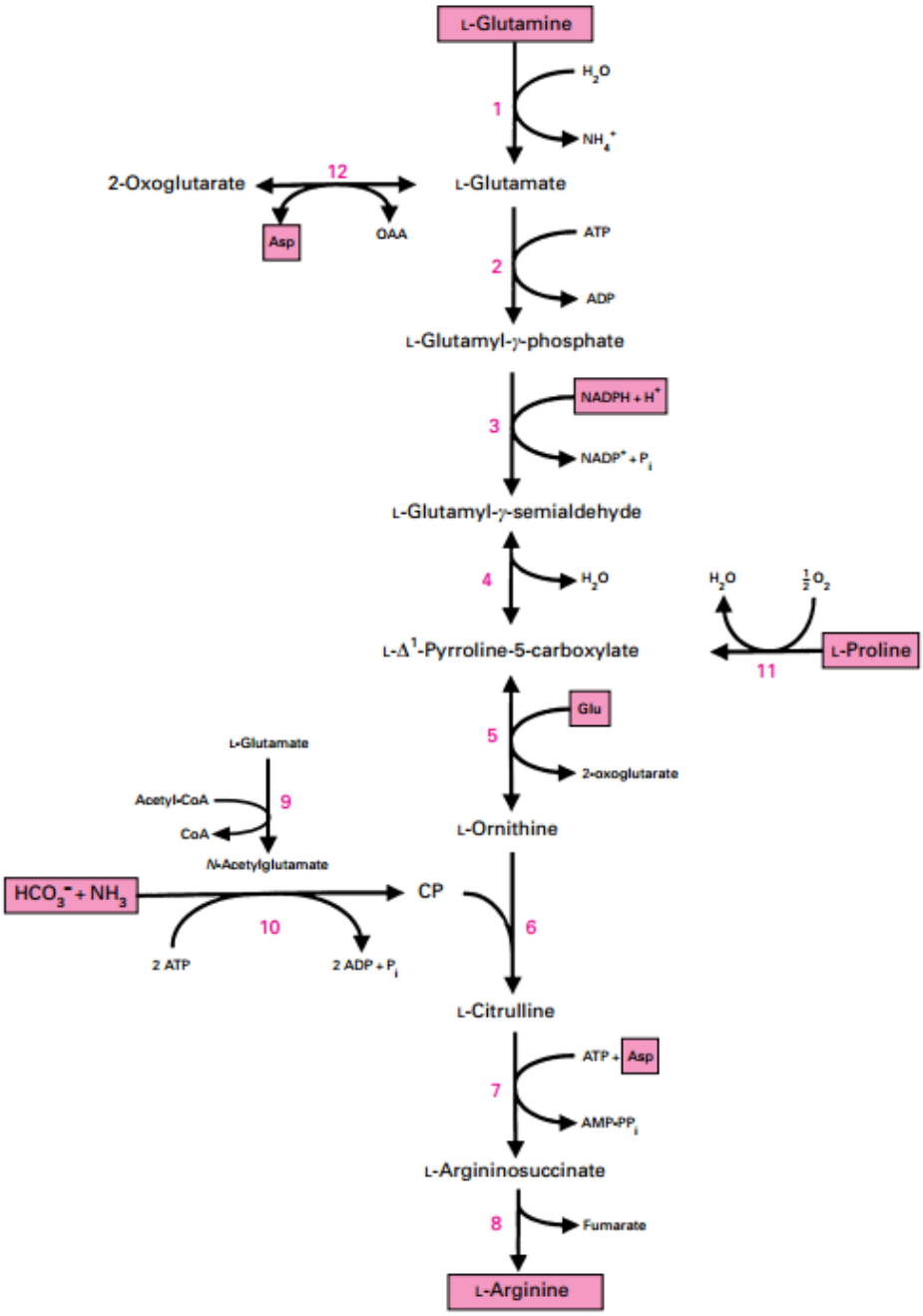

Figure 2.1 Pathways of arginine synthesis. 1, phosphate-dependent glutaminase (EC 3.5.1.2); 2 and 3, P5C synthetase (EC number not assigned); 5, ornithine aminotransferase (OAT; EC 2.6.1.13); 6, ornithine carbamoyltransferase (OCT; EC 2.1.3.3); 7, argininosuccinate synthase (AS; EC 6.3.4.5); 8, argininosuccinate lyase (AL; EC 4.3.2.1); 9, N-acetylglutamate synthase (EC 2.3.1.1); 10, carbamoyl-phosphate synthase I (ammonia) (CPS I; EC 6.3.4.16); 11, proline oxidase (EC number not assigned); 12, aspartate aminotransferase (EC 2.6.1.1). Abbreviations: OAA, oxaloacetate; CP, carbamoyl phosphate. Adapted from Wu and Morris (1998).

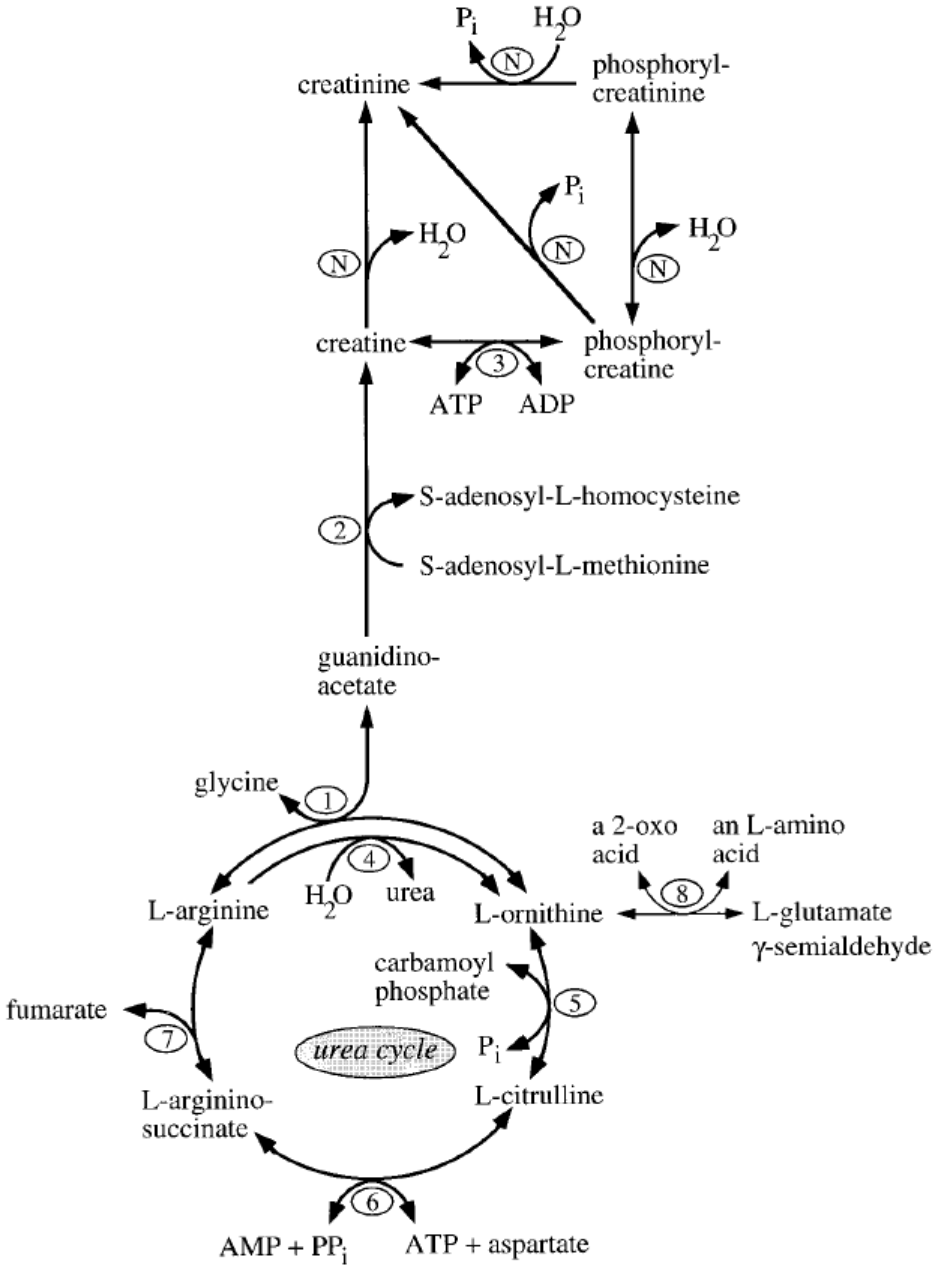

Figure 2.2 Schematic representation of the reactions and enzymes involved in vertebrate creatine and creatinine metabolism. The respective enzymes are denoted by numbers: 1, L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1); 2, S-adenosyl- L-methionine:N-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2); 3, creatine kinase (CK; EC 2.7.3.2); 4, arginase (L-arginine amidinohydrolase; EC 3.5.3.1); 5, ornithine carbamoyltransferase (EC 2.1.3.3); 6, argininosuccinate synthase (EC 6.3.4.5); 7, argininosuccinate lyase (EC 4.3.2.1); 8, L-ornithine:2-oxo-acid aminotransferase (OAT; EC 2.6.1.13); N, nonenzymatic reaction. Adapted from Wyss Kaddurah-Daouk (2000).

