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GUANIDINOACETIC ACID AS FEED SUPPLEMENT TO BROILER CHICKENS

Thesis submitted in fulfillment of the requirements
for the joint degree of Doctor (PhD) of Bioscience Engineering:
Animal Science and Aquaculture

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GUANIDINOAZIJNZUUR ALS VOEDERSUPPLEMENT VOOR VLEESKIPPEN

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ABBREVIATIONS

A	assay
AA	amino acid
AD	arginine deficient
ADFI	average daily feed intake
ADG	average daily gain
ADP	adenosine diphosphate
AGAT	L-arginine:glycine amidinotransferase
AID	apparent ileal digestible
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
Arg	L-arginine
ATP	adenosine triphosphate
BHMT	betaine-homocysteine methyltransferase
BMM	breast muscle myopathies
BSE	bovine spongiform encephalopathy
BW	body weight
BWG	body weight gain
CK	creatine kinases
CMH	creatine monohydrate
CP	crude protein
Cr	creatine
CrN	creatinine
CRT1	creatine transporter 1
CS	cold stress
Cys	L-cysteine
dArg	digestible arginine
DDGS	distillers' dried grains with solubles
dLys	digestible lysine
DM	dry matter
DMG	dimethylglycine
DNA	desoxyribonucleic acid
EC	European Commission
EE	ether extract
EPEF	European Production Efficiency Factor
EU	European Union
F:G	feed to gain ratio
FI	feed intake
GAA	guanidinoacetic acid
GAMT	N-guanidinoacetate methyltransferase
GLM	general linear model
Gly	glycine

Gly_{equi}	glycine equivalents
GNMT	glycine N-methyltransferase
GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulphide
HA	high altitude
HCy	homocysteine
HDL	high density lipoprotein
HPA	hypothalamic-pituitary-adrenal
HS	heat stress
HSpS	heat stress prior to slaughter
IGF-I	insulin-like growth factor-I
Iso	L-isoleucine
Leu	L-leucine
Lys	L-lysine
M+C	methionine + cysteine
MAT	methionine adenosyltransferase
MBM	meat and bone meal
MDA	malondialdehyde
ME	metabolizable energy
Met	L-methionine
MS	methionine synthase
MTHF	5,10-methylene-tetrahydrofolate
MTHFR	5,10-methylene-tetrahydrofolate reductase
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
ND	nutrient density
NO	nitric oxide
NRC	National Research Council
NSP	non-starch polysaccharides
OS	oxidative stress
PBM	poultry by-product meal
PC	phosphatidylcholine
PCA	principal component analysis
PCr	phosphocreatine
PEMT	phosphatidylethanolamine N-methyltransferase
PSE	pale-soft-exudative
RH	relative humidity
RNA	ribonucleic acid
RSM	rapeseed meal
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SBM	soybean meal

SEM	standard error of mean
Ser	L-serine
SFM	sunflower meal
SGK-1	serum- and glucocorticoid-inducible kinase 1
SID	standard ileal digestible
SLC6A8	plasma membrane Na^+/Cl-dependent creatine transporter
SOD	superoxide dismutase
T	temperature(s)
TBARS	thiobarbituric acid reactive species
TCr	total creatine
THF	tetrahydrofolate
Thr	L-threonine
TN	thermo-neutral
TSAA	total sulfur amino acids
UA	uric acid
UCP	uncoupling protein
Val	L-valine
VLDL	very low density lipoprotein levels
WB	wooden breast
WHC	water holding capacity
WS	white striping

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General background

Poultry meat consumption has become increasingly popular, and hence broiler production is yet to grow in the time to come (Mottet and Tempio, 2016). However, broiler production faces many challenges (Porter, 2016). The use of animal protein such as poultry by-product meal (PBM) and meat and bone meal (MBM) in diets has being prohibited in EU (Regulation (EC) No 999/2001) or is decreasing, depending on the region, which means that some nutrients are less present in the diet. For example, creatine (Cr), essential for energy conservation in muscle cells, can only be found in animal protein sources. However, the body can synthesize Cr by a two-step pathway (Wyss and Kaddurah-Daouk, 2000). Decades ago it was established that human vegetarians have lower Cr levels than non-vegetarians (Delanghe et al., 1989). Hence, it is believed that fast-growing broiler chickens fed vegetable diets may benefit from dietary supplementation of Cr, or its direct precursor guanidinoacetic acid (GAA). Thus, dietary GAA may improve cellular bioenergetics by stimulating Cr loading. It is therefore appealing to know whether dietary GAA might have different effects on broiler performances depending on the metalizable energy (ME) or nutrient density (ND) of the diet. Further, the *de-novo* synthesis of Cr in the body or the synthesis via dietary GAA shows many interactions with several amino acids such as L-arginine (Arg) and L-methionine (Met) (Curt et al., 2015). Regarding the former, in fact, the use of GAA may spare Arg, and regarding the latter, Met is converted to S-adenosyl methionine (SAM) that is used to methylate GAA rendering Cr. It means that the efficacy of supplementation of GAA may depend on the Met provision in the diet and its methylation potential. Furthermore, broiler production is most growing in (sub)tropical regions, and concomitantly the impact of climate change worldwide increases the likelihood for heat stress (HS) in poultry production (Mottet and Tempio, 2016). Heat exposure has a significant impact on well-being and production (Lara and Rostagno, 2013). The existence of feathers on the body, absence of sweat glands, and high metabolic rate of modern strains makes broilers very susceptible to high temperatures (T). Dietary solutions have been generally proposed as being effective and relatively cheap (Renaudeau et al., 2012). Supplementing broilers with GAA may boost energy metabolism. This is of utmost importance since heat stressed broilers reduce their feed consumption and cellular energy demand is increased. Also, dietary GAA may spare Arg and thus leave Arg available for other metabolic functions such as conversion to nitric oxide (NO), which in turn can support tolerance to HS. Altogether, GAA may confer several benefits to heat stressed broilers. The growth enhancing potential of GAA in broilers fed vegetable diets has been well established (e.g. Lemme et al., 2007b; Michiels et al., 2012). This feed additive for broilers is available in many parts of the world. However, seeing the many interactions with other nutrients, many questions remain. Also, the efficacy of the feed additive may depend on the context, which needs further elucidation in order to use the additive in a proper way.

Objectives and outline of the thesis

In general, this PhD thesis aims to increase our understanding of how GAA interacts with other nutrients in the diet of broiler chickens, and what and if larger benefits for performance are to be expected when used in specific conditions such as HS. Further, this thesis wants to generate additional insights in the mode of action by exploring the metabolism of GAA and Cr, and the potential antioxidant action in the broiler chicken.

The **specific objectives** of the present study are:

> [1] to study the interaction of GAA and ND on performance and Cr loading in broiler chickens,
>
> [2] to study the interaction of GAA and Met on performance and Cr loading in broiler chickens,
>
> [3] to study the effect of GAA on tolerance of finisher broilers subjected to a chronic cyclic HS model, with interest in performance indices, antioxidant potential and understanding of GAA and Cr metabolism.

In **Chapter 1**, a literature review is presented. It comprises relevant data on endogenous Cr synthesis, degradation and regulation and the involvement of Arg, glycine (Gly) and Met. Further, the feed additive GAA is presented, and literature was searched to compile the described effects on performance and carcass yield and composition, Cr loading in muscle, meat quality and myopathies, oxidative status and other traits in various poultry.

Chapter 2 describes experimental work dealing with the interaction of dietary GAA and ND. The trial aimed to investigate the main and interactive effects of ND and GAA supplementation in corn-soybean (SBM) diets on animal performance and carcass characteristics and organ weights, and traits of the energy metabolism in breast muscle of broiler chickens.

Further, it was hypothesized that availability of Met, as the single precursor of SAM, could play an important role in endogenous GAA conversion to Cr, next to other factors essential in the re-methylation and folate cycles. Thus, **Chapter 3** deals with an experiment with similar protocols as in Chapter 2, but here the interaction between Met; being deficient, adequate or surfeit, and GAA supplementation in corn-SBM diets was investigated.

Various arguments were made to believe that benefits of GAA would be more prominent when birds are subjected to HS, this because of: 1/ improved energy status, due to Cr's pivotal role in energy homeostasis, and 2/ improved Arg metabolism, due to Arg sparing effects. These hypotheses were tested in heat stressed finisher broilers in a model of chronic cyclic HS, detailed in **Chapter 4**.

For the last experimental part, **Chapter 5**, tissue samples from the experiment in Chapter 4 were taken to assess the effect of feeding GAA to broilers subjected to HS during the finisher period on oxidative status and GAA and Cr metabolism in key organs. Data were subjected to analysis of variance, and additionally data were used in a Principal Component Analysis (PCA) to explore the correlation structure across variables.

Finally, a general discussion (**Chapter 6**) relates all data and are put in perspective. In order, following items are addressed: highlights of our findings, the partitioning into the muscle Cr loading and Arg sparing effect by feeding GAA, the effects on carcass yield and composition and breast meat quality, the relation of the efficacy of GAA and other nutrients in the diet, opportunities for use of GAA in certain conditions, practical use of GAA in formula for broilers, and future perspectives.

CHAPTER 1

GUANIDINOACETIC ACID AS FEED SUPPLEMENT IN POULTRY

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GUANIDINOACETIC ACID AS FEED SUPPLEMENT IN POULTRY

1.1. POULTRY PRODUCTION AND CHALLENGES

The world has now over 23 billion poultry - about three birds per person on the planet (FAOSTAT, 2016), which is about five times more than 50 years ago. They are kept and raised in a wide range of production systems, and provide mainly meat, eggs and manure for crop fertilisation. Poultry meat and eggs are among the most common animal source foods consumed at the global level, through a wide diversity of cultures, traditions and religions, making them key to food security and nutrition. Poultry makes a substantial contribution to food security and nutrition, providing energy, protein, and essential micro-nutrients to humans, with short production cycles and the ability to convert a wide range of agri-food by-products and wastes into meat and eggs edible by humans (Mottet, 2017).

The growth of the global livestock sector is expected to continue as global human population is estimated to reach 9.6 billion in 2050. In this context, Alexandratos and Bruisma (2012) projected that the demand for animal source food could grow by 70% between 2005 and 2050. Poultry meat is expected to have the highest growth, with 121%. By 2050, the annual growth rate of poultry meat production is estimated to reach 1.8% at global level, and 2.4% in developing countries. Most of the growth will be driven by Asia (Mottet and Tempio, 2016). However, at the same time the poultry industry is facing several challenges (Porter, 2016; Choct, 2016; Hafez, 2016; Méda et al., 2019; Rogiewicz and Slominski, 2019; Tixier-Boichard, 2018), some of which are outlined here:

- Impact on environment and global warming
- Sustainability and availability of raw materials
- Ethical aspects of limits to genetic selection
- Need for reducing use of antibiotics
- Production in hot climates
- Quality of end-products, e.g. breast muscle myopathies (BMM)
- Consumer oriented production
- Animal welfare, e.g. feather pecking, lameness, footpad lesions, etc.

In order to thrive, the poultry industry has to reply adequately to these challenges. These are the avenues for sustainable development of poultry production; encompassing economic, environmental, social and institutional governance aspects (Vaarst et al., 2015).

1.2. FEED INGREDIENTS AND FUTURE PROSPECTS

Several of these challenges connect closely to nutritional concepts for poultry. Feed formula for poultry are not fixed, but dynamic amidst changing supply and prices of raw materials and additives and alterations in the socio-economical context. Since the turning of the millennium numerous trends have re-shaped, and are ongoing, the feed ingredient market. Some of these trends, that may have impact on the potential use of GAA as feed additive, will be detailed here.

In the aftermath of the Bovine spongiform encephalopathy (BSE)-crisis in the late 90's primarily in the United Kingdom but also in other EU countries, the EU decided to ban animal protein sources such as PBM and MBM (Table 1.1) in diets of food-producing animals (Regulation (EC) No 999/2001). Ever since 2005, the EU gradually reverted this legislation, though all animal protein remained prohibited for ruminants, with few exceptions. Regarding pigs and poultry, products such as fishmeal (Table 1.1), blood products from non-ruminants, and animal phosphorous sources are to date allowed under strict conditions. The conditions adhered to this may differ across EU countries. However, for example in Belgium, these conditions are hard to comply with if the feed mill also produces ruminant feeds, hence currently only 7 feed mills that produce ruminant feeds have approval to use fishmeal or blood products from non-ruminants for poultry feeds. There are more feed mills that do not produce ruminant feeds but do produce non-ruminant feed with fishmeal or blood product from non-ruminants. In Iran there is no such a limitation. In addition, fish meal has become relatively expensive over the years. As a consequence, the use of animal protein, at least in most EU countries, has been abolished rougly two decades ago. Rodehutscord et al. (2002) stated that the most critical consequence of this ban on animal by-products on the long term, and in face of the limited world-wide phosphate stores, is the irreversible disappearance of phosphorus from the food chain. According to the same authors, the gap in protein and minerals caused by the ban could easily be replaced by plant proteins. However, animal proteins also contain nutrients not found in vegetable sources, such as Cr. Although the body can synthesize Cr, it can be questioned whether the endogenous synthesis of Cr is sufficient for fast-growing broilers fed complete vegetable diets. Thus, the formulation of diets for poultry without animal protein sources is done to comply with regulations, or in areas where access to this sources is scarce, or as an attempt to add value to the final customer. To note, fishmeal and for sure animal by-products are still used in Iran, though at limited inclusion levels. Mainly in the north of Iran locally produced fish meal is used, but inclusion levels are limited to 3% in the diet. The inclusion level is limited because the quality (amino acid composition) is very variable and not all feed mills are able to analyse every batch of fish meal. Other feed mills that do routine analysis of incoming ingredients can use it up to somewhat higher levels.

The production of biofuels together with the search for new proteins either replacing animal protein (fish meal) and/or overseas produced SBM has brought various new raw materials on the front. Bioethanol production will reach 134.5 billion litres by 2024

(Choct, 2016). Bioethanol production yields ethanol, CO_2, and distillers' dried grains with solubles (DDGS) in approximately equal proportions (1/3 each). Therefore, the bioethanol production will generate 120.6 Mt of DDGS by 2024. DDGS is high in non-starch polysaccharides (NSP) but contains around 32% crude protein (CP), if derived from wheat (Table 1.1). Likewise, the global production of biodiesel is projected to reach 39 billion litres by 2024 (Choct, 2016). This will consume 13% of all vegetable oils produced in the world by 2024. Rapeseed is an important source for biodiesel production in Europe, but the cultivation of rapeseed is mainly driven to supply demand for oil in human. The EU produced 19.6 million tonnes of rapeseed in 2018, though over 3 tonnes lower than the average of the previous 5 years (Rogiewicz and Slominski, 2019). Low-glucosinolate rapeseed meal (RSM) or canola, obtained after separation of the oil, is commonly used in poultry nutrition as an economically viable alternative to SBM. However, it still cannot fully replace SBM due to the presence of antinutritive factors (NSP, tannins and phytate), lower ME value, and lower and less consistent amino acid (AA) digestibility (Table 1.1). Another by-product from oil seeds is sunflower meal (SFM) (Table 1.1), which similarly is high in NSP and low in ME. The inclusion levels in broiler diets are therefore limited, but improvements in processing conditions, development of high-protein meals or protein concentrates, and the application of feed enzymes will eventually mitigate these limitations. Nonetheless, the replacement of SBM by DDGS, RSM and SFM may result in changes in inherent diet concentrations of AA, not in the least of Arg. Inclusion of RSM (digestible Arg:digestible Lys (dArg:dLys)=121%) and SFM (dArg:dLys=258%) may decrease or increase, respectively, the Arg content of the diet, since in SBM dArg:dLys is 124%. Further, the trend is to apply low(er) CP diets for broilers. There are many reasons to do so: to lower the overseas protein dependency (reduce soybean/SBM imports), reduce N-excretion of the bird (Méda et al., 2019), and for animal welfare reasons (e.g. footpad dermatitis, Van Harn et al., 2019). Indeed, the excretion of nitrogenous compounds has negative effects on the environment because the excreted N is dispersed into environmental water, soil and air. N in manure can be applied reasonably for fertilizing agricultural land, but in too high amounts, this practice risks N leakage into the groundwater (Verstegen and Jongbloed, 2003). Ammonia emissions from livestock enterprises have been associated with a number of environmentally damaging effects, which include soil acidification, eutrophication, formation of fine particulates, and secondary emissions of nitrous oxide (Martínez-Lagos et al., 2013). In addition, ammonia emission affects human and animal health as well as the acceptance of livestock farming by the public due to unpleasant odors (Aneja et al., 2009). Altogether, with increased use of alternative protein sources and a move to lower CP concentrations to achieve financial and environmental benefits such as reduction of ammonia emission, the need to incorporate crystalline AA to maintain optimal dietary profiles is rising and changing. In that perspective, GAA, as an Arg sparing source, may have its benefits.

Energy is a component of poultry diets, representing about half of the expenses that producers have to pay for broilers' feed (Cook, 1987). At the same time, a part of this energy is wasted in the body by heat increment produced during the utilization of

different chemical components (Noblet at al., 2010). Past selection for fast-growing broilers has increased considerably growth rates, the birds reach their slaughter weight earlier than ever before. This has reduced the resource use of the bird, mainly because during the shorter growth cycle, less energy is now needed to maintain the body functions. This improved energy efficiency has considerably reduced the feed consumption of the birds and therefore improved the environmental sustainability of broiler production. However, selection for improved energy efficiency may have reached its limits in broiler chickens (Tallentire et al., 2018). These authors showed that future continued selection for maximum energy efficiency in broilers would only slightly reduce environmental burdens as compared to current fast-growing broilers, i.e. the greenhouse gas emissions (CO_2 equiv.) and agricultural land use associated with feed production may be reduced not more than 8%. As such, nutritionists are always looking for novel approaches to improve availability of energy for broiler chickens. One mechanism is to store the flow of energy in the body within the cells mediated through the formation of high-energy phosphate bonds (Lemme et al., 2007a). On this note, GAA, as precursor of Cr may increase energy utilization in broilers. In view of world-wide efforts to reduce greenhouse gas emissions, improving energy utilization in producing animal protein for human is paramount.

Table 1.1. Nutrient composition of some protein-rich raw materials for poultry (g/kg, unless otherwise stated) (Centraal Veevoederbureau, 2019).

Raw material	Soybean meal (SBM), High protein	Meat and bone meal (MBM), Low fat	Fish meal, High protein	Distillers' dried grains with soluble (DDGS), From wheat	Rapeseed meal (RSM), Conventional	Sunflower meal (SFM), Dehulled
Dry matter (DM)	878	943	917	916	897	901
Ash	65	392	132	46	80	66
Crude protein (CP)	489	455	707	324	383	368
Ehter extract (EE)	13	87	91	-	18	9
Non-starch polysaccharides (NSP)	217	9	-24	360	322	390
Metabolizable energy (ME), kcal/kg	2203	1926	3384	-	1754	1514
Lysine (SID Lys)	30.3 (26.7)	21.9 (16.4)	53.7 (45.7)	6.8 (3.3)	21.1 (16.4)	12.9 (10.5)
Methionine (SID Met)	6.8 (6.2)	5.9 (4.6)	19.8 (16.4)	4.9 (3.7)	7.7 (6.7)	8.1 (7.4)
Threonine (SID Thr)	19.1 (15.8)	13.7 (9.3)	29.7 (24.0)	10.4 (6.5)	16.9 (12.3)	13.6 (10.3)
Isoleucine (SID Iso)	22.5 (19.6)	11.8 (8.8)	29.7 (24.6)	11.6 (8.6)	14.9 (11.7)	15.1 (12.8)
Arginine (SID Arg)	36.7 (33.0)	34.2 (26.3)	41.7 (35.4)	12.9 (9.7)	23.4 (19.9)	29.8 (27.1)
Leucine (SID Leu)	37.7 (32.8)	25.0 (18.8)	51.6 (43.9)	24.3 (19.2)	26.8 (21.5)	23.2 (19.4)
Valine (SID Val)	23.5 (20.2)	18.2 (13.5)	34.6 (28.7)	13.9 (9.9)	19.5 (15.0)	18.0 (14.9)
Glycine (SID Gly)	21.0 (17.7)	71.5 (51.5)	45.9 (35.4)	12.6 (8.2)	19.9 (15.5)	20.9 (14.9)
Serine (SID Ser)	24.9 (21.5)	17.3 (11.4)	28.3 (22.1)	14.2 (10.1)	16.9 (12.8)	15.8 (12.3)

1.3. CREATINE: BIOSYNTHESIS, FUNCTION AND REGULATION

1.3.1. The creatine and phosphocreatine system as a high energy shuttle

Cr is the common name of *N*-carbamimidoyl-*N*-methylglycine or methylguanidoacetic acid (CAS No. 57-00-1, molecular formula: $C_4H_9N_3O_2$, molecular weight 131.1 g/mol) (Fig 1.1). Cr is known for its stimulatory effects on muscle function and energetics, and it contributes to a transient intracellular storage of metabolic energy by the phosphate high energy bond exchange operated with adenosine triphosphate (ATP) and catalysed by creatine kinases (CK) (Wyss et al., 2007). Phosphocreatine (PCr), also referred to as creatine phosphate, represents the phosphorylated form of Cr (Fig. 1.1) and is a convenient form of energy storage in tissues that have a high and rapidly fluctuating energy requirement such as muscle. This reversible reaction catalysed by CK is shown in Fig. 1.2. So when energetic needs are high at ATP requiring sites (muscular exercise, plasma membrane Na^+/K^- ATPase), PCr can restore the high energy phosphate bond to adenosine diphosphate (ADP) to regenerate ATP which then becomes directly available for ATP driven events. The Cr and PCr system thus act as a high energy shuttle. Two thirds of intramuscular Cr is PCr, with the remaining being free Cr (Kreider et al., 2017). About 95% of Cr is located in skeletal muscle, and the remaining 5% is stored in the brain, liver, kidneys, and testes. Meanwhile, most of the energy is consumed by these organs and tissues (Persky and Brazeau, 2001).

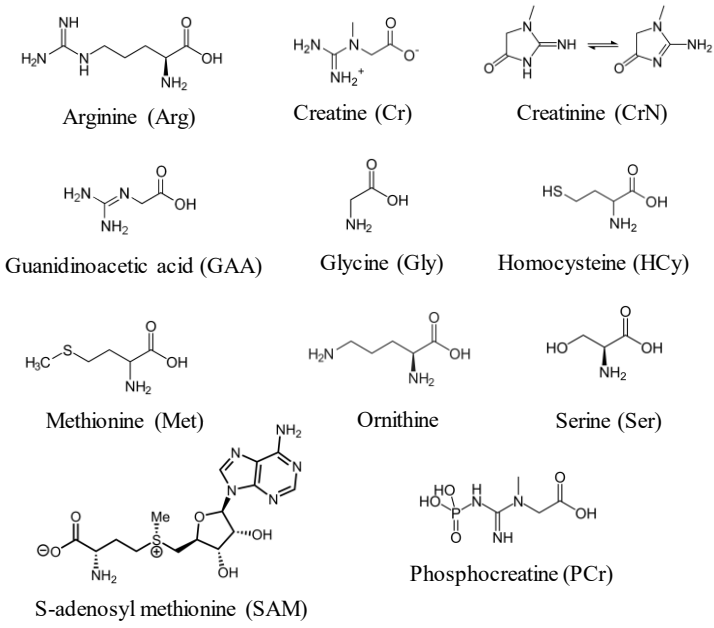


Fig 1.1. Amino acids and derivatives involved in creatine metabolism. Abbreviations used in the thesis between brackets.

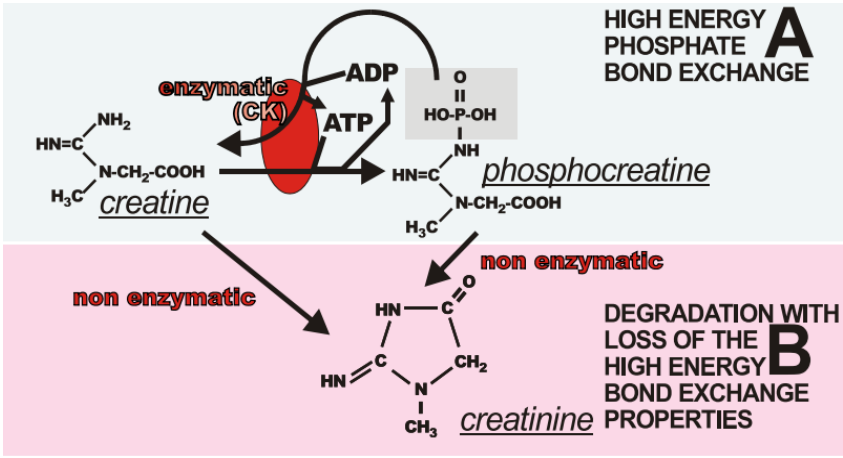


Fig. 1.2. Creatine, phosphocreatine and creatinine: enzymatic (A) and non-enzymatic (B) conversions. Abbreviations are CK, creatine kinases; ADP, adenosine diphosphate; ATP, adenosine triphosphate (Curt et al., 2015).

1.3.2. Creatine biosynthesis, transport, degradation and regulation

The endogenous synthesis of Cr requires three AA: Arg, Gly, and Met (Fig. 1.1) and two enzymes; L-arginine:glycine amidinotransferase (AGAT, EC 2.1.4.1; encoded by *GATM*) and N-guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2; encoded by *GAMT*), along with a transporter, SLC6A8 (also called creatine transporter 1, CRT1; encoded by *SLC6A8*). In a first step, the amidino group from Arg is transferred to the amino group of Gly, yielding L-ornithine and GAA, a reaction catalyzed by AGAT and occurring mainly in the kidney, more specifically in the mitochondria intermembrane space and to a lesser extent in the cytoplasm of cells (Tormanen, 1990) (Fig. 1.3). It has been suggested that AGAT is a critical control step in Cr biosynthesis (Da Silva et al., 2009; Guthmiller et al., 1994). In a second step, GAMT induces the GAA methylation on the original Gly nitrogen, using SAM as the methyl donor to form S-adenosyl homocysteine (SAH) and Cr (Da Silva et al., 2009; Walker, 1979; Stead et al., 2006) (Fig. 1.3). This step occurs essentially in the liver. Alternatively, these reactions can take place in other cells such and pancreas or kidney. After endogenous synthesis or in turn via nutritional supply and intestinal absorption (Wyss and Schulze, 2002; Balsom, 1994), Cr may be released in the blood stream. Circulating Cr may enter cells via the specific plasma membrane Na^+/Cl-dependent creatine transporter SLC6A8, highly expressed in brain, intestine and skeletal muscle (Ostojic, 2017).

Cr and PCr may be subject to a non-enzymatic and irreversible dehydration and cyclization to form creatinine (CrN) (Fig. 1.2). In accordance with *in vitro* studies, an almost constant fraction of the body Cr (1.1%/d) and PCr (2.6%/d) is converted non-enzymatically into CrN *in vivo* in mammals, giving an overall conversion rate for the total Cr pool (Cr+PCr) of ~1.7%/d (Walker, 1979). It is important to understand that the total Cr pool appears to be converted to CrN at a constant rate. This end product, CrN,

freely diffuses out the cell to be removed ultimately in the urine. CrN is freely filtered across the glomerulus and neither reabsorbed nor metabolized by the kidney. Then, urinary excretion of CrN physiologically depends on the muscular mass and is also routinely used as a marker of renal function, the alteration of which results in excessive retention of CrN in blood. Urinary excretion of CrN is also used for nutritional studies in animals since it is not influenced by nutrient intake and diet composition, if not provided via the diet (e.g. Chen et al., 1995). However, related to birds, Hasegawa et al. (2017) demonstrated that CrN excretion is lower when Arg or Met are limiting, but does not increase when birds are fed beyond Arg and Met requirements. Further, limited data from bird studies suggest more discrepancies from metabolism in mammals. It was suggested that in birds, Cr is mostly excreted in urine before it is converted to CrN so levels of plasma CrN are low (Lierz 2003), which is confirmed by data from EFSA that clearly show interspecies differences (chicken *vs*. pig) in this respect. Bell and Freeman (1971) reported that CrN is excreted by glomerular filtration and reabsorbed in the tubules. Both mechanisms appear to keep the plasma concentration constant, and postprandial elevations have not been observed (Lumeij and Remple, 1991). In general, it should be borne in mind that the Cr:PCr cycle is expected to be impaired in disorders of Cr biosynthesis and transport through a reduced supply in Cr. This reduced substrate availability for CK may be, however, corrected by dietary supplementation in Cr and by other appropriate strategies to an extent depending on the underlying defect.

Endogenous *de-novo* synthesis of Cr is modulated by several factors. A major regulation is operated at the level of AGAT (Fig. 1.3). AGAT is subject to negative feedback exerted by high levels of the intermediate and end products L-ornithine and Cr, respectively (Curt et al., 2015). Arg and GAA have only 'apparent' repressor activity. They exert no effect on AGAT expression by themselves but are readily converted to Cr, which then acts as the true repressor (Wyss and Schulze, 2002). On the opposite, a low nutritional supply in Cr is associated with a sustained increase in AGAT activity contributing to Cr homeostasis (Wyss and Kaddurah-Daouk, 2000). SLC6A8 is regulated by extracellular Cr levels in a time-dependent manner, with negative control by excess Cr occurring more rapidly than positive regulation by Cr deficiency (Fig. 1.3). SLC6A8 is also sensitive to inhibition by GAA (Wyss and Kaddurah-Daouk, 2000). Recently, it has been unexpectedly found that human female and male populations might be differentiated as regards to urine but not blood values including Cr:CrN ratio and GAA:CrN ratio values. These gender differences observed in healthy populations were proposed to result from differential effects of testosterone and estrogen in adolescents and adults, and by estrogen effects in prepubertal age on SLC6A8 function (Joncquel-Chevalier et al., 2015). Other hormonal effects affecting Cr biosynthesis include stimulatory effects of growth hormone and thyroxine on AGAT (McGuire et al., 1980). On the other hand, an important finding lies in the regulation of SLC6A8 by the adenosine monophosphate-activated protein kinase (AMPK) (Li et al., 2010a). This protein kinase is a sensor of cellular energetic status and is known to couple substrate transport to capacity of cells to yield energy. AMPK is activated in conditions of energy depletion. It was shown by Li et al. (2010a) to inhibit SLC6A8 via the mammalian target

of rapamycin (mTOR) pathway. The biological significance of this observation is that when cells are in energy depletion, Cr which buffers ATP levels is no longer necessary (because of the lack of ATP) and as a result its cellular uptake by SLC6A8 is consequently reduced. A striking parallelism between the enzymes involved in vertebrate Cr metabolism (AGAT, GAMT, CK) is that they all are sensitive to modification and inactivation by sulfhydryl reagents (Fujioka et al., 1992). However, there is no reason to believe that modification by sulfhydryl reagents (e.g., glutathione disulphide (GSSG)) represents a unifying mechanism for the *in vivo* regulation of AGAT, GAMT, and CK (Curt et al., 2015). These hormone and emerging regulatory aspects completing metabolic regulations of Cr biosynthesis are given in Fig. 1.4.

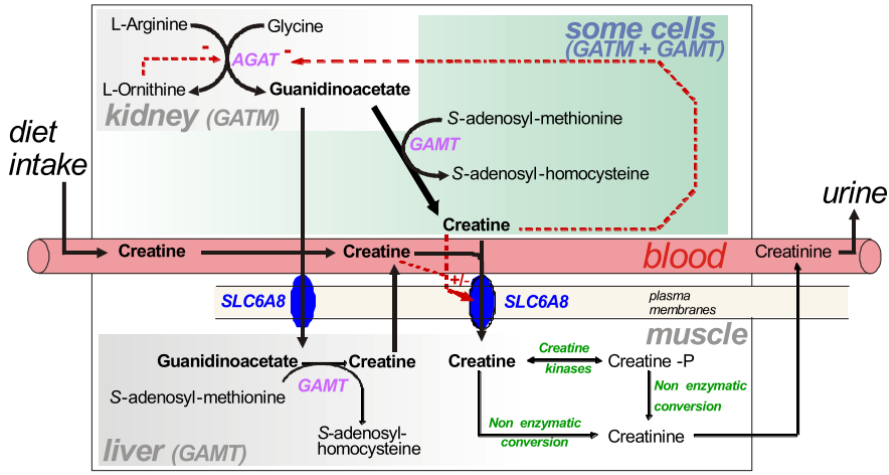


Fig. 1.3. Creatine (Cr) biosynthesis, metabolism and major regulation. In a first step, the amidino group from L-arginine is transferred to the amino group of glycine, yielding L-ornithine and guanidinoacetic acid (GAA), a reaction catalyzed by L-arginine:glycine amidinotransferase (AGAT), encoded by *GATM*, and occurs mainly in kidney. AGAT is subject to a negative feedback exerted by high levels of intermediate and end product, L-ornithine and Cr, respectively, as is shown by broken red lines. In a second step, N-guanidinoacetate methyltransferase (GAMT), encoded by *GAMT*, induces the GAA methylation using S-adenosyl methionine as the methyl donor to form S-adenosyl homocysteine and Cr. This step occurs mainly in liver. Alternatively, these reactions can take place in other cells such and pancreas or kidney. Cr from *de-novo* synthesis or from diet source transits by blood and is up-taken by tissues or cells expressing the specific plasma membrane Na^+/Cl-dependent Cr transporter SLC6A8 (encoded by *SLC6A8*) responsible for intracellular incorporation of Cr. SLC6A8 is regulated by extracellular Cr levels, with negative control by excess Cr occurring more rapidly than positive regulation by Cr deficiency. In tissues that have a high and rapidly fluctuating energy requirement, the Cr and phosphocreatine system act as a buffer to generate ATP by virtue of creatine kinases. Cr and phosphocreatine may be irreversibly and non-enzymatically degraded to creatinine, which in turn is devoided via urine (Curt et al., 2015).

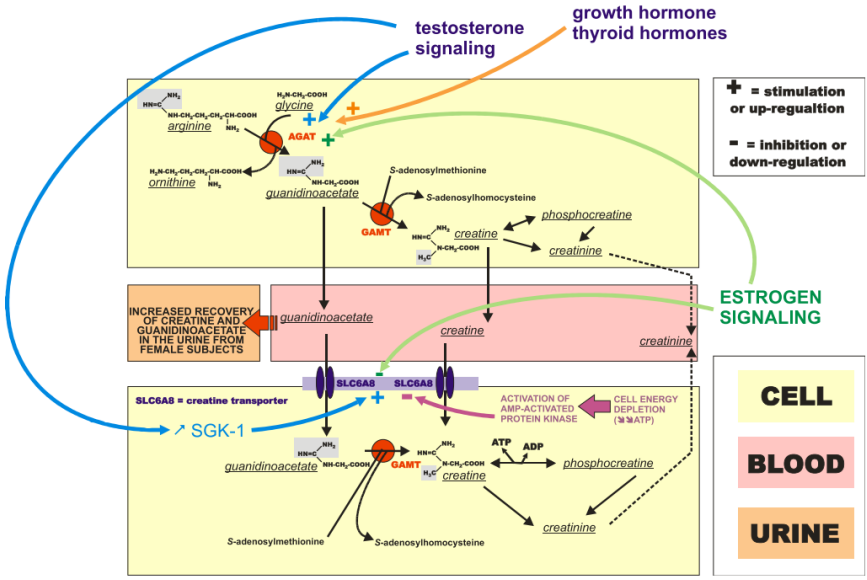


Fig. 1.4. Hormonal and emerging regulatory aspects of creatine (Cr) metabolism. Mechanisms conveyed by sex hormones are in part currently hypothetic and might explain some unexpected gender differences recently observed in the metabolic patterns observed in the urinary excretions of Cr and guanidinoacetic acid (expressed *vs*. urinary creatinine) in a series of more than 6000 subjects. Abbreviations are as in Fig. 1.3. SGK-1: serum- and glucocorticoid-inducible kinase 1 (Curt et al., 2015).

1.3.3. Other relevant physiological functions of creatine

Another physiological function of Cr that may have relevance for production animals is the attenuation of acute stress responses by quenching superoxide anions and other aqueous reactive species (Lawler et al., 2002, Deminice and Jordao, 2012). It is well known that excessive production of reactive species is harmful to normal metabolism, which may cause cellular damage resulting from lipid peroxidation, protein oxidation and DNA modification. Sestili et al. (2006) showed that added Cr, at concentrations comparable to those attainable in plasma upon oral supplementation of Cr in human, exerted direct antioxidant activity in cultured mammalian cells exposed to various oxidizing agents. Investigations showed protective effects of Cr exposure on oxidatively injured mitochondrial DNA (Guidi et al., 2008) and against RNA-damaging agents (Fimognari et al., 2009). Hence, it was reported that short-term Cr supplementation (5 g/d for 6 days) decreases reactive oxygen species content *in vivo* in rat skeletal muscle, possibly due to the direct action of Cr on scavenging superoxide anion radicals (Guimarães-Ferreira, 2014). Also, Deminice and Jordao, (2012) have demonstrated the protective effects of Cr against oxidative stress (OS) induced by a single bout of moderate aerobic exercise in rats, evidenced by increases in total plasma antioxidant capacity and muscle glutathione (GSH), a major cellular antioxidant. The latter corroborates with Young et al. (2010) who reported the capacity of Cr exposure to up-

regulate the thiol redox system, of which GSH is an important component. Cr may also exert antioxidant outcomes through its primary action on cellular energy status. The putative benefits of Cr in a number of muscular, neurological, and cardiovascular diseases such as gyrate atrophy have been generally attributed and not surprisingly to the Cr-induced buffering of cellular ATP levels, whose fall would lead to the accumulation of intracellular Ca^{2+}, and stimulation of formation of reactive species leading to tissue oxidative damage (Persky and Brazeau, 2001). On the contrary, Aksentijevic et al. (2014) questioned any physiologically relevant antioxidant activity of Cr in oxidatively challenged mice heart. Nonetheless, it is well known that fast-growing broilers need antioxidant protection (Aurousseau, 2002), and that some circumstances like HS can induce OS in poultry (Akbarian et al., 2016). It is therefore appealing to know whether dietary GAA through Cr loading may protect broiler's tissues from oxidative damage. However, guanidine compounds, like GAA, can induce the formation of free radicals (review by Hiramatsu, 2003). Zugno et al. (2006, 2008) showed that GAA administration to the rat brain led to a decrease of the non-enzymatic antioxidant capacity likely due to oxidation of sulfhydryl groups, leading to lower GSH levels. Yet, in healthy men, dietary GAA did not impact markers of oxidative status, apart from an elevation of fasting plasma superoxide dismutase (SOD) activity (Ostojic, 2015). It is thus equivocal what the effect would be of dietary GAA on the oxidative status of animals.

In the context of use in human, bodybuilding effects of Cr have been accounted for in part by intracellular water retention rather than by significant increase in muscle mass and function due to osmolyte properties of Cr (e.g. Ziegenfuss et al., 2002). For example, Alfieri et al. (2006) demonstrated that hypertonicity induced Cr uptake in C2C12 cultured muscle cells but also that Cr can act like the well-established compatible osmolytes betaine, taurine and *myo*-inositol in protecting the cells against hypertonic stress. Returning to the subject, selection towards increased growth rate and breast yield over the last 30 years has increased the incidence of breast meat abnormalities in broilers, amongst them pale-soft-and-exudative (PSE)-like meat (Petracci et al., 2015). The PSE-like condition not only impairs appearance, but reduces the ability of meat to hold and bind water during processing and storage. Thus, as Cr supplementation has been shown to result in increased cellular hydration (Juhn, 1999); Cr or GAA feeding could positively impact water-holding capacity (WHC) of meat. BMM that more recently appeared in broiler meat at sometimes prominent incidence will be dealt with in section 1.4.2.3.

1.3.4. Interaction between creatine and amino acid metabolism

As discussed above, the endogenous synthesis of Cr requires three AA: Arg, Gly and Met (Fig. 1.1. and Fig. 1.5.). Arg and Gly are substrates used for formation of GAA, whereas SAM derived from Met is donating the methyl group to GAA to yield Cr.

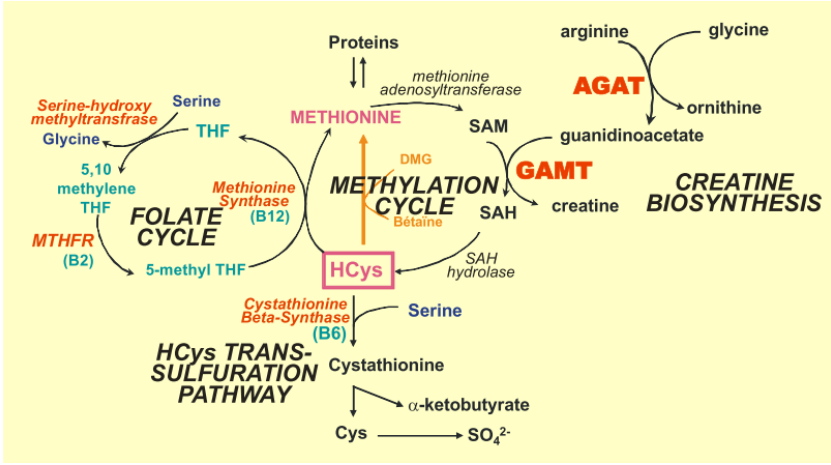


Fig. 1.5. Creatine biosynthesis, re-methylation cycle, folate cycle and transsulfuration pathways. Vitamin requirements of steps involved in homocysteine metabolism are indicated between brackets. Abbreviations are as in Fig. 1.3. Cys, L-cysteine; DMG, dimethylglycine ; HCys, homocysteine; THF, tetrahydrofolate; MTHFR, 5,10-methylene-tetrahydrofolate reductase; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine (Curt et al., 2015).

1.3.4.1. Arginine

Arg or 2-amino-5-guanidinopentanoic acid (CAS No. 7200-25-1; molecular formula: $C_6H_{14}N_4O_2$; molecular weight 174.2 g/mol) is generally considered either the fourth or fifth-limiting amino AA for broiler chickens (Han et al., 1992; Fernandez et al., 1994; Waguespack et al., 2009). The lack of *de-novo* synthesis of Arg due to the urea cycle being not functional in birds means that Arg is an indispensable AA to birds and needs to be supplied by the diet. Arg is an essential AA for broiler growth, but is classified as well as functional AA due to its other roles for broiler nutrition and health beyond sole incorporation in peptides (Wu and Morris, 1998). For example, Arg is source for NO, a potent vasodilator that directly relaxes vascular smooth muscle and modulates or inhibits the production and release of vasoconstrictors such as serotonin and endothelin-1. Next, L-ornithine is derived from Arg, and by virtue of ornithine decarboxylase it is converted into putrescine, and further metabolized to the bioactive polyamines spermidine and spermine whereby SAM donates successively propylamino groups. And of course, Arg is paramount to Cr biosynthesis (Fig. 1.5) (Wu, 2009). Yet, crystalline Arg is available in an economically viable form for the animal feed industry (EU Register of Feed Additives, Category 3, functional group C; https://ec.europa.eu/food/safety/animal-feed/feed-additives/eu-register_en), if least cost formulation dictates. Lower CP formulations, increased use of by-product ingredients such as DDGS or RSM (Parsons and Baker, 1983), and a reduction of animal derived protein sources in feed formulations all result in decreased inherent Arg concentrations in the diet of broilers. In contrast, increased growth rate of modern broilers (Havenstein et al., 2003) mandate higher dietary Arg requirements. Therefore, dietary strategies fuelling the non-protein functions might

be promising solutions to spare Arg, thereby allowing a greater proportion of this AA to be used for muscle protein synthesis. Feeding GAA to broiler chickens by-passes the first step of Cr synthesis and hence may spare Arg. Dilger et al. (2013) and Lemme et al. (2018b) provided GAA equivalencies relative to dietary Arg based on performance data of broilers. Both groups conducted Arg titration series, either or not supplemented with GAA (1.2 g/kg, Dilger et al., 2013; 0.6 g/kg, Lemme et al., 2018b) and constructed response curves for performance indices. Dilger et al. (2013) found an equivalency of 140% (on weight basis) (i.e. 100 g GAA is equivalent to 140 g Arg) for feed to gain ratio (F:G), whereas Lemme et al. (2018b) reported equivalencies of 116 and 77% for body weight gain (BWG) and F:G, respectively. These large differences in estimation of Arg equivalency, for example for F:G, may be related to level of GAA used, experimental setup, age of birds, basal deit composition, level of performance and Arg taken to deduce equivalency. Actually, determination of Arg equivalency may be rather an attempt to allocate matrix values to the GAA additive. However, any effect on performance encompasses much more than Arg sparing *per se*, but also enhanced Cr loading and ergogenic (see 1.3.1.) and antioxidant (see 1.3.3.) effects are involved. To note, as the molar weights of Arg and GAA are 174.2 and 117.1 g/mol, respectively, and that one molecule of Arg is consumed to produce one molecule of GAA, then theoretically, the metabolic equivalency can be maximum 149%. Notwithstanding that, the use of GAA may become more crucial in circumstances such as high altitude (HA) (Khajali and Wideman, 2010; Khajali et al., 2013) and HS (Brake, 1988) that increase Arg requirements.

1.3.4.2. Glycine

Gly (CAS No. 56-40-6; molecular formula: $C_2H_5NO_2$; molecular weight 75.1 g/mol) can be metabolized from Ser with cooperation of tetrahydrofolate (THF) (Fig. 1.5). This reaction can be reversed by adding methyl from THF. For poultry, it is generally assumed that this interconversion of Gly and Ser is not limited in metabolism (Akrabawi and Kratzer, 1968; Sugahara and Kandatsu, 1976). Therefore, they are usually assessed together to determine the physiological value of a diet, for example as Gly equivalents (Gly_{equi} (g/kg) = Gly (g/kg) + (0.7143 × Ser (g/kg)); Siegert and Rodehutscord, 2019). Gly can also be metabolized from threonine (Thr) via two pathways which mainly occur in the liver. The mitochondrial enzyme threonine dehydrogenase (EC, 1.1.1.103) produces Gly from Thr with 2-amino-3-ketobutyrate as an intermediate metabolic step, which further reacts to Gly, acetyl-CoA, and aminoacetone (Davis and Austic, 1994). It has been shown in pigs, rats and chickens that this is the major pathway which accounts for about 80% of Thr degradation (Ballèvre et al., 1990; Davis and Austic, 1994). Another pathway of producing Gly is metabolic conversion of choline (Soloway and Stetten, 1953). Glyoxylate in combination with alanine is a further source of Gly. Gly is thus not an indispensable AA to broilers. One of physiological functions of Gly and Ser, like any other AA, is incorporation in protein synthesis. An important pathway in birds that consumes Gly is uric acid (UA) production, the end product of nitrogen metabolism here. Every mole of UA synthesized requires 1 mole of Gly. It would seem that high

protein rather than low protein intakes would require more Gly. However, current paradigm states that Gly_{equi} represents the first-limiting non-essential AA in poultry diets, and may limit potential to reduce CP, which largely depends on other dietary constituents such as Thr and choline (Siegert and Rodehutscord, 2019). These authors conclude that dietary CP can be reduced to ~15–16% in diets for up to 21d-old broiler chicken without affecting growth performance compared to responses to diets with currently common CP concentrations by considering Gly_{equi} in the diet formulation. Similarly to Arg, one can argue that dietary GAA may spare Gly as one molecule of Gly is consumed to produce one molecule of GAA. Or said alternatively, GAA maybe more efficacious in low CP diets if not (entirely) controlled for Gly_{equi}. Establishing experimentally Gly sparing effects *per se*, however, is more delicate, considering that Gly is not an indispensable AA. Nonetheless, several studies have shown that Cr concentration in the chicken *pectoralis major* increases when Gly is supplemented to diets (Ngo et al., 1977; Ospina-Rojas et al., 2013).

1.3.4.3. Methionine

Met or 2-amino-4-(methylthio) butanoic acid (CAS No. 59-51-8; molecular formula: $C_5H_{11}NO_2S$; molecular weight 149.2 g/mol) is an indispensable AA with a significant non-protein requirement. In avian species, Met is classified as a first-limiting AA because it is limited in plant protein sources and because there is a strong requirement for it to support feather growth (via Cys, Fig. 1.5) and protein synthesis (Bunchasak, 2009). Therefore, dietary supplementation with feed-grade Met in chickens has a long history, and various forms are on the market (e.g. DL-methionine, L-methionine, and DL-2-*hydroxy*-(4-methylthio) butanoic acid) (EU Register of Feed Additives, Category 3, functional group C; https://ec.europa.eu/food/safety/animal-feed/feed-additives/eu-register_en). Met is not only incorporated into protein but also in high demand for essential methylation reactions via the re-methylation cycle (Fig. 1.5). This cycle functions to transfer methyl groups in order to synthesize critical metabolites as well as to regulate gene expression. Approximately 80% of dietary Met is directed to the liver during first-pass (Riedijk et al., 2007) and 85% of methylation reactions occur in the liver (Lu and Mato, 2012). Met that is partitioned toward transmethylation is first adenylated to form SAM, the primary biological methyl donor, which is demethylated to SAH, which is in equilibrium with homocysteine (HCy) (Fig. 1.5) (Mudd and Poole, 1975). It is thought that the vast majority of transmethylation occurs to synthesize Cr and phosphatidylcholine (PC) (Bertolo and McBreairty, 2013), as well as to methylate DNA and proteins. McBreairty et al. (2013) demonstrated that hepatic Met partitioning toward transmethylation is readily altered by a portal infusion of GAA. It was shown that Cr synthesis may consume as much as 40% in adult human (Stead et al., 2006) and 63-77% in neonatal piglets (Brosnan et al., 2009) of all the labile methyl groups used, but no data are available for chicken. Re-methylation replenishes Met upon transferring a methyl group to HCy from the dietary methyl donors, 5-methyl-tetrahydrofolate (folate) and betaine (Fig. 1.5). Folate regenerates Met by donating a methyl group to HCy via methionine synthase (MS). Betaine, which is an irreversible product of choline, re-

methylates HCy via betaine/homocysteine methyltransferase. The potential for these dietary methyl donors to affect Met availability is great; indeed, ~25% of whole body Met flux is derived from re-methylation in piglets (Bauchart-Thevret et al., 2009). Therefore, dietary GAA may inflict Met and the re-methylation cycle by diverting more Met to SAM, reducing its use for other purposes, increasing risk for HCy accumulation (hyperhomocysteinemia), and potentially depleting the sources of methyl groups in the body like folate, betaine, and choline and co-factors such as vitamin B12 (Fig. 1.5). In broilers, Lemme et al. (2010b) showed that with adequate Met supply, dietary 0.8 g/kg GAA improved F:G while at Met deficiency no such effect was observed, concluding that Met deficiency may limit availability of methyl groups for GAA methylation. Though, Cr supplementation was also not effective at low dietary Met. Hence, it remains equivocal whether GAA efficacy relies on Met provision.

1.4. GUANIDINOACETIC ACID AS FEED SUPPLEMENT

1.4.1. Guanidinoacetic acid shows interesting properties for application as feed additive

To boost muscle Cr loading, Cr supplementation may be used. However, thermal instability is the main issue that limits the use of Cr as a feed additive in poultry diets (Vranes et al., 2017; Khajali et al., 2020). Poultry diets are most often subjected to feed processing (pelleting or extruding), which undergo heat exposure of minimum 70°C and this impacts the stability of Cr. Similarly, Cr in animal protein sources may be subject to destruction. Cr in animal protein sources is already highly variable (Ringel et al., 2007; Lemme et al., 2011). Recently, Li and Wu (2020) analysed various animal protein sources and different fishmeal qualities for their content in Cr, CrN, and PCr. In fishmeals, Cr metabolites were mostly present as CrN and PCr, e.g. contents were 3575 and 1751 mg/kg, 5412 and 6150 mg/kg, and 4151 and 2403 mg/kg for fishmeal Menhaden, fishmeal Peruvian anchovy, and fishmeal Southeast Asian miscellaneous marine, respectively. Other animal protein sources showed lower levels. Assuming a high fishmeal inclusion of 10% in the diet, the molar contribution of Cr+PCr is still lower than for example 0.6 g/kg GAA inclusion. The stability of GAA supplemented to a complete feed for chickens and storage of 3 months was studied (EFSA, 2016). After mixing feed ingredients, the feed was conditioned and pelleted at two different T (70 or 80°C). The mash feed (before conditioning) was analysed to contain 535 mg/kg GAA wheras after pelleting at 70 and 80°C, the GAA content was 562 and 531 mg/kg and no losses were detected after 3 months. Supplementation with GAA rather than Cr is then justified because it is more heat stable, and in addition has lower cost and high bioavailability (Baker, 2009). Oral delivery is a good approach because the compound is rapidly and completely absorbed from the gastrointestinal tract (Tossenberger et al., 2016), being transformed into Cr (Michiels et al., 2012). Tossenberger et al. (2016) reported an ‘apparent’ post-absorptive utilization of 76.2% for GAA at 0.6 g/kg and 45.6% at 6.0 g/kg in broilers; whereas Lemme et al. (2018a) showed complete utilization of digested GAA at Arg deficiency and 0.6 g/kg GAA, while this declined with 1.2 g/kg

GAA to 86%. When in addition Arg was increased, the utilisation was 90 and 70% for 0.6 and 1.2 g/kg GAA, respectively. Indeed, with higher Arg in the diet, the *de-novo* synthesis may have a larger portion in total GAA availability which means that apparent utilization of dietary GAA diminishes. It would thus be an elegant way to synthesize Cr and conserve the AA involved in its synthesis (Ostojic et al., 2014a). GAA is the common name of N-(aminoimino-methyl)-glycine, also called glycocyamine (CAS No. 352-97-6; molecular formula: $C_3H_7N_3O_2$; molecular weight 117.1 g/mol). GAA are white crystals, is moderately soluble in water due to self-aggregation, and X-ray crystallography indicates GAA molecules are in zwitterionic form in aqueous solutions (Vranes et al., 2017). When dissolved in water, the pH turns to be neutral.

1.4.2. Use of guanidinoacetic acid in poultry

1.4.2.1. Effects on performance and carcass yield and composition in broilers

Web of Science and PubMed databases were searched for all studies testing GAA in poultry. The first papers appeared in 2007 (Lemme et al., 2007ab), and in last years the frequency of publications is increasing, not only addressing broilers but also breeders, layers, turkey, quail and ducks. Outcomes were used to present data in graphs. Therefore, animal performances of broilers fed GAA were expressed relative to control birds (control=100%). Then, linear dose-response effects were tested by simple linear regression using dietary GAA as independent variable and response relative to control as dependent variable with constant set at 100%. GAA dosage ranged between 0.20 and 2.25 g/kg of diet, with 0.6 and 1.2 g/kg found mostly, in line with legal provisions (see 1.4.4.7.) and commercial recommendations. Few reports tested higher dosages but these were not used for making the graphs and regressions. Some reports, such as EFSA (2009) contain multiple experiments, hence various datapoints are shown in the graphs, sometimes for the same dosage. It is of utmost importance to highlight that in all studies GAA was added on-top of the basal formulation (control treatment).

Fig. 1.6 shows the effects of dietary GAA on average daily gain (ADG). First, large variation across studies was found with outspoken improvements of daily growth as well as decreases (most datapoints, i.e. the ADG relative to control treatment, are between 95 and 110%). Extremely high responses were found by Dilger et al. (2013) because of using Arg deficient diets. Remaining variation might be due to differences in duration of the study, ingredient and nutrient composition of basal diet, sex, breed, and environmental conditions. Nonetheless, overall, the linear regression was significant with positive slope, i.e. ADG relative to control increases 3.50% per g GAA in one kg of diet. For example, if we take a target as-hatched daily growth of 66.2 g/d for a 39-day rearing period (Aviagen, 2019. Ross 308: performance objectives), then the application of 0.6 and 1.2 g/kg increases daily growth with 1.39 and 2.78 g/d, respectively. Using GAA in challenge models as HA (Ahmadipour et al., 2018ab) and HS (Amiri et al., 2019) appears to elicit responses slightly beyond average, but not for cold stress (CS) (Faraji et al., 2019; Kodambashi et al., 2017; Nasiroleslami et al., 2018), a model to induce ascites.

Opposite to ADG, feed intake was not affected by inclusion of GAA in the diet (Fig. 1.7). Apart from 2 observations, all responses fall within 94 and 104% of the non-supplemented control treatment, and the total mean was 99.8% (not significantly different from 100%, one-sample t-test). Most remarkable is the effect on feed efficiency (F:G, Fig. 1.8). Here, the regression line has a negative slope equalling to -3.05%. For the same scenario as discussed above, this would mean that a F:G of 1.58 can be reduced with 2.9 and 5.8 points at 0.6 and 1.2 g/kg GAA, respectively.

Not all reports have mentioned mortality. Effects of GAA were extremely variable across studies (22 to 400% relative to control), and no conclusion can be drawn here.

To make a more proper comparison of feeding GAA with control birds, we selected 10 experiments and showed them in a radar (Fig. 1.9). The idea was to select only studies compliant to following criteria: 1/ treatments 0, 0.6 and 1.2 g/kg GAA were included in the experiment; 2/ no deviating environmental conditions such as HS, CS or HA, or deviating feed formulation such as Arg deficiency; and 3/ application was for minimum 35 days, including starter to finisher. This presentation allows easy visual comparison of supplementation levels across these selected studies. So, the radar shows that the blue lines (ADG at 0.6 and 1.2 g/kg GAA) run parallel with the exception of Fosoul et al. (2018) and to lesser extent Kodambashi et al. (2017), where surprisingly the higher dosage resulted in lower growth benefits. Here, mean for 0.6 and 1.2 g/kg GAA) is 101.6 and 101.1%, which is for the former similar to Fig. 1.6 but for the latter dramatically lower then Fig. 1.6. Lines for ADFI suggests no effect by 0.6 g/kg GAA (99.8%), but some reduction when feeding 1.2 g/kg GAA (98.8%). Again these findings are slightly different than the previous graph (Fig. 1.7). On the contrary, numbers for F:G are more in line with above. This means a reduction by 1.6 and 2.8% for 0.6 and 1.2 g/kg GAA, respectively.

Carcass yield and composition have been studied in few experiments. EFSA (2009) reported measurements for carcass weight, breast weight and abdominal fat (all in absolute weight) for 4 experiments. Results for carcass and breast weight were rather inconsistent, either showing no effect, or changes for intermediate dosage within range 0 to 1.5 g/kg GAA compared to control. Abdominal fat appears to be lowered with higher GAA in the diet, in particular with >1.0 g/kg GAA, but also here outcomes were not unequivocal. Further, Michiels et al. (2012) demonstrated that supplementation of GAA linearly improved breast meat yield (29.4, 30.4, and 30.7% for 0, 0.6, and 1.2 g/kg GAA, respectively). Next, Heger et al. (2014) showed that supplementation of GAA could improve breast meat yield, but carcass yield and leg yield were not affected. Zhang et al. (2019) who studied feeding GAA at 0.6 and 1.2 g/kg during 14d pre-slaughter and whereby broilers were experiencing transport stress during summer did not find effects on carcass traits. The most extensive work on GAA and carcass yield and composition was done by Córdova-Noboa et al. (2018ab), however these authors used Ross708 male broilers and assessed these parameters at the age of 51 to 55d (~4-5 kg broilers). In Córdova-Noboa et al. (2018a), GAA supplementation did not alter carcass yield and

composition, but effects on *pectoralis major* and breast meat were dependent on the inclusion of PBM in the diet. With PBM in diet, GAA elevated yield of these parts. Córdova-Noboa et al. (2018b) who studied the effect of supplementation of GAA in broilers fed either corn or sorghum based diets showed an improvement in breast meat yield at 55d of age when adding GAA to corn diets, but not when added to sorghum diets.

Overall, it can thus be concluded that benefits for growth are predominantly caused by better feed efficiency, with most marked dose-responses on feed efficiency, and that GAA offers potential to increase breast meat yield.

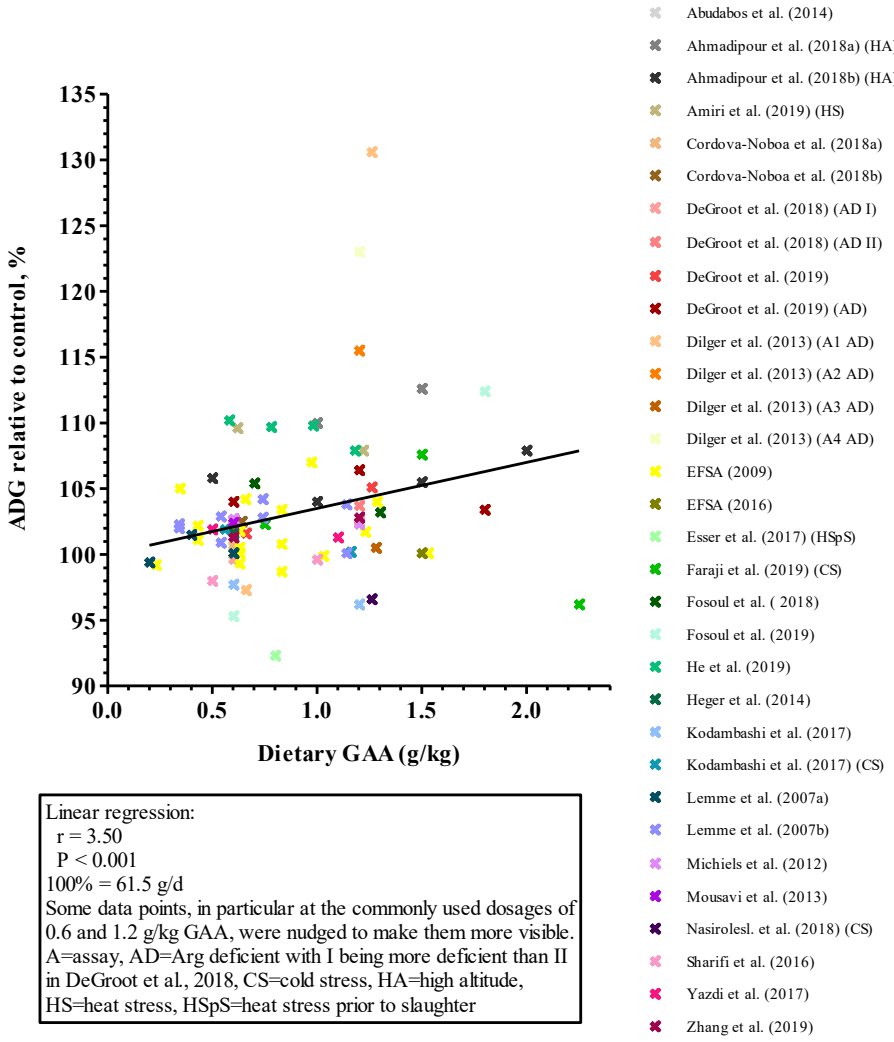


Fig. 1.6. Effect of dietary GAA on daily growth (ADG) relative to control treatment (=100%) in broiler chickens.