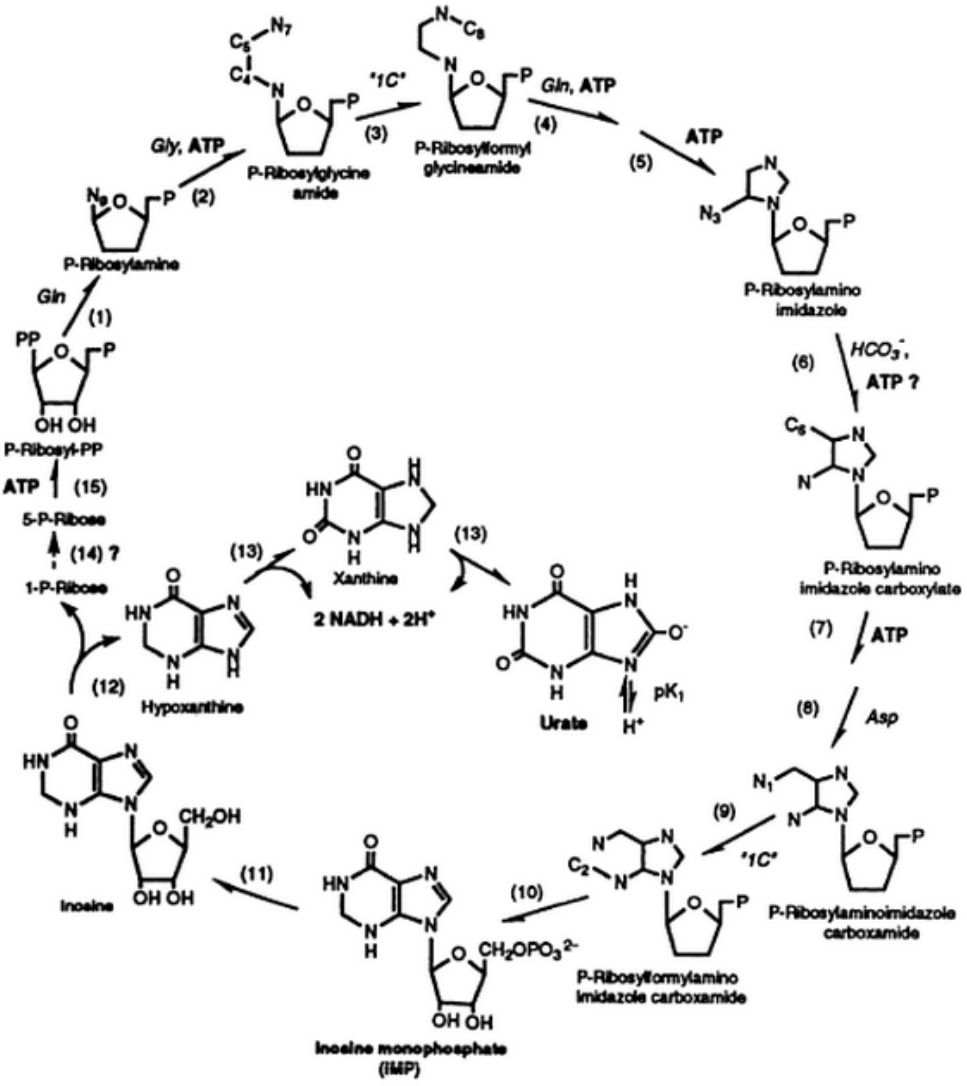

Figure 2.3 Biosynthesis of urate. The enzymes involved include: 1, glutamine phosphoribosyl pyrophosphate amidotransferase (PRPP amidotransferase); 2, glycineamide ribonucleotide synthetase (GAR synthetase); 3, glycineamide ribonucleotide transformylase (GAR transformylase); 4, formylglycieamide ribonucleotide amidotransferase (FGAR amidotransferase); 5, aminoimidazole ribonucleotide synthetase (AIR synthetase); 6, aminoimidazole ribonucleotide carboxylase (AIR carboxylase); 7, N-succinylcarboxamide aminoimidazole ribonucleotide synthetase (SAICAR synthetase); 8, adenylosuccinate lyase (adenylosuccinase); 9, aminoimidazole carboxamide ribonucleotide transformylase (AICAR transformylase); 10, IMP cyclohydrase (IMP synthetase, inosinicase); 11, 5'-ribonucleotide phosphohydrolase (5'-nucleotidase); 12, nucleoside phosphohydrolase (nucleoside phosphorylase); 13, xanthine: NAD+ oxidoreductase (xanthine dehydrogenase, XDH); 14, phosphopentomutase; and 15, ATP:ribose-5-P pyrophosphotransferase (5'phosphoribosyl pyrophosphokinase). Adapted from Campbell (1995).

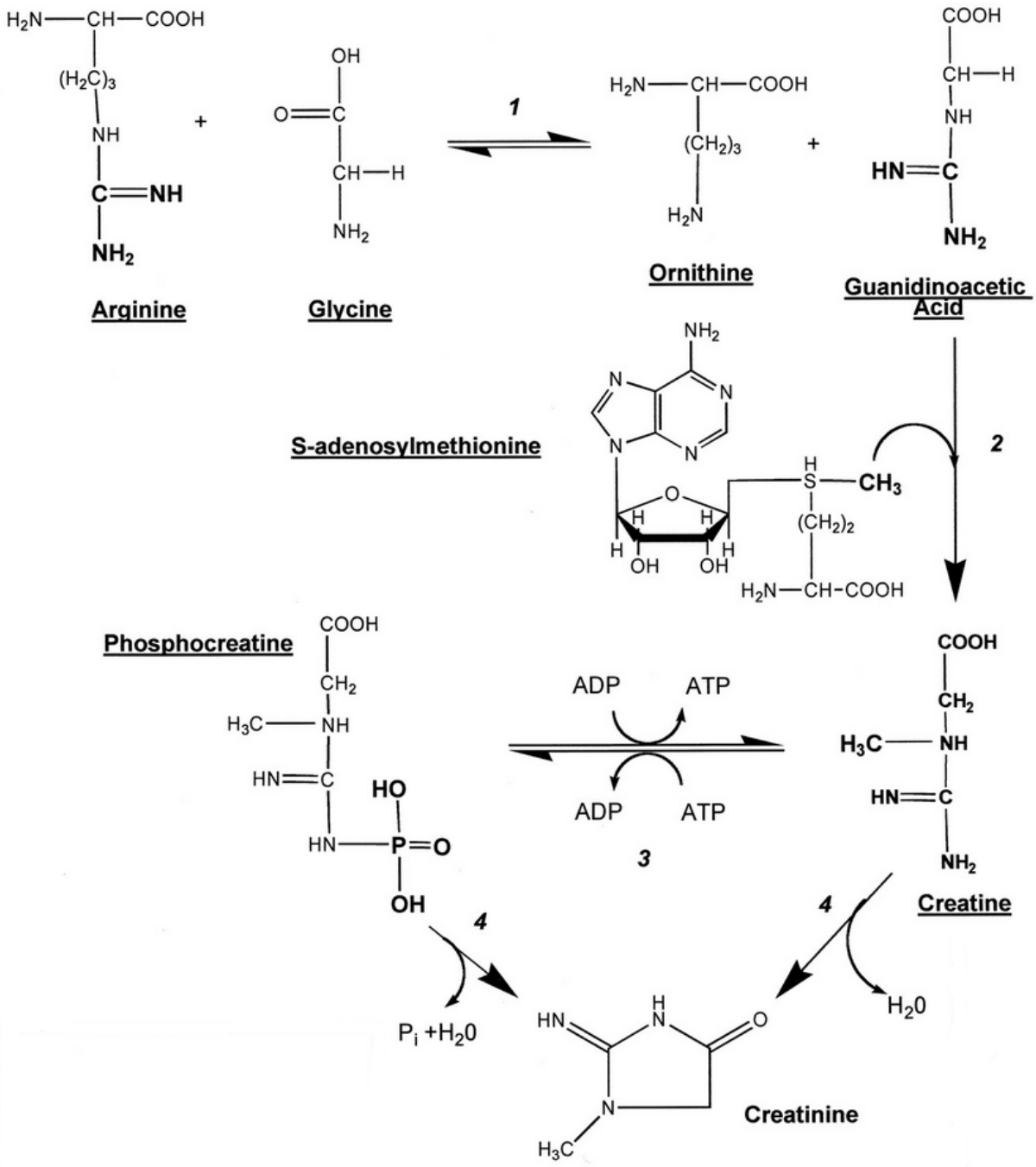

Figure 2.4. Pathway of creatine metabolism. 1, L-arginine:glycine amidinotransferase (AGAT) 2, S-adenosyl- L-methionine:N-guanidinoacetate methyltransferase (GAMT) 3, creatine kinase (CK) 4, spontaneous. Adapted from Persky and Brazeau (2001).

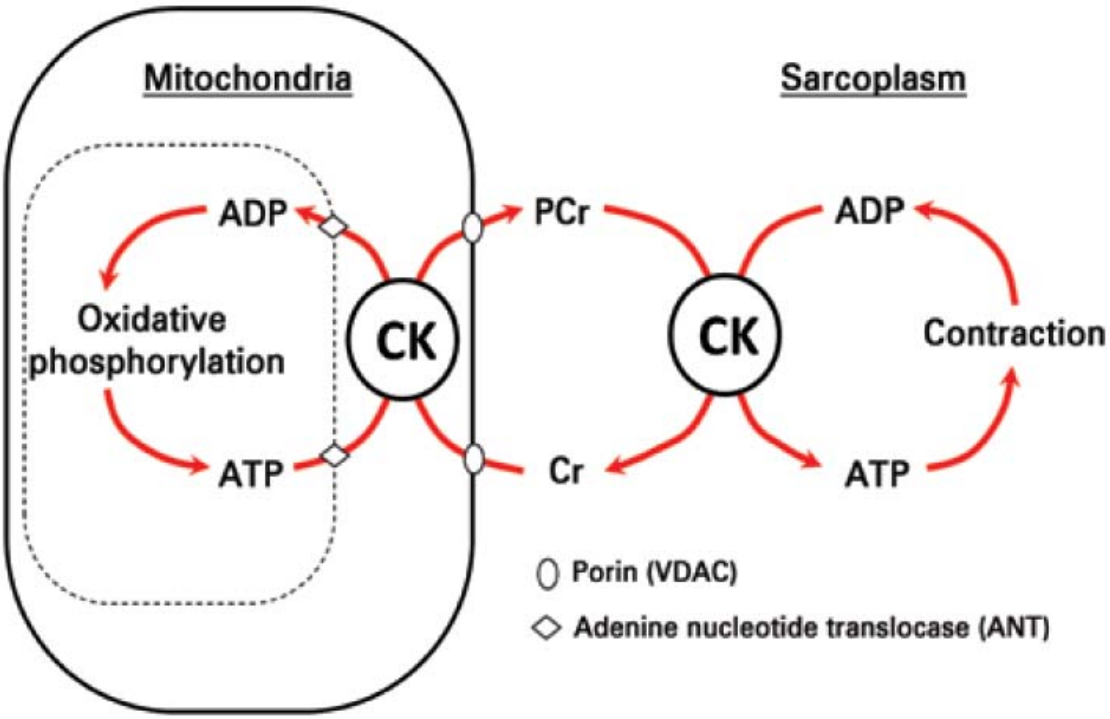

Figure 2.5 Phosphocreatine "shuttle" system. Adapted from Guimaraes-Ferreira (2014).