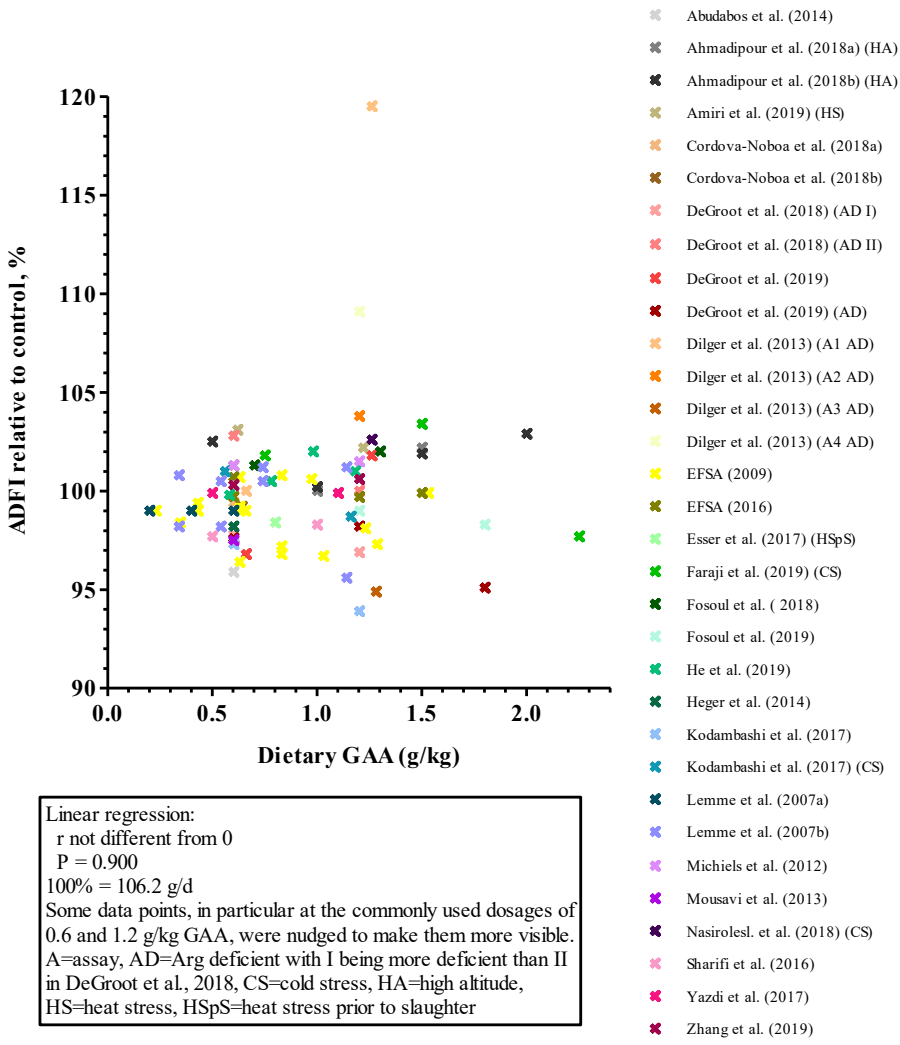


Fig. 1.7. Effect of dietary GAA on daily feed intake (ADFI) relative to control treatment (=100%) in broiler chickens.

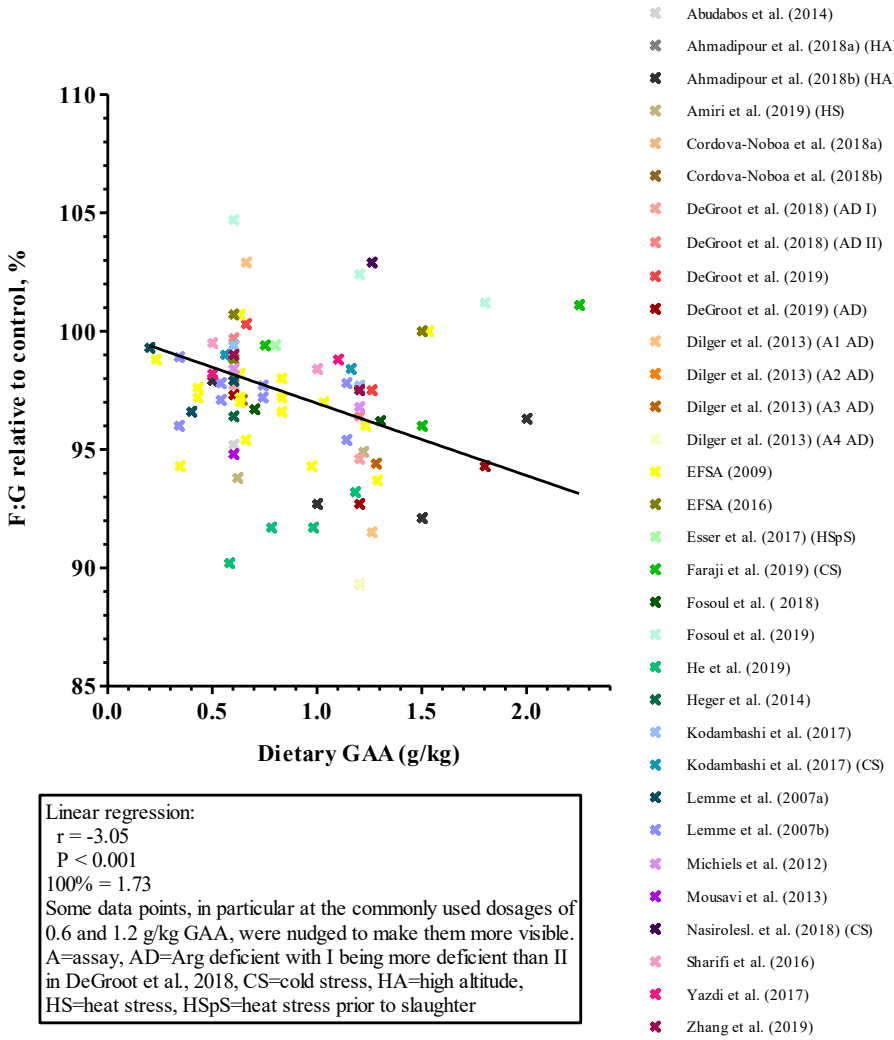


Fig. 1.8. Effect of dietary GAA on feed efficiency (F:G) relative to control treatment (=100%) in broiler chickens.

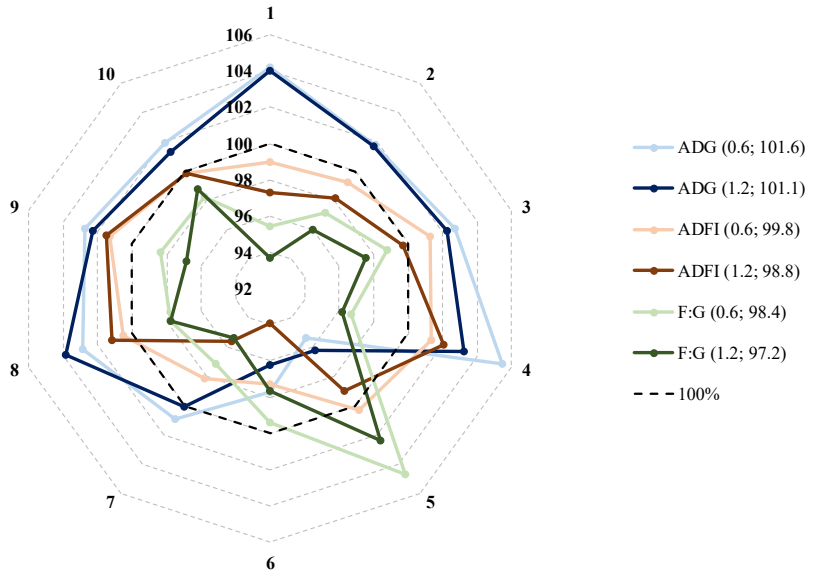
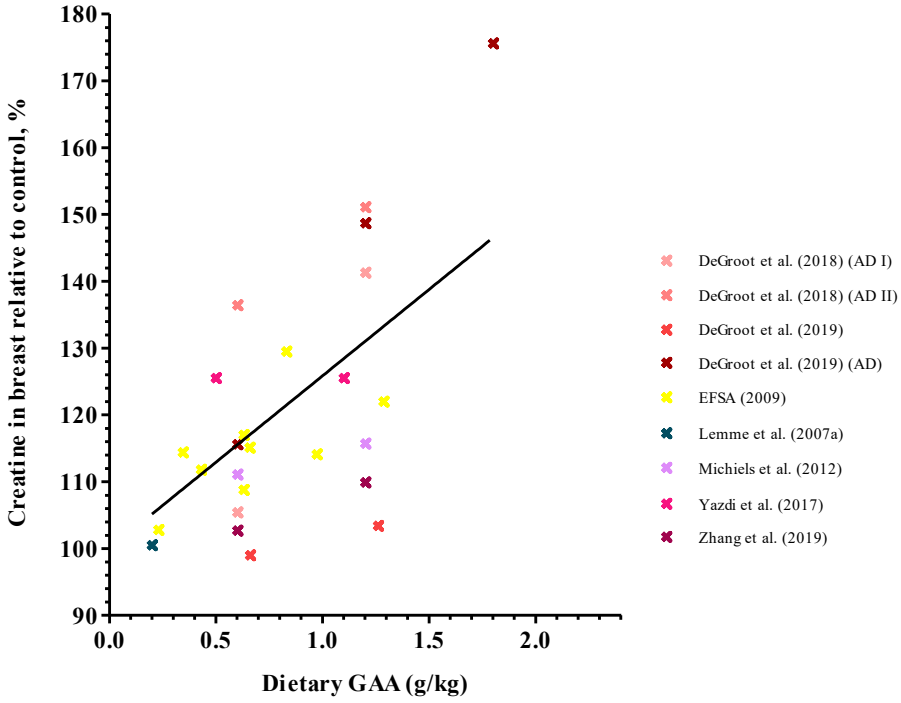


Fig. 1.9. Radar presentation of relative effect of 0.6 and 1.2 g/kg GAA level to control treatment (=100%, bold broken line) for ADG, ADFI and F:G (%) in 10 comparisons. Legend highlights GAA dosage and mean value across 10 comparisons. References: 1, EFSA (2009) I; 2, EFSA (2009) II; 3, EFSA (2016); 4, Fosoul et al. (2018); 5, Fosoul et al. (2019); 6, Kodambashi et al. (2017); 7, Lemme et al. (2007b) I; 8, Lemme et al. (2007b) II; 9, Michiels et al. (2012); 10, Yazdi et al. (2017). Criteria for selecting these studies were: 1/ treatments 0, 0.6 and 1.2 g/kg GAA were included in the experiment; 2/ no deviating environmental conditions such as heat stress, cold stres or high altitude or deviating feed formulation such as Arg deficiency; and 3/ application was for minimum 35 days, including starter to finisher.

1.4.2.2. Effects on creatine loading in breast muscle of broilers

Dietary GAA can be digested and utilized to generate Cr by methylation in the liver. Despite the fact that higher body Cr can exert increased negative feedback on AGAT, Cr load in muscle is stimulated by dietary GAA. This is clearly demonstrated in Fig. 1.10. Only few studies report Cr levels in breast muscle of broilers fed GAA. This muscle Cr refers to total Cr (Cr+PCr). The steep rise in muscle Cr (+25.9% per g GAA in one kg of diet) indicates a high capacity for Cr loading. The highest values were obtained in Arg deficient conditions (DeGroot et al., 2018, 2019). Data from Ringel et al. (2007) and Tossenberger et al. (2016) suggest that at high dietary GAA (>2.0 g/kg GAA) muscle Cr may reach asymptotic values approximately 130% to control (not shown in Fig. 1.10).



Linear regression:
r = 25.9
P < 0.001
100% = 4105 mg/kg FM
Some data points, in particular at the commonly used dosages of 0.6 and 1.2 g/kg GAA, were nudged to make them more visible.
A=assay, AD=Arg deficient with I being more deficient than II in DeGroot et al., 2018, CS=cold stress, HA=high altitude, HS=heat stress, HSpS=heat stress prior to slaughter

Fig. 1.10. Effect of dietary GAA on breast muscle Cr relative to control treatment (=100%) in broiler chickens.

1.4.2.3. Effects on breast meat quality and myopathies in broilers

It is well known that meat quality is related to the energy status in muscle at slaughter (Mir et al., 2017). After slaughter, with the stop of blood circulation and oxygen provision, the energy metabolism of skeletal muscle turns into glycolysis and lactic acid accumulation, resulting in a decrease in pH value. As higher Cr load in muscle may sustain post-mortem ATP buffering, it was hypothesized that pre-slaughter dietary Cr or GAA may reduce lactic acid accumulation and pH drop in meat (James et al., 2002b). Also, Cr supplementation has been shown to result in increased cellular hydration (Juhn, 1999), which could positively impact WHC of meat. Findings in the pig have been inconsistent (e.g. Cr or creatine monohydrate, CMH: Berg et al., 1999; James et al., 2002a; GAA: Wang et al., 2012).

In broilers, only few authors addressed meat quality aspects. EFSA (2009) found in one experiment that after slaughter on d41, breast muscle pH 4h post-mortem was lower with the two highest GAA doses (0.4 and 0.6 g/kg), but the relevance of this finding was not indicated. Drip loss was not affected by treatment. Opposite to that, in a second experiment, drip loss was higher (2.61%) in 1.5 g/kg GAA than control (1.99%), while L* value was higher suggesting lighter colouring. Also Michiels et al. (2012) reported an increase of L* value, together with higher b* (more yellow), slightly lower pH24h, and slightly reduced WHC, more specifically increased press loss. These authors found no effect on oxidative stability of myoglobin and fat upon simulated retail display. Esser et al. (2018) (7d-old birds) found no effect on breast meat characteristics such as pH, press loss, cooking loss, shear force, and colour. In the study of Zhang et al. (2019) birds were either or not experiencing transport stress during summer, and the potential of 0.6 and 1.2 g/kg GAA on alleviating the negative effects of this stress on meat and textural characteristics were evaluated. It appeared that the high dosage only ameliorated partially the negative effects on drip loss. Córdova-Noboa et al. (2018a), feeding Ross708 males up to 55d of age, found that dietary inclusion of GAA did not affect meat quality parameters. Again, Córdova-Noboa et al. (2018b), testing GAA broilers fed either corn or sorghum based diets, found a reduced ultimate breast pH (at 51 and 55d of age), and lower b* at 51d of age, however depending on the cereal used in diet.

Emerging concerns have come related to the presence of BMM such as wooden breast (WB) and white striping (WS) (Kuttappan et al., 2016; Barbut, 2019). While the incidence of BMM is erratic and not observed in all regions of the world, it has become an important issue for the poultry industry. This may be related to the selection of genotypes exhibiting faster growth rates with higher breast yields, and concomitant inadequate vascularization of growing muscle (Petracci et al., 2015). Although the incidence rates and meat quality effects have been well characterised, underlying etiology of these diseases remains to be fully determined. For example, Abasht et al. (2016) found that WB affected tissues possess a unique metabolic signature, like elevated levels of hypoxanthine, xanthine, and urate molecules, the generation of which can contribute to altered redox homeostasis. In this context, Arg and GAA have received interest to prevent BMM. Regarding Arg, Aviagen (2019) (Breast muscle myopathy) concluded from their own studies that higher levels of Arg than recommended (120% dArg:dLys *vs.* 107% dArg:dLys) could provide some benefit in lowering BMM, but the effects were not observed consistently, suggesting the response is likely multifactorial. Bodle et al. (2018) found that with increased dArg:dLys the WB score dropped from 1.83 to 1.49, changes that were the result of lower incidence of severe cases of WB. Concerning GAA, Córdova-Noboa et al. (2018ab) found that GAA could decrease the incidence and severity of WB of broilers exceeding 4 kg of BW, which is a BW that is higher than common standards in Belgium or Iran. These authors stated that WB may be a consequence of reduced glycogen in muscle and higher ultimate pH post-mortem, both effects counteracted by dietary GAA, together with the ATP buffering effect of elevated Cr. Aviagen (2019) (Breast muscle myopathy) tested GAA and found slight

improvements in WB at 49d, but not at 56d, but with no effect on WS, despite higher breast meat yield.

Altogether, it looks that GAA has no effect or, and in this case opposite to what could be anticipated, reduces ultimate pH slightly associated with lower WHC. However, these effects were small and the practical relevance may be questioned. Also different proxies are used for WHC, for example using no external force (drip loss) vs. using external force (press loss) which may it render more difficult to draw conclusions. Next, meat colour turns more light by feeding GAA. Interestingly, GAA shows potential to combat BMM, then notably WB.

1.4.2.4. Effects on oxidative status

Wang et al. (2016) found conclusive evidence for antioxidant outcomes by feeding GAA. Cherry Valley ducks fed 0.5 g/kg GAA exhibited reduced malondialdehyde (MDA), a marker for lipid peroxidation and increased serum glutathione peroxidase (GPx) activity and GSH in blood, whereas in liver the activity of catalase, SOD, and GPx and GSH increased. They concluded that GAA can improve the body's antioxidative capacity to some extent due to the ability of GAA to increase the level of the Cr in the body; though as we know Cr is mainly present in skeletal muscle, tissues that these authors did not investigate for antioxidant indexes. Further, in broilers, dietary GAA supplementation increased liver GPx and decreased serum MDA level (Nasiroleslami et al. 2018).

1.4.2.5. Interaction with other dietary factors and environment in broilers

Several studies have investigated the interaction of dietary GAA with other nutrients in factorial arrangements, with metabolizable energy (ME), nutrient density (ND), and Arg receiving most attention, and in one study with T, actually CS (Table 1.1.).

In particular the interaction with ME is interesting. As dietary GAA may improve cellular bioenergetics by stimulating Cr loading, it can be conceived that dietary GAA might have different effects on broiler performances depending on the energy level of the diet. This hypothesis was tested by Abudabos et al. (2014), Fosoul et al. (2018), Heger et al. (2014) and Mousavi et al. (2013) by feeding broilers 2 or 3 levels of GAA (0, 0.6 and 1.2 g/kg) added to diets decreasing in ME level. Mousavi et al. (2013) using ME steps of 150 kcal/kg (~5% reduction) found that the ME level had an obvious positive effect on ADG, F:G and ME efficiency, but no interaction with GAA level was found, except for F:G and ME intake per kg of carcass. It was shown that GAA improved these traits only at the highest ME level. It appears that GAA affects to a lower extent performances at suboptimal dietary ME levels. These authors suggested that with higher energy levels more rapid BWG and muscle growth are achieved and that in this respect a higher Cr provision may contribute to a more efficient utilization of dietary nutrients and energy, resulting in improved ADG and particularly F:G. Similarly, Abudabos et al. (2014) found that birds receiving control or 25 kcal/kg less ME had more benefit on growth when

supplemented with GAA as compared to treatments with more ME reduction. Fosoul et al. (2018) found that only supplementation of GAA at 1.2 g/kg decreased the negative effects of feed ME reduction (-150 kcal/kg) on BWG across starter, grower and the entire production, i.e. energy retention as fat and total energy were increased when birds received low ME diets supplemented with 1.2 g/kg GAA. Finally, Heger et al. (2014) using small graded reductions of ME found no interaction. Thus, it appears that interaction between GAA and ME can only be achieved with substantial reduction of ME and the highest application dosage of GAA (1.2 g/kg). To conclude, it remains elusive whether and how much ME dietary GAA could spare, and possibly GAA is most efficient at recommended ME. Yazdi et al. (2017) studied the interaction between GAA and ND in broilers fed a wheat-SBM diet. The levels of ND chosen were low, medium, and high, whereby levels of all nutrients were adjusted to 3 ME levels (2800, 2950, and 3100 kcal/kg) to have constant energy to nutrient ratios for each feeding period. ND had a marked effect on performance whereas the effect of GAA was limited to F:G in grower phase. Significant interaction was observed only for feed intake (FI) during starter period. It suggests that FI decreases or increases with graded GAA for the low and medium ND and high ND, respectively. Again, this could emphasize that GAA is more efficacious with high nutrient dense diets rather than diluted diets. However, in this study no other interactions were seen, and also ND levels were kept constant across rearing phases.

Interestingly, Amiri et al. (2019) tested the hypothesis that efficacy of GAA would depend on dietary CP level (normal *vs.* low, i.e. 100 and 90% of commercially recommended levels, respectively). The groups were reared under cyclic HS, i.e. 8h at 34°C with 53-62% relative humidity (RH), apart from one group, a positive control, under thermo-neutral (TN). It showed that the effect of GAA on ADG was slightly better at low CP levels, ADG for normal and low CP with 0, 0.6, and 1.2 g/kg GAA was 50.8, 55.7, and 54.8, and 50.6, 54.1, and 56.8 g/d, respectively. In Arg deficient diets, the interaction between GAA and supplemental Arg was tested by DeGroot et al. (2018). These authors could not demonstrate interactions for performance, but showed significant interactions for various metabolites in muscle and blood. They concluded that dietary GAA may spare Arg when Arg deficient diets are fed to young broiler chicks. Further, 1.2 g/kg GAA was capable of ameliorating the effects of an Arg deficiency on growth performance and muscle phosphagen and glycogen concentrations caused by a dietary Arg deficiency, with outcomes due to this treatment closely matching responses of chicks fed an Arg adequate diet. Kodambashi et al. (2017) found some interaction for weight gain and other endpoints between GAA or Arg and environmental T. They studied GAA and Arg levels in split plot by T, i.e. 0, 0.6, and 1.2 g/kg GAA and 0.86 and 1.72 g/kg Arg with Arg levels close to equimolar level with GAA, with TN (23°C) and CS (17°C) as contrasting environmental conditions. CS reduced weight gain markedly, and the significant interaction elucidated that not GAA but Arg improved weight gain at CS but not at TN. Hence, also the 'equivalent' groups GAA at 1.2 g/kg and 1.72 g/kg Arg differed in weight gain, with the latter outdoing the former. Finally, under CS conditions, Nasiroleslami et al. (2018) showed that F:G was both worsened by

both 0.6 g/kg GAA (2.14) and 0.6 g/kg betaine (2.12) as compared to control (1.97), but not by the combination (2.06); leaving a difficult interpretation of these outcomes. In case of GAA, this was due to simultaneously lower growth and higher feed consumption.

Table 1.1. Overview of studies with dietary GAA in a factorial design in broilers testing interaction with other dietary factors or environmental conditions.

References	Breed	Experimental design	Main result
Abudabos et al. (2014)	Ross 308 male (0 to 42d)	GAA with metabolisable energy (ME) (2 × 4 factorial arrangement including 0 and 0.6 g/kg GAA and 4 levels ME, control minus 25 kcal/kg in steps)	Significant interaction between GAA and ME was shown on FI and BWG in total period, suggesting that birds receiving control and 50 or 75 kcal/kg less consumed less feed when supplemented with GAA and birds receiving control or 25 kcal/kg less gained more weight when supplemented with GAA.
Amiri et al. (2019)	Ross 308 male (0 to 42d	GAA with CP (3 x 2 factorial arrangement plus 1, including 0, 0.6, and 1.2 g/kg GAA and normal and low CP with all groups under cyclic HS, i.e. 8h at 34°C with 53-62% RH, apart from one group, positive control, under thermo-neutral	Significant interaction for overall ADG, indicating that the effect of GAA on this response was slightly better at low dietary CP levels.
Córdova-Noboa et al. (2018a)	Ross 708 male (0 to 55d)	GAA with poultry by-product meal (PBM) (2 × 2 factorial arrangement including 0 and 0.6 g/kg GAA and PBM at 0 and 5% of diet)	Significant interaction between supplementation of GAA and PBM on breast meat, *pectoralis major,* cooking loss and GAA in liver was detected.
Córdova-Noboa et al. (2018b)	Ross 708 male (0 to 55d)	GAA with 2 grain sources (2 × 2 factorial arrangement including 0 and 0.6 g/kg GAA and corn *vs*. sorghum as cereal in diet)	Significant interaction between supplementation of GAA and 2 different grains on breast meat, *pectoralis major,* leg quarters, a^*, b^* cooking loss and GAA in breast muscle was reported.
DeGroot et al. (2018)	Ross 708 male (0 to 22d)	GAA with Arg (3 × 2 factorial arrangement plus 1 including 0, 0.6, and 1.2 g/kg GAA and supplemental Arg at 0 and 1.6 g/kg, with basal level corresponding to 8.4 g/kg SID Arg, apart form one group, positive control with 3.2 g/kg supplemental Arg)	Significant interaction between supplementation of GAA and Arg on total Cr in muscle, on Arg, histidine, isoleucine, lysine, phenylalanine, threonine, valine, glutamine, α-aminobutyric acid, citrulline, 3-methylhistidine, phosphoserine, phosphoethanolamine, on GAA in serum, on UA in plasma, and on glucose in blood was shown.
Fosoul et al. (2018)	Ross 308 male (0 to 35d)	GAA with ME (3 × 2 factorial arrangement including 0, 0.6, and 1.2 g/kg GAA with or without ME reduction)	Significant interaction between supplementation of GAA and ME on F:G and BWG, retained carcass fat, carcass gross energy retained as carcass fat, efficiency of dietary ME retention, net energy for production (carcass energy retained per kg feed intake), heat production per kg feed intake was detected.
Heger et al. (2014)	Ross 308 male (0 to 35d)	GAA with ME (2 × 5 factorial arrangement including 0 and 0.6 g/kg GAA with ME of 100, 99, 98, 97 and 96% of ME requirement of recommended)	GAA decreases voluntary feed intake, improved efficiency of energy utilization and increased breast meat yield, but no interaction with ME occurred.

Kodambashi et al. (2017)	Ross 308 (0 to 35d)	GAA and Arg levels in split plot by T (0, 0.6, and 1.2 g/kg GAA and 0.86 and 1.72 g/kg Arg; Arg levels close to equimolar levels with GAA, with thermo-neutral (23°C) and CS (17°C)	Significant interaction between supplemented diet and T on weight gain, feed intake, haematocrit, H/L, ascites, RV/TV, carcass and bursa (% of the body) and villus height, villus surface area was shown.
Mousavi et al. (2013)	Cobb 500 (0-40d)	GAA with ME (2 × 3 factorial arrangement including 0 and 0.6 g/kg GAA with ME of 100, 95 and 90% of ME of ME requirement of recommended)	Significant interaction between supplementation of GAA and ME on F:G and ME efficiency per kg of carcass, and on small intestine (% of the body) was observed.
Nasiroleslami et al. (2018)	Cobb male (0 to 42d)	GAA with betaine in CS condition (2 × 2 factorial arrangement including 0 and 0.6 g/kg GAA with 0 and 0.6 g/kg betaine)	Significant interaction between supplementation of GAA and betaine on overall F:G and plasma level of CK was detected.
Yazdi et al. (2017)	Ross 308 (0-42d)	GAA with nutrient density (3 × 3 factorial arrangement including 0, 0.6, and 1.2 g/kg GAA and 3 levels; low, medium and high nutrient density of the diet)	Significant interaction between GAA and nutrient density in diet was observed on FI during 1–10d period.

1.4.2.6. Effects of guanidinoacetic acid in other poultry

Reduced semen quality and fertility rate are common issues in breeders. The decrease in semen quality is associated with dysfunction of Sertoli cells and defective spermatogenesis. Cr may play an important role in proper functioning of Sertoli cells and energy metabolism of sperm. Hence, Tapeh et al. (2017) reported that supplementation of 1.2 g/kg GAA of diet resulted in improvements in some semen quality traits (total sperm number, sperm forward motility, plasma membrane functionality) from broiler breeder roosters. Also, senile rooster fertility rate was increased by dietary GAA. They concluded that GAA has the capability to diminish or delay age related sub-fertility in commercial broiler breeder roosters. Moreover, Sharideh et al. (2016) showed that 1.2 g/kg GAA and Arg has the potential to improve fertility of broiler breeder hens due to increased sperm penetration in the inner perivitelline layer which is promising to improve fertility at the later phase of the egg production period. Though, adding GAA and Arg to diet had no effect on egg production. Hence hatchability was dependent on dietary GAA; feeding 0.8 (83.8%) and 1.2 g/kg GAA (84.0%) increased massively hatchability as compared to control (65.7%) and 0.4 (65.6%) g/kg GAA (EFSA, 2016). Recently, Epstein et al. (2018) studied the effect of supplemantry GAA on nutrient deposition in the egg and the consequent effect on breeding performance and hatchling quality. They used 26-week old Cobb500 broiler breeder hens and roosters and reared them into individual cages and kept under standard conditions for 32 weeks. By supplementing 0.0, 0.5, 1.0, 1.5 and 2.0 g/kg GAA in diet, Cr levels in treatment group compared to control were elevated by 33%, 73%, 46% and 106% after 11 weeks, respectively. Laying percentage was improved and progenies of the broiler breeder hens fed with 0.0 and 1.5 g/kg GAA showed a significant increase in BW at 20-d for the latter group. Their results show that GAA supplementation in broiler breeder diet has beneficial effects on laying and progeny growth performance, associated to elevated levels of Cr in the hatching egg.

Khakran et al. (2018) investigated the effects of GAA addition to corn-SBM based diets on laying hens performances. Results showed that GAA addition had no effects on performance but 1.71 g/kg GAA addition reduced egg weight compared with control groups. Also, supplementing 1.14 g/kg GAA increased levels of luteinizing hormone and follicle stimulating hormone compared with control groups at 42 and 84 days. They concluded that GAA addition is not an appropriate strategy for improving performance of laying hens. Since this is the only study in layer, more studies are needed to confront these outcomes.