Chapter 3

EFFICACY OF GUANIDINOACETIC ACID ON GROWTH AND MUSCLE PHOSPHAGENS WHEN INCLUDED IN ARGININE-DEFICIENT DIETS

Abstract

Guanidinoacetic acid is formed from Arg and is the immediate precursor to creatine (Cre). A study was conducted to determine the ability for GAA to spare Arg in broiler chicks fed an Arg-deficient practical diet. A common basal diet (0.84% digestible Arg) was supplemented with combinations of Arg (0 or 0.16%) and GAA (0, 0.06, or 0.12%) to form a 2 × 3 factorial arrangement of dietary treatments. Additionally, the basal diet was supplemented with 0.32% Arg (1.16% digestible Arg) to serve as a positive control. Diets were fed in mash form to 8 replicate pens of 5 chicks per treatment from d 8 to 22 post-hatch, with measurements including growth performance, blood and tissue metabolite concentrations, and clinical outcomes. Supplementation of Arg increased ($P < 0.05$) BWG from d 15 to 22 post-hatch, with the PC diet eliciting greater ($P < 0.05$) BWG than the NC diet. Supplementation of either Arg or GAA increased ($P < 0.05$) gain:feed ratio (G:F) from d 15 to 22 and d 8 to 22 post-hatch, with NC-fed birds exhibiting greater ($P < 0.05$) G:F than PC-fed birds. An interactive effect ($P < 0.05$) was observed as serum GAA was more responsive to supplementation of GAA compared with Arg. Interactive effects ($P < 0.05$) due to Arg and GAA supplementation were also observed for concentrations of muscle total creatine, whole breast creatinine, and nearly every AA in serum, and PC-fed birds had greater ($P < 0.05$) responses for nearly all blood and tissue outcomes compared with NC-fed birds. Very few effects were noted for clinical chemistry and hematological outcomes from this study. Overall, these observations indicate that GAA

supplementation alleviated the growth-depressive effects of an Arg deficiency (i.e., GAA spared Arg) and positively affected muscle phosphagen concentrations in broiler chicks fed practical diets.

Introduction

As prices of commodity crops rise due to poor weather conditions, and with increased product demand, producers increasingly look for alternative feed ingredient to use as substitutes. These non-traditional feed ingredients can be highly variable, however, resulting in the possibility of lower protein and amino acid concentrations compared with traditional ingredients. With the increased use of alternative protein sources, along with diets formulated with lower crude protein levels, there is a necessity for the inclusion of crystalline amino acids. Arginine is considered the fifth limiting amino acid for broiler chickens (Fernandez et al., 1994; Han et al., 1992; Waguespack et al., 2009), but is not currently commercially available. Combined with lower crude protein formulations with alternative ingredients, increased growth rate of modern broilers (Havenstein et al., 2003), and a lack of *de novo* synthesis of Arg (Tamir and Ratner, 1963a), supplementation of dietary Arg may be needed for optimal growth (Han et al., 1992). Without a commercially-available source of Arg, the poultry industry may soon have a need for an Arg replacement, and one way to achieve this may be providing a feed additive that can spare the use of Arg.

Guanidinoacetic acid (GAA) is a naturally occurring compound that is either synthesized *in vivo* from Arg and Gly via arginine:glycine amidinotransferase (AGAT) or produced via chemical synthesis. After formation or absorption from the small intestine (2009; Ostojic et al., 2013), GAA is converted into creatine (Cre) via guanidinoacetic methyltransferase (GAMT) and

then phosphorylated to phosphocreatine (PCr), which is necessary to maintain energy homeostasis in muscle cells (Wyss and Kaddurah-Daouk, 2000). Therefore, GAA may be important not only for sparing Arg, but also to help maintain overall energy homeostasis in the bird.

The Arg-sparing capacity of GAA was previously studied (Edwards Jr. et al., 1958; Savage and O'Dell, 1960), but this work was conducted almost exclusively using purified diets. More recent research (Dilger et al., 2013) indicates that GAA improves feed efficiency when supplemented to Arg-deficient practical diets. Because GAA is the precursor of Cre, the sparing effect of GAA may be due to the decreased need for GAA synthesis for Cre production. Based on previous evidence that GAA-supplementation of Arg-deficient diets elicited an improved growth response, we sought to quantify the dose-response relationship of GAA on growth performance and tissue metabolite concentrations when included in practical broiler chicken diets containing varying concentrations of Arg.

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 Diets

Two-hundred eighty male Ross 708 chicks (Hoovers Hatchery, Rudd, IA) were maintained in thermostatically-controlled starter 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 14-d feeding period.

Chicks received a diet adequate in all nutrients (NRC, 1994) from d 2 to 7 post-hatch. Following an overnight fast, chicks were weighed, wing-banded, and assigned to treatments on d 8 post-hatch such that the average initial pen weights were not different among all treatments. Eight replicate pens of 5 chicks received one of 7 treatment diets during a 14-d study (d 8 to 22 post-hatch). Chicks and feeders were weighed on d 8, 15, and 22 post-hatch, and body weight gain, feed intake, and gain:feed ratio were calculated for each replicate pen of chicks.

The Arg-deficient basal diet (0.84% digestible Arg) consisted primarily of corn, soybean meal, distillers dried grains with solubles (DDGS), and corn gluten meal (**Table 3.1**). Vitamin and mineral premixes, as well as crystalline amino acids, were incorporated into the basal diet to meet or exceed requirements for broiler chicks (NRC, 1994), with the exception of Arg (**Table 3.2**). A common basal diet was supplemented with 2 levels of Arg (0 or 0.16%) and 3 levels of GAA (0, 0.06, or 0.12%) to form a 2 × 3 factorial arrangement of dietary treatments, with the 0% Arg and 0% GAA supplemented diet serving as the negative control. Additionally, the basal diet was supplemented with 0.32% Arg (1.16% digestible Arg) to serve as a positive control. Study diets were fed in mash from in a single feeding phase that lasted from d 8 to 22 post-hatch.

Sample Collection

On d 22 post-hatch, one bird per pen was randomly chosen and euthanized via an intracardiac injection of 390 mg/ml sodium pentobarbital at 0.2 ml/kg BW to facilitate rapid collection of breast muscle tissue. A muscle biopsy sample, ranging from 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 60 s). 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. Following rapid muscle biopsy collection, the remainder of each breast muscle was excised and stored on ice in a sealed bag prior to storage at -20°C.

Only upon successful muscle collection were remaining birds in each pen euthanized by CO_2 asphyxiation. A pooled blood sample from 2 of the remaining birds per pen was collected from the heart into evacuated tubes containing either EDTA, heparin, or no additive to preserve whole blood, serum, and plasma samples, respectively. Samples for plasma were stored on ice and serum was allowed to clot at room temperature for no more than 2 h before samples were processed and stored. Serum and plasma samples were separated by centrifugation at 1,300 x g at 20°C and 4°C, respectively, and allocated into separate tubes and stored at -80°C.

Chemical Analyses

Muscle PCr, free Cre, and ATP concentrations were quantified (Harlan Laboratories, Itingen, Switzerland) using fully-validated procedures. In brief, muscle biopsy samples were freeze-dried, powdered, and following extraction of the powder with perchloric acid (PCA), an aliquot of 25 µL of neutralized PCA extract (corresponding to 250 µg of dried muscle) was used for the simultaneous determination of ATP and PCr. For determination of Cre, the neutralized extract was diluted 1:5 with assay buffer, and in a single run, undiluted extract was used for the analysis of Cre. The analytical method was based on enzymatic determinations, which ultimately resulted in either reduction of NADP to NADPH (ATP, PCr) or oxidation of NADH to NAD (Cre), 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 test muscle biopsy samples. Final data included the absolute concentration of each Cre-related metabolite (ATP, PCr, and Cre), along with calculation of absolute concentration of total Cre (tCre; PCr plus free Cre) and relative proportions (PCr:ATP and PCr:total Cre).

Whole breast samples were analyzed (AlzChem AG, Trostberg, Germany) for GAA, total Cre, Crn, and homocysteine concentrations using fully-validated procedures (data not shown). Serum and plasma were analyzed for amino acids, Cre, Crn, GAA, and homocysteine using standardized procedures (Baylor University, Huston, TX). Whole blood was submitted to the University of Illinois Urbana-Champaign Veterinary Diagnostic Laboratory for analysis of hematological and clinical pathology parameters. Blood biochemistry was assayed using an automated spectrophotometric method on a Hitachi 917 analyzer (Roche, Indianapolis, IN), while hematological parameters were assessed using a combination of automated and manual procedures.

Statistical Analysis

Data were analyzed as a 2-way ANOVA using the GLM procedure of SAS (SAS Inst., Cary, NC). Dietary supplemental Arg and GAA concentrations were independent variables in this model. When interactive effects were noted, means separation was conducted using a Tukey's adjustment. Additionally, a two-tailed Dunnett's test was used to compare the positive control with all other treatments. Overall treatment effects with a probability of $P < 0.05$ were accepted as statistically significant.

Results

Overall, formulation objectives were achieved in terms of creating an Arg-deficient basal diet, and graded supplementation of Arg and GAA was realized in the final diets (**Table 3.2**).

Growth Performance

Body weight gain d 15 to 22 and d 8 to 22 post-hatch increased ($P < 0.05$) due to Arg supplementation, with the negative control (NC; 0% supplemental Arg and GAA) diet exhibiting reduced ($P < 0.05$) BWG compared with the PC (**Table 3.3**). Gain:feed ratio (G:F) increased ($P < 0.05$) with increasing dietary Arg or dietary GAA supplementation during d 15-22 and d 0-22 post-hatch.

Tissue Analysis

Muscle PCr concentrations independently increased ($P < 0.05$) due to dietary addition of either Arg or GAA (**Table 3.4**). Supplementation of the basal diet with 0.16% Arg and 0.12% GAA increased ($P < 0.05$) PCr concentrations by 46% compared with the PC diet. The ratio of PCr:ATP also independently increased ($P < 0.05$) due to dietary addition of either Arg or GAA. Supplementation of the basal diet with 0.16% Arg and 0.12% GAA resulted in PCr:ATP ratio that was increased ($P < 0.05$) 72% over that of the PC diet. Graded GAA supplementation increased muscle tCre concentrations, but the effect was more pronounced in diets containing added Arg (interaction, $P < 0.05$). The addition of 0.16% Arg and 0.12% GAA resulted in tCre levels that were increased ($P < 0.05$) 26% compared with the PC diet. The ratio of PCr:tCre increased independently ($P < 0.05$) due to dietary addition of GAA. Muscle GAA was measured

but not reported due to most samples containing GAA concentrations below the detection limit (<5 mg/kg).