Another three studies were carried out by Lemme et al. (2010a) in turkeys. In the first study with 4 levels of GAA (0. 0.3, 0.6, and 0.9 g/kg) next to positive control with MBM, over a 12 week fattening period, they did not see any effect on performance. In the second study with 3 levels of GAA (0, 0.4, and 0.8 g/kg) over a 21 week fattening period, they showed that supplemental GAA reduced F:G at 0.8 g/kg GAA due to reduction in FI. Breast meat (% of carcass) with GAA at 0.4 and 0.8 g/kg was improved with 1.4 and 2.0

%, respectively. Also, Cr loading in breast muscle at 0.8 g/kg GAA was enhanced. The third study with 18 weeks rearing and 4 levels of GAA (0, 0.6, 0.8, and 1.2 g/kg) showed improvements in BWG and FI at 0.8 g/kg, rather than F:G which was not affected at any level. The highest ratio of PCr:ATP was reported in the same treatment.

It has been reported that adding GAA to the diet (1.4 g/kg) of breeders of meat-type quails increased GAA, Cr and CrN content in eggs (Murakami et al., 2014). This study showed that GAA was successfully absorbed by the avian gastrointestinal tract and there was a carry-over on the reproductive tract fluid. They also showed that dietary GAA supplementation of breeders of meat-type quails not only increases Cr level in eggs, but also in progeny muscle, which resulted in better post-natal progeny performance.

Raei et al. (2019) found that the highest laying was obtained with 1.8 g/kg GAA, but optimum egg weight, egg mass, shell and yolk weight were observed at 1.2 g/kg GAA in laying Japanese quails.

Supplementation of GAA plus Met, positively influenced Mulard duck's performance. It was advised to add Met in combination with GAA up to 4 g/kg to support its anabolic effect on muscle growth potential and protein synthesis (Ibrahim et al., 2019). Wang et al. (2016) reported that GAA supplementation could increase the activity of antioxidant enzymes and the content of GSH in serum and liver in Cherry Vally ducks, but did not find significant effects on performance (up to 1.0 g/kg GAA).

1.4.2.7. Legal framework regarding the use of guanidinoacetic acid in animal feeding in EU

In the EU, feed additives fall within the scope of EC Regulation 1831/2003. GAA is to be found in category 3, i.e. nutritional additives, functional group C, i.e. amino acids, their salts and analogues (EU Register of Feed Additives, Category 3, functional group C; https://ec.europa.eu/food/safety/animal-feed/feed-additives/eu-register_en). GAA is currently authorized for application in chickens for fattening, weaned piglets, and pigs for fattening with a minimum and maximum content of 0.6 and 1.2 g/kg of complete feed with a moisture content of 12% (Code 3c372, Commision Implenting Regulation (EU) 2016/1768 of 4 October 2016).

CHAPTER 2

GUANIDINOACETIC ACID SUPPLEMENTATION IN BROILER CHICKENS FED CORN-SOYBEAN DIETS AFFECTS PERFORMANCE IN THE FINISHER PERIOD AND ENERGY METABOLITES IN BREAST MUSCLE INDEPENDENT OF DIET NUTRIENT DENSITY

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CHAPTER 2

GUANIDINOACETIC ACID SUPPLEMENTATION IN BROILER CHICKENS FED CORN-SOYBEAN DIETS AFFECTS PERFORMANCE IN THE FINISHER PERIOD AND ENERGY METABOLITES IN BREAST MUSCLE INDEPENDENT OF DIET NUTRIENT DENSITY

2.1. ABSTRACT

GAA is the single immediate endogenous precursor of Cr, which in turn can be converted to PCr that is a convenient form of energy storage in animal tissues. It was hypothesized that dietary GAA would have different effects on performance and energy metabolites in breast muscle depending on ND of corn-SBM diets. A total of 540 day-old male Ross 308 broilers were allocated to 9 dietary treatments with 6 replicates (10 birds each) in a 3 × 3 factorial arrangement with 3 levels of ND (low, 2800; medium, 2950 and high, 3100 kcal ME/kg; and with the other nutrients being constant relative to ME) and with 3 levels of GAA (0, 0.6 and 1.2 g/kg) for 42 days. In the starter and grower period, increasing levels of ND improved body weight (BW), ADG, ADFI and F:G, with the exception of ADFI in the starter period. GAA supplementation did not change these performance characteristics. All performance indicators responded markedly to increasing ND in the finisher period, whereas the highest GAA level reduced ADFI compared to control (156 *vs*. 162 g/d) and concomitantly F:G (1.81 *vs*. 1.93). No interactive effects were noted for any performance trait. The high ND level resulted in a higher breast yield at d42, associated with higher fat content and darker colour compared to the other ND levels, whereas GAA supplementation did not affect carcass and breast traits. At the end of the experiment, Cr was elevated when feeding GAA at 1.2 g/kg (5455 *vs*. 4338 mg/kg fresh muscle). To conclude, ND had a substantial effect on performance and carcass traits, whereas the effect of GAA was limited to F:G in the finisher period and independent of diet ND level.

2.2. INTRODUCTION

Cr is naturally present in vertebrate cells, serving as an energy shuttle where high energy phosphates are brought to sites of rapid ATP utilization, particularly in cells with high energy demands such as muscle cells (Brosnan et al., 2009). Thus, Cr and PCr function as a backup to the ADP/ATP system for the purpose of storing and mobilizing energy

when required on short term. About 1.7% of the Cr pool is irreversibly converted to CrN each day and excreted in the urine (Walker, 1979). Therefore, replacement of this Cr lost as CrN seems to be necessary. The source of Cr in animals is by *de-novo* synthesis through a two-step pathway or it can be obtained from the diet, like from animal protein sources. GAA is the only metabolic intermediary product and precursor of Cr. GAA is synthesized from Arg and Gly by transferring the amidino group from Arg to Gly, catalysed by AGAT and takes place mainly in the kidney and pancreas (Wyss and Kaddurah-Daouk, 2000). Subsequently, after transport to the liver, GAA is methylated to Cr with SAM as the methyl group donor and by the action of GAMT (Daly, 1985; Komoto et al., 2003). Under pure vegetable feeding conditions, all Cr must be derived by *de-novo* synthesis and it is assumed that the potential of *de-novo* synthesis for Cr supply is limiting. For example, Cr levels were found to be lower in human vegetarians, who are deprived of exogenous Cr supply, than in a reference population (Delanghe et al., 1989). Hence, in view of the decreasing amounts of protein from animal origin included in animal feeds, supplementation with Cr or its precursor GAA might help in restoring the Cr load in tissues. Cr as a feed additive shows some drawbacks, such as thermal instability and high cost, compared to GAA, which is more stable and less expensive (Baker, 2009). GAA might therefore be more suitable for use in animal nutrition. GAA might particularly be favorable in young fast-growing chicks because of their high need to supply Cr to growing muscles (Brosnan et al., 2009) and because the regeneration of ATP from the Cr and PCr system appears to be of paramount importance in the cardiac energy management of fast-growing broilers (Nain et al., 2008). Furthermore, GAA is the immediate precursor of Cr that requires only a methyl-group transfer from SAM, and does not consume Arg. Accordingly, Baker (2009) hypothesized that dietary GAA could spare dietary Arg, in the same manner as dietary Cr, evidenced by recent work of Dilger et al. (2013) and DeGroot et al. (2018, 2019). In broilers, it was shown that in all-vegetable diets dietary supplementation with 0.6 or 1.2 g/kg diet improved animal performances, with the effect on F:G being most consistent, concomitant with a reduction in FI, in particular in the finisher phase and at 1.2 g/kg, and enhanced breast meat yield with no or minor effects on meat quality parameters (Abudabos et al., 2014; EFSA, 2009; Kodambashi et al., 2017; Michiels et al., 2012; Ringel et al., 2008ab).

As dietary GAA seems to improve cellular bioenergetics by stimulating Cr synthesis (Ostojic, 2015), it can be conceived that dietary GAA might have different effects on broiler performances depending on the energy level of the diet. This hypothesis was amongst others tested by Mousavi et al. (2013) and Abudabos et al. (2014) by feeding broilers 2 levels of GAA (0 and 0.6 g/kg) added to diets decreasing in ME level. In the former study using ME steps of 150 kcal/kg ME, the ME level had an obvious positive effect on ADG, F:G and ME efficiency, but no interaction with GAA level was found, except for F:G and ME intake per kg of carcass. It was shown that GAA improved these traits only at the highest ME level. It appears that GAA does not affect performances at suboptimal dietary ME levels. The authors suggested that with higher energy levels more

rapid weight gain and muscle growth are achieved and that in this respect a higher Cr provision may contribute to a more efficient utilization of dietary nutrients and energy, resulting in improved ADG and particularly F:G. Similarly, Abudabos et al. (2014) found that birds receiving control or 25 kcal/kg less ME had more benefit on growth when supplemented with GAA as compared to treatment with more ME reduction. As changing solely dietary ME may cause imbalances between energy and protein in the diet, potentially masking any interaction with GAA as shown in Mousavi et al. (2013) and Abudabos et al. (2014), Yazdi et al. (2017) investigated the interaction between GAA and ND in the diet, when birds were fed wheat-SBM basal diets. Dietary ND is one of several nutritional factors that has a significant impact on growth of broiler chickens. In addition, manipulation of ND has been shown to affect growth performance, carcass quality (Jones and Wiseman, 1985) and animal health with respect to the occurrence of metabolic disorders (Scott, 2002). In general, feed intake increases with dilution of ND, but birds may have difficulty maintaining energy intake with high levels of dilution (Nielsen, 2004), which may negatively affect growth rate. Hence, it is appealing to study the interaction between ND and GAA. Thus, the aim of the present trial was to investigate the main and interactive effects of ND and GAA supplementation in corn-SBM diets on animal performance and carcass characteristics and organ weights, and traits of the energy metabolism in breast muscle of broiler chickens. Indeed, in Iran it is more common to apply corn-SBM than wheat-SBM in broiler diets. The reasons are: 1/ wheat is mainly used for human nutrition so imported corn is more available for animal nutrition; 2/ energy for animal feeds is costly since there is no supply of lard and limited supply of vegetable oils, so energy in animal feeds is enhanced by corn, and 3/ no aversion exists for yellow coloured broiler meat. Wheat middlings are also use in Iran. Therefore, also in the study at Ghent University corn-SBM was used. However, a previous study in our group tested the same hypothesis in wheat-SBM diets (Yazdi et al., 2017).

2.3. MATERIAL AND METHODS

The experiment was conducted with the approval of the Animal Care Committee of the Ferdowsi University of Mashhad (Mashhad, Iran).

2.3.1. Animals and housing

In this experiment, a total of 540 male day-old Ross 308 broiler chickens from a local hatchery (Fariman, Mashhad, Iran) were used. Broilers were randomly distributed to 54 floor pens (10 chickens each; 100 × 100 cm pen dimensions; 0.1 m^2/bird). Each pen was equipped with 1 pan feeder, nipple waterer and the floor was covered with wood shavings (5 cm thickness). The lighting program was 21L:3D during the whole experimental period and temperature scheme was according to breeder guidelines. Animals and housing facilities were inspected three times daily. A complete randomized design experiment with a 3 × 3 factorial arrangement comprised of 3 levels of ND and 3 levels

of GAA. The 3 levels of ND were: low, 2800; medium, 2950 and high, 3100 kcal ME/kg; and with the other nutrients being constant relative to ME. The 3 supplementary levels of GAA were 0, 0.6 and 1.2 g/kg. Starter (0 to 10 d of age), grower (10 to 25 d of age), and finisher (25 to 42 d of age) diets were provided in mash form and the diets per rearing period were formulated to meet or exceed Ross308 requirements (Aviagen, 2007; Ross 308: broiler nutrition specifications). Prior to the trial, samples of stored batches of main ingredients (SBM 44: DM, 893, CP, 462, EE, 15, and ash, 70 g/kg; and corn: DM, 839, CP, 80, EE, 36, and ash, 14 g/kg) were analyzed for proximate and AA contents. These values were used in least-cost formulation of the experimental diets. The source of GAA was CreAMINO® (GAA, feed grade >96.0%, made available by Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany, at the time of the study). Ingredient and calculated compositions are presented in Table 2.1. Feed and drinking water was provided *ad libitum*. Weight gain, F:G, F:G (adjusted for mortality), and mortality were measured on d10, d25, and d42. Stunted birds were removed at the end of the grower period.

Table 2.1. Ingredient and nutrient composition of experimental diets with varying levels of nutrient density and guanidinoacetic acid (GAA) fed to male broiler chickens[1]

Period	Starter (d0-10)			Grower (d10-25)			Finisher (d25-42)		
Nutrient density	L	M	H	L	M	H	L	M	H
Ingredient, g/kg									
Corn	552.1	534.0	441.0	556.5	613.8	590.2	615.8	655.1	584.4
SBM CP44	381.2	408.9	464.4	332.4	317.4	343.7	293.8	301.7	335.7
Soybean oil	10.0	11.8	44.8	10.0	10.0	28.4	10.0	10.0	45.3
Dicalcium phosphate	15.3	16.1	17.5	11.6	12.6	13.4	10.7	11.4	12.5
Limestone	13.6	14.3	14.3	12.4	13.0	13.4	10.8	11.4	11.6
Sodium chloride	2.2	3.6	3.9	3.2	3.4	3.6	3.2	3.4	3.7
L-Lysine-HCl	2.4	2.3	1.6	0.2	0.2		0.1	0.4	
DL-Methionine	3.4	3.5	3.7	1.7	2.1	2.3	1.6	1.6	1.9
Vitamin premix[2]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[3]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sand	13.1			65.0	22.0		49.0		
GAA[4]	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2
Calculated composition									
ME, kcal/kg	2800	2950	3100	2800	2950	3100	2800	2950	3100
Crude protein, g/kg	220	231	246	196	206	217	183	190	199
Ether extract, g/kg	34.0	35.3	68.8	32.8	33.9	51.3	35.8	36.7	70.2
Ca, g/kg	9.7	10.2	10.7	8.0	8.4	8.8	7.4	7.8	8.2

P dig., g/kg	4.6	4.8	5.1	4.0	4.2	4.4	3.6	3.8	4.0
Na, g/kg	1.8	1.9	2.0	1.7	1.8	1.9	1.7	1.8	1.9
Total Lys, g/kg	13.2	13.9	14.6	11.0	11.6	12.2	9.2	9.7	10.2
Total Met, g/kg	4.7	4.9	7.3	5.1	5.6	5.9	4.6	4.7	5.1
Total Met+Cys, g/kg	9.9	10.3	10.9	8.4	8.8	9.3	7.2	7.5	8.0
Total Met+Cys / Total Lys	0.75	0.74	0.75	0.76	0.76	0.76	0.78	0.77	0.78

[1] Nutrient density: L = low density, M = medium density, H = high density

[2] Providing per kg of diet: vitamin A (all-trans-retinol), 11000 IU; vitamin D3, 4500 IU; vitamin E (a-tocopherol), 65 IU; vitamin K3 (menaquinone), 3 mg; thiamine, 2.5 mg; choline, 1600 mg; riboflavin, 6.5 mg; pantothenic acid, 18.0 mg; pyridoxine, 3.2 mg; cyano-cobalamin, 0.02 mg; niacin, 60 mg; biotin, 0.18 mg; folic acid, 1.9 mg

[3] Providing per kg of diet: I, 1.25 mg; Cu, 16 mg; Se, 0.30 mg; Co, 1.0 mg; Mn, 120 mg; Zn, 110 mg; Fe, 20 mg and ethoxyquin, 33mg

[4] Each diet was divided into three equal portions and GAA was added to each part at a dose of 0, 0.6 and 1.2 g/kg on top and mixed to provide the nine diets for each feeding period

2.3.2. Sampling procedures

At the end of starter, grower and finisher period, one chicken per pen with a weight close to the average weight of the pen was selected for sampling. First, immediately after slaughter, right *pectoralis major* samples were taken, frozen in liquid nitrogen, and stored at −80°C pending analyses for biochemical parameters and energy metabolites. Next, birds were dissected and following parts were collected and weighed: whole breast, wings, thighs, drumsticks, abdominal fat, heart, liver, pancreas, spleen, gizzard, duodenum, jejunum, ileum, and bursa of Fabricius. Abdominal fat was not measurable at d10.

2.3.3. Analysis of ATP, ADP, AMP, Cr and CrN in right *pectoralis major*

The concentrations of adenosine phosphates (ATP, ADP and AMP), Cr and CrN in breast muscles were evaluated by reverse-phase-HPLC according to Liu et al. (2006) and Zhang et al. (2010), and modified. In brief, a frozen muscle sample (200 mg) was cut and homogenized for 1 min in 1.0 mL ice-cold 0.4 mol/L $HClO_4$ and kept on an ice bath for 10 min, then the homogenate was centrifuged for 10 min at 3600 *g* and 4°C. The supernatans (850 µL) was collected and adjusted to a pH of 6.5 with 850 µL of 0.6 mol/L K_2HPO_4. This mixture was vortexed for 20 s and centrifuged for 5 min. The supernatans was filtered with 0.45 µm Durapore membrane polyvinylidene fluoride filters (Millipore Corp). The filtrate (1.5 mL) was harvested, stored at -20°C and analyzed within 24h. The separation of muscle ATP, ADP, AMP, Cr and CrN was performed using an Alliance HPLC system Waters-2695 (Waters Corporation, Milford, MA), equipped with an integrated auto sampler. Chromatographs were recorded with a Waters-2996 PDA UV detector. The HPLC conditions were set as follows: a) Purospher STAR RP-18e column (2504.6 mm i.d., 5 µm) with a column T of 30° C; b) mobile phase, acetonitrile: 50 mmol/L KH_2PO_4 buffer solution (v:v, 10:90, pH adjusted to 6.5 for ATP, ADP, AMP and 6.8 for Cr and CrN using 1 mol/L KOH) at a flow rate of 0.8mL/min; c) detection wavelength was 254 nm and injection volume was 20 µL; and d) sample and mobile phase water were prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA). All standards were purchased from Sigma-Aldrich Inc. A volume of 20 µL of each standard sample was used for HPLC analysis. The standard curve and regression equations were administrated.

2.3.4. Breast meat quality measurements

Skinless left breast samples were used for colour and pH determination and subsequently analyzed for DM, CP, EE and ash according to AOAC (1990), whereas right breast samples were used for press loss, cooking loss and shear force (Al-Owaimer et al., 2014). Postmortem pH (t=0.5 h and t=24 h after storing at 4 °C) was determined at 1.5 cm depth of the breast muscle. Colour L*, a*, and b* values were measured on breast muscle samples after 24 h postmortem stored at 4 °C with a Minolta CR-410 apparatus (Konica

Minolta). After 30 min equilibration to room T, colour was measured in 3 different parts (top, middle and bottom) of the left breast and the average value was used. Press loss was defined as the proportionate weight loss of a breast sample stored for 24 h at 4°C. Therefore, 300 mg minced meat sample was accurately weighed on Whatman filter paper grade 1, and then loaded with a 2 kg metal disc for 5 min. Cooking loss was determined after overnight storage at 4°C. It was quantified as proportionate weight loss of a sample after cooking in an open plastic bag in a water bath at 80°C for 60 min followed by cooling in cold-running tap water for 15 min. Next, shear force was determined on cylindrical cores (diameter, 1.27 cm), taken from cooked samples by perpendicular shearing to the longitudinal orientation of the right muscle fibers using a TA.XT plus (Stable Micro System, England).

2.3.5. Statistical analysis

Data were analyzed using the GLM procedure of SAS Enterprise Guide 7 (SAS Institute, Cary, USA) with a model containing the fixed effects of GAA supplementation, diet ND and their interaction term. Means were separated by Duncan's multiple range test. The data were assumed to be statistically significant when $P<0.05$. Mortality was analysed by using the non-parametric Kruskal-Wallis test.

2.4. RESULTS

2.4.1. Broiler performances

In the starter, grower and finisher periods, the high level of ND significantly improved BW and ADG as compared to the two other levels of ND ($P<0.05$) (Table 2.2). Overall (d0-42), there was a significant ADG response when ND increased ($P<0.05$). ADFI was not affected by ND in the starter period, in contrast to the grower and finisher periods; a linear increase of ADFI was found with increasing ND ($P<0.05$). The highest GAA supplementation level reduced ADFI compared to the group with 0.06 g/kg GAA in the diet in the finisher period (156 *vs*. 165 g/d; $P<0.05$). For all rearing periods, the highest level of ND reduced F:G as compared to the lower levels of ND ($P<0.05$). No effect of GAA supplementation was observed for this performance trait except for the finisher period, whereby GAA at 0.12 g/kg improved F:G compared to control and 0.6 g/kg GAA group (1.81 *vs*. 1.93; $P<0.05$). For the whole period, the highest level of ND and GAA lowered the F:G by 0.16 and 0.09 compared to the respective lowest level. Total mortality was only 1.35% and did not differ between treatments. Consequently, the European Production Efficiency Factor (EPEF) gradually increased with increasing ND ($P<0.05$), whereas GAA had no effect on EPEF. Importantly, for none of the performance traits, an interaction between ND and GAA was found.

2.4.2. Carcass traits and organ weights

The effect of ND and GAA supplementation on carcass traits in male broilers on d10, d25 and d42 is shown in Table 2.3. At d10, relative thigh weight increased concomitant with the diet ND level ($P<0.05$), whereas at d25 wing/BW and abdominal fat/BW decreased and increased, respectively, with increasing ND (all $P<0.05$). In opposite, GAA increased wing yield at d25 ($P<0.05$). At the end of the study, ND increased whole breast weight ($P<0.05$). Relative breast weight was increased upon feeding the high and medium ND level compared to the low ND level ($P<0.05$). No other carcass cuts were affected by the main factors and again no interactions were observed. In general absolute organ weight increased with higher dietary ND (Table 2.4), corroborating higher BW for birds fed high ND as shown above. These effects were most pronounced at d25 and d42. In contrast, whenever a significant effect of ND on relative organ weight was observed it showed a decreasing effect, except for heart at d25. For example, at all sampling points, it was shown that relative gizzard weight decreased with higher dietary ND (all $P<0.05$). GAA had only an effect on the bursa of Fabricius at d10 ($P<0.05$).

2.4.3. Breast meat quality traits

Proximate analysis of breast muscle at d42 did not reveal treatment effects (Table 2.5), with the exception of EE that was affected by ND. Next, press loss was lower for the medium compared to the low level of ND ($P<0.05$). Colour L* was lower for the high compared to the low ND level ($P<0.05$), suggesting darker breast when feeding a high ND diet. GAA had no effect on breast meat quality parameters, but an interaction was found between ND and GAA for press loss ($P<0.05$). GAA had no effect on press loss at the low dietary ND, whereas in medium ND 1.2 g/kg GAA showed higher press loss compared to control and 0.6 g/kg GAA (Fig. 2.1). At the high ND level, an opposite effect could be perceived, though effects were not significant.

Table 2.2. Effect of nutrient density (ND)[1] and guanidinoacetic acid (GAA) supplementation on BW, ADG, ADFI, F:G, mortality[2] and EPEF[3] in male broiler chickens[4]

Item	ND			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	ND	GAA	ND × GAA	ND		GAA	
											Linear	Quadratic	Linear	Qaudratic
Starter (d0-10)														
BW d0, g	40.4	40.0	40.2	40.0	40.3	40.4	0.29	0.370	0.578	0.422	0.497	0.212	0.314	0.785
BW d10, g	241[b]	246[b]	266[a]	247	253	252	3.7	<0.001	0.432	0.997	<0.001	0.078	0.385	0.510
ADG, g/d	20.0[b]	20.6[b]	22.6[a]	20.7	21.3	21.2	0.36	<0.001	0.468	0.992	<0.001	0.096	0.414	0.525
ADFI, g/d	26.4	26.2	26.2	25.9	26.6	26.3	0.25	0.873	0.183	0.962	0.629	0.298	0.298	0.099
F:G	1.32[a]	1.27[b]	1.17[c]	1.26	1.25	1.25	0.013	<0.001	0.790	0.638	<0.001	0.106	0.721	0.782
Grower (d10-25)														
BW d25, g	879[b]	918[b]	1040[a]	950	953	934	13.5	<0.001	0.569	0.436	<0.001	0.015	0.560	0.714
ADG, g/d	45.5[b]	48.0[b]	55.3[a]	50.3	50.0	48.7	0.87	<0.001	0.405	0.411	<0.001	0.035	0.376	0.799
ADFI, g/d	88.0[b]	91.1[ab]	94.3[a]	92.3	91.4	89.7	1.40	0.011	0.418	0.509	0.002	0.955	0.220	0.883
F:G	1.93[a]	1.90[a]	1.74[b]	1.84	1.84	1.88	0.024	<0.001	0.433	0.389	<0.001	0.033	0.367	0.541
Finisher (d25-42)														
BW d42, g	2340[c]	2448[b]	2725[a]	2482	2519	2511	29.8	<0.001	0.647	0.873	<0.001	0.023	0.710	0.910
ADG, g/d	79.6[c]	85.4[b]	93.4[a]	84.4	86.3	87.7	1.41	<0.001	0.254	0.578	<0.001	0.501	0.263	0.915
ADFI, g/d	157.2[b]	162.1[ab]	164.4[a]	161.5[ab]	164.9[a]	156.3[b]	2.50	0.047	0.042	0.153	0.028	0.506	0.111	0.061
F:G	1.97[a]	1.92[a]	1.77[b]	1.93[a]	1.93[a]	1.81[b]	0.030	<0.001	0.011	0.504	<0.001	0.220	0.030	0.115
Total (d0-42)														
ADG, g/d	54.7[c]	57.8[b]	63.9[a]	58.1	59.0	59.3	0.64	<0.001	0.393	0.487	<0.001	0.066	0.495	0.945
ADFI, g/d	98.1	101.6	102.3	101.1	102.2	98.7	1.30	0.060	0.171	0.151	0.032	0.408	0.155	0.177
F:G	1.92[a]	1.91[a]	1.76[b]	1.90[a]	1.89[a]	1.81[b]	0.018	<0.001	0.004	0.129	<0.001	0.022	0.036	0.152
Mortality, %	1.1	0.7	2.2	0.7	0.6	2.8		0.232	0.194	0.117				
EPEF	287[b]	306[b]	359[a]	312	317	324	6.6	<0.001	0.463	0.599	<0.001	0.046	0.448	0.757

The values are means of 18 replicates per level of main factor

[1] Nutrient density: L = low density, M = medium density, H = high density

[2] Mortality data within treatment were not normally distributed and hence treatments were compared using the non-parametric Kruskal-Wallis test. No linear or quadratic responses for main factors were computed.
[3] European Production Efficiency Factor: [viability d0-42 (%) * BW d42 (kg) * 100] / [age (d) * F:G d0-42]
[4] Values with different superscripts within a row and main factor are significantly different at $P < 0.05$

Table 2.3. Effect of nutrient density (ND)[1] and guanidinoacetic acid (GAA) supplementation on carcass traits (% of body weight) in male broiler chickens on day 10, 25 and 42 [2]

Item	ND			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	ND	GAA	ND × GAA	ND		GAA	
											Linear	Quadratic	Linear	Quadratic
Day 10														
Breast	16.9	17.2	17.6	16.9	17.6	17.1	0.65	0.407	0.389	0.524	0.180	0.905	0.717	0.182
Thigh	15.6^{b}	15.7^{ab}	16.3^{a}	16.0	15.4	16.1	0.31	0.024	0.280	0.090	0.025	0.391	0.938	0.020
Wing	6.9	6.5	6.7	6.9	6.5	6.6	0.31	0.361	0.332	0.308	0.516	0.210	0.310	0.286
Drum	11.1	10.9	10.8	10.9	10.0	10.9	0.37	0.647	0.959	0.383	0.256	0.350	0.450	0.126
Day 25														
Breast	20.8	20.4	20.1	21.0	20.0	20.3	0.35	0.804	0.636	0.927	0.493	0.973	0.507	0.458
Thigh	17.7	18.0	17.3	17.6	17.7	17.7	0.41	0.127	0.857	0.558	0.302	0.068	0.634	0.789
Wing	6.6^{a}	6.3^{a}	5.8^{b}	5.9^{b}	6.2^{ab}	6.6^{a}	0.29	0.007	0.028	0.389	0.003	0.626	0.013	0.989
Drum	14.2	14.2	14.1	14.0	14.4	14.0	0.36	0.903	0.335	0.322	0.564	0.750	0.740	0.325
Abdominal fat	0.65^{b}	0.85^{ab}	0.89^{a}	0.75	0.80	0.82	0.11	0.036	0.847	0.902	0.010	0.314	0.501	0.808
Day 42														
Breast	24.1^{b}	25.6^{a}	26.8^{a}	26.1	24.9	25.4	0.35	0.001	0.068	0.369	<0.001	0.829	0.365	0.098
Thigh	19.7	19.5	19.1	19.4	19.4	19.4	0.35	0.540	0.998	0.772	0.275	0.742	0.950	0.921
Wing	6.2	5.7	5.6	5.7	6.0	5.8	0.45	0.296	0.641	0.542	0.137	0.424	0.657	0.415
Drum	13.1	14.6	13.6	14.3	14.1	13.9	0.86	0.153	0.281	0.633	0.490	0.125	0.110	0.825
Abdominal fat	0.90	0.97	0.87	0.94	0.95	0.84	0.108	0.522	0.445	0.271	0.672	0.262	0.293	0.466

The values are means of 18 replicates per level of main factor

[1] Nutrient density: L = low density, M = medium density, H = high density

[2] Values with different superscripts within a row and main factor are significantly different at $P<0.05$

Table 2.4. Effect of nutrient density (ND)[1] and guanidinoacetic acid (GAA) supplementation on absolute (weight) and relative (weight/BW) weight of organ in male broiler chickens on day 10, 25 and 42 [2]

Item	Day 10						Day 25						Day 42					
	Weight			Weight/BW			Weight			Weight/BW			Weight			Weight/BW		
	ND[3]	GAA	ND x GAA	ND[4]	GAA[4]	ND x GAA	ND[3]	GAA	ND x GAA	ND[4]	GAA	ND x GAA[5]	ND[3]	GAA	ND x GAA	ND[4]	GAA	ND x GAA
Heart	**						***			**			**					
Liver	T						***						**					
Pancreas							T						**			T		
Spleen										*								
Gizzard		T		**			***			*			*			*		
Duodenum	**					T	***			T			**			T		
Jejunum	***						***					*	***					
Ileum	*						***						**					
Bursa of Fabricius	T	T		*	**		**											

[1] Nutrient density: L = low density, M = medium density, H = high density

[2] Significant effects of main factors (ND and GAA) and their interaction are indicated; T, 0.1>P≥0.05; *, P<0.05; **, P<0.01; ***, P<0.0011

[3] Significant effects indicated linear increase in absolute weight upon increasing ND

[4] Significant effects indicated linear decrease in relative weight upon increasing ND or GAA, except for heart at d25 where relative weight increases upon increasing ND

[5] Interactive effect for jejunum shows that GAA lowers relative weight of jejunum in low level of ND, whereas it increases relative weight of jejunum in high level of ND

Table 2.5. Effect of nutrient density (ND)[1] and guanidinoacetic acid (GAA) supplementation on breast meat quality parameters in male broiler chickens on day 42 [2]

Item	ND			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	ND	GAA	ND × GAA	ND		GAA	
											Linear	Quadratic	Linear	Quadratic
Breast meat proximate analysis														
DM, %	24.2	24.4	24.6	24.6	24.2	24.3	0.22	0.338	0.343	0.249	0.168	0.977	0.295	0.354
Ash, %	1.06	0.98	1.04	1.00	1.03	1.05	0.044	0.487	0.691	0.874	0.782	0.211	0.403	0.758
EE, %	1.00	1.01	1.32	1.27	1.03	1.04	0.101	0.047	0.163	0.763	0.035	0.200	0.143	0.296
CP, %	21.5	21.7	21.7	21.7	21.5	21.6	0.20	0.747	0.730	0.335	0.618	0.600	0.634	0.533
Breast meat characteristics														
pH after 0.5h	6.24	6.21	6.28	6.26	6.18	6.29	0.128	0.943	0.832	0.329	0.943	0.490	0.904	0.620
pH after 24h in cooler	5.75	5.78	5.79	5.86	5.87	5.85	0.127	0.974	0.227	0.364	0.811	0.836	0.110	0.337
Press loss, %	23.0^{a}	17.6^{b}	18.7^{ab}	20.2	18.3	20.9	1.44	0.035	0.451	0.027	0.063	0.070	0.884	0.320
Cooking loss, %	33.1	33.1	32.8	33.3	32.8	32.9	0.70	0.930	0.857	0.804	0.716	0.884	0.625	0.741
Shear force, N	25.7	25.2	27.4	24.0	25.9	28.4	2.57	0.836	0.528	0.523	0.671	0.508	0.260	0.948
Colour a*	5.3	5.4	5.6	5.4	5.4	5.4	0.24	0.391	0.999	0.124	0.231	0.394	0.881	0.982
Colour b*	15.8	15.1	15.2	15.2	13.4	15.4	0.74	0.375	0.995	0.137	0.181	0.681	0.966	0.995
Colour L*	60.9^{a}	59.2^{ab}	57.8^{b}	58.7	59.5	59.6	1.33	0.027	0.660	0.887	0.005	0.856	0.442	0.722

The values are means of 18 replicates per level of main factor

[1] Nutrient density: L = low density, M = medium density, H = high density

[2] Values with different superscripts within a row and main factor are significantly different at $P<0.05$

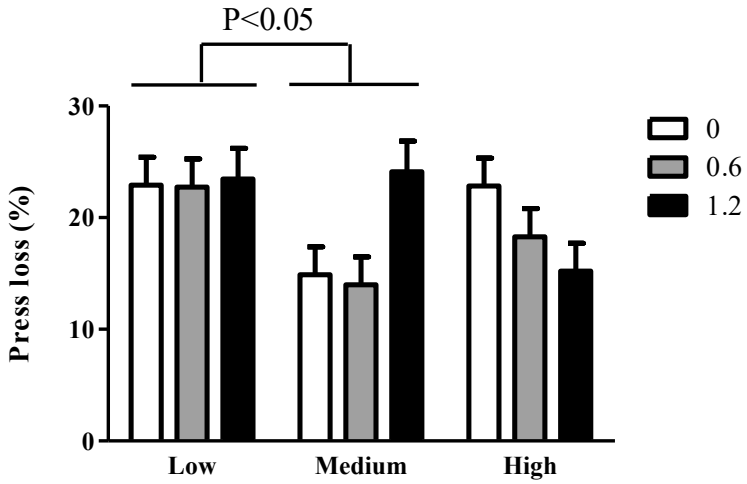

Fig 2.1. Effect of nutrient density (low, medium and high density) and guanidinoacetic acid (0, 0.6 and 1.2 g/kg) supplementation and their interaction on press loss of breast muscle in male broiler chickens on day 42. The values are means of the 6 replicates per treatment.