Blood Analysis

Serum Arg concentrations were unchanged by graded GAA in diets containing 0% added Arg, but increased 38% due to addition of 0.12% GAA in diets containing 0.16% added Arg (interaction, $P < 0.05$; **Table 3.5**). Interactive effects ($P < 0.05$) were also observed for His, Ile, Lys, Phe, and Val, which decreased an average of 34 and 7% due to addition of 0.12% GAA when included in diets containing 0.0 or 0.16% added Arg, respectively. Glutamine was the only non-essential AA that exhibited an interaction ($P < 0.05$), with all other non-essential AA (except Asn) decreasing ($P < 0.05$) due to Arg supplementation. Additionally, Ala decreased ($P < 0.05$) due to graded addition of GAA, regardless of dietary Arg concentration.

Graded GAA supplementation increased serum GAA concentrations, but the effect was more pronounced in diets containing added Arg (interaction, $P < 0.05$). Moreover, addition of either 0.06 or 0.12% GAA increased ($P < 0.05$) serum GAA by at least 323% when compared with the PC diet. Serum Cre concentrations independently increased ($P < 0.05$) due to dietary addition of either Arg or GAA. Blood Crn was measured, but not reported, due to all samples containing Crn levels below detection limits (< 83 μM). Serum concentrations of citrulline, phosphoserine, and phosphoethanolamine increased due to GAA addition to Arg-unsupplemented diets, but were unchanged when GAA was added to diets containing supplemental Arg (interaction, $P < 0.05$). Opposite interactive effects ($P < 0.05$) were observed for alpha-aminobutyric acid and 3-methylhistidine, which decreased due to GAA addition to Arg-unsupplemented diets, but were unchanged when GAA was added to diets containing

supplemental Arg. Serum ornithine increased ($P < 0.05$) 117% due to Arg supplementation, and a main effect ($P < 0.05$) of GAA supplementation was observed for taurine. Finally, serum 1-methylhistidine decreased ($P < 0.05$) by 18 and 27% due to supplementation with either 0.16% Arg or 0.12% GAA, respectively.

Clinical chemistry and hematological outcomes were also affected by dietary treatments (**Table 3.6**). An interaction ($P < 0.05$) was observed for glucose, with 0.12% added GAA increasing glucose concentrations by 2.7% in Arg-unsupplemented diets and decreasing glucose concentrations by 4.1% in Arg-supplemented diets. Plasma phosphorus concentrations and Glu dehydrogenase activity were both decreased ($P < 0.05$) due to Arg supplementation, and Glu dehydrogenase activity was also decreased ($P < 0.05$) due to GAA supplementation. In terms of hematological responses, blood protein concentrations decreased ($P < 0.05$) due to supplementation of either Arg or GAA (**Table 3.7**). As a proportion of total blood leukocytes, heterophils decreased ($P < 0.05$), while lymphocytes increased ($P < 0.05$), due to graded GAA supplementation.

Discussion

The study was designed to test the efficacy of GAA for supporting growth and restoring muscle phosphagen status in fast-growing broiler chicks fed an Arg-deficient diet based on practical ingredients. Combined with a general lack of effects on hematological and clinical chemistry outcomes, this research provides clear and direct evidence of GAA efficacy in practical Arg-deficient diets fed to broiler chicks.

The improvement in growth performance indicates that we effectively created an Arg deficiency in the chicks, but were also able to alleviate that deficiency with increased Arg

supplementation. Improvements in growth performance when GAA was added to Arg-deficient diets are in agreement with previous research conducted by Savage and O'Dell (1960) and Dilger et al. (2013). Although there was no BWG response due to GAA supplementation, this was also in agreement with previous research (Dilger et al., 2013). Improvements in FCR likely occurred because Arg was spared from serving as a precursor for Cre synthesis (Almquist et al., 1941) and was therefore available for alternative functions throughout the body; a theory that is in agreement with the research conducted by Edwards et al. (1958). In general, GAA was able to improve, and in some instances restore, growth performance of birds receiving Arg-deficient diets containing practical ingredients common to the U.S. poultry industry.

In our study, dietary Arg deficiency decreased concentrations of muscle PCr compared with the PC diet. Because PCr is used to maintain muscle energy homeostasis (Walker, 1979), the decrease in PCr means that muscles in Arg-deficient birds were less able to maintain energy homeostasis, and therefore may have reduced muscle performance. Regardless of Arg supplementation, GAA increased PCr such that they were never different from the PC. This indicates that GAA improves energy homeostasis in muscles cells when supplemented in an Arg-deficient diet. Relative to the NC diet, tCre concentrations were increased due to 0.12% GAA supplementation by 64% and 59% in diets containing 0.84% and 1.00% Arg, respectively. These results corroborate recent observations by Michiels et al. (2012), who reported that Cre concentrations in breast meat increased by 16% due to supplementation of 0.12% GAA in an Arg-adequate diet. Because the maintenance of PCr is based on Cre being phosphorylated to PCr and exported to sites of ATP usage (Brosnan and Brosnan, 2010; Guimarães-Ferreira, 2014), the increased tCre concentration may indicate the increased capacity for PCr due to increased overall Cre concentrations. Also, both the absolute concentration and relative ratio (to ATP) of PCr and

tCre were improved with GAA supplementation, indicating in both cases that GAA supplementation may increase the ability of cells to regenerate ATP more effectively. Because of the increase in muscle metabolite concentrations, we can conclude that dietary GAA is successfully absorbed and metabolized to synthesize Cre with efficacy greater than dietary Arg, which indicates that GAA supplementation spares dietary Arg (Wyss and Kaddurah-Daouk, 2000).

Supplementation of GAA reduced plasma AA concentrations in birds, with more drastic decreases observed in diets containing 0.84% digestible Arg. This indicates that as the Arg deficiency was alleviated, essential AA concentrations in plasma decreased. Zimmerman and Scott (1965) reported a similar trend in BWG and plasma AA concentrations as dietary lysine approached the requirement. The improvement in BWG and decrease in plasma AA can be explained in the context of the ideal protein concept, which is based on the premise that optimal performance is limited by availability of the scarcest resource. In these diets, Arg is the most-limiting amino acid, and as the body has no AA storage, any excess AA remaining after attaining maximal growth are transported via the blood to the liver for deamination and subsequent oxidation to generate ATP. Therefore, GAA added to diets containing either 0.84 or 1.00% Arg may spare Arg by diminishing the need for Arg to serve as a precursor for Cre synthesis, therefore making more Arg available for incorporation into lean tissue along with excess AA and decreasing blood AA concentrations.

In addition to decreased growth in general, evidence from blood chemistry and hematological outcomes indicate that protein degradation may have occurred in Arg-deficient birds. Blood 3-methylhistidine decreased with dietary supplementation of GAA to Arg-unsupplemented diets, but were unchanged when GAA was supplemented to containing

supplemented Arg. Supplementation of GAA also decreased heterophils as the largest proportion of total leukocytes, while dietary Arg concentration had no effect. Heterophil proportions were reduced with GAA supplementation an average of 35% when birds were supplemented with 0.12% GAA compared with 0.0% GAA. There was also a reciprocal change in lymphocyte percentages, the normal circulating leukocyte (Weiss et al., 2010), as heterophil percentage decreased. The combination of decreased 3-methylhistidine concentrations and heterophil percentages in birds with increased GAA supplementation indicates that Arg deficiency may cause minor changes in whole-body protein degradation. Although 3-methylhistidine is an indication of skeletal protein degradation (Young and Munro, 1978), it is typically related to body weight loss. However, when combined with the high proportion of white blood cells as heterophils, a sign of muscle damage (Weiss, et al., 2010), there may be some protein degradation that occurs in Arg-deficient birds. Therefore, GAA supplementation may reduce metabolic stress (i.e., protein degradation) caused by dietary Arg deficiency.

The alleviation of an Arg deficiency is associated with changes in Arg-related metabolites. Blood ornithine (Orn) concentrations were increased with graded Arg supplementation, but GAA supplementation had no effect. The production of Orn may increase with Arg supplementation due to the increased production of urea by avian arginase (Fernandes and Murakami, 2010; Tamir and Ratner, 1963a); this could be quantified further if blood urea had been measured. Increased blood Orn concentrations may also indicate that more GAA and Orn are formed from Arg and Gly due to Arg supplementation (Walker, 1979). Supplementation of GAA decreased Orn concentrations by at least 33% and 12% in the 0.84% Arg diet and 1.00% Arg diet with 0.0% GAA, respectively. This was expected because the supplementation of GAA spares dietary Arg most in Arg deficient diets. Blood citrulline (Cit) levels were increased with

the supplementation of GAA, although to a much lower effect when dietary Arg was closer to adequate concentrations. This could be due to increased nitric oxide production, which also produces Cit (Wu and Morris Jr., 1998), but also a decreased conversion of Cit to Arg (Tamir and Ratner, 1963b) in chicks with a decreased deficiency. Serum Cit concentrations increased dramatically with GAA supplementation in Arg-unsupplemented diets, indicating that Cit levels may be a way to measure Arg deficiency and that GAA effectively reduced the deficiency by sparing Arg in unsupplemented diets.

Overall, it can be concluded from this study that dietary GAA may spare Arg when Arg-deficient diets are fed to young broiler chicks. Our results indicate that 0.12% supplemental GAA was capable of ameliorating the effects of an Arg deficiency on growth performance and muscle phosphagen concentrations caused by a dietary Arg deficiency, with outcomes due to this treatment closely matching responses of chicks fed an Arg-adequate diet. While Arg-sparing effects of GAA were evident in Arg-deficient diets based on practical ingredients, further studies are warranted to determine whether GAA can also improve outcomes in chicks consuming an Arg-adequate diet**.**

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Tables

Table 3.1. Basal diet formulation and nutrient composition

Ingredient, %	**Value**
Corn	57.57
Soybean Meal	10.03
DDGS	12.04
Corn Gluten Meal	11.54
Soy Oil	3.01
Salt	0.40
Limestone	1.50
Dicalcium Phosphate	1.71
Vitamin Premix[1]	0.20
Mineral Premix[2]	0.15
Choline Chloride	0.20
Titanium Dioxide	0.40
L-Lysine HCl	0.70
DL-Methionine	0.25
L-Isoleucine	0.06
L-Threonine	0.18
L-Tryptophan	0.05
Calculated Proximates	
Crude Protein, %	21.3
Calcium, %	10.0
Phosphorus (total), %	6.8
Phosphorus (available), %	4.5
AME_N, kcal/kg	3,148

	Calculated		
Amino Acids, %	Total	Digestible[3]	Analyzed
Arg	9.6	8.4	9.9
Ile	8.6	7.6	8.4
Leu	25.3	23.1	24.1
Lys	12.4	11.4	11.9
Met	6.8	6.4	6.2
Met+Cys	10.4	9.2	9.9
Thr	8.7	7.3	8.6
Trp	2.3	1.9	-
Val	10.0	8.6	9.3

[1]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.

[2] Provided as milligrams per kg of diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; Se, 0.1 from Na_2SeO_3.

[3]Acquired from AMINODat® 4.0 (Evonik Industries AG, Hanau-Wolfgang, Germany).

Table 3.2. Analyzed composition of negative and positive control treatments[1]

Nutrient	Negative Control (NC)	Positive Control (PC)
Crude Protein, %	21.36	22.96
Crude Fat, %	5.78	5.87
Crude Fiber, %	2.33	2.20
Ash, %	6.00	6.53
GAA, mg/kg[2]	< 1.0	< 1.0
Creatine, mg/kg[2]	< 1.0	< 1.0
Folic Acid, mg/kg	0.94	0.92
Choline, mg/kg	1,030	1,070
Betaine, mg/kg	6,800	7,200
Amino Acids, %		
Essential		
Arg	1.01	1.31
His	0.52	0.52
Ile	0.86	0.86
Leu	2.49	2.49
Lys	1.21	1.22
Met	0.68	0.66
Phe	1.10	1.1
Thr	0.87	0.89
Val	0.96	0.96
Non-Essential		
Ala	1.45	1.43
Asp	1.55	1.56
Cys	0.38	0.38
Glu	3.87	3.87
Gly	0.76	0.76
Pro	1.57	1.56
Ser	1.02	1.01

[1]The basal diet was formulated to contain 0.96% total Arg (0.84% digestible Arg) and was analyzed to contain 0.99% Arg.

[2]Analyzed values were below the stated detection limit.

[3]Amino acids values were standardized to a dry matter content of 88%.

Table 3.3. Growth performance of chicks fed Arg-deficient diets[1]

	Dietary Treatment[2]										
Suppl. Arg, %	**0.00**	**0.00**	**0.00**	**0.16**	**0.16**	**0.16**	**0.32**		***P*-values[6]**		
Variable ***GAA, %***	**0.00[3]**	**0.06**	**0.12**	**0.00**	**0.06**	**0.12**	**0.00[4]**	**SEM[5]**	**Arg**	**GAA**	**Arg×GAA**
Body weight, g											
d8	99.3	99.4	99.4	99.3	99.5	99.5	99.3	2.83	1.000	0.998	1.000
d15	299.8	285.4	286.3	293.6	299.7	300.4	301.1	10.02	0.626	0.901	0.507
d22	627.9	626.2	641.0	642.0	656.3	662.2	668.5	16.37	0.076	0.593	0.888
BW gain, g/chick											
d 8-15	200.5	186.0	186.9	194.3	200.2	200.9	201.8	7.52	0.444	0.831	0.305
d 15-22	328.1*	340.9	354.7	348.4	356.6	361.8	367.4	8.99	0.008	0.094	0.759
d 8-22	528.6	526.8	541.6	542.7	556.8	562.7	569.2	14.39	0.037	0.515	0.857
Feed intake, g/chick											
d 8-15	301.4	278.9	278.6	282.1	295.1	283.1	286.9	9.37	0.851	0.466	0.130
d 15-22	514.0	516.3	512.4	509.4	518.7	503.3	523.6	13.60	0.771	0.747	0.902
d 8-22	815.6	797.7	791.0	791.5	813.8	791.3	810.8	20.98	0.975	0.725	0.590
Gain:feed, g/kg											
d 8-15	663.8	666.8	668.9	687.0	680.9	711.4	697.4	17.94	0.100	0.566	0.691
d 15-22	637.9*	661.4	693.0	683.9	690.5	718.1	701.9	15.43	0.003	0.011	0.746
d 8-22	647.3*	661.5	684.3	685.1	686.9	710.9	700.5	13.63	0.004	0.047	0.865

*Mean value for this treatment was different from the PC diet ($P < 0.05$).

[1]Values are means of 8 replicate pens of 5 chicks during the feeding period 8 to 22 d post-hatch. Arg = arginine, BW = body weight, GAA = guanidinoacetic acid.

[2]The basal diet was formulated to contain 0.96% total Arg (0.84% digestible Arg) and was analyzed to contain 0.99% Arg.

[3]Negative control treatment.

[4]Positive control treatment.

[5]Standard error of the mean that applies to all 7 dietary treatments.

[6]*P*-values apply only to the first 6 treatments that were included in the 2-way ANOVA.

Table 3.4. Muscle analyses of creatine-related metabolites of chicks fed Arg-deficient diets[1]

	Dietary Treatment[2]										
Suppl. Arg, %	**0.00**	**0.00**	**0.00**	**0.16**	**0.16**	**0.16**	**0.32**		***P*-values[6]**		
Variable ***GAA, %***	**0.00[3]**	**0.06**	**0.12**	**0.00**	**0.06**	**0.12**	**0.00[4]**	**SEM[5]**	**Arg**	**GAA**	**Arg×GAA**
PCr, mmol/kg DW	37.6*	56.1	91.0	61.7	82.4	108.7*	74.4	8.14	0.001	< 0.001	0.859
ATP, mmol/kg DW	37.8	38.0	41.0	38.1	40.8	34.1	40.1	2.36	0.617	0.709	0.111
PCr:ATP	0.99*	1.54	2.20	1.68	2.04	3.24*	1.88	0.24	0.001	< 0.001	0.515
Free Cre, mmol/kg DW	68.9	56.3	59.5	54.7	76.5	67.3	64.7	7.81	0.741	0.837	0.094
Total Cre, mmol/kg[7]	106.6a*	112.4a*	150.6b	116.5a	158.9b	176.0b*	139.2	7.13	< 0.001	< 0.001	0.044
PCr/total Cre, %	34.5	51.0	60.5	52.2	51.9	61.7	53.7	5.40	0.105	0.007	0.216

*Mean value for this treatment was different from the PC diet ($P < 0.05$).

[1]ATP = adenosine triphosphate, Cre = creatine, Crn = creatinine, DW = dry weight, GAA = guanidinoacetic acid, PCr = phosphocreatine, WW = wet weight.

[2]The basal diet was formulated to contain 0.96% total Arg (0.84% digestible Arg) and was analyzed to contain 0.99% Arg.

[3]Negative control treatment.

[4]Positive control treatment.

[5]Standard error of the mean that applies to all 7 dietary treatments.

[6]*P*-values apply only to the first 6 treatments that were included in the 2-way ANOVA.

[7]Calculated as PCr plus free Cr.

Table 3.5. Blood amino acid and metabolite concentrations (μM) of chicks fed Arg-deficient diets

	Dietary Treatment[1]										
Suppl. Arg, %	**0.00**	**0.00**	**0.00**	**0.16**	**0.16**	**0.16**	**0.32**		***P*-values[5]**		
Variable ***GAA, %***	**0.00[2]**	**0.06**	**0.12**	**0.00**	**0.06**	**0.12**	**0.00[3]**	**SEM[4]**	**Arg**	**GAA**	**Arg x GAA**
Essential											
Arginine	155.8^{ab*}	136.9^{a*}	143.8^{a*}	199.1^{bc*}	244.6^{cd*}	274.0^{d*}	364.9	15.35	< 0.001	0.132	0.019
Histidine	127.0^{c*}	109.1^{bc}	79.6^{ab}	80.9^{ab}	74.1^{ab}	73.4^{a}	84.7	8.03	< 0.001	0.005	0.046
Isoleucine	118.4^{b*}	89.4^{a}	82.9^{a}	79.6^{a}	82.9^{a}	76.8^{a}	77.3	4.26	< 0.001	0.001	0.001
Leucine	434.3*	354.4	334.5	370.3	337.4	318.4	341.1	16.92	0.002	0.001	0.279
Lysine	579.0^{c*}	470.6^{bc*}	272.5^{a}	297.3^{ab}	237.8^{a}	262.5^{a}	274.3	43.58	< 0.001	0.001	0.007
Methionine	196.4	181.3	179.6	177.0	158.8	162.0	161.6	10.64	0.017	0.219	0.973
Phenylalanine	217.5	184.1	184.4	188.3	198.8	186.1	172.9	8.67	0.021	0.130	0.042
Threonine	2,093*	1,839*	1,333	1,410	1,243	1,175	1,254	113.18	< 0.001	0.001	0.054
Valine	245.1^{b*}	173.3^{a}	160.9^{a}	164.3^{a}	158.3^{a}	148.6^{a}	150.8	8.33	< 0.001	< 0.001	0.001
Non-Essential											
Alanine	1,328*	1,148	1,077	1,055	991.1	950.1	927.3	71.08	0.001	0.047	0.559
Asparagine	20.9	43.5	60.3	41.8	59.6	35.0	44.6	21.79	0.862	0.618	0.512
Aspartic acid	221.9*	220.6*	185.6	176.1	162.6	179.1	157.1	13.32	0.001	0.463	0.141
Cysteine	85.6*	80.9	75.1	75.0	75.8	72.5	72.9	3.34	0.021	0.149	0.478
Glutamine	1,243^{b}	1,514^{c*}	1,180^{b}	1,095^{ab}	1,070^{ab}	882.6^{a*}	1,095	54.66	< 0.001	< 0.001	0.033
Glutamic acid	266.0*	250.5	254.4	224.9	229.9	254.4	224.5	9.43	0.009	0.323	0.103
Glycine	731.3*	696.3*	632.0*	520.5	521.8	517.0	476	38.27	< 0.001	0.399	0.456
Proline	832.8	883.9	886.4	756.3	748.0	745.3	768.5	52.37	0.027	0.895	0.792
Serine	936.5*	918.0*	800.0	721.9	680.5	652.3	715.0	48.08	< 0.001	0.098	0.628
Tyrosine	409.6	422.0	365.0	329.9	363.6	337.5	326.9	26.80	0.024	0.308	0.622
Other Metabolites[4]											
Alpha-aminobutyric	71.9^{c*}	34.8^{a*}	50.5^{b*}	42.5^{ab*}	37.5^{ab*}	36.4^{ab*}	20.4	3.31	< 0.001	< 0.001	< 0.001
Citrulline	2.75^{a*}	9.0^{b}	15.9^{c*}	15.8^{c*}	14.9^{c*}	16.8^{c*}	9.5	1.07	< 0.001	< 0.001	< 0.001
Creatine	14.2*	22.3	37.1	19.8	26.0	43.2*	28.3	3.31	0.006	< 0.001	0.930
Guanidinoacetic acid	0.39^{d}	3.85^{c*}	9.84^{b*}	0.48^{d}	4.91^{c*}	13.95^{a*}	0.91	0.54	< 0.001	< 0.001	0.001
1-methylhistidine	38.1*	30.4	24.6	26.6	27.4	22.8	20.5	2.87	0.001	0.014	0.196
Ornithine	11.0*	7.0*	7.38*	20.1*	17.3*	17.6*	28.0	1.78	< 0.001	0.117	0.936
Phosphoserine	20.6^{a}	19.0^{a}	70.8^{b*}	75.9^{b*}	75.5^{b*}	68.9^{b*}	20.6	3.47	< 0.001	< 0.001	< 0.001
Phosphoethanolamine	2.75^{ab}	2.25^{a}	4.13^{ab*}	14.9^{c*}	4.38^{b*}	4.50^{b*}	1.63	0.48	< 0.001	< 0.001	< 0.001
Taurine	362.9	297.8	340.5	381	288.8	311.8	286.9	29.82	0.074	0.038	0.734
3-methylhistidine	16.3^{c*}	12.6^{b}	7.13^{a}	9.25^{ab}	7.25^{a}	8.88^{ab}	11.0	1.05	0.001	0.001	0.001

[a-d]Means within a row lacking a common superscript letter differ ($P < 0.05$).