2.4.4. Energy metabolites

The effect of ND and GAA supplementation on concentration of ATP, ADP, AMP, Cr and CrN in breast muscles of male broiler chickens at d10, d25 and d42 are shown in Table 2.6. The effect of dietary ND was not significant on ATP, ADP, AMP, Cr and CrN concentrations of broiler chickens' breast muscle in the starter, grower and finisher periods with the exception of AMP concentration at the end of the starter period ($P<0.05$) and CrN at d42 ($P<0.05$). AMP was higher for high than for medium ND diets at d10. GAA showed an effect on ATP at d25 and ADP at d42 (both $P<0.05$), though difficult to interpret. At d42 Cr was elevated when feeding GAA at 1.2 g/kg compared to no GAA in the diet (5455 *vs.* 4338 mg/kg fresh muscle, $P<0.05$). No interactive effects were observed for any energy metabolite. We should add that data for Cr and CrN at d25 are very different from other sampling days and deviating for example Chapter 3, Table 3.5., which may suggest that these analyses were not accurate.

Table 2.6. Effect of nutrient density (ND) and guanidinoacetic acid (GAA) supplementation on energy metabolites in male broiler chicken breast at day 10, 25 and 42

Item	ND			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	ND	GAA	ND× GAA	ND		GAA	
											Linear	Quadratic	Linear	Quadratic
Day 10														
ATP, µmol/g FM[3]	3.01	3.14	3.16	3.01	3.13	3.16	0.271	0.820	0.837	0.136	0.596	0.899	0.627	0.883
ADP, µmol/g FM	0.83	0.88	0.87	0.86	0.91	0.81	0.087	0.726	0.438	0.432	0.509	0.503	0.466	0.270
AMP, µmol/g FM	0.64[ab]	0.61[b]	0.78[a]	0.64	0.67	0.71	0.076	0.026	0.541	0.543	0.025	0.081	0.291	0.853
Cr, mg/kg FM	5473	5077	4860	5065	5159	5187	365.1	0.158	0.915	0.264	0.041	0.760	0.476	0.731
CrN, mg/kg FM	50.0	52.6	50.5	49.2	55.4	48.4	4.91	0.800	0.189	0.527	0.948	0.496	0.829	0.062
Day 25														
ATP, µmol/g FM	2.90	2.89	3.23	3.01[ab]	3.36[a]	2.65[b]	0.248	0.328	0.030	0.648	0.362	0.521	0.240	0.022
ADP, µmol/g FM	0.99	0.98	1.31	1.00	1.28	0.99	0.201	0.115	0.175	0.915	0.093	0.274	0.907	0.091
AMP, µmol/g FM	0.71	0.67	0.80	0.74	0.68	0.76	0.073	0.099	0.362	0.381	0.152	0.100	0.706	0.097
Cr, mg/kg FM	2646	2557	2471	2560	2742	2372	167.5	0.508	0.051	0.143	0.290	0.963	0.378	0.028
CrN, mg/kg FM	21.9	21.9	21.3	20.7	23.7	20.6	1.89	0.911	0.121	0.075	0.687	0.930	0.795	0.048
Day 42														
ATP, µmol/g FM	3.70	3.80	3.68	3.48	3.60	4.10	0.468	0.945	0.241	0.309	0.904	0.538	0.154	0.465
ADP, µmol/g FM	1.37	1.30	1.33	1.62[a]	1.10[b]	1.27[ab]	0.212	0.918	0.018	0.787	0.923	0.734	0.040	0.021
AMP, µmol/g FM	0.49	0.67	0.53	0.52	0.59	0.58	0.096	0.076	0.686	0.901	0.739	0.016	0.388	0.482
Cr, mg/kg FM	4713	4644	4743	4306[b]	4338[b]	5455[a]	475.6	0.974	0.014	0.320	0.887	0.760	0.010	0.098
CrN, mg/kg FM	41.8	35.9	31.0	33.1	35.7	40.1	4.48	0.040	0.225	0.433	0.010	0.932	0.063	0.874

The values are means of 18 replicates per level of main factor

[1] Nutrient density: L = low density, M = medium density, H = high density

[2] Values with different superscripts within a row and main factor are significantly different at $P<0.05$

[3] Fresh muscle

2.5. DISCUSSION

The study was designed to evaluate the response of broiler chickens to GAA added to corn-SBM based diets with different ND on performance, carcass traits, organ weights and muscle energy metabolites. Contrary to our expectations, no two-way interactions were found for performance indices, which suggests that effects of GAA were independent of dietary ND. In all phases of feeding, when ND increased in the diet, BW and ADG increased substantially. As such, results with regard to ND are in line with Yazdi et al. (2017) using wheat-SBM diets, but an improvement in F:G by GAA supplementation was only seen in the grower period in that study.

2.5.1. Nutrient density positively affects performance

Improvements in growth performances by increasing ND of the diet are in agreement with Brickett et al. (2007), Li et al. (2010b) and Yazdi et al. (2017). In the latter study, using diets with similar formulated ME levels, higher ND in the diet improved growth and feed efficiency, and FI was linearly increased by ND in the starter, but not in the finisher period and a quadratic response was seen in the grower period. Concerning FI, this was opposite to Brickett et al. (2007) who found a larger reduction in FI when birds were older upon higher ND in the diet, corroborating to the assertion that birds increase feed consumption to compensate for lower dietary energy to meet requirements. Birds have the ability to regulate feed intake based on the dilution of ND within certain limits (Nielsen, 2004). The ability to compensate for diluted ND is affected by age (young chickens especially within first two weeks are less able to compensate), feed form (mash or pellet; with mash it is more difficult to compensate), light schedule etc. At the end of the study we found a higher breast yield, from 24 to 27% relative to BW. Brickett et al. (2007) did not find this, but observed small effects on other portions such as wing and abdominal fat. Not surprisingly, we also found that most organ weights were heavier with higher ND, which is obviously related to the higher BW. Nonetheless, a consistent effect on relative gizzard weight suggests that higher ND resulted in relative smaller gizzards. It is well established that coarse diets stimulate gizzard development (Zaefarian et al., 2016), with concomitant improvements in gut motility and health. Hence, our findings intuitively might feel contradictory, but one can also speculate that gizzards develop more in low ND diets because birds need to propel more digestive capacity, also diverting more energy to the grinding process. Next to the higher breast yield with higher ND, breast meat was found to contain more fat, have darker colour and higher WHC. These relations of breast meat quality parameters were also observed in the work of Michiels et al. (2014) where indoor and outdoor raised birds were opposed. Also, Fanatico et al. (2007) showed that high ND decreased the moisture loss of broiler meat, which suggests superior WHC.

2.5.2. Guanidinoacetic acid decreases feed to gain ratio in the finisher period associated with higher creatine levels in breast

This study confirmed that supplementation with GAA can improve F:G in the finisher period or overall, corroborating previous studies (e.g. Abudabos et al., 2014; EFSA, 2009; Michiels et al., 2012; Ringel et al., 2008ab). However, in contrast to other studies, this occurred only when fed at 1.2 g/kg. The reduction in F:G for the total period was 9 points and this is larger the 5.8 points suggested in Chapter 1, section 1.4.2.1. In our study, the effect of GAA supplementation on BWG was not significant throughout the experiment. Improvement in growth performance when GAA was added to the diets at 1.2 g/kg is in agreement with Michiels et al. (2012) who showed that in all-vegetable diets, when considering the entire rearing period, birds fed GAA compared to the negative control group performed better, mainly because of better feed efficiency in the finisher period. It seems that GAA supplementation is more beneficial at higher growth rates. Improvements in F:G likely occurred because of two reasons. Firstly, Arg could be spared from serving as a precursor for Cr synthesis and hence could be available for alternative functions throughout the body. This hypothesis is in agreement with the research conducted by Dilger et al. (2013). Secondly, when 1.2 g/kg GAA was added to the finisher diet, ADFI decreased. A similar response was reported by Lemme et al. (2010a) who showed that GAA supplementation reduced FI in turkeys, in some studies reported in EFSA (2009), at least for the whole rearing period, and similarly by Kodambashi et al. (2017). In contrast, EFSA (2009) (some studies), Michiels et al. (2012), Mousavi et al. (2013), Ringel et al. (2008ab), Yazdi et al. (2017) found no effect of GAA on FI. Variation in response for FI was already discussed in Chapter 1, section 1.4.2.1. A reduction in FI might be explained by the hepatic energy status theory that is related to feeding behavior. Friedman and coworkers conducted a series of experiments that illustrate this theory in rats. The synergistic stimulation of feeding from the combined treatment of the fructose analogue 2,5-anhydro-D-mannitol, which is able to trap hepatic phosphate and thus decreases ATP, and methylpalmoxirate, an inhibitor of carnitine palmitoyltransferase that transports fatty acids into mitochondria, was related to a decreased hepatic ATP:ADP ratio and phosphorylation potential when rats were fed a diet with equal energy from carbohydrate and fat (Ji and Friedman, 1999; Ji et al., 2000). In other words, it means that a lower hepatic energy status from the oxidation of fuels accelerates feeding. Alternatively, we hypothesize that dietary GAA to broilers might enhance ATP and/or PCr in the liver, and hitherto by negative feedback reduce FI. This might have occurred in the finisher phase, though we have no proof for this effect of GAA on liver energy status. As a result of reduced FI at similar growth rate, F:G decreases. Maintenance of growth is likely associated with the Arg sparing effect, and the restoration of Cr load in growing muscle tissues and enhanced cell management. Elaborating further on the hepatic energy status theory, it can be conceived that an interaction between ND and GAA should occur, since reduced ND might reduce oxidative fueling in the liver, hence increases in ATP and/or PCr in the liver by GAA might be more beneficial. We did observe that when comparing ADFI of the 1.2 g/kg

GAA treatment with the control diet, the reduction in FI was higher in the low (-8%) compared to the medium and high (-2 to -4%) ND level, with higher growth rates for the supplemented treatments (+1 to +2.1 g/d). Although these differences were not significant, it lends to speculate that increases in hepatic energy status by GAA might be higher with lower ND, thus resulting in more negative feedback on feed intake. To recall, the ME levels used in the present study were lower or equal compared to recommendations for this strain in the finisher period (3100-3200 kcal/kg). These findings are analogues to the results of Yazdi et al. (2017) in which a lower FI also occurred with higher GAA supplementation in low and medium ND diets, however only in the starter phase. It remains to be established whether dietary GAA interferes with the hepatic energy status in broilers. Furthermore, Mousavi et al. (2013) and Abudabos et al. (2014) tested 2 levels of GAA (0 and 0.6 g/kg) added to diets only differing in ME levels. In the former study, no interaction was found, except for F:G and ME intake per kg of carcass whereby it was shown that GAA did improve these traits only at the high ME level. Numerically, it could be seen that 0.6 g/kg GAA caused a greater reduction in FI at normal ME level compared to 90% of normal ME level. Abudabos et al. (2014) found only an interaction for FI in the grower period and entire period. At recommended ME level, 0.6 g/kg GAA seemed to reduce FI similar to the -50 and -75 kcal/kg ME treatments, whereas the treatment -25 kcal/kg ME showed the opposite.

In line with previous studies, we provide evidence that dietary GAA elevates the breast muscle Cr load. In our study, supplementing GAA to an all-vegetable diet resulted in a dose-related increase of Cr concentrations in breast meat at the end of the experiment, which means that supplemental GAA is an efficient precursor in terms of Cr incorporation into the muscle. Also, it appeared that ADP decreased, whereas ATP increased in breast muscle, though the latter was not significant, further supporting increased energy status in this tissue. It should be mentioned that Cr concentrations in breast meat represent both phosphorylated and non-phosphorylated Cr in this study. This finding is in line with a linear increase of muscle Cr levels with increasing dietary GAA provision as observed by EFSA (2009), Lemme et al. (2007a) and Ringel et al. (2008a). Michiels et al. (2012) showed that the PCr:ATP ratio was significantly higher for the birds on the 1.2 GAA g/kg diet compared with the control group. The PCr:ATP ratio was positively related to the Cr content (Michiels et al., 2012). GAA supplementation had no effects on breast meat characteristics of 42 day-old broilers, which corroborates partially to the findings of Michiels et al. (2012). In that study, GAA increased lightness, which was only numerically the case in our study, and is in line with earlier research of Young et al. (2004) where broilers were supplemented with Cr via drinking water, or Sthal et al. (2003) providing dietary Cr, and more recently of Esser et al. (2017) in GAA fed broilers after 2 days of HS. With regard to press loss, contrasting results are reported. In our study, an interaction was found between the two main factors. GAA had no effect on press loss at the low dietary ND, whereas in medium ND, 1.2 g/kg GAA showed higher press loss compared to the control and 0.6 g/kg GAA treatments. At the high ND level, an opposite effect was observed, though differences were not significant. The high ND

offers a diet with 3100 kcal ME per kg diet, which resembles the 3150 kcal ME/kg level of the finisher diet in Michiels et al. (2012), hence results in that and the present study are contradictory. Furthermore, Esser et al. (2017) found no effect of 0.8 g/kg GAA on press loss, but cooking loss was decreased. This warrants further investigation. Another finding in our study is that dietary GAA decreased relative bursa weight at the end of the starter period. Kodambashi et al. (2017) found that supplementary Arg (1.72 g/kg) reduced relative bursa weight compared to control, but not the supplementation of an 'equivalent' amount of GAA (1.2 g/kg).

2.6. CONCLUSION

Contrary to our hypothesis, the effects of dietary GAA supplementation were not influenced by the ND of the diet. Dietary GAA improved performances in terms of F:G in the finisher period and breast meat yield, likely associated with increased Cr concentration in the breast muscle. Nonetheless, the effects on other energy metabolites were not consistent. Most notably were the substantial effects on performance and carcass traits of the dietary ND in all rearing phases. It is concluded that GAA can improve F:G but cannot compensate for reduced ND in the diet.

2.7. ACKNOWLEDGEMENTS

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CHAPTER 3

EFFECTS OF METHIONINE AND GUANIDINOACETIC ACID SUPPLEMENATION ON PERFORMANCE AND ENERGY METABOLITES IN BREAST MUSCLE OF MALE BROILER CHICKENS FED CORN-SOYBEAN DIETS

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CHAPTER 3

EFFECTS OF METHIONINE AND GUANIDINOACETIC ACID SUPPLEMENATION ON PERFORMANCE AND ENERGY METABOLITES IN BREAST MUSCLE OF MALE BROILER CHICKENS FED CORN-SOYBEAN DIETS

3.1. ABSTRACT

GAA is the single endogenous precursor of Cr, and the latter plays a critical role in energy homeostasis of cells. Since GAA is endogenously converted to Cr by methylation, it was hypothesized that the effects of dietary GAA supplementation might depend on Met provision in corn-SBM diets. A total of 540 day-old male Ross 308 broilers were allocated to 9 dietary treatments with 6 replicates (10 birds each) in a 3 × 3 factorial arrangement with 3 graded levels of supplementary Met (+0.4 g/kg per level) whilst Cys was equal across groups, resulting in a low, medium and high level of total sulfur AA (TSAA), and with 3 levels of GAA (0, 0.6 and 1.2 g/kg), for 42 days. Increasing levels of supplemental Met enhanced performance indices in all rearing periods, apart from no effect on F:G in the grower and FI in the finisher period. Final BW was 8.8 and 14.6% higher in medium and high Met diets, respectively, as compared to the low level. Relative breast weight and protein content in muscle on d25 linearly increased with increasing levels of Met. At low and high Met levels, growth in the finisher phase was negatively affected by supplementing GAA at 1.2 g/kg. It is suggested that disturbances in methylation homeostasis and/or changes in Arg metabolism might explain these findings. At the end of the grower phase, muscle Cr content was higher when feeding GAA at 0.6 and 1.2 g/kg (4464 and 4472, respectively, *vs.* 4054 mg/kg fresh muscle, in control). To conclude, the effects of dietary GAA supplementation were influenced by the dietary Met level only in the finisher period, which urges for proper sulfur AA formulation of diets when feeding GAA.

3.2. INTRODUCTION

The beneficial effects of supplemental GAA to poultry diets have been demonstrated in many experiments. At 0.6 or 1.2 g/kg GAA in the feed, a consistently lower F:G, in some cases concomitant with a reduction in FI, enhanced breast meat yield with no or minor effects on meat quality parameters, and a reduction in myopathy incidence have been reported (Abudabos et al., 2014; Cordova-Noboa et al., 2018ab; DeGroot et al., 2018;

EFSA, 2009; Kodambashi et al., 2017; Michiels et al., 2012; Ringel et al., 2008ab; Chapter 2). It is assumed that performance enhancements arrive from restoration of Cr load in tissues (EFSA, 2009; Michiels et al., 2012), an Arg sparing effect (DeGroot et al., 2018; Dilger et al., 2013), stimulation of myogenic differentiation and muscle growth (Wang et al., 2018), stimulation of hormonal release and neuromodulation, and an adjustment of oxidant-antioxidant status (Ostojic, 2016). GAA is the only metabolic intermediary product and precursor of Cr. GAA is synthesized from Gly and Arg by transferring the amidino group from Arg to Gly, catalysed by AGAT and taking place mainly in the kidney and pancreas (Wyss and Kaddurah-Daouk, 2000). This reaction results in GAA and L-ornithine. Subsequently, after transport to the liver, GAA is methylated to Cr with SAM as the methyl group donor and by the action of GAMT (Daly, 1985). In addition to its primary function as a constituent of proteins, Met can be activated by ATP to deliver SAM (Mato et al., 2013). The reaction is catalyzed by the enzyme methionine adenosyltransferase (MAT), two main MAT isoforms, MATI and MATIII, are expressed in the mammalian liver (Mato et al., 2013). SAM is the common co-substrate involved in methyl group transfers, transsulfuration and aminopropylation. More than 200 methyltransferase enzymes have been described. Quantitatively, Cr, PC and sarcosine synthesis, mediated by enzymes GAMT, phosphatidylethanolamine N-methyltransferase (PEMT) and glycine N-methyltransferase (GNMT), respectively, are considered the most important ones. Though the latter reaction is more a kind like shunt to balance excessive hepatic SAM.

To note, 85% of methylation reactions occur in the liver (Lu and Mato, 2012). More particularly, it was shown that Cr synthesis may consume as much as 40% in adult human (Stead et al., 2006) and 63-77% in neonatal piglets (Brosnan et al., 2009) of all the labile methyl groups used, but no data are available for chicken. The resulting compound of methylation reactions is SAH, which is then reversibly hydrolyzed to HCy and adenosine. HCy is a key intermediate because it can be re-methylated to Met, by either employing betaine or 5-methyltetrahydrofolate (MTHF), using the enzymes betaine-homocysteine methyltransferase (BHMT) and MS, respectively, with the former to be found more important than the latter in broiler (Pillai et al., 2006), or can condense with homoserine to form cystathionine and then Cys. The folate cycle involves folates, Ser, Gly and vitamin B12. Betaine can be produced from choline by an irreversible two-step oxidation reaction that occurs in liver and kidney. Donation of propylamino groups from SAM is employed to produce the polyamine series with spermidine and spermine being both essential for cell division, protein synthesis, and tissue growth. Taking together, SAM is a central metabolite that is competitively used for multiple metabolic pathways, and it appears that hepatic SAM levels are tightly regulated by a concerted action of enzymes involved in the re-methylation and folate cycles and in the transsulfuration pathway.

From the above it can be hypothesized that availability of Met, as the single precursor of SAM, could play an important role in endogenous GAA conversion to Cr, next to other

factors essential in the re-methylation and folate cycles. In broilers, Lemme et al. (2010b) showed that with adequate Met supply, dietary 0.8 g/kg GAA improved F:G while at Met deficiency no such effect was observed, concluding that Met deficiency may limit availability of methyl groups for GAA methylation. Though Cr supplementation was also not effective at low dietary Met. Hence, it remains equivocal whether GAA efficacy relies on Met provision. Therefore, the aim of the present trial was to investigate the main and interactive effects of Met, being deficient, adequate or surfeit, and GAA supplementation in corn-SBM diets on animal performance, organ weights, and traits of the energy metabolism in breast muscle of broiler chickens.

3.3. MATERIAL AND METHODS

The experiment was conducted with the approval of the Animal Care Committee of the Ferdowsi University of Mashhad (Mashhad, Iran).

3.3.1. Animals and housing

A total of 540 male day-old Ross 308 broiler chicks from a local hatchery (Fariman, Mashhad, Iran) were used. Broilers were randomly distributed to 54 floor pens (10 chicks each; 0.1 m^2/bird) and each pen was equipped with 1 pan feeder and nipple waterer. The floor was covered with wood shavings (5 cm thickness). The lighting program was 21L: 3D during the whole experimental period period and temperature scheme was according to breeder guidelines. Animals and housing facilities were inspected three times daily. A complete randomized design experiment was run with a 3 × 3 factorial arrangement comprised of 3 levels of supplemental Met (MetAMINO, >99%, Evonik Degussa GmbH, Hanau-Wolfgang, Germany) and 3 levels of supplemental GAA (CreAMINO®, GAA, feed grade >96.0%, made available by Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany, at the time of the study). As Cys was equal across groups, the 3 levels of supplemental Met correspond to 3 levels of TSAA, i.e. low, 0.4 g/kg less than requirement for TSAA (Aviagen, 2014; Ross 308: broiler nutrition specifications) (further referred to as L); medium, equal to requirement for TSAA (M); and high, 0.4 g/kg more than requirement for TSAA (H). The 3 supplementary levels of GAA were 0, 0.6 and 1.2 g/kg. Starter (0 to 10 d of age), grower (10 to 25 d of age), and finisher (25 to 42 d of age) diets were provided in mash form and the diets per rearing period were formulated to meet or exceed Ross308 recommendations 2014 (Aviagen, 2014). Prior to the trial, samples of stored batches of main ingredients (SBM: DM, 893; CP, 462; EE, 15; and ash, 70 g/kg; and corn: DM, 839; CP, 80; EE, 36; and ash, 14 g/kg) were analyzed for proximate and AA contents. These values were used in least-cost formulation of the experimental diets. Ingredient and calculated compositions are presented in Table 3.1. Weight gain, FI, F:G (adjusted for mortality), mortality, and EPEF were measured on d10, d25, and d42. Stunted birds were removed at the end of the grower period.

Table 3.1. Ingredient and nutrient composition of experimental diets with three levels of methionine (Met) and guanidinoacetic acid (GAA) fed to male broiler chickens[1]

Period	Starter (d0-10)			Grower (d10-25)			Finisher (d25-42)		
Methionine	L	M	H	L	M	H	L	M	H
Ingredient, g/kg									
Corn	600.7	600.2	599.7	640.6	640.1	639.6	647.6	647.8	647.7
SBM CP46	344.1	344.2	344.3	297.5	297.6	297.7	280.7	280.2	280.0
Soybean oil	10.7	10.7	10.7	20.6	20.6	20.6	34.6	34.5	34.4
Dicalcium phosphate	15.4	15.4	15.4	13.6	13.6	13.6	11.4	11.4	11.4
Limestone	14.3	14.3	14.3	13.3	13.3	13.3	12.3	12.3	12.3
Sodium chloride	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
L-Lysine-HCl	3.0	3.0	3.0	2.9	2.9	2.9	2.6	2.6	2.6
DL-Methionine	3.4	3.8	4.2	3.1	3.5	3.9	2.4	2.8	3.2
L-Threonine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix[2]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix[3]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
GAA[4]	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2	0, 0.6, 1.2
Calculated composition, g/kg									
ME, kcal/kg	3000	3000	3000	3100	3100	3100	3200	3200	3200
Crude protein, g/kg	220	223	227	201	204	208	191	192	194
Ether extract, g/kg	35.2	35.2	35.3	46.3	46.4	46.4	60.4	60.5	60.6
Ca, g/kg	9.6	9.6	9.6	8.7	8.7	8.7	7.8	7.8	7.8
P dig., g/kg	4.8	4.8	4.8	4.4	4.4	4.4	3.9	3.9	3.9
Na, g/kg	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Total Lys, g/kg	14.5	14.5	14.5	13.2	13.2	13.2	11.9	11.9	11.9
Total Met, g/kg	7.0	7.4	7.8	6.4	6.8	7.2	5.6	6.0	6.4
Total Met+Cys, g/kg	10.4	10.8	11.2	9.5	9.9	10.3	8.6	9.0	9.4
Total Met+Cys / total Lys	0.71	0.74	0.77	0.72	0.75	0.78	0.72	0.76	0.79

[1] Methionine levels: L = low, 0.4 g/kg less than requirement for TSAA; M = medium, equal to requirement for TSAA; and H = high, 0.4 g/kg more than requirement for TSAA.

[2] Providing per kg of diet: vitamin A (all-trans-retinol), 11000 IU; vitamin D3, 4500 IU; vitamin E (a-tocopherol), 65 IU; vitamin K3 (menaquinone), 3 mg; thiamine, 2.5 mg; choline, 1600 mg; riboflavin, 6.5 mg; pantothenic acid, 18.0 mg; pyridoxine, 3.2 mg; cyano-cobalamin, 0.02 mg; niacin, 60 mg; biotin, 0.18 mg; folic acid, 1.9 mg.
[3] Providing per kg of diet: I, 1.25 mg; Cu, 16 mg; Se, 0.30 mg; Co, 1.0 mg; Mn, 120 mg; Zn, 110 mg; Fe, 20 mg and ethoxyquin, 33mg.
[4] Each diet was prepared in one batch, which was divided in three equal portions to add GAA at a dose of 0, 0.6 and 1.2 g/kg on top.

3.3.2. Sampling procedures

At the end of starter and grower period, one chicken per pen with a weight close to the average weight of the pen, and after 6h of removal of feeders in the pen, was selected for sampling. Birds were gently handled, and fixated in a calm manner prior to decapitation. After bleeding, first and instantaneously, right *pectoralis major* samples were taken, frozen in liquid nitrogen, and stored at −80°C pending analyses for energy metabolites. Next, birds were dissected and following parts were collected and weighed: whole breast, wings, thighs, drumsticks, abdominal fat, heart, pancreas, duodenum, jejunum, and ileum. Abdominal fat was not measurable on d10.

3.3.3. Analysis of ATP, ADP, AMP, Cr and CrN in right *pectoralis major*

The concentrations of adenosine phosphate (ATP, ADP and AMP), Cr and creatinine (CrN) in breast muscles of sampled birds on d10 and d25 were evaluated as indicated in Chapter 2.

3.3.4. Breast muscle proximate analysis

Skinless left breast samples of birds at d25 were analyzed for DM, CP, EE and ash according to AOAC (1990).

3.3.5. Statistical analysis

Data were analyzed using the GLM procedure of SAS Enterprise Guide 7 (SAS Institute, Cary, USA) with a model containing the fixed effects of Met and GAA supplementation, and their interaction term. Means were separated by Duncan's multiple range test. Further, orthogonal contrasts were applied to explore linear and quadratic effects of the main factors. The data were assumed to be statistically significant when $P<0.05$. Mortality was analysed by using the non-parametric Kruskal-Wallis test.