[*]Mean value for this treatment was different from the PC diet ($P < 0.05$).

[1]The basal diet was formulated to contain 0.96% total Arg (0.84% digestible Arg) and was analyzed to contain 0.99% Arg.

[2]Negative control treatment.

[3]Positive control treatment.

[4]Standard error of the mean that applies to all 7 dietary treatments.

[5]*P*-values apply only to the first 6 treatments that were included in the 2-way ANOVA.

Table 3.6. Plasma clinical pathology outcomes of chicks fed Arg-deficient diets

	Dietary Treatment[1]										
Suppl. Arg, %	**0.00**	**0.00**	**0.00**	**0.16**	**0.16**	**0.16**	**0.32**		***P*-values[5]**		
Variable ***GAA, %***	**0.00[2]**	**0.06**	**0.12**	**0.00**	**0.06**	**0.12**	**0.00[3]**	**SEM[4]**	**Arg**	**GAA**	**Arg x GAA**
Albumin, g/dL	1.10	1.13	1.14	1.1	1.00	1.08	1.05	0.05	0.240	0.559	0.430
Glucose, mg/dL	232.1[ab]	244.6[b]	238.3[ab]	241.5[ab]	220.4[a]	231.5[ab]	236.8	5.36	0.267	0.725	0.011
Asp aminotransferase, U/L	136.75	148.25	143.88	135	131.75	146.38	145	7.37	0.417	0.459	0.407
Phosphorus, mg/dL	7.60	8.39	7.98	7.53	7.20	7.39	7.68	0.27	0.027	0.701	0.136
Calcium, mg/dL	10.69	10.85	10.68	10.61	10.28	10.85	10.45	0.21	0.501	0.647	0.214
Glu dehydrogenase, U/L	6.78	4.76	5.40	4.95	3.33	3.69	4.81	0.66	0.007	0.025	0.956
Creatine kinase, U/L	2,106	1,908	2,586	2,672	2,133	2,379	2,178	303.98	0.622	0.280	0.416

[a-b]Means within a row lacking a common superscript letter differ ($P < 0.05$).

[*]Mean value for this treatment was different from the PC diet ($P < 0.05$).

[1]The basal diet was formulated to contain 0.96% total Arg (0.84% digestible Arg) and was analyzed to contain 0.99% Arg.

[2]Negative control treatment.

[3]Positive control treatment.

[4]Standard error of the mean that applies to all 7 dietary treatments.

[5]*P*-values apply only to the first 6 treatments that were included in the 2-way ANOVA.

Table 3.7. Hematology outcomes of chicks fed Arg-deficient diets

	Dietary Treatment[1]										
Suppl. Arg, %	**0.00**	**0.00**	**0.00**	**0.16**	**0.16**	**0.16**	**0.32**		***P*-Value[5]**		
Variable ***GAA, %***	**0.00[2]**	**0.06**	**0.12**	**0.00**	**0.06**	**0.12**	**0.00[3]**	**SEM[4]**	**Arg**	**GAA**	**Arg x GAA**
Packed cell volume, %	28.7	28.2	29.1	28.3	28.5	28.9	28.5	0.86	0.994	0.682	0.895
Protein, g/dL	3.36	3.03	3.15	3.18	2.86	2.99	3.03	0.96	0.009	0.003	0.995
Differential cell counts, $10^3/\mu L$											
Total leukocytes	10.65	12.88	11.08	9.86	9.99	13.97	9.89	1.95	0.972	0.455	0.270
Heterophils	5.14	4.93	3.88	4.88	4.35	4.62	4.33	0.76	0.738	0.549	0.605
Lymphocytes	4.57	5.93	5.82	3.75	3.98	7.69	4.34	1.57	0.965	0.190	0.392
Monocytes	0.35	0.57	0.30	0.43	0.53	0.80	0.43	0.21	0.488	0.674	0.287
Eosinophils	0.25	0.44	0.16	0.21	0.24	0.28	0.18	0.09	0.591	0.325	0.071
Basophils	0.48	1.07	0.94	0.74	0.92	0.81	0.67	0.27	0.950	0.246	0.591
Differential cell proportions, %[6]											
Heterophils	61.0	40.0	35.3	51.0	44.0	37.7	44.0	5.63	0.172	0.001	0.330
Lymphocytes	28.4	44.5	52.2	35.9	40.9	49.6	43.9	6.37	0.272	0.008	0.571
Monocytes	3.67	4.60	2.75	5.13	5.25	5.40	4.71	1.86	0.504	0.878	0.836
Eosinophils	2.80	2.50	1.57	2.57	2.29	2.29	2.25	0.67	0.833	0.361	0.598
Basophils	5.43	8.00	8.38	6.67	7.88	6.57	6.00	1.73	0.836	0.263	0.568
Subjective observations[7]											
Reactive lymphocytes	1/7	4/6	2/8	3/8	3/8	3/7	2/7				
Immature erythrocytes	-	-	-	-	-	-	1/7				
Vacuoles in heterophils	-	5/6	-	-	6/8	4/7	-				
Hypogranulated heterophils	-	1/6	-	-	-	-	-				
Granulated lymph	-	1/6	-	-	2/8	1/7	-				

[1]The basal diet was formulated to contain 0.96% total Arg (0.84% digestible Arg) and was analyzed to contain 0.99% Arg.
[2]Negative control treatment.
[3]Positive control treatment.
[4]Standard error of the mean that applies to all 7 dietary treatments.
[5]*P*-values apply only to the first 6 treatments that were included in the 2-way ANOVA.
[6]Differential cell counts expressed as a proportion of total leukocytes detected.
[7]Values represent number of positive observations per total number of samples analyzed.

Chapter 4

EFFICACY OF GUANIDINOACETIC ACID ON GROWTH AND MUSCLE PHOSPHAGENS WHEN INCLUDED IN BOTH ARGININE-DEFICIENT AND ARGININE-ADEQUATE DIETS

Abstract

Two studies were conducted to test the Arg-sparing efficacy of GAA in supporting growth performance and muscle phosphagen homeostasis in broiler chicks. In both studies, 12 replicate pens of 6 chicks received dietary treatments beginning at d 2 post-hatch. At conclusion of each study, muscle biopsy samples were collected within 60 s of euthanasia for analysis of creatine (Cre)-related metabolites. In study 1, Arg-deficient starter and grower basal diets (0.97 and 0.84% digestible Arg, respectively) were supplemented with 0 (negative control, NC), 0.06, 0.12, or 0.18% guanidinoacetic acid (GAA), or supplemental Arg (positive control, PC; 0.37% and 0.32% L-Arg in starter and grower phases, respectively). Final BW and overall BW gain were increased ($P < 0.05$) by 0.12% GAA compared with the NC diet, and overall gain:feed (G:F) was increased ($P < 0.05$) in the same fashion. Supplementation of 0.12% GAA also increased ($P < 0.05$) phosphocreatine (PCr) and total Cre concentrations, as well as the PCr:ATP ratio, compared with the NC diet. In study 2, Arg-adequate starter and grower basal diets (1.32 and 1.19% digestible Arg, respectively) were supplemented with 0 (negative control, NC), 0.06, or 0.12% GAA, 0.12% Cre monohydrate (PC1), or salmon protein (PC2; containing total Arg concentrations equal to those of the NC diet in each phase). Overall G:F was increased ($P < 0.05$) by PC1 compared with the NC, but GAA supplementation had no effect. However, 0.12% GAA increased ($P < 0.05$) concentrations of PCr and total Cre, as well as the PCr:ATP ratio,

relative to the NC (Arg-adequate) diet. Collectively, these data indicate that GAA can be used to replace Arg in practical diets containing either deficient or adequate concentrations of Arg when fed to broiler chicks.

Introduction

As prices of commodity crops rise, the use of feed ingredient substitutes and alternatives increase. These alternative feed ingredients may be more variable, however, compared with traditional ingredients, resulting in possible reduced protein and amino acid concentrations in animal diets. This variability in protein levels, along with a decreasing level of crude protein in diet formulation, necessitates the inclusion of crystalline amino acids. Arginine is the fifth limiting amino acid for broiler chickens (Fernandez et al., 1994; Han et al., 1992; Waguespack et al., 2009), but is not commercially available. Along with the increased growth rate of modern broilers (Havenstein et al., 2003), lack of *de novo* synthesis of Arg (Tamir and Ratner, 1963), and the possibility of reduced protein quality in production diets, supplementation of dietary Arg may be needed for optimal growth (Han et al., 1992). Without a commercially-available source of Arg, the poultry industry may soon have a need for an Arg replacement, providing a feed additive that can spare the use of Arg as a possible solution.

Guanidinoacetic acid (GAA) was previously studied for its Arg-sparing effects using purified diets (Edwards Jr. et al., 1958; Savage and O'Dell, 1960), and it was reported to be comparable to Cre supplementation, and recent work further supports the ability for GAA to spare Arg when included in diets based on practical ingredients (Dilger et al., 2013). As the immediate precursor of Cre, GAA appears to cause feedback inhibition of its synthesizing enzyme, arginine:glycine aminotransferase (AGAT) (Guimarães-Ferreira, 2014; Walker, 1979).

However, only limited information exists regarding the dose-dependent ability for GAA to spare Arg in practical broiler diets, especially in terms of Cre-related metabolites in muscle (i.e., phosphagens). In terms of Arg-adequate diets, current research indicates GAA improves growth performance (Michiels et al., 2012; Mousavi et al., 2013), but outcomes related to muscle phosphagen status are unavailable.