3.4. RESULTS

3.4.1. Broiler performances

Increasing levels of Met enhanced growth in the starter period (linear effect, $P<0.001$ for BW and ADG), feed intake (linear effect, $P<0.001$), whereas F:G decreased (linear effect, $P<0.05$) (Table 3.2). Birds fed the L diets performed less than the M and H groups. Supplemental GAA had no effect on performance in the starter period, except for a linear decrease in F:G ($P<0.05$). At the end of the grower period, BW was 4.6% higher in H birds as compared to M ($P<0.05$), while in turn M outperformed L birds by 4.9% ($P<0.05$). In line with this, a significant linear effect of increasing Met level in the diet was noticed for ADG ($P<0.001$) and ADFI ($P<0.01$). F:G was not significantly affected by supplemental Met, in spite of numerically large differences. Further, supplemental

GAA did not affect growth in this period but lowered F:G ($P<0.001$; 1.68 *vs*. 1.85 for GAA at 0.6 and 1.2 g/kg *vs*. control), and linearly reduced feed intake ($P<0.05$). For both starter and grower period, no interaction effect of Met and GAA was observed.

In the finisher period, a linear dose-response was seen for final BW, ADG and F:G (all $P<0.001$) by supplemental Met. Final BW and ADG were affected by dietary GAA, whereby the birds fed GAA at 0.6 g/kg showed higher final BW as compared to other groups, and birds receiving 1.2 g/kg GAA exhibited lower growth rates than the others groups. FI and F:G were not affected by GAA supplementation. The Met × GAA interaction effect was significant for BW d42 and ADG (Fig. 3.1A). Although differences among GAA groups were not significant within Met levels, it is clear that the 1.2 g/kg GAA group had a lower ADG in the H and L diets compared to the control and the 0.6 g/kg GAA supplementation, whereas in the M diets ADG was highest for the 1.2 g/kg GAA group. The results for the total period (d0-42) were corroborating to the finisher period. Here, in addition, ADFI was significantly lower for GAA 1.2 g/kg than for the other levels ($P<0.05$). Fig 3.1B shows that ADG for GAA at 1.2 g/kg in the H group was lower compared to GAA at 0.6 g/kg (-6.1%, $P<0.05$), whereas the differences among GAA groups were not significant within the L and M diets. The EPEF value linearly increased with increasing Met level (Table 3.2, $P<0.001$), but was not affected by supplemental GAA.

Table 3.2. Effect of methionine levels (Met)[1] and guanidinoacetic acid (GAA) supplementation on BW, ADG, ADFI, FCR, mortality[2] and EPEF[3] in male broiler chickens[4]

Item	Met			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	Met	GAA	Met × GAA	Met		GAA	
											Linear	Quadratic	Linear	Quadratic
Starter (d0-10)														
BW d0, g	37.0	37.2	36.8	37.0	37.1	36.8	0.091	0.187	0.655	0.950	0.315	0.101	0.695	0.397
BW d10, g	219[b]	232[a]	233[a]	228	228	228	1.864	<0.001	0.998	0.995	<0.001	0.099	0.950	0.986
ADG, g/d	18.2[b]	19.5[a]	19.7[a]	19.1	19.1	19.1	0.187	<0.001	0.996	0.995	<0.001	0.118	0.935	0.981
ADFI, g/d	23.8[b]	24.7[a]	25.0[a]	24.8	24.5	24.2	0.147	<0.001	0.187	0.825	<0.001	0.217	0.096	0.974
F:G	1.31	1.27	1.27	1.30	1.29	1.27	0.007	0.057	0.131	0.981	0.036	0.208	0.046	0.902
Grower (d10-25)														
BW d25, g	792[c]	831[b]	869[a]	813	843	836	7.632	<0.001	0.158	0.901	<0.001	0.978	0.202	0.316
ADG, g/d	38.1[b]	39.8[b]	42.3[a]	39.0	40.9	40.4	0.498	0.002	0.224	0.893	<0.001	0.663	0.243	0.296
ADFI, g/d	66.8[b]	69.1[b]	70.9[a]	71.6[a]	68.2[b]	67.1[b]	0.630	0.020	0.006	0.839	0.008	0.863	0.003	0.286
F:G	1.77	1.75	1.69	1.85[a]	1.68[b]	1.68[b]	0.018	0.106	<0.001	0.942	0.101	0.498	<0.001	0.015
Finisher (d25-42)														
BW d42, g	2320[c]	2524[b]	2658[a]	2486[b]	2548[a]	2468[b]	22.85	<0.001	0.009	0.023	<0.001	0.227	0.735	0.188
ADG, g/d	89.5[c]	99.2[b]	104.9[a]	98.2[a]	99.9[a]	95.6[b]	1.129	<0.001	0.032	0.035	<0.001	0.212	0.376	0.275
ADFI, g/d	184	190	180	186	189	179	2.126	0.153	0.126	0.184	0.500	0.112	0.242	0.170
F:G	2.07[a]	1.92[b]	1.72[c]	1.92	1.91	1.88	0.053	<0.001	0.881	0.930	<0.001	0.678	0.805	0.870
Total (d0-42)														
ADG, g/d	54.4[c]	59.2[b]	62.4[a]	58.3[b]	59.8[a]	57.9[b]	0.544	<0.001	0.009	0.024	<0.001	0.232	0.736	0.189
ADFI, g/d	104	107	104	107[a]	107[a]	102[b]	0.900	0.192	0.043	0.183	0.924	0.108	0.049	0.262
F:G	1.91[a]	1.81[b]	1.67[c]	1.84	1.78	1.76	0.021	<0.001	0.181	0.960	<0.001	0.605	0.215	0.736
Mortality, %	5.2	2.8	2.8	4.4	1.7	4.7		0.231	0.879	0.448				
EPEF	272[c]	325[b]	378[a]	308	338	329	7.39	<0.001	0.105	0.870	<0.001	0.697	0.593	0.394

The values are means of 18 replicates per level of main factor.

[1] Methionine levels: L = low, 0.4 g/kg less than requirement for TSAA; M = medium, equal to requirement for TSAA; and H = high, 0.4 g/kg more than requirement for TSAA.

[2] Mortality data within treatment were not normally distributed and hence treatments were compared using the non-parametric Kruskal-Wallis test. No linear or quadratic responses for main factors were computed.

[3] European Production Efficiency Factor: [viability d0-42 (%) * BW d42 (kg) * 100] / [age (d) * FCR d0-42].

[4] Values with different superscripts within a row and main factor are significantly different at (P<0.05).

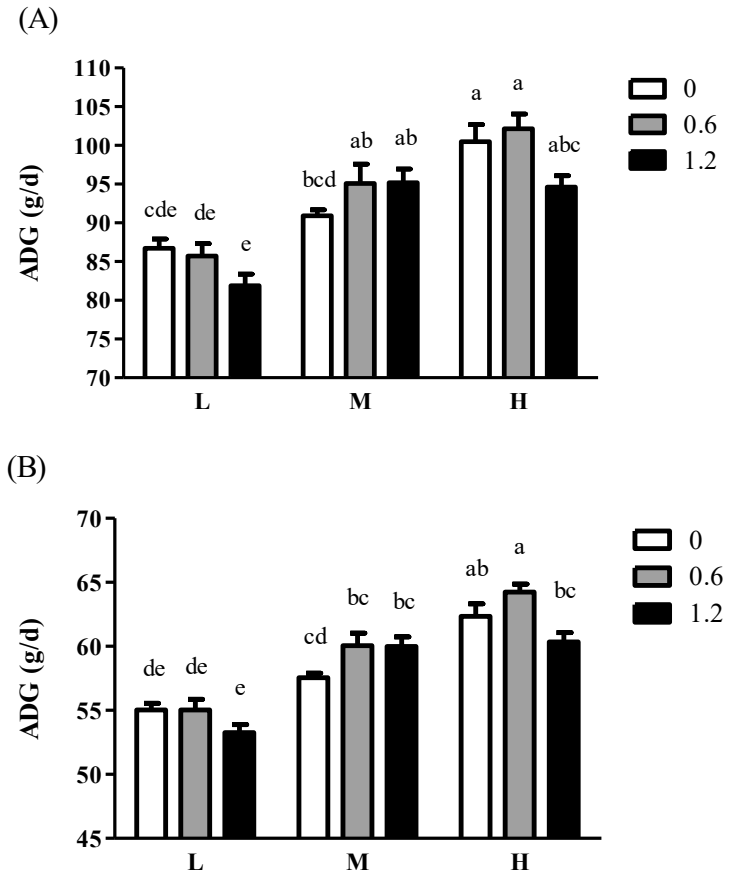

Fig 3.1. Effect of methionine and guanidinoacetic acid (GAA) supplementation on average daily gain (ADG) of male broiler chickens for periods d25-42 (A) and d0-42 (B). Bars with different superscripts are significantly different at P<0.05. Methionine levels: L = low, 0.4 g/kg less than requirement for TSAA; M = medium, equal to requirement for TSAA; and H = high, 0.4 g/kg more than requirement for TSAA. GAA supplementary levels: 0, 0.6 and 1.2 g/kg.

3.4.2. Carcass traits and organ weights

Carcass traits of sampled birds on d10 and d25 are given in Table 3.3. Relative breast weight increased concomitant with higher Met in the diet in a linear manner for both sampling points (P<0.05), while for relative thigh weight only at d25 a linear increase was found with increasing Met level (P<0.05). In contrast, relative wing and drum weight at d10 and abdominal fat weight at d25 was linearly reduced, and also in quadratic manner for the latter with increasing Met level (P<0.05). GAA in the diet did not affect any of the carcass traits at d10 or d25.

Regarding relative organ weights, solely at d10 a few significant effects were found (data not shown). Relative pancreas weight linearly decreased with increasing Met level (0.59, 0.53 and 0.50%, for L, M and H groups, respectively, $P<0.05$), but GAA had no effect on this organ weight. Similarly, relative duodenum and ileum weight was dose-dependently lowered by dietary Met (linear effect, all $P<0.05$). Interestingly, an interaction was found for relative duodenum weight ($P<0.05$). The post-hoc evaluation showed that within birds fed 0.6 g/kg GAA, feeding low Met had higher relative duodenum weight (1.97%) *vs*. feeding medium Met (1.54%, $P<0.05$ compared to low Met).

3.4.3. Breast muscle proximate analysis

Proximate analysis of breast muscle on d25 was not affected by supplementary GAA. Conversely, the dietary Met level showed linear and quadratic effects depending on the parameter. DM content was higher in M and H groups as compared to L ($P<0.05$).

3.4.4. Energy metabolites

There was no effect of dietary Met on any of the energy metabolites (ATP, ADP, AMP, Cr and CrN), with the exception of a linear effect on the ATP concentration at d25 ($P<0.05$) (Table 3.5). A higher Met level resulted in a lower ATP concentration. GAA showed obvious effects on multiple energy metabolites on both sampling days. At d10, ATP, Cr, CrN, and ATP:AMP all showed a linear increase with higher dietary GAA (linear effect, $P<0.05$). Cr levels in breast muscle of GAA 1.2 g/kg fed birds were significantly higher than in the other groups (+12.7 and +6.5% compared to control and GAA 0.6 g/kg, respectively). For birds at d25, linear effects with incremental GAA were noticed for AMP, Cr and CrN (all, $P<0.05$). Cr in breast muscle was higher in both GAA groups compared to control (+10.1%; $P<0.05$), whereas no difference between GAA groups occurred. No treatment interactions were observed for any energy metabolite.

Table 3.3. Effect of methionine levels (Met)[1] and guanidinoacetic acid (GAA) supplementation on carcass traits (% of body weight) in male broiler chickens on day 10 and 25 [2]

Item	Met			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	Met	GAA	Met × GAA	Met		GAA	
											Linear	Quadratic	Linear	Quadratic
Day 10														
Breast	16.5[b]	16.6[b]	17.8[a]	17.1	16.9	16.9	0.85	<0.001	0.791	0.196	<0.001	0.068	0.675	0.685
Thigh	15.8	15.4	16.1	15.9	15.4	16.0	0.93	0.168	0.257	0.072	0.443	0.105	0.960	0.123
Wing	7.9[a]	6.9[b]	6.8[b]	7.3	7.3	7.0	0.66	0.001	0.522	0.561	<0.001	0.051	0.371	0.676
Drum	12.1[a]	11.2[b]	10.8[b]	11.8	11.4	11.0	0.89	0.003	0.121	0.876	0.001	0.372	0.057	0.958
Day 25														
Breast	18.5[b]	19.1[b]	19.6[a]	19.1	19.1	19.1	0.62	0.013	0.966	0.066	0.004	0.881	0.833	0.918
Thigh	16.8	17.2	17.6	17.2	17.1	17.3	0.51	0.054	0.905	0.095	0.018	0.990	0.738	0.812
Wing	5.6	5.9	5.9	5.8	5.8	5.8	0.48	0.352	0.947	0.391	0.245	0.367	0.751	0.940
Drum	14.5	14.6	14.4	14.2	14.4	14.9	1.25	0.924	0.416	0.897	0.889	0.702	0.183	0.703
Abdominal fat	0.96[a]	0.79[b]	0.81[b]	0.87	0.88	0.81	0.086	0.002	0.343	0.239	0.005	0.022	0.328	0.397

The values are means of 18 replicates per level of main factor.

[1] Methionine levels: L = low, 0.4 g/kg less than requirement for TSAA; M = medium, equal to requirement for TSAA; and H = high, 0.4 g/kg more than requirement for TSAA.

[2] Values with different superscripts within a row and main factor are significantly different at ($P < 0.05$).

Table 3.4. Effect of methionine (Met)[1] and guanidinoacetic acid (GAA) supplementation on breast muscle proximate analysis (%) in male broiler chickens on day 25 [2]

Item	Met			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	Met	GAA	Met × GAA	Met		GAA	
											Linear	Quadratic	Linear	Quadratic
DM	23.6[b]	23.9[a]	24.0[a]	23.9	23.8	23.8	0.23	0.034	0.783	0.786	0.032	0.312	0.168	0.202
Ash	1.12	1.18	1.20	1.16	1.17	1.17	0.096	0.418	0.967	0.584	0.189	0.732	0.810	0.915
EE	1.07	1.15	1.08	1.09	1.12	1.10	0.092	0.076	0.770	0.314	0.702	0.025	0.819	0.519
CP	21.5	21.8	21.8	21.5	21.8	21.7	0.26	0.061	0.159	0.582	0.018	0.180	0.536	0.785

The values are means of 18 replicates per level of main factor

[1] Methionine levels: L = low, 0.4 g/kg less than requirement for TSAA; M = medium, equal to requirement for TSAA; and H = high, 0.4 g/kg more than requirement for TSAA.

[2] Values with different superscripts within a row and main factor are significantly different at ($P<0.05$).

Table 3.5. Effect of methionine levels (Met)[1] and guanidinoacetic acid (GAA) supplementation on energy metabolites in male broiler chicken breast at day 10 and 25 [2]

Item	Met			GAA (g/kg)				Significance						
	L	M	H	0	0.6	1.2	SEM	Met	GAA	Met × GAA	Met		GAA	
											Linear	Quadratic	Linear	Quadratic
Day 10														
ATP, µmol/g FM[3]	3.93	3.75	3.94	3.69	3.88	4.05	0.385	0.390	0.075	0.320	0.919	0.190	0.024	0.934
ADP, µmol/g FM	1.36	1.41	1.30	1.44	1.35	1.29	0.223	0.708	0.490	0.442	0.790	0.553	0.256	0.735
AMP, µmol/g FM	0.90	0.92	0.91	0.85	0.96	0.98	0.118	0.674	0.124	0.099	0.813	0.425	0.062	0.484
Cr, mg/kg FM	4355	4111	4084	3942[b]	4168[b]	4441[a]	336.5	0.317	0.045	0.143	0.201	0.549	0.016	0.896
CrN, mg/kg FM	57.7	58.0	62.1	51.3	57.6	68.8	13.25	0.813	0.078	0.450	0.577	0.777	0.023	0.710
ATP:AMP	3.1	2.9	3.2	2.7	3.1	3.4	0.59	0.653	0.098	0.174	0.939	0.544	0.035	0.968
Cr:CrN	108.9	82.3	74.3	91.9	96.9	76.8	34.06	0.195	0.572	0.184	0.089	0.594	0.463	0.482
Cr:ATP[4]	8.5	8.4	8.1	8.3	8.3	8.5	0.65	0.627	0.926	0.113	0.383	0.720	0.791	0.784
Day 25														
ATP, µmol/g FM	3.67	3.54	3.15	3.18	3.70	3.50	0.420	0.093	0.113	0.453	0.040	0.550	0.203	0.108
ADP, µmol/g FM	1.24	1.16	1.92	1.22	1.18	1.18	0.302	0.891	0.960	0.618	0.764	0.674	0.801	0.881
AMP, µmol/g FM	0.87	0.93	1.00	0.85	0.93	1.02	0.119	0.223	0.062	0.242	0.102	0.916	0.022	0.957
Cr, mg/kg FM	4210	4371	4409	4054[b]	4464[a]	4472[a]	298.0	0.479	0.026	0.186	0.289	0.706	0.021	0.191
CrN, mg/kg FM	55.3	56.5	60.9	46.3[b]	60.1[ab]	66.2[a]	19.83	0.766	0.052	0.956	0.494	0.822	0.013	0.571
ATP:AMP	3.4	3.7	3.1	3.0	3.8	3.2	1.05	0.674	0.443	0.278	0.575	0.516	0.622	0.248
Cr:CrN	96.0	111.5	85.1	126.2	83.1	83.2	39.66	0.516	0.107	0.435	0.645	0.307	0.065	0.277
Cr:ATP[4]	9.1	9.6	10.6	10.1	9.6	9.6	0.98	0.215	0.739	0.928	0.063	0.715	0.510	0.659

The values are means of 18 replicates per level of main factor

[1] Methionine levels: L = low, 0.4 g/kg less than requirement for TSAA; M = medium, equal to requirement for TSAA; and H = high, 0.4 g/kg more than requirement for TSAA.

[2] Values with different superscripts within a row and main factor are significantly different at ($P<0.05$).

[3] Fresh muscle.

[4] Ratio calculated when both are expressed in µmol/g FM

3.5. DISCUSSION

It was hypothesized that the effects of supplemental GAA might depend on the provision of Met, which delivers SAM for methylation of GAA to synthesize Cr. Surprisingly, no interaction effects were found between Met level and supplemental GAA, apart from growth in the finisher phase and relative pancreas weight in 10-day old birds.

3.5.1. Methionine supplementation level affects performance

Corroborating with ample literature, increasing supplemental Met improved performance and increased relative breast and thigh weight and breast muscle protein content (e.g. Conde-Aguilera et al., 2013; Drazbo et al., 2015; Wen et al., 2017). It is well known that Met supplementation improves the AA balance and consequently promotes growth by increasing protein synthesis and decreasing fat synthesis, leading thereby to enhance feed efficiency. Effects of graded levels of Met were particularly notable in the finisher period. Concerning TSAA responses, it has been demonstrated that TSAA requirements for maximal gain to feed often exceed weight gain requirements (e.g. Baker et al., 1996; Mack et al., 1999), thus corroborating with our findings. This would be even more prominent with modern high breast meat yielding broiler strains. Aviagen (2014) (Ross 308: broiler nutrition specifcations) recommends a ratio M+C/Lys of 0.76 and 0.78 (apparent ileal digestible, AID level) for grower and finisher, respectively, and Spek (2018) suggests minimal M+C/Lys ratios of 0.73-0.74 (maximum growth-maximum feed efficiency) (SID level), for both grower and finisher. In our study, this ratio was approximately 0.75 (AID level) for the M groups in both the grower and finisher period, suggesting that there might have been little room for improvement beyond Met supplementary level in M groups, though it occurred. Further, a pronounced Met effect on abdominal fat in 25-day old broilers was found. Indeed, the addition of Met has been correlated with the tendency to have less total body fat (Rostagno et al., 1995). The anabolic effect of Met is partly due to increased substrate supply at the sites of protein synthesis, however recent insights highlight its action as signal molecule in the regulation of protein metabolism. Del Vesco and Gasparino (2013) demonstrated that insulin-like growth factor-I (IGF-I) gene expression in the liver was higher in animals fed Met supplementation than in animals fed control diet, but growth hormone receptor and IGF-I gene expression in breast muscle tissue was not affected. However, opposite to our study, graded Met levels in their study were below or equal to requirements. Further, feeding high Met diets compared to low Met diets gave alterations in gene expression in breast muscle of chickens; it increased the expression of the anabolic factors IGF-I and mTOR, while it decreased the expression of *atrogin-1 and forkhead box O 4* involved in protein degradation (Wen et al., 2014). Other studies have confirmed that in the fast-growing chicken supplementary Met promotes anabolism and reduces catabolic processes favouring muscle protein synthesis (e.g. Del Vesco et al., 2015; Wen et al., 2017), though others did not found conclusive evidence (e.g. Zeitz et al., 2019).

3.5.2. Guanidinoacetic acid affects performance as influenced by supplementation level

In the current study, a lack of effect of GAA supplementation on BWG in starter and grower periods was seen, in contrast to 0.6 g/kg GAA in finisher period. Next to that, GAA linearly reduced F:G in starter and grower period, in some part due to a reduction in FI, particularly for the 1.2 g/kg GAA groups. Overall, 0.6 g/kg GAA improved growth, but not when fed at 1.2 g/kg, and only numerical, nonetheless substantial (6 and 8 points reduction for 0.6 and 1.2 g/kg GAA, respectively), improvements for feed efficiency were found. The fact that 1.2 g/kg GAA did not ameliorate growth might depend on the interaction with dietary Met, and is discussed below. The beneficial effects of supplementary GAA on performance have been demonstrated in many other reports (e.g. Abudabos et al., 2014; Cordova-Noboa et al., 2018ab; EFSA, 2009; Michiels et al., 2012; Ringel et al., 2008ab, Chapter 1, section 1.4.2.1). Similar to Chapter 2, data suggest that feeding GAA at 1.2 g/kg reduces FI, and it was even more pronounced in the current study. However, when we only consider groups fed adequate Met, performance indices for birds fed 0.6 and 1.2 g/kg GAA in all rearing phases were very similar; and compared to control, ADFI was numerically almost equal (-1.0%), lower (-6.1%) or higher (+5.4%) in starter, grower and finisher period, respectively, whereas growth was notably higher for GAA fed birds as compared to control in grower (+3.6%) and finisher (+4.6%). This urges for a cautious interpretation of the data, but irrespective of that, it can be stated that when Met is adequate, performance is improved by supplemental GAA.

The effect of supplementing the diet with GAA on feed intake remains equivocal, and as illustrated in the current study, it might depend on other nutritional factors, possibly explaining part of the contradictory results elicited in literature. Lemme et al. (2010a) showed that GAA supplementation reduced FI in turkeys, and FI reduction was also found in some studies reported in EFSA (2009), at least for the whole rearing period, and similarly by Kodambashi et al. (2017). In contrast, EFSA (2009) (some studies), Michiels et al. (2012), Mousavi et al. (2013), Ringel et al. (2008b), Yazdi et al. (2017) found no effect of GAA on FI; congruent to the overall conclusion made in Chapter 1, section 1.4.2.1. In the current study dietary GAA elevated the breast muscle Cr load at d10 and d25 (here not measured at d42), whereas in Chapter 2 a significant increase was only found on d42. Many studies confirmed the Cr loading effect of GAA (EFSA, 2009; Michiels et al., 2012; Lemme et al., 2007a; and Ringel et al., 2008b). This finding indicates that GAA is an efficient precursor in terms of Cr loading to improve energy homeostasis in muscle cells. It should be mentioned that Cr concentrations in breast meat represent both phosphorylated and non-phosphorylated Cr in this study.

3.5.3. Effect of guanidinoacetic acid was dependent on dietary methionine in the finisher period

First it should be noted that no interactive effects between dietary Met and GAA were found in the starter and grower phase. As GAA reduced F:G, it is fair to state that for the

period d0-25, this beneficial effect was independent on dietary Met. In line with the hypothesis, there was the interaction between dietary Met and GAA for growth in the finisher phase, showing that at deficient and surfeit Met, GAA at 1.2 g/kg negatively affected growth. Lemme et al. (2010b) concluded that 0.8 g/kg supplemental GAA was not effective in improving F:G at deficient Met, whereas it enhanced feed efficiency at adequate Met supply. As the negative effect on growth did not occur with adequate Met level, it can be excluded that this effect was solely due to failure to provide labile methyl groups and/or hyperhomocysteinemia. Indeed, feeding broilers at current GAA supplementation levels or even at 3 g/kg failed to prove accumulation of HCy (EFSA, 2016). Recently, Robinson et al. (2016a) concluded that in neonatal piglets transmethylation is sacrificed to maintain protein synthesis when Met is limiting, which is opposite to our speculation, though it could depend on physiological state and species. Nonetheless, our findings are still surprising considering that the graded levels of Met in our study (+0.4 g/kg) were way smaller than for example in the study of Lemme et al. (2010b).

Various studies have shown that GAA instigates a reduction in FI, mostly in the finisher phase, whilst maintaining growth (e.g. Chapter 2). Here we found that growth was negatively affected when birds were fed 1.2 g/kg GAA at a deficient or surfeit Met level. As outlined above, in the case of deficient Met, this could be explained by a decreased Met availability for growth. The results with 1.2 g/kg GAA and high Met are more difficult to interpret. Nonetheless, several assumptions can be made. First, as Met activates hepatic MATIII, more SAM might be synthesized at high Met level. This might lead to higher SAH production, as with feeding 1.2 g/kg GAA ample GAA is available for methylation. It is known that SAH is inhibitory for most methyltransferases. Thus other methylation reactions such as DNA methylation or conversion of phosphatidylethanolamine to PC might be disbalanced in these peculiar circumstances. Here, it is suggested that homeostasis in furnishing various methylation pathways might be disturbed, rather than methylation potential *per se*, in line with the conclusion made by Robinson et al. (2016b). Secondly, when the hepatic content of Met is high, this AA is not only rapidly converted to SAM by MATIII, but also the catalysis by GNMT acts as a shunt to remove SAM by reaction of SAM with Gly into SAH and sarcosine as products. Moreover, SAM inhibits MTHFR, and consequently the synthesis of MTHF, which in turn shows a negative feedback to GNMT. Thus, taking together, possible higher SAM production might result in higher sarcosine levels, even it is believed that sarcosine is readily demethylated. In a study by Kalmar et al. (2012) it was found that feeding DMG to broiler chickens numerically, but consistently, showed reduced FI. DMG levels in plasma were dramatically increased, which leads to the speculation that sarcosine levels might have been uploaded as well, though such data were not given in that paper. Finally, polyamine synthesis might be affected as well. Higher Cr downregulates kidney AGAT activity in an effort to minimize Arg metabolization to GAA when Cr is adequate. Liu et al. (2015) showed lower AGAT expression in porcine kidney when feeding GAA. In our study, in birds sampled at d25, we found that Cr levels in breast muscle of H groups were 10% lower when supplemented with 1.2 g/kg GAA

as compared to 0.6 g/kg, and this was only 10% higher than controls. It may indicate lower incorporation efficiency of Cr in muscle tissues when surfeit Met is combined with 1.2 g/kg GAA. Another consequence of this negative feedback mechanism entails that less L-ornithine is synthesized. The net effect on L-ornithine availability is not clear, since L-ornithine in birds is also, and probably mostly, derived by action of arginase. Nonetheless, in the recent paper by DeGroot et al. (2018), numerical reductions were found in plasma ornithine with graded GAA, but this occurred in Arg deficient diets. By virtue of ornithine decarboxylase, L-ornithine is converted into putrescine, and further metabolized to the bioactive polyamines spermidine and spermine whereby SAM donates successively propylamino groups. Gonzalez-Esquerra and Leeson (2006) found that duodenal spermidine was positively correlated with FI under TN conditions, whereas a positive association was found between spermine and FI when birds were subjected to HS, also depending on age of birds. Further, one can argue that GAA is able to spare Arg, and might render Arg more available for L-ornithine production. It can be even said that more Arg would potentially enable more NO production, which in turn has shown to deplete hepatic SAM (Ruiz et al. 1998). To summarize, the above mechanisms might have contributed to the observed lower FI and reduced growth, but as no direct evidence is provided here, this warrants further investigation. Eventually, there is a link with the hepatic energy status theory that was suggested to affect feed intake when using GAA in Chapter 2. Finally, Michiels et al. (2012) found that GAA at 1.2 g/kg remarkably elevated IGF-I circulatory concentrations. Knowing that Met is also able to enhance IGF-I expression in liver (Del Vesco and Gasparino, 2013) and that excess IGF-I may compromise glucose homeostasis or may decrease growth hormone release due to inhibitory feedback (Scanes, 2009), it can be conceived that the additional effect of GAA at 1.2 g/kg and high Met might have impacted hypothalamic-pituitary-adrenal (HPA) axis in some way or another.

3.6. CONCLUSION

Under the conditions of the current study, the effects of dietary GAA supplementation were only influenced by the dietary Met level in the finisher period. We showed that at deficient and surfeit Met, GAA at 1.2 g/kg negatively affected growth. It is suggested that disturbances in methylation homeostasis and/or changes in Arg metabolism could explain these findings. A deeper understanding of the relation between feeding GAA and liver metabolic functions is warranted. Overall, positive outcomes of feeding GAA might depend on proper formulation of other factors in the diet, not in the least to sulfur AA provision, methyl donors, and vitamins like B9 and B12.

3.7. ACKNOWLEDGEMENTS

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CHAPTER 4

GUANIDINOACETIC ACID SUPPLEMENATION IMPROVES FEED CONVERSION IN BROILERS SUBJECTED TO HEAT STRESS ASSOCIATED WITH MUSCLE CREATINE LOADING AND ARGININE SPARING

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CHAPTER 4

GUANIDINOACETIC ACID SUPPLEMENATION IMPROVES FEED CONVERSION IN BROILERS SUBJECTED TO HEAT STRESS ASSOCIATED WITH MUSCLE CREATINE LOADING AND ARGININE SPARING

4.1. ABSTRACT

It was hypothesized that dietary GAA, the precursor of Cr, would be beneficial to heat stressed finisher broilers because of improved cellular energy status and Arg sparing effects. A total of 720 one-day-old male Ross 308 broilers were allocated to 3 treatments: 0, 0.6 or 1.2 g/kg GAA added to complete corn-SBM diets and fed for 39d, with 12 replicates (20 birds each) per treatment. A chronic cyclic HS model (T to 34°C with 50-60% RH for 7h daily) was applied in finisher phase (d25-39). Samples were taken on d26 and 39 to determine thrombocytes, white blood cells, corticosterone, protein and AA in blood and Cr, PCr and ATP in breast muscle. In addition, meat quality was assessed on d40, after overnight fasting. GAA at 1.2 g/kg decreased F:G compared to control in grower phase (1.32 *vs*. 1.35) due to slightly reduced FI. In finisher period, 0.6 and 1.2 g/kg GAA reduced FI more pronounced (1.1 and 3.3%, respectively), whilst F:G improved substantially (1.76, 1.66 and 1.67 for control, 0.6 and 1.2 g/kg GAA, respectively). Mortality outcomes highlight that GAA-feeding improved survival during HS, in particular towards the end, supported by lower panting frequency (linear effect). Plasma Arg was higher with increasing dietary GAA (+18.3 and +30.8% for 0.6 and 1.2 g/kg GAA, respectively) on d26 and a trend for linear increase was detected on d39. This suggests enhanced availability of Arg for other metabolic purposes than *de-novo* GAA formation. Examination of breast muscle revealed at both sampling days increases of PCr (linear effect at d26 and effect at d39), free Cr (at d39), total creatine (TCr) (both days), and, PCr:ATP ratio (linear effect at d26 and effect at d39) with increasing dietary GAA. GAA supplementation improved feed conversion and survival during chronic cyclic HS; associated with enhanced breast muscle energy status and Arg sparing effect.