We hypothesized that GAA supplementation would elicit positive effects on growth performance when included in Arg-deficient, practical broiler diets, with additional benefits involving muscle phosphagens 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

Three-hundred sixty male Ross 708 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 two feeding phases, starter (d 0 to 14) and grower (d 14 to 28 post-hatch, study 1; d 14 to 27 post-hatch, study 2), beginning at 2 d post-hatch. On study d 14, birds were moved from starter (0.06 m^2/bird) to grower (0.09 m^2/bird) battery pens. Twelve

replicate pens of 6 chicks received each of the 5 study diets in each study. To determine BWG, FI, and gain:feed ratio, chicks and feeders were weighed on d 0, 14, and 28 of study 1, and on d 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 were recorded 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 (1994), with the exception of Arg (**Table 4.1**). Dietary treatments included a common Arg-deficient basal diet (1.08 and 0.95% total Arg in starter and grower phases, respectively) supplemented with 0.0 (negative control, NC), 0.06, 0.12, or 0.18% GAA, or L-Arg (positive control, PC; 0.37 and 0.32% L-Arg in starter and grower phases, respectively).

Study 2. An Arg-adequate basal diet was formulated to meet or exceed requirements for broiler chicks for all nutrients (1994) (Table 4.1). Dietary treatments included a common Arg-adequate basal diet (1.42 and 1.28% total Arg in starter and grower phases, respectively) supplemented with 0 (negative control, NC), 0.06, or 0.12% GAA, 0.12% Cre monohydrate (PC1), or salmon protein (PC2). The salmon protein-containing diet was formulated to provide total Arg concentrations equal to those of the NC diet in each phase of this study. Additionally, the PC1 and PC2 were designed to provide the same amount of digestible Cre.

Muscle Biopsy Collection

Two birds per pen with BW closest to the pen average were identified for muscle collection. 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 samplings, an alternate bird with a BW next closest to the pen average was used. In total, a muscle sample from only 1 bird per pen was collected and analyzed.

To begin the procedure, a selected bird was injected intramuscularly (on the contralateral side of the psoas 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 8-12 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, flapping, or dead) recorded every 2 min. No birds died as a result of anesthesia prior to euthanasia, and alternate birds were selected 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 390 mg/ml sodium pentobarbital at 0.2 ml/kg BW to facilitate rapid collection of breast muscle tissue. A muscle biopsy sample, ranging from 1-5 g of wet tissue, was collected within 30 s of euthanasia and immediately immersed directly in liquid nitrogen until gas elaboration ceased (up to 60 s). 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 Cre-related Metabolites in Muscle

Muscle phosphocreatine (PCr), free creatine (Cre), and ATP were measured simultaneously by an external laboratory (Harlan Laboratories, Itingen, Switzerland) using fully-validated procedures. In brief, muscle biopsy samples were freeze-dried, powdered, and following extraction of the powder with perchloric acid (PCA), an aliquot of 25 μL of neutralized PCA extract (corresponding to 250 μg of dried muscle) was used for the simultaneous determination of ATP and PCr. For determination of Cre, the neutralized extract was diluted 1:5 with assay buffer, and in a single run, undiluted extract was used for the analysis of Cre. The analytical method was based on enzymatic determinations, which ultimately resulted in either reduction of NADP to NADPH (ATP, PCr) or oxidation of NADH to NAD (Cre), 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 test muscle biopsy samples. Final data included the absolute concentration of each Cre-related metabolite (ATP, PCr, and Cre), along with calculation of absolute concentration of total Cre (tCre; PCr plus free Cre) and relative proportions (PCr:ATP and PCr:total Cre).

Statistical Analysis.

All data were analyzed as a RCBD 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

served as the experimental unit for all response variables. Treatments were compared using means separations procedures using least squares means. Overall treatment effects with a probability of $P < 0.05$ were accepted as statistically significant.

Results

In general, all formulation objectives were achieved (**Table 4.2**).

Study 1

Growth Performance: The PC diet increased ($P < 0.05$) final BW by 10% and daily BWG d 14-28 and d 0-28 by 11 and 10%, respectively, compared with the NC diet (**Table 4.3**). Supplementation with 0.12% GAA increased ($P < 0.05$) both final BW and daily BWG (d 0-28) by 6% compared with the NC diet. In each case, responses to 0.12% GAA supplementation were statistically equivalent to the PC diet. Comparatively, supplementation with 0.06% or 0.18% GAA only 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 gain:feed ratio (G:F) was increased ($P < 0.05$) by 10% on average in birds fed the PC diet, compared with the NC diet, during each feeding phase and over the entire 28-d feeding period. Moreover, G:F was increased ($P < 0.05$) with increasing concentrations of GAA during both the starter (6, 11, and 8%, respectively) and finisher (3, 6, and 7%, respectively) periods, with equivalent effects noted when comparing the 0.12% GAA-supplemented diet with the PC diet during each feeding period. Overall, only 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. Supplementation of either 0.12 or 0.18% GAA increased ($P < 0.05$) PCr concentrations by 66 and 105%, respectively, when compared with the NC diet (**Table 4.4**). Supplementation of 0.12 or 0.18% GAA also increased ($P < 0.05$) the PCr:ATP ratio by 77 and 108%, respectively, when compared with the NC diet. Total Cre 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 tCre concentration that was equal to the PC diet, and addition of 0.18% GAA caused tCre to be 11% greater ($P < 0.05$) than tCre concentrations elicited by the PC diet. There were no differences between treatments for absolute concentrations of ATP or relative proportions of PCr:tCre.

Study 2

Growth Performance: Body weight gain of chicks receiving Arg-adequate diets did not respond to the supplementation of GAA or Cre, with no significant differences study d 0-14 or d 0-27 (**Table 4.5**). During d 14-27, BWG was greater ($P < 0.05$) in chicks fed diets supplemented with either GAA (11% higher) or Cre (PC1; 8% higher) compared with the salmon protein-containing diet (PC2). There were no effects of dietary supplementation on feed intake during any feeding period in this study.

Mortality-corrected gain:feed ratio was increased ($P < 0.05$) by addition of 0.12% GAA as compared with the NC diet from study d 0-14 and d 14-27. Supplementation of Cre 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 d 0-14, it also produced the lowest G:F for d 14-27 and d 0-27.

Overall, only the Cre-supplemented diet (PC1) increased ($P < 0.05$) G:F during the entire 27-day feeding study compared with the NC diet.

Tissue Analysis. Supplementation of 0.12% GAA increased ($P < 0.05$) absolute PCr concentrations by 26% compared with the NC diet, and 0.12% GAA supplementation elicited PCr concentrations that were not different from either the PC1 or PC2 diets. The relative ratio of PCr:ATP was increased ($P < 0.05$) by 24% due to 0.12% GAA supplementation compared with the NC diet. Muscle tCre concentrations were increased ($P < 0.05$) by 18% due to 0.12% GAA supplementation relative to the NC diet. Supplementation of GAA at 0.06% elicited comparable tCre concentrations compared with the PC2 diet, but caused a reduced ($P < 0.05$) concentration of PCr as a proportion of tCre when compared with all other treatments.

Discussion

The present studies were designed to test the efficacy of GAA for supporting growth and improve 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 tCre concentrations to meet or exceed those of the Arg-adequate PC diet. In Arg-adequate diets, GAA supplementation also markedly increased G:F, such that diets containing 0.12% GAA elicited growth performance responses that were overall equivalent to the Cre-supplemented and salmon protein-supplemented PC diets. Moreover, responses due to GAA were evident in muscle phosphagen concentrations as 0.12% GAA

supplementation caused the PCr and tCre concentrations to meet or exceed those of the Cre-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) and Dilger et al. (2013). Unlike previous observations, however, supplementation of 0.12% GAA elicited both final BW and overall BWG improvements that were greater than the NC diet and equal to the PC diet. Moreover, G:F was improved as observed previously (Dilger et al., 2013). Our data strongly indicate an Arg-sparing effect (Almquist et al., 1941), which would allow Arg to be used for functions other than Cre synthesis, most notably muscle growth (Edwards Jr. 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 0.12% GAA increased concentrations of Cre-related metabolites in chicken breast muscle, with PCr and tCre concentrations increasing 66 and 49%, respectively, compared with the NC diet in Study 1. When compared with the PC diet, PCr and tCre 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 Cre concentrations with GAA supplementation. Because of the tandem increases in muscle PCr and tCre concentrations, we can assume that dietary GAA is successfully absorbed and metabolized to Cre 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 Cre synthesis.

Muscle PCr and tCre concentrations increased by 26 and 18%, respectively, due to supplementation of 0.12% GAA to the Arg-adequate diet when compared with the NC diet; these observations are also supported by recent research from Michiels et al. (2012). The increase in muscle metabolites, especially PCr and 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 the down-regulation of AGAT caused by Cre (Wyss and Kaddurah-Daouk, 2000), and because the production of Cre from GAA is unregulated and continues until all GAA is consumed or excreted (da Silva et al., 2009). Supplemental GAA bypasses enzymatic regulation and permits increased concentrations of Cre-related metabolites in muscle, which is a unique outcome for this nutritional additive. In this way, tCre 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 decreased 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. Because 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 Cre via a methyltransferase step (Wyss and Kaddurah-Daouk, 2000), and both muscle PCr and tCre concentrations increased due to GAA supplementation, it is assumed that

GAA was synthesized to Cre with high efficacy, thereby producing results very similar to direct Cre supplementation. The advantage of using GAA, however, is that this chemically-synthesized metabolite is cheaper to produce and more stable than creatine monohydrate when used in broiler diets.

Both studies resulted in improvements in BWG 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 increasing BWG (Dilger et al., 2013). In the Arg-adequate diet, however, the rate of muscle protein synthesis should be equivalent because all diets contained adequate quantities of Arg, yet muscle concentrations of Cre-related metabolites continued to increase due to GAA supplementation. While that did not manifest as an improvement in BWG in our study, this phenomenon has been purported to increase muscle growth (Ingwall, 1976; Ingwall and Wildenthal, 1976). Whereas 0.12% GAA supplementation elicited increases in the PCr:ATP ratio in both Arg-deficient and Arg-adequate diets, improvements in the Arg-deficient diet were three times greater than in the Arg-adequate diet. With either dietary Arg status, GAA supplementation increased the concentration of Cre observed in the muscle, and this effect was predominantly through alteration of PCr concentrations. However, improvements in PCr:ATP ratio due to 0.12% GAA supplementation were greater for the Arg-deficient NC diet (1.45) than for the Arg-adequate NC diet (2.57), thereby directly implicating mild Arg-deficiency in decreased muscle phosphagen 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 Cre-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 AGAT activity to better quantify the Arg-sparing effects of GAA. Moreover, investigating whether GAA supplementation alters muscle protein synthesis *in vivo* is warranted, especially in direct relation to Cre supplementation.

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Tables

Table 4.1. Formulation and calculated nutrient composition for 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
DDGS	10.00	12.00	-	-	-	-
Corn Gluten Meal	8.50	10.60	-	-	-	-
Salmon Protein	-	-	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[2]	0.20	0.20	0.20	0.20	0.20	0.20
Mineral Premix[3]	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
Calcium, %	1.07	0.94	1.06	1.04	0.97	0.95
Phosphorus (total), %	0.75	0.69	0.74	0.77	0.68	0.70
Phosphorus (available), %	0.51	0.46	0.49	0.55	0.44	0.50
AME_N, kcal/kg	3,073	3,199	3,050	3,050	3,150	3,150
Digestible Amino Acids, %[4]						
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]NC = negative control, PC2 = salmon protein-containing positive control.

[2]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.
[3]Provided as milligrams per kg of diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; Se, 0.1 from Na_2SeO_3.
[4]Acquired from AMINODat® 4.0 (Evonik Industries AG, Hanau-Wolfgang, Germany).

Table 4.2. Analyzed composition of dietary treatments and ingredient (%, as-is basis)[1]

	Study 1[2]				Study 2[3]						
	NC		PC		NC		PC1		PC2[4]		Salmon[5] Protein
Nutrient	**Starter**	**Grower**	**Starter**	**Grower**	**Starter**	**Grower**	**Starter**	**Grower**	**Starter**	**Grower**	
Crude Protein	22.56	23.55	23.55	21.33	22.6	19.9	23.2	20.6	24.1	21.8	82.8
Crude Fat	6.9	6.8	6.8	8.1	7.0	7.1	6.7	7.8	6.3	7.6	2.67
Crude Fiber	2.8	2.7	2.7	2.9	2.9	2.3	2.7	2.3	1.9	1.9	1.20
Ash	6.5	6.1	6.1	5.8	6.4	6.0	6.0	6.2	6.5	6.2	13.2
Amino Acids[6]											
Essential											
Arg	1.05	0.98	1.47	1.25	1.54	1.31	1.45	1.36	1.47	1.31	4.98
His	0.51	0.48	0.52	0.47	0.58	0.53	0.54	0.54	0.49	0.47	1.03
Ile	0.95	0.87	0.92	0.85	0.97	0.85	0.89	0.87	0.86	0.81	1.15
Leu	2.36	2.47	2.30	2.49	2.01	1.79	1.89	1.83	1.70	1.56	2.30
Lys	1.35	1.21	1.43	1.20	1.42	1.23	1.38	1.27	1.36	1.20	2.91
Met	0.68	0.56	0.66	0.58	0.61	0.59	0.66	0.61	0.68	0.62	1.37
Phe	1.09	1.08	1.08	1.08	1.17	1.01	1.10	1.03	0.97	0.85	1.56
Thr	0.98	0.93	0.99	0.87	0.97	0.84	0.98	0.87	0.96	0.84	1.96
Val	1.05	0.98	1.07	0.96	1.13	0.97	1.06	0.99	1.04	0.95	1.65
Non-Essential											
Ala	1.34	1.37	1.31	1.37	1.12	1.03	1.06	1.05	1.29	1.24	6.09
Asp	1.69	1.58	1.72	1.54	2.38	2.04	2.23	2.12	2.00	1.82	4.75
Cys	0.36	0.35	0.36	0.35	0.39	0.35	0.36	0.36	0.31	0.28	0.21
Glu	3.88	3.86	3.84	3.83	4.19	3.64	3.95	3.75	3.63	3.27	7.58
Gly	0.74	0.70	0.75	0.68	0.94	0.81	0.89	0.83	1.69	1.61	14.37
Pro	1.49	1.59	1.47	1.57	1.36	1.25	1.27	1.28	1.50	1.45	6.31
Ser	1.01	0.99	1.00	0.99	1.11	1.00	1.06	1.03	1.02	0.95	2.99

[1]NC = negative control treatment, PC = positive control treatment.

[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]Salmon protein is a commercially available product called Lipromar Salmon Protein.

[6]Amino acid values standardized to a dry matter content of 88%.

Table 4.3. Growth performance of chicks fed Arg-deficient diets (Study 1)[1]

	Dietary Treatment[2]					
Variable	**NC**	**0.06% GAA**	**0.12% GAA**	**0.18% GAA**	**PC**	**SEM**
Body weight, g						
d 0	33.2	33.2	33.2	33.2	33.2	0.01
d 14	259.5	269.2	273.1	262.9	273.7	5.13
d 28	950.8^{a}	987.9^{abc}	1,010.4^{bc}	982.3^{ab}	1,042.4^{c}	19.83
Daily BW gain, g/chick						
d 0-14	16.2	16.9	17.1	16.4	17.2	0.37
d 14-28	49.4^{a}	51.0^{a}	52.6^{ab}	51.2^{a}	54.8^{b}	1.18
d 0-28	32.8^{a}	34.1^{abc}	34.9^{bc}	33.9^{ab}	36.0^{c}	0.71
Daily feed intake, g/chick						
d 0-14	23.8	23.5	22.5	22.4	22.9	0.51
d 14-28	76.0	75.6	76.1	72.5	77.6	1.27
d 0-28	49.4	48.2	48.5	47.0	49.5	0.80
Gain:feed, g/kg						
d 0-14	675.3^{a}	715.5^{b}	749.5^{c}	730.2^{bc}	749.7^{c}	10.03
d 14-28	643.3^{a}	661.0^{a}	680.8^{b}	685.8^{b}	706.0^{c}	7.14
d 0-28	646.0^{a}	663.7^{a}	696.6^{bc}	685.3^{ab}	712.6^{c}	8.79
Mortality, # of chicks						
d 0-14	3	3	5	2	5	
d 14-28	2	5	1	5	1	
d 0-28	5	8	6	7	6	

[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-30 d post-hatch. Arg = arginine, BW = body weight, GAA = guanidinoacetic acid, NC = negative control treatment, PC = positive control treatment.

[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.

Table 4.4. Muscle analyses of creatine-related metabolites of chicks fed Arg-deficient diets (Study 1)[1]

	Dietary Treatment[2]					
Variable	**NC**	**0.06% GAA**	**0.12% GAA**	**0.18% GAA**	**PC**	**SEM**
ATP, mmol/kg DW	35.93	36.90	34.40	35.84	33.73	0.90
PCr, mmol/kg DW	52.81[a]	62.42[a]	87.69[b]	108.17[c]	87.60[b]	4.25
PCr:ATP ratio	1.45[a]	1.72[a]	2.56[b]	3.02[c]	2.62[b]	0.12
Free Cre, mmol/kg DW	35.64[a]	39.88[a]	43.85[ab]	47.11[b]	51.73[b]	3.39
Total Cre, mmol/kg DW[3]	88.45[a]	102.29[b]	131.54[c]	155.28[d]	139.33[c]	4.33
PCr:total Cre, %	58.93	61.37	66.80	69.68	62.69	2.96

[a-d]Means within a row lacking a common superscript letter differ ($P < 0.05$).

[1]ATP = adenosine triphosphate, Cre = creatine, DW = dry weight, GAA = guanidinoacetic acid, PCr = phosphocreatine, NC = negative control treatment, PC = positive control treatment

[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 Cre.

Table 4.5. Growth performance of chicks fed Arg-adequate diets (Study 2)[1]

	Dietary Treatment[2]					
Variable	**NC**	**0.06% GAA**	**0.12% GAA**	**PC1[3]**	**PC2[4]**	**SEM**
Body weight, g						
d 0	34.3	34.3	34.3	34.3	34.3	0.24
d 14	431.1	438.7	449.3	440.5	448.4	9.42
d 27	1,418.1	1,440.4	1,490.2	1,451.3	1,378.3	26.81
Daily BW gain, g/chick						
d 0-14	28.4	28.9	29.6	29.0	29.6	0.67
d 14-27	75.6^{ab}	76.9^{b}	79.2^{b}	77.7^{b}	71.5^{a}	1.51
d 0-27	51.3	52.1	53.9	52.5	51.4	1.21
Daily feed intake, g/chick						
d 0-14	36.7	36.0	37.3	35.2	33.7	0.87
d 14-27	97.1	96.0	100.2	95.5	94.1	1.76
d 0-27	66.6	64.5	67.8	65.0	62.8	1.32
Gain:feed, g/kg						
d 0-14	772.6^{a}	785.4^{ab}	801.3^{bc}	818.4^{c}	851.3^{d}	9.55
d 14-27	719.5^{a}	727.9^{ab}	738.7^{bc}	746.0^{c}	703.7^{a}	6.10
d 0-27	729.8^{ab}	727.3^{a}	748.5^{bc}	759.9^{c}	740.2^{abc}	7.01
Mortality, # of chicks						
d 0-14	1	7	4	1	7	
d 14-27	2	5	3	2	1	
d 0-27	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. Arg = arginine, BW = body weight, GAA = guanidinoacetic acid, NC = negative control treatment.

[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.

Table 4.6. Muscle analyses of creatine-related metabolites fed Arg-adequate diets (Study 2)[1]

	Dietary Treatment[2]					
Variable	**NC**	**0.06% GAA**	**0.12% GAA**	**PC1[3]**	**PC2[4]**	**SEM**
ATP, mmol/kg DW	27.71	28.36	27.84	30.04	27.73	1.14
PCr, mmol/kg DW	70.27[a]	65.40[a]	88.44[b]	96.24[b]	86.95[b]	5.75
PCr:ATP ratio	2.57[a]	2.30[a]	3.18[b]	3.23[b]	3.11[b]	0.18
Free Cre, mmol/kg DW	58.30[a]	88.19[b]	63.31[a]	76.06[b]	79.37[b]	4.99
Total Cre, mmol/kg DW[5]	128.57[a]	153.60[bc]	151.74[b]	172.30[d]	166.32[cd]	4.71
PCr:total Cre, %	54.66[b]	41.92[a]	58.28[b]	55.83[b]	51.66[b]	3.28

[a-d]Means within a row lacking a common superscript letter differ ($P < 0.05$).

[1]ATP = adenosine triphosphate, Cre = creatine, DW = dry weight, GAA = guanidinoacetic acid, NC = negative control treatment, PCr = phosphocreatine.

[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 Cre.