4.2. INTRODUCTION

Heat exposure affects poultry production on a world-wide basis and has a significant impact on well-being and production. The existence of feathers on the body, absence of sweat glands, and high metabolic rate of modern strains makes broilers very susceptible to high T. HS, therefore occurs when the amount of heat produced by an animal surpasses the animal's capacity to dissipate the heat to its surrounding environment (Lara and

Rostagno, 2013). The physiological consequences of HS are numerous and can be summarized as: increased body core T, reduced voluntary FI, depressed immunity, alteration of the electrolyte balance and blood pH, impairment in endocrine and reproductive functions, decreased energy availability to cells, alteration in the digestion and metabolism of various nutrients, disruption in the structure and function of intestinal epithelium, alteration of the normal and protective microbiota, and increased circulatory cortisol and corticosterone levels (Lin et al., 2006; Syafwan et al., 2011; Renaudeau et al., 2012; Lara and Rostagno, 2013; Nawab et al., 2018). Consequently, a number of mitigation strategies have been proposed (e.g. Syafwan et al., 2011; Renaudeau et al., 2012; Loyau et al., 2015). As mentioned, one of the primary consequences of heat discomfort is the reduction of FI. In practice, nutritionists may formulate more energy dense and higher fat diets in summer periods to counteract the reduced intake and to lower body heat increment (Renaudeau et al., 2012). Next, it was shown that during acute HS the cellular energy demand increases (Yang et al., 2010) and that during chronic HS mitochondrial ATP generation is reduced (Azad et al., 2010). Also, HS induces higher utilization of muscle energy reserves in the form of glycogen (Gonzalez-Esquerra and Leeson, 2006). On this note, it could be perceived that enhancing the cellular Cr/PCr energy-shuttle system might offer substantial benefits for the broiler subjected to HS. Indeed, this system functions as a backup to the ADP/ATP-cycle to store and mobilize energy when required on short notice (Wyss and Kaddurah-Daouk, 2000). Importantly, it has been repeatedly shown in broilers that dietary GAA is efficiently converted to Cr (Chapter 1), and increases Cr loading in muscle and the ratio PCr:ATP which serve as markers for cellular energy status, concomitant with improved performances in broilers under TN conditions (e.g. Lemme et al., 2007b; EFSA, 2009; Michiels et al., 2012; Chapter 2 and 3). Furthermore, Chamruspollert et al. (2004) reported that higher T slowed Arg metabolism, negatively affecting Cr synthesis pathways, which was evidenced by reduced Cr and CrN levels in excreta.

Another primary physiological response during HS is the increased blood flow to the body surface or upper respiratory tract to dissipate internal body heat (Kregel et al., 1988; Yahav et al., 1997; Zhu et al., 2014). Therefore, the blood flow to some visceral organs is significantly reduced (Horowitz, 2003). In this respect, Arg plays a pivotal role as it is the nitrogenous precursor for the endogenous synthesis of NO by nitric oxide synthase (Wu and Meininger, 2009). NO is a potent vasodilator that directly relaxes vascular smooth muscle and modulates or inhibits the production and release of vasoconstrictors such as serotonin. On a similar note, NO plays an important role in glucose transport into the muscle during muscular contraction (Balon and Nadler, 1997). It was shown that increasing Arg concentrations promotes NO synthesis in a variety of cells. Higher Arg bioavailability might thus be beneficial for heat stressed broilers, as it has been demonstrated in heat stressed Pekin ducks (Zhu et al., 2014). In similar situations of low oxygen supply, as evident in HA, it was shown that the Arg requirement for male broilers up to 3-weeks of age was higher than NRC recommendations for optimal growth (1.53 *vs.* 1.25%) and that maximal plasma NO levels were obtained at 1.56% Arg in the diet (Khajali et al., 2010). Further, available evidence shows that NO is required for appetite

modulation in animals. A low availability of NO blocks the orexigenic effect of neuropeptide Y, ghrelin, orexin A and growth hormone in rats (Farr et al., 2005). Thus, Arg via its impact on enhanced plasma NO levels could be important in shaping FI, which is a major constraint for productivity of broilers under HS conditions. However, it should be recalled that increased ghrelin has a reducing effect on FI in birds, and the effect of orexin A is controversial (Song et al., 2013). On the other hand, increased NO restores the leptin-induced decrease in FI in rats (Squadrito et al., 1994; Calapai et al., 1998) and chickens (Yang and Denbow, 2007). Nonetheless, Wang et al. (2014) demonstrated that dietary Arg can be converted to NO to modulate feeding behaviour in ducks. Further, conclusively, it was demonstrated that dietary supplemented GAA is able to spare Arg in broilers (Dilger et al., 2013; DeGroot et al., 2018) due to the fact that less endogenous GAA is synthesized. Consequently, more Arg would be available for its protein and non-protein functions, such as precursor for NO and polyamines. Regarding the latter, L-ornithine is the precursor of putrescine, which in turn is converted to the polyamines spermidine and spermine by merging with recurrent propylamino groups from SAM. Interestingly, lower plasma ornithine was reported in birds raised at high ambient T (Balnave et al., 1999).

Altogether, it was hypothesized that dietary supplementation with GAA, as precursor of Cr, would be beneficial to heat stressed finishing broilers because of: 1/ improved energy status, due to Cr's pivotal role in energy homeostasis; and 2/ improved Arg metabolism, due to Arg sparing effects. These hypotheses were tested in heat stressed finishing broilers in a model of chronic cyclic HS (Akbarian et al., 2014). Further, breast meat quality was assessed as previously was shown that dietary Cr in pigs could delay muscle pH decline postmortem, with a possible positive effect on WHC (James et al., 2002b) and that HS may affect negatively meat quality aspects (Song and King, 2015). Though vast literature is available on the use of GAA in broilers, only few addressed its application under HS conditions. Amiri et al. (2019) showed that dietary GAA improved birds' growth in a cyclic HS model, which was more pronounced in low CP diets as compared to normal protein diets, whereas Esser et al. (2017) indicated that carcass and breast yield at slaughter was elevated in GAA fed broilers when they were subjected to 48h of 32°C prior to slaughter.

4.3. MATERIALS AND METHODS

4.3.1. Animals, housing and diets

The experiment was carried out according to the guidelines of the Ethics Committee of The Faculty of Veterinary Medicine, Ghent University (Belgium) for the humane care and use of animals in research (reference EC2016/49). A total of 720 day-old male Ross 308 broilers (Broeierij Vervaeke-Belavi, Tielt, Belgium) were allocated to 3 dietary treatments with 12 pen replicates of 20 birds each (density of 14.7 birds/m^2), according to a completely randomized design. Prior to arrival, the solid floor was covered with fresh wood shavings (1.5 kg/m^2) and no additional filling or cleaning of the floor was

executed during the trial. The light schedule was 23L:1D and 18L:6D (18L from 4:00 am to 10:00 pm) in the periods d0-7 and beyond, respectively. Stable T was 34°C at setting, and linearly decreased to 22°C by d25. During the first 5 days of trial additional infra-red lamp heating (one per pen) was used. From d25 onwards, a specific T and moisture regime was followed to induce chronic cyclic HS (Fig. 4.1). The chronic cyclic HS model was applied in the entire finisher phase only. The basal T was 22°C. Between 8:00 and 9:00 am, the T was gradually increased to 34°C and this high T was then maintained for 7 hours (until 4:00 pm). After that, the T was decreased to the basal level, again taking one hour. For practical reasons, at days of sampling, the HS cycle and lightening scheme was shifted two hours earlier. Air humidity was kept between 50 and 60% RH during the HS episodes by nebulization of water. The broilers were vaccinated the 1st day of age against Newcastle Disease and Infectious Bronchitis at the hatcheries facilities. At 18 days of age the vaccination against Newcastle Disease was repeated with Nobilis ND Clone 30 by spraying.

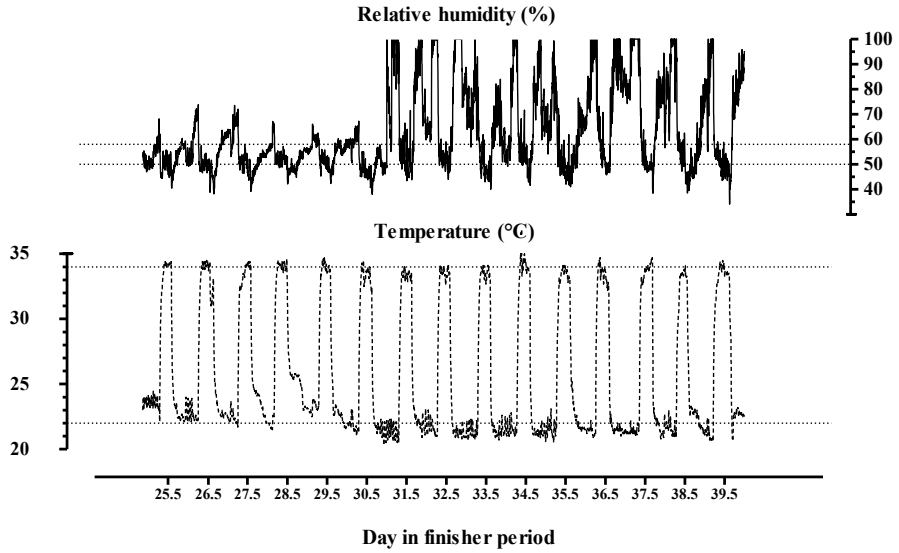

Fig. 4.1. Relative humidity (upper line, right Y-axis) and temperature (lower line, left Y-axis) from d25 until end of experiment, d39. The chronic cyclic HS model was implemented from d25 till d39. During time of low temperatures (22°C), RH tended to increase on some days, depending on outside weather conditions and build-up moisture in stable. On d28 and 29, low temperatures were higher than 22°C because of high outside temperatures.

The dietary treatments were: 0, 0.6 or 1.2 g/kg GAA (CreAMINO®, GAA, feed grade >96.0%, made available by Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany, at the time of the study) added to complete corn-SBM diets, referred to as CON, GAA0.6 and GAA1.2, respectively (Table 4.1). Birds were fed a starter (d0-10), grower (d10-25) and finisher (d25-39) diet. The strategy of high energy (high fat) diet formulation was applied. Starter feed was pelleted on a 2.5x20 mm die, whereas grower

and finisher diets were pelleted on a 3.0x35 mm die. Basal diets were formulated according to Ross308 Guidelines (Aviagen, 2014: Ross 308: broiler nutrition specifications) for energy, digestible AA, Ca and P, and according to National Research Council (NRC) (1994), Centraal Veevoederbureau (2016) and Ross308 Guidelines (Aviagen, 2014: Ross 308: broiler nutrition specifications), for other nutrients. Choline levels in starter and grower were set slightly higher (approx. 30 %) than Ross308 Guidelines (Aviagen, 2014) to ensure re-methylation pathways and improve Cys availability for GSH synthesis in the HS finisher period. Proximate and AA composition of major ingredients was determined to serve diet formulation. Experimental diets were analysed for proximate and AA composition, GAA, Cr, CrN, and choline as described by Michiels et al. (2012) and DeGroot et al. (2018). Feeds and water were given *ad libitum* throughout the trial until d39. On d39, animals were fasted overnight. Pen live-weight and feed refusals were recorded at d0, 10, 25, and 39. Weight gain, FI, F:G (adjusted for mortality and calculated as total FI divided by total gain including weight of lost birds), mortality and EPEF were measured on d10, 25 and 39. Stunted birds were removed at the end of the starter and grower period.

4.3.2. Measurements and sampling during acute and chronic heat stress

Panting frequency was measured on d27 and 38 during the HS protocol (starting >4h after inducing HS on that respective day; 2 *at random* chickens per pen and day) based on video recordings. Panting frequency was counted as the number of breaths per minute using a timer. On d26 (acute, HS for one day) and d39 (chronic, HS for 14 days), one animal per pen with weight close to average weight of the pen was selected. Sampling started >4h after inducing HS on that day. Rectal T was determined immediately with a digital thermometer inserted minimum 3 cm in the cloaca. Next, birds were placed in a dark box for 10 min in the stable, followed by induction of euthanasia by 40 mg pentobarbiturate per kg BW intramuscularly (left breast). Then, blood was taken by puncture in heart with an 80 mm needle 22 G, and collected in two K_2EDTA-tubes and one plain tube. One K_2EDTA-tube was used for fixation of blood by the addition of Transfix® reagent (Caltag Medsystems, Ltd., UK) according to the manufacturer's instructions, pending analysis for thrombocytes and white blood cell differentials, whereas the unfixed K_2EDTA blood sample was employed for harvesting plasma, followed by storage at -80°C, pending analysis of blood protein and AA. Serum was stored at -20°C and used for corticosterone quantification. Immediately after euthanasia, the skin was removed from the breast, and a 2 cm flat piece of the right breast (*pectoralis major*, middle of right breast in length, and 2 cm away from median, from 0.5 cm in depth) were transferred to liquid N_2. Snap frozen tissue samples were broken, collected in precooled cryovials and submerged in liquid N_2 and stored at -80°C pending analysis of energy metabolites.

Table 4.1. Ingredient and analysed composition of corn-soybean based basal diets for the starter (d0-10), grower (d10-25) and finisher (d25-39) phase (as-is basis)[1].

Item	Dietary treatment								
	Starter			Grower			Finisher		
Supplemental GAA, g/kg	0.0	0.6	1.2	0.0	0.6	1.2	0.0	0.6	1.2
Ingredients, g/kg									
Corn	559.6	559.6	559.6	574.8	574.8	574.8	609.2	609.2	609.2
SBM Hipro	279.1	279.1	279.1	273.6	273.6	273.6	207.8	207.8	207.8
Corn gluten meal							3.50	3.50	3.50
Full fat toasted soybeans	80.0	80.0	80.0	50.0	50.0	50.0	50.0	50.0	50.0
Animal fat	11.1	11.1	11.1	52.4	52.4	52.4	53.6	53.6	53.6
Soybean oil	20.0	20.0	20.0	5.0	5.0	5.0	5.0	5.0	5.0
Dicalciumphosphate	16.6	16.6	16.6	13.0	13.0	13.0	10.0	10.0	10.0
Limestone	10.5	10.5	10.5	10.8	10.8	10.8	11.0	11.0	11.0
DL-methionine	4.0	4.0	4.0	3.6	3.6	3.6	2.7	2.7	2.7
L-lysine HCl	3.2	3.2	3.2	2.4	2.4	2.4	2.6	2.6	2.6
Sodium chloride	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Sodium bicarbonate	2.6	2.6	2.6	2.5	2.5	2.5	4.0	4.0	4.0
L-threonine	1.9	1.9	1.9	1.4	1.4	1.4	1.0	1.0	1.0
L-valine	0.9	0.9	0.9	0.5	0.5	0.5			
L-arginine	0.6	0.6	0.6	0.02	0.02	0.02	0.1	0.1	0.1
3-phytase (500 FTU/kg)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Coccidiostatic[2]	0.5	0.5	0.5	0.5	0.5	0.5	0.1	0.1	0.1
Vitamin and Mineral premix[3]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Choline chloride 50S	2.3	2.3	2.3	2.4	2.4	2.4	0.8	0.8	0.8
GAA[4]		0.6	1.2		0.6	1.2		0.6	1.2
Calculated nutrient composition									
ME, kcal/kg	3000	3000	3000	3120	3120	3120	3220	3220	3220
AID Met+Cys, g/kg	9.5	9.5	9.5	8.7	8.7	8.7	8.0	8.0	8.0
AID Lys, g/kg	12.8	12.8	12.8	11.5	11.5	11.5	10.2	10.2	10.2
AID Thr, g/kg	8.6	8.6	8.6	7.7	7.7	7.7	6.8	6.8	6.8
AID Arg, g/kg	13.4	13.4	13.4	12.3	12.3	12.3	10.9	10.9	10.9
AID Val, g/kg	9.6	9.6	9.6	8.7	8.7	8.7	7.9	7.9	7.9

Analysed nutrient composition									
Dry matter, g/kg	885	888	886	884	885	885	884	884	883
Crude ash, g/kg	57	58	57	53	52	53	46	47	47
Crude protein, g/kg	218	222	225	206	212	211	192	202	198
Ether extract, g/kg	71	72	72	93	92	92	94	93	93
Ca, g/kg	9.5	9.7	9.6	8.6	8.5	8.7	7.4	7.4	7.5
P, g/kg	6.8	6.9	6.9	5.8	5.8	5.9	5.0	5.1	5.0
Amino acids, g/kg									
Met	6.8	6.8	6.9	6.4	6.1	6.3	5.7	5.6	5.8
Cys	3.4	3.4	3.4	3.3	3.2	3.3	3.2	3.2	3.1
Lys	14.3	14.2	14.4	13.2	12.8	13.3	11.5	11.6	11.7
Thr	10.2	10.0	10.2	9.3	9.1	9.3	8.3	8.3	8.2
Arg	15.2	15.0	15.0	13.8	13.6	13.8	12.1	12.2	11.9
Val	11.3	11.1	11.2	10.3	10.2	10.3	9.1	9.3	9.1
$Gly_{equivalents}$	17.1	16.7	16.7	15.9	15.8	16.1	14.6	14.9	14.6
GAA, mg/kg	<1	512	1269	<1	581	1200	<1	597	1179
Creatine, mg/kg	<1	<1	<1	<1	<1	<1	<1	<1	<1
Creatinine, mg/kg	<1	<1	<1	<1	<1	<1	<1	<1	<1
Choline, mg/kg	2210	2360	2020	2040	2280	2260	1400	1440	1540

[1] Diet formulation was based on analysed values for corn, soybean meal (48% CP), full fat soybeans and corn gluten meal (61% CP) provided by Evonik, and matrix values for other ingredients provided by DSM Nutritional Products Belgium (updated matrix by January 2016) (Deinze, Belgium); [2] Coccidiostatic; salinomycin in starter and grower, and narasin in finisher diet; [3] Vitamin and trace elements premix providing per kg of diet: vitamin A (retinyl acetate), 10000 IU; vitamin D3 (cholecalciferol), 2500 IU; vitamin E (dl-α-tocopherol acetate), 50 mg; vitamin K3 (menadione), 1.5 mg; vitamin B1 (thiamin), 2.0 mg; vitamin B2 (riboflavin), 7.5 mg; niacin, 35 mg; D-pantothenic acid, 12 mg; vitamin B6 (pyridoxine-HCl), 3.5 mg; vitamin B12 (cyanocobalamin), 20 µg; folic acid, 1.0 mg; biotin, 0.2 mg; choline chloride, 460 mg; Fe ($FeSO_4.H_2O$), 80 mg; Cu ($CuSO_4.5H_2O$), 12 mg; Zn (ZnO), 60 mg; Mn (MnO), 85; I ($Ca(IO_3)_2$), 0.8 mg; Co ($Co_2CO_3(OH)_2$), 0.77 mg; Se (Na_2O_3Se), 0.15 mg; [4] Added on top of basal diet.

4.3.3. Sampling for meat quality

On d39, remaining chickens were fasted overnight. Then, on d40, one bird per pen with weight close to average weight of the pen was euthanized by electrical stunning followed by exsanguination. pH of right breast muscle was measured upon slaughter. Eviscerated carcasses were transferred to the chilling room (4°C) immediately after slaughter. pH of right breast muscle was measured again 24h post-mortem, together with colour measurements (Michiels et al., 2012, 2014). Finally, the breast muscle was excised and vacuumed stored at -20°C pending measurements of thawing loss (Michiels et al., 2012), and lipid oxidative stability during simulated retail display. Thaw loss was the proportionate weight loss of a sample before frozen vacuum storage at −20°C and after overnight thawing at 4°C. Regarding oxidative stability, defined subsamples of the *pectoralis major* that had been frozen and thawed were wrapped in oxygen-permeable polyethylene film and displayed under fluorescent light (1,000 lux) for 7 days at 4°C. Lipid oxidation was assessed by measuring thiobarbituric acid reactive species (TBARS) using the distillation method described by Grotto et al. (2007), and was expressed as micrograms of MDA per gram of meat.

4.3.4. Blood analyses

Thrombocytes and white blood cell differentials were done on fixated blood samples as outlined by Seliger et al. (2012). This automated analysis of chicken blood is based on flow cytometry using an anti-CD45 monoclonal antibody in combination with selected subset specific markers. Serum corticosterone concentration, as marker for stress, was also analysed following Dehnhart et al. (2003).

4.3.5. Protein and amino acids in plasma

The entire AA profile in plasma was determined by means of LC-MS/MS. Sample preparation included a simple protein precipitation and addition of the suitable internal standards (stable isotopic labeled) prior to analysis. Albumin was determined via photometric colour test at 570/660 nm, whereas total protein was determined via the biuret reaction photometrically at 540/660 nm. UA was determined via an enzymatic colour test (uricase/PAP-method) at 660/800 nm. Albumin, UA, and total protein were assayed using an AU 5800 Beckman Coulter.

4.3.6. Energy metabolites in breast muscle

ATP, PCr, and free Cr were determined as described by DeGroot et al. (2018, 2019) in muscle biopsies. The analytical method was based on enzymatic determinations in freeze dried muscle, which ultimately resulted in either reduction of NADP to NADPH (for ATP and PCr) or oxidation of NADH to NAD (for free Cr). Final data included the absolute concentration of each Cr-related metabolite (ATP, PCr, and free Cr), along with calculation of absolute concentration of total Cr (TCr=PCr+Cr) and relative proportions

(PCr:ATP). As muscle ATP is an indicator for the muscle tissue content in the sample, the PCr:ATP ratio is more appropriate than absolute PCr values since it cancels out variance due to differences in biopsy samples in their contents of blood and connective tissue (Harris et al., 1992).

4.3.7. Statistical analysis

Statistical analytical techniques appropriate to a completely randomized design were used (SAS Enterprise Guide 7, SAS Institute, Cary, USA). The experimental unit for all performance variables was pen. For physiological variables one animal per pen was taken and similarly pen was then considered as experimental unit. Data were checked for normality by Kolmogorov-Smirnov and Shapiro-Wilk tests and for homogeneity of variances by Levene's test. All parameters were analysed by a one-way ANOVA model or the non-parametric Kruskall-Wallis test, if appropriate, with GAA supplementation being the fixed effect. Linear and quadratic contrasts were included as well. Post-hoc Tukey test was used to compare means in case model was significant. Level of $P<0.05$ was considered significant, $0.05<P< 0.10$ was considered a trend. Further, referring to mortality, survival curves were constructed using the Kaplan-Meier representation and statistically analysed by the log-rank test for trend (GraphPad Prism 5.00, San Diego, USA).

4.4. RESULTS

4.4.1. Diets, bird performance, breast meat quality and rectal temperature

The analysed GAA concentration in the GAA diets was close to the intended dose, whereas the control diets contained no GAA (<1 mg/kg; Table 4.1). Broiler growth was high, primarily in the starter and grower phase but reduced in the HS finisher period (Table 4.2). Final BW exceeded 3 kg in all treatments. Until d25 (starter and grower phase), when birds were raised under standard conditions, BW was not affected by GAA application, however, feeding 1.2 g/kg GAA decreased F:G as compared to control (1.30 *vs*. 1.27, $P<0.05$, and linear effect, $P<0.05$), in part caused by a linear reduction in FI ($P<0.05$). In the finisher period, when HS was prevailing, 0.6 and 1.2 g/kg GAA reduced FI by 1.1 and 3.3%, respectively, (linear effect, $P<0.05$) associated with large improvements of F:G as compared to control (1.76, 1.66 and 1.67 for control, 0.6 and 1.2 g/kg GAA respectively, $P<0.05$). The effect on F:G was significant for both the linear and quadratic contrast. This resulted in a better F:G for the entire 39d-period when feeding GAA ($P<0.05$), again with a linear and quadratic outcome indicating no further improvement beyond the 0.6 g/kg GAA level. Mortality in finisher period for control was as high as 6.1%, due to implemented HS. In all rearing phases, absolute values for mortality were lowest with graded supplemental GAA, but this did not reach significance ($P>0.05$). Fig. 4.2. though, demonstrates that time-dependent survival of birds tended to be altered by treatment ($P=0.071$), in particular it shows that towards the end of the finisher period, mortality raised sharply for CON while survival remained higher for

GAA-fed broilers. In line with this, the composite measure EPEF gradually increased with graded dietary GAA, and this value was significantly improved by 1.2 g/kg GAA as compared to control ($P<0.05$) (Table 4.2). Panting frequency was determined during the periods of HS on d27 and 38. A linear effect indicated that panting frequency was lowered in GAA supplemented broilers on d38 as compared to CON ($P<0.05$) (Table 4.2), and this was also the case for the mean (linear effect, $P<0.05$). Regarding breast meat quality characteristics on d40, including oxidative stability during simulated retail display, no treatment effects were found (Table 4.3). On d26 and 39, birds were sampled at the end of the HS period on the respective day. Of these birds, rectal T confirmed HS whereby hyperthermia was more prone on d39 (older birds, chronic HS), however it was not influenced by treatment (Fig. 4.3).

Table 4.2. Effect of guanidinoacetic acid (GAA) supplementation on BW, ADG, ADFI, F:G, mortality, European Production Efficiency Factor (EPEF) and panting in male broilers subjected to chronic cyclic heat stress in the finisher phase[1]. (n=12)

Item	Dietary treatment [2]			SEM	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
d 0 to 10 (starter)							
Initial BW, g	48.1	48.1	48.1	0.05	0.993	0.921	0.954
Final BW, g	344	339	341	1.8	0.625	0.563	0.440
ADG, g/d	29.4	29.5	29.3	0.18	0.627	0.687	0.383
ADFI, g/d	29.9	29.5	29.5	0.11	0.188	0.137	0.283
F:G	1.02	1.02	1.01	0.005	0.858	0.664	0.735
Mortality[3], %	1.3	1.3	0.8	-	0.855	-	-
d 10 to 25 (grower)							
Final BW, g	1555	1540	1547	5.3	0.500	0.520	0.326
ADG, g/d	80.5	80.0	80.3	0.30	0.771	0.797	0.504
ADFI, g/d	109[a]	107[ab]	106[b]	0.4	0.022	0.006	0.705
F:G	1.35[a]	1.34[a]	1.32[b]	0.004	0.001	<0.001	0.575
Mortality[3], %	2.1	0.9	0.8	-	0.470	-	-
d 0 to 25							
ADG, g/d	59.2	59.0	59.5	0.25	0.749	0.656	0.541
ADFI, g/d	76.8	75.8	75.3	0.27	0.067	0.025	0.580
F:G	1.30[a]	1.29[ab]	1.27[b]	0.004	0.002	0.001	0.730
Mortality[3], %	3.3	2.1	1.7	-	0.522	-	-
d 25 to 39 (finisher)							
Final BW, g	3018	3064	3032	15.5	0.475	0.716	0.247
ADG, g/d	103	107	105	1.1	0.228	0.449	0.123
ADFI, g/d	180[a]	178[ab]	174[b]	0.9	0.027	0.008	0.725
F:G	1.76[a]	1.66[b]	1.67[b]	0.014	0.002	0.003	0.043
Mortality[3], %	6.1	4.7	3.4	-	0.375	-	-
d 0 to 39							
ADG, g/d	75.3	76.7	76.4	0.40	0.340	0.299	0.298
ADFI, g/d	111	110	109	0.4	0.129	0.045	0.949
F:G	1.48[a]	1.44[b]	1.43[b]	0.005	<0.001	<0.001	0.047
Mortality[3], %	9.2	6.7	5.0	-	0.329	-	-
EPEF[4]	476[b]	510[ab]	517[a]	7.1	0.036	0.016	0.337
Panting, #/min							
d27	185	160	160	6.5	0.202	0.122	0.366
d38	190	185	163	5.0	0.062	0.028	0.392
Mean (d27, d38)	187	173	162	4.4	0.055	0.017	0.839

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a row are significantly different at $P<0.05$; [3] Mortality data within treatment were not normally distributed, and hence treatments were compared using the non-parametric Kruskal-Wallis test, [4] European Production Efficiency Factor; [viability d0-39 (%) * BW d39 (kg) * 100] / [age (d) * F:G d0-39].

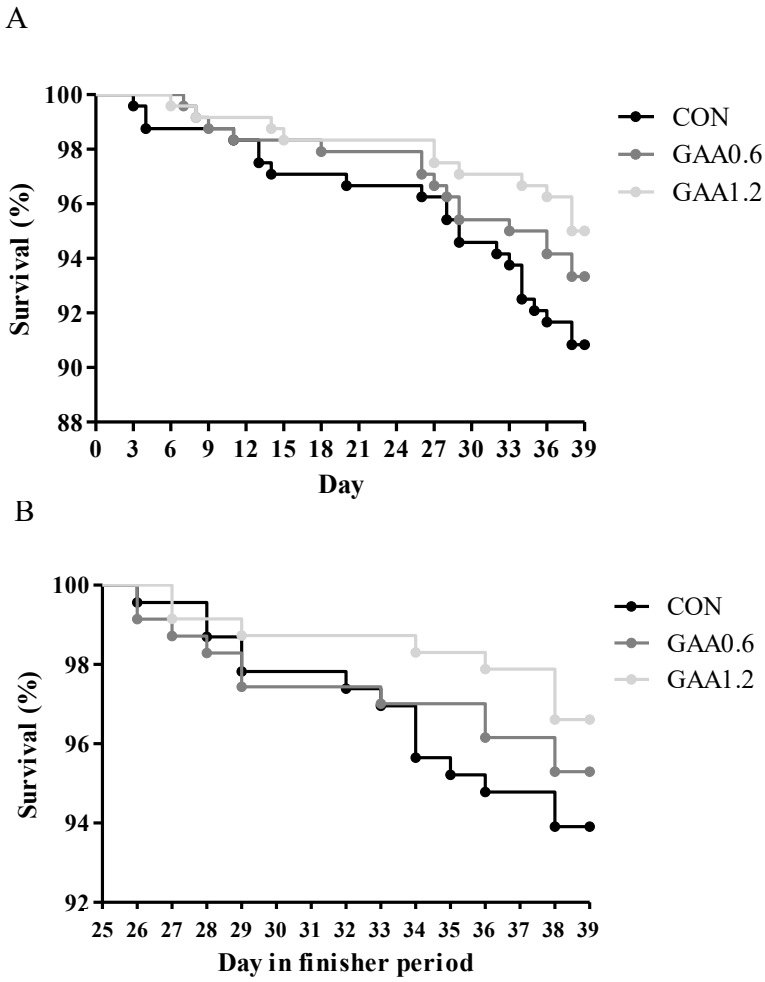

Fig. 4.2. Effect of guanidinoacetic acid (GAA) supplementation on survival curves using the Kaplan-Meier representation for total period (A; P=0.071) and for finisher period (B; P=0.169) in male broilers subjected to chronic cyclic heat stress. CON, GAA0.6 and GAA1.2 with dietary GAA at 0, 0.6 and 1.2 g/kg. (n=12).

Table 4.3. Effect of guanidinoacetic acid (GAA) supplementation on breast meat characteristics of male broilers subjected to chronic cyclic heat stress in the finisher phase. Breast meat samples were taken from birds after overnight fast on d40[1]. (n=12)

Item	Dietary treatment [2]			SEM	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
pH t0h	6.3	6.3	6.2	0.03	0.593	0.310	0.990
pH t24h	6.0	6.0	5.9	0.19	0.249	0.100	0.840
Colour							
CIE L*	59.7	58.9	60.1	0.55	0.689	0.757	0.428
CIE a*	1.0	1.0	1.1	0.11	0.923	0.727	0.851
CIE b*	14.4	14.3	14.2	0.28	0.963	0.789	0.962
Thawing loss, %	4.1	4.6	3.8	0.24	0.398	0.689	0.197
TBARS, µg MDA/g[3]	0.41	0.44	0.53	0.04	0.416	0.208	0.725

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a row are significantly different at P<0.05; [3] TBARS was determined after 7 days of simulated retail display under light at 4°C in samples that were previously stored at -20°C.

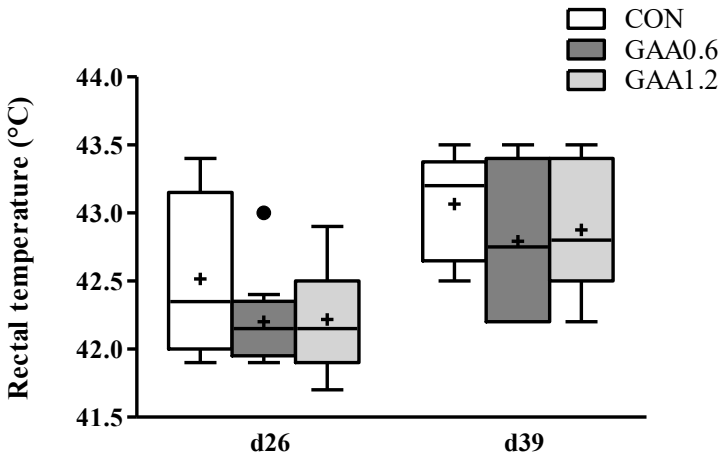

Fig. 4.3. Effect of guanidinoacetic acid (GAA) supplementation on rectal T in male broilers subjected to chronic cyclic heat stress in the finisher phase on d26 (P=0.139) and d39 (P=0.343). Box and with median, 25th and 75th percentiles, and whiskers extending from the upper and lower edge of the box, which represents the interquartile range (IQ), to the highest and lowest values which are no greater than 1.5 times the IQ range, out values - circles, values between 1.5 and 3 times the IQ range; + denotes mean. CON, GAA0.6 and GAA1.2 with dietary GAA at 0, 0.6 and 1.2 g/kg. (n=12).

4.4.2. Thrombocytes, white blood cell differentials and corticosterone in blood

Data on thrombocytes and immune cells in blood were found to be very variable, but multiple significant effects were seen on d26 (Table 4.4). Thrombocytes showed a linear decrease with increasing GAA in the diet (P<0.05). Further, GAA supplementation linearly decreased the number of lymphocytes on d26, caused by linear decreases in T-

cells (both P<0.05). Altogether, number of leukocytes was substantially reduced by feeding GAA on d26 (P<0.05). When comparing d39 with d26, the number of heterophils increased while the number of lymphocytes remained unchanged resulting in a higher heterophil/lymphocyte ratio. Serum corticosterone was only affected by treatment at d26, without post-hoc differences (P=0.05) (Fig. 4.4).

Table 4.4. Effect of guanidinoacetic acid (GAA) supplementation on thrombocytes and white blood cell differentials in blood of male broilers subjected to chronic cyclic heat stress in the finisher phase at d26 and 39[1]. (n=12)

Item	Dietary treatment [2]			SEM	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
Thrombocytes, #/µl							
d26	72500	56700	51000	4200	0.088	0.040	0.552
d39	53800	50800	49000	2000	0.648	0.358	0.909
Heterophils, #/µl							
d26	9000	7700	6800	490	0.215	0.089	0.866
d39	12000	11200	8800	800	0.235	0.104	0.666
Monocytes, #/µl							
d26	1200	790	430	170	0.194	0.076	0.946
d39	1620	780	730	190	0.100	0.052	0.336
Lymphocytes, #/µl							
d26	12200^{a}	10000^{ab}	8200^{b}	560	0.009	0.003	0.857
d39	10000	10200	8000	500	0.126	0.090	0.268
T-cells, #/µl							
d26	10300^{a}	8600^{ab}	6800^{b}	470	0.006	0.002	0.989
d39	8500	8600	6900	420	0.186	0.121	0.336
B-cells, #/µl							
d26	1900	1460	1370	127	0.189	0.103	0.517
d39	1490	1580	1030	105	0.064	0.063	0.152
Total leukocytes, #/µl							
d26	22400^{a}	18600^{ab}	15500^{b}	870	0.002	0.001	0.798
d39	23600	22100	17500	1300	0.117	0.048	0.571
Heterophils/lymphocytes							
d26	0.8	0.8	0.9	0.05	0.904	0.734	0.749
d39	1.2	1.1	1.1	0.07	0.703	0.476	0.652

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a row are significantly different at P<0.05.

4.4.3. Protein and amino acids in plasma

Neither UA, albumin or total protein in plasma was significantly affected by treatment, on either sampling day; though a trend for linear decrease for UA is observed for d26 (Table 4.5). Among all plasma AA and dipeptides shown in Table 4.5, only Arg, carnosine and Gly were affected by treatment. Blood Arg increased linearly with increasing dietary GAA (+18.3 and +19.9% for 0.6 g/kg GAA on d26 and d39, respectively, both not significantly different from control, and +30.8 and +33.6% for 1.2 g/kg GAA, only significantly different from control at d26 due to higher variance on d39). Carnosine was reduced by supplementing the diet with GAA on d39 (P<0.05), with treatment 0.6 g/kg GAA showing the lowest value, and ultimately showing a significant quadratic effect (P<0.05). Also for this treatment at d39, plasma Gly content was elevated

compared to control (P<0.05), whereas GAA at 1.2 g/kg exhibited an intermediate value; and again this resulted in a quadratic effect (P<0.05).

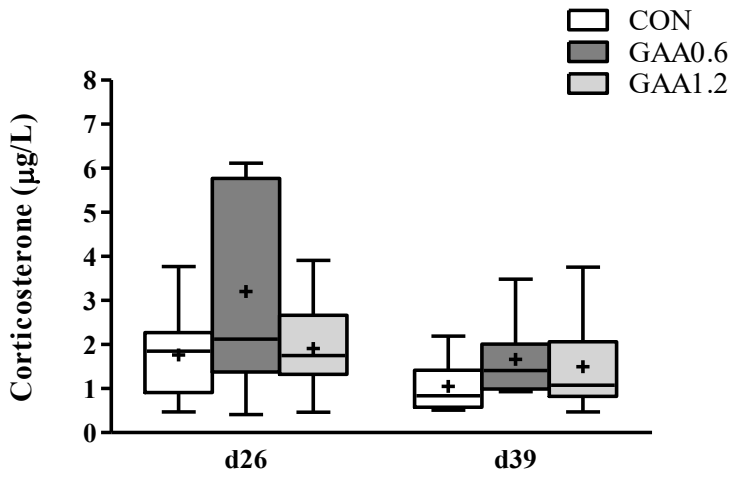

Fig. 4.4. Effect of guanidinoacetic acid (GAA) supplementation on serum corticosterone in male broilers subjected to chronic cyclic heat stress in the finisher phase on d26 (P=0.050) and d39 (P=0.192). Box and with median, 25th and 75th percentiles, and whiskers extending from the upper and lower edge of the box, which represents the interquartile range (IQ), to the highest and lowest values which are no greater than 1.5 times the IQ range; + denotes mean. CON, GAA0.6 and GAA1.2 with dietary GAA at 0, 0.6 and 1.2 g/kg. (n=12).

4.4.4. Energy metabolites in breast muscle

Examination of breast muscle samples revealed at both sampling days increases of PCr (P<0.05, linear effect at d26 and P<0.05 at d39), free Cr (P<0.05 at d39), TCr (P<0.05, both days), and, PCr:ATP (P<0.05, linear effect at d26 and P<0.05 at d39) with increasing dietary GAA (Table 4.6). Muscle Cr levels appeared to be lower at d39 as compared to d26, whereas the opposite was seen for PCr and PCr:ATP.

Table 4.5. Effect of guanidinoacetic acid (GAA) supplementation on protein and amino acids in plasma of male broilers subjected to chronic cyclic heat stress in the finisher phase at d26 and 39[1]. (n=12)

Item	Dietary treatment [2]			*SEM*	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
Uric acid, mmol/l							
d26	342	320	271	15.1	0.147	0.057	0.666
d39	233	274	251	9.2	0.184	0.409	0.100
Albumin, g/l							
d26	11	10	10	0.2	0.196	0.096	0.483
d39	10	10	10	0.1	0.783	0.903	0.494
Total protein, g/l							
d26	30	28	28	0.5	0.315	0.217	0.376
d39	28	29	28	0.4	0.816	0.718	0.602
Alanine, µmol/l							
d26	768	761	692	29.5	0.521	0.304	0.626
d39	452	445	442	15.9	0.967	0.800	0.963
α-Aminobut. acid µmol/l							
d26	18	16	18	0.8	0.657	0.987	0.363
d39	14	13	13	0.5	0.896	0.671	0.851
Arginine, µmol/l							
d26	263[b]	311[ab]	344[a]	12.8	0.029	0.009	0.752
d39	226	271	302	16.9	0.185	0.070	0.847
Aspargine, µmol/l							
d26	106	118	98	8.1	0.592	0.628	0.357
d39	46	74	63	6.2	0.172	0.256	0.132
Aspartic acid, µmol/l							
d26	74	74	68	6.2	0.912	0.727	0.808
d39	91	81	78	3.4	0.264	0.124	0.596
Carnosine, µmol/l							
d26	54	44	47	2.4	0.243	0.264	0.206
d39	54[a]	40[b]	46[ab]	1.9	0.012	0.100	0.011
Citrulline, µmol/l							
d26	5.4	6.8	6.8	0.48	0.438	0.272	0.509
d39	9.1	10.9	9.4	0.51	0.293	0.759	0.127
Cystine, µmol/l							
d26	46	49	46	1.5	0.628	0.975	0.339
d39	51	54	51	1.5	0.430	0.897	0.200
Glutamine, µmol/l							
d26	883	929	783	33.7	0.196	0.224	0.179
d39	806	806	804	21.2	0.342	0.970	0.146
Glutamic acid, µmol/l							
d26	187	177	158	6.7	0.204	0.080	0.793
d39	177	176	181	3.6	0.843	0.682	0.681
Glycine, µmol/l							
d26	384	413	375	12.9	0.466	0.776	0.232
d39	349[b]	411[a]	387[ab]	10.2	0.039	0.113	0.041
Histidine, µmol/l							
d26	38	34	38	1.1	0.306	0.985	0.127
d39	39	41	43	1.5	0.581	0.301	0.967
Hydroxyproline, mol/l							
d26	145	174	158	6.2	0.164	0.384	0.090
d39	156	137	148	6.1	0.472	0.610	0.267
Isoleucine, µmol/l							

d26	60	55	63	2.5	0.443	0.702	0.226
d39	67	76	66	2.9	0.343	0.958	0.147
Leucine, µmol/l							
d26	157	151	170	6.1	0.452	0.387	0.361
d39	169	179	152	5.4	0.133	0.195	0.120
Lysine, µmol/l							
d26	120	90	102	6.6	0.191	0.278	0.142
d39	165	151	141	18.9	0.595	0.314	0.921
Methionine, µmol/l							
d26	60	65	64	2.4	0.725	0.551	0.596
d39	62	74	67	3.0	0.286	0.471	0.159
Ornithine, µmol/l							
d26	22	21	23	1.1	0.704	0.943	0.407
d39	26	33	25	2.0	0.227	0.907	0.088
Phenylalanine, µmol/l							
d26	96	90	96	2.4	0.487	0.953	0.229
d39	109	119	112	4.5	0.246	0.647	0.109
Proline, µmol/l							
d26	334	336	344	13.9	0.948	0.759	0.914
d39	237	235	234	8.7	0.989	0.882	0.977
Serine, µmol/l							
d26	505	522	434	20.9	0.194	0.166	0.239
d39	412	454	449	11.4	0.261	0.185	0.334
Taurine, µmol/l							
d26	155	217	185	14.9	0.240	0.407	0.141
d39	105	91	101	8.5	0.800	0.851	0.334
Threonine, µmol/l							
d26	429	415	393	18.3	0.721	0.928	0.425
d39	339	357	375	22.8	0.509	0.249	0.999
Tryptophan, µmol/l							
d26	67	59	63	1.9	0.325	0.429	0.203
d39	82	88	83	1.9	0.341	0.936	0.146
Tyrosine, µmol/l							
d26	131	155	150	6.1	0.245	0.250	0.219
d39	142	150	153	8.9	0.871	0.614	0.893
Valine, µmol/l							
d26	95	88	102	3.1	0.197	0.352	0.122
d39	103	111	95	4.1	0.309	0.459	0.181

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a row are significantly different at $P<0.05$.

Table 4.6. Effect of guanidinoacetic acid (GAA) supplementation on breast meat creatine/phosphocreatine system of male broilers subjected to chronic cyclic heat stress in the finisher phase at d26 and 39[1]. (n=12)

Item	Dietary treatment [2]			*SEM*	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
ATP, µmol/g DM							
d26	26	27	26	0.6	0.703	0.898	0.410
d39	29	29	28	0.5	0.472	0.387	0.388
Phosphocreatine, µmol/g DM							
d26	49	55	65	3.0	0.085	0.029	0.748
d39	85^{b}	95^{ab}	99^{a}	2.2	0.016	0.005	0.532
Free creatine, µmol/g DM							
d26	111	128	129	3.9	0.097	0.056	0.310
d39	45^{b}	57^{a}	61^{a}	1.9	0.001	<0.001	0.245
Total creatine, µmol/g DM							
d26	160^{b}	183^{a}	194^{a}	3.9	<0.001	<0.001	0.357
d39	130^{b}	151^{a}	160^{a}	3.4	<0.001	<0.001	0.274
Phosphocreatine:ATP							
d26	1.9	2.1	2.6	0.13	0.080	0.033	0.488
d39	3.0^{b}	3.3^{ab}	3.7^{a}	0.9	0.008	0.002	0.785

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a row are significantly different at P<0.05.

4.5. DISCUSSION

4.5.1. Guanidinoacetic acid maintains growth performance and improves feed conversion and survival in heat stressed broilers

In agreement with our hypotheses, this study confirmed that dietary supplementation with GAA is beneficial to heat stressed finishing broilers because of muscle Cr loading and Arg sparing. Besides that, prominent and important improvements in feed efficiency, survival, panting frequency, lymphocyte numbers, and muscle energy metabolites in finisher period were observed. Here, HS was confirmed by exuberant high panting frequencies and rectal T found in broilers during the daily episodes of high T in the finisher period.

In the current study, no effect of GAA supplementation on BWG in the starter, grower or finisher period were seen which is in contrast to several studies under TN (Chapter 1) and to one study under HS conditions. Under HS conditions, Amiri et al. (2019), who studied the efficacy of GAA supplementation at different dietary CP levels, described linearly improved final BW and overall ADG by supplementing GAA. Nonetheless, the current findings are in agreement with Chapter 2 in which the interaction between GAA and ND under TN conditions was addressed. Under heat challenge birds seek homeostasis and hitherto energy is diverted to restrict body T increases by ways such as panting behaviour, leading to less energy available for growth promotion (Gaughan et al., 2009), and in addition energy is lost due to the occurrence of mitochondrial dysfunction which causes redox energy dissipation as heat instead of being used for ATP synthesis (Mujahid et al., 2007). Furthermore, the response of heat stressed broilers is

commonly associated with less appetite and lower feed consumption, which is an evident defence mechanism in order to decrease heat increment and to sustain homeothermy (Lara and Rostagno, 2013). Altogether, lower growth rates are typically found during HS due to reduced FI and reduced feed efficiency, hence here we found that GAA had the potential to allow even higher reduction of FI whilst still keeping BW.

Notably, findings for ADFI showed a reduction in broiler chickens fed diets supplemented with 1.2 g/kg GAA during the grower and finisher period, but it corroborates to Chapter 2 and 3. In different studies performed under TN conditions, inconsistent results have been obtained with regard to the influence of GAA on FI (Chapter 1, section 1.4.2.1). In Chapter 2 we related this reduced feed consumption to the hepatic energy status theory. Essentially, we speculated that GAA supplementation might improve hepatic cellular energy status, reflected by higher ATP:ADP, and hence reducing appetite, congruent to the findings by Ji and Friedman (1999) in rats. Furthermore, it is well known that AMPK is the main sensor of cellular energy status, and is activated in response to energy stress to restore energy balance by inhibiting ATP-consuming processes and promoting ATP-generating pathways. AMPK is allosterically activated through fluctuations in the AMP:ATP in the liver (Corton et al., 1994, 1995). Hence, again, GAA feeding might enhance cellular energy in liver and thus lower AMPK stimulation, altering energy metabolism. Interestingly, Hu et al. (2019) illustrated that dexamethasone treatment of broilers decreased growth performance, and amongst others upregulated liver mRNA levels of AMPK and downregulated liver mRNA cholesterol 7 alpha-hydroxylase, the latter enzyme being essential for bile acid synthesis. Similarly, as HS was shown to upregulate the HPA-axis (Star et al., 2008), HS birds may show similar changes in liver metabolism. Potentially, GAA may impede upregulation of AMPK, and more importantly, it may counteract reduced cholesterol 7 alpha-hydroxylase expression. The latter would mean that fat digestion can be improved, which is of utmost importance as HS has repeatedly been shown to reduce digestibility of nutrients (Bonnet et al., 1997). Altogether, it could mean that GAA improves dietary energy utilization, even more under HS conditions. Actually, this can be confirmed as drastic improvements in F:G for the finisher period were observed in this study. Here, supplementation with GAA improved F:G in grower and particularly in finisher period, thus when HS was prevailing, and overall, as such corroborating to many previous studies (EFSA, 2016; Michiels et al., 2012; Chapter 2 and 3). To say, F:G in finisher period was improved by no less than 10 and 11 points in 0.6 and 1.2 g/kg GAA fed birds, respectively. Very much in agreement with that, Amiri et al. (2019) showed 12 points reduction in F:G at 1.2 g/kg GAA in finisher period under HS conditions, and thus far beyond average improvements outlined in Fig. 1.8 (Chapter 1).

Although mortality rates in finisher and total period did not show significant differences, rates were considerably lower with graded levels of supplemented GAA and detailed survival curves are leading to similar conclusions. Mortality in finisher period accounted for 6.1, 4.7 and 3.4% in 0, 0.6, and 1.2 g/kg GAA, respectively, undoubtedly illustrating that HS impacted survival. Survival curves for the finisher period shows that GAA

feeding limited mortality mainly towards the end of the study. This corroborates to the linear reduction in panting frequency on d38, and not on d27. Panting occurs heavily during episodes of HS, and hence here it increased when birds age and HS is increasing. Respiratory frequency of birds at TN would be between 40-80, depending on age and metabolism. Even so, but only numerically, rectal T was reduced by GAA mostly on d38, by 0.3 to 0.4°C from 43.1°C for control birds, but due to high variation across birds not significantly. To note, rectal T of birds at TN fluctuates between 40.5 and 41.5°C. These figures support the idea that heat tolerance was improved by feeding GAA, in particular when HS gets a chronic nature. However, it can also be said that GAA-fed broilers were likely better prepared for the implementation of HS by the previous feeding period, hereby referring to improved feed conversion in the grower period.

4.5.2. Guanidinoacetic acid has minimal effect on the birds' stress response, but may spare arginine

Supplementation of GAA had no effect on heterophils or heterophil/lymphocyte ratio, the latter described as marker for HS, and thus in contrast with findings from DeGroot et. al (2018). These authors reported that supplementation of GAA decreased heterophils as the largest proportion of leukocytes. However, in our study, supplementation of GAA on d26 decreased total leukocytes caused by decreases in lymphocytes and the latter in turn because of decreasing T-cells. These findings indicate that GAA may alter cell-mediated immune responses, in particular in the acute phase of HS. The full interpretation of these findings warrants further research. Also in our study, treatment had no (d39) or just significant (d26) effect on corticosterone. In fact, corticosterone in GAA-fed chickens was increased at 0.6 g/kg GAA during acute HS. Notably, as illustrated in Fig. 4.4 variation across birds for the 0.6 g/kg GAA group was exceptionally high. It should also be noted that high levels of corticosterone can only be maintained for short periods, less than one hour, to cope with the immediate stressor, *in casu* high T. When birds are chronically exposed to high T, concentration of corticosterone may decline after the initial stage (Etches et al., 2008). Together, it indicates that GAA feeding has minimal effect on the birds' stress response.

Supplementation of GAA did not affect plasma AA or dipeptide concentrations, apart from Arg, Gly and carnosine. The Arg sparing effect was demonstrated by higher plasma Arg levels, only significant on d26, but though clearly numerical on d39. Similarly, Dilger et al. (2013) argued that GAA-feeding must be able to save Arg for other metabolic functions than Cr synthesis. In light of the occurrence of HS, the role of Arg as precursor for NO is pivotal. During HS, one of the primary physiological autonomic responses is to increase blood flow to the body surface or upper respiratory tract to dissipate internal body heat (Kregel et al., 1988; Yahav et al., 1997). Therefore, the blood flow to some visceral organs is significantly reduced (Horowitz, 2003). NO is a potent vasodilator that directly relaxes vascular smooth muscle and modulates or inhibits the production and release of vasoconstrictors such as serotonin. Higher Arg bioavailability might thus be beneficial for heat stressed broilers, as has been demonstrated in heat

stressed Pekin ducks (Zhu et al., 2014). Nonetheless, we failed to prove that GAA-feeding could result in higher plasma NO. We attempted to measure plasma NO, but apparently not all commercial available kits are reliable, as was also found by Chapman and Wideman (2006). Further, our findings indicate that 0.6 g/kg GAA increased plasma Gly at d39. This increase amounted to 15%, and interestingly, Ser was also increased by 10%. Since Gly and Ser are interconvertible, this suggests a potential Gly_{equiv} sparing effect for this treatment, although it does not appear to be the case on d26, nor for 1.2 g/kg GAA at d39 (Siegert and Rodehustcord, 2019). Nonetheless, if feeding GAA evokes lower endogenous GAA synthesis, then similarly to the effect on Arg, Gly_{equiv} should be less consumed for this purpose. However, as Gly and Ser are not essential AA, these effects may have less impact on birds' metabolism. Anyway, one may argue that in the context of HS this could lead to higher productivity. Indeed, HS may accentuate some Gly_{equiv}-consuming metabolic processes. UA synthesis is a major consumer of Gly in the bird (Leeson and Summers 2003). UA can be used as indicator of AA utilization in broilers (Donsbough et al., 2010). HS is believed to alter the balance between muscle anabolic and catabolic reactions. In fact, it was shown that the expression of IGF-I, phosphatidyl-inositol 3-kinase, and p70S6 kinase associated with protein synthesis was lower, whereas the expression of muscle ring-finger protein-1 and MAFbx associated with protein degradation were higher in HS condition (Zuo et al., 2015). Likely this can lead to higher UA production, and thus Gly need. Another pathway consuming Gly is the contribution to the synthesis of porphyrins, such as haem, that is formed from the condensation of succinyl-Co-A and Gly. The formation of each haem group dissipates eight Gly molecules. Thus, Gly is involved in the formation of haem-containing compounds, such as myoglobin, haemoglobin, and cytochromes (Meléndez-Hevia et al., 2009). On this note, HS typically reduces blood haematocrit. Finally, HS may induce OS resulting in higher needs for the endogenous antioxidant GSH, that is composed of Gly, glutamate, and Cys, the latter also needing Ser for its endogenous synthesis (Akbarian et al., 2016). After all, Gly_{equiv} is supposed to be the first limiting non-essential AA in broiler chickens (Siegert and Rodehustcord, 2019). It remains to be confirmed whether GAA is able to promote Gly metabolic functions, possibly advantaging broilers under HS. Carnosine, a dipeptide formed by histidine and β-alanine declined by supplementing the diet with GAA on d39. Carnosine is believed to serve as a free radical scavenger in animal tissues. Manhiani et al. (2011) reported that an increase in carnosine levels in brain, breast and thigh of broilers in response to stress could be temporarily, and may decrease after a certain time. Also, but in horses, Dunnett et al. (2002) found higher plasma carnosine concentrations at 5 to 30 min and lower carnosine concentrations at 120 min after inducing exercise as stressor. Carnosine values returned to normal values after 1 d. Differences in plasma carnosine content among animals exposed to stress may be accounted for by the difference in species, stress duration time and intensity. It shows that interpretation of such data are peculiar, and very much time-dependent. Though, it remains hard to relate GAA and Cr metabolism with carnosine, and also, actually, muscle serves at the major storage for carnosine, which was not determined here.

4.5.3. Energy metabolites are enhanced in breast muscle by feeding guanidinoacetic acid

It is well established that supplementation of GAA increases concentrations of Cr-related metabolites in chicken breast muscle (Chapter 1, section 1.4.2.2, Chapter 2, Chapter 3). Here, supplementing GAA to the diets of birds under HS condition resulted in dose-related elevations for PCr, free Cr, TCr and PCr:ATP, supposedly substantially contributing to the better feed conversion observed in the finisher phase. These muscle Cr and PCr outcomes are in line with Lemme et al. (2007b), EFSA (2009), Michiels et al. (2012), DeGroot (2018, 2019) and Chapter 2 and 3. Because maintenance of PCr is based on Cr being phosphorylated to PCr and exported to sites of ATP usage (Brosnan and Brosnan, 2010; Guimarães-Ferreira, 2014), the increased total Cr concentration may indicate increased capacity for PCr loading. Interestingly, all effects on PCr, free Cr, TCr and PCr:ATP were found linear, suggesting that no saturation of Cr storage and metabolites thereof occurred within the GAA dosage range applied, as also indicated in Chapter 1 that saturation may happen beyond 2 g/kg GAA. PCr:ATP ratio is believed to be an indicator for the buffering capacity for ATP hydrolysis upon fast energy usage. The effects were largely maintained throughout the 14-day HS finisher period, and definitely support better feed efficiency as was demonstrated. Also, higher PCr and TCr concentrations simultaneously in muscle, underlines that dietary GAA is successfully absorbed and metabolized to Cr and is thus an efficient precursor in terms of Cr incorporation into the muscle. The increases of the ratio of PCr:ATP in breast muscle at d39 were 10 and 23% at 0.6 and 1.2 g/kg GAA, respectively.

4.6. CONCLUSION

GAA supplementation improved feed conversion ratio and survival, with the largest benefits in the finisher period when birds were subjected to chronic cyclic HS. This was associated with enhanced energy status as shown in breast muscle, both in the acute and chronic phase of HS. Finally, dietary GAA exhibited an Arg and potentially a Gly sparing effect, which might offer additional benefits for heat stressed finisher broilers.

4.7. ACKNOWLEDGEMENTS

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CHAPTER 5

EFFECTS OF FEEDING GUANIDINOACETIC ACID ON OXIDATIVE STATUS AND CREATINE METABOLISM IN BROILERS SUBJECTED TO CHRONIC CYCLIC HEAT STRESS IN FINISHER PHASE

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CHAPTER 5

EFFECTS OF FEEDING GUANIDINOACETIC ACID ON OXIDATIVE STATUS AND CREATINE METABOLISM IN BROILERS SUBJECTED TO CHRONIC CYCLIC HEAT STRESS IN FINISHER PHASE

5.1. ABSTRACT

In this experiment, the effect of supplementing GAA to broilers subjected to HS during the finisher period on oxidative status and GAA and Cr metabolism in key organs was studied. A total of 720 day-old male Ross 308 broilers were allocated to 3 treatments: 0, 0.6 or 1.2 g/kg GAA was added to complete corn-SBM dietS and fed for 39d, with 12 replicates (20 birds each) per treatment. A chronic cyclic HS model (T to 34°C with 50-60% RH for 7h daily) was applied in the finisher phase (d25-39). Samples from one bird per pen were taken on d26 and 39 to assess energy metabolites and parameters of oxidative status in blood, breast muscle, liver, kidney and heart. GAA and Cr in plasma were linearly increased by feeding GAA on either sampling day, illustrating efficient absorption and methylation. Energy metabolism in muscle was greatly supported as visible by increased Cr, PCr:ATP and glycogen stores providing an improved capacity for readily available PCr which may be of particular relevance when oxidative phosphorylation can be limited, e.g. HS. Blood cholesterol levels linearly decreased by graded GAA (effect on d26, trend on d39), which may suggest that GAA affects AMPk in liver. The lipid peroxidation marker MDA, and the antioxidant enzymes SOD and GPx showed no alterations by dietary GAA in plasma. Opposite to that, SOD activity in breast was linearly lowered when feeding GAA (trend on d26, effect on d39). It may highlight an indirect antioxidant effect of dietary GAA due to Cr loading protecting muscle function, in particular after chronic HS. To conclude, in addition to previous findings, beneficial performance in heat stressed broilers by GAA may be associated with enhanced energy metabolism resulting in improved oxidative status.

5.2. INTRODUCTION

Dietary GAA has been shown to affect Cr metabolic pathways resulting in increased cellular Cr levels and hitherto body performance in various animal species (e.g. DeGroot et al., 2019; Zeng et al., 2018; Li et al., 2018) and humans (e.g. Ostojic et al., 2014b). In chicken, it is well established that supplementation of GAA dose-dependently increases concentrations of Cr-related metabolites in breast muscle (Lemme et al., 2007ab; EFSA, 2009; Michiels et al., 2012; DeGroot et al.; 2018, 2019; Chapter 2, 3 and 4). In the muscle

cell, elevated muscle Cr provides substrate for PCr formation in times of rest which diffuses to sites of ATP usage (Brosnan and Brosnan, 2010; Guimarães-Ferreira, 2014), for immediate re-phosphorylation of ADP back to ATP. Elevated PCr and TCr concentrations provide evidence that dietary GAA is successfully absorbed and metabolized to Cr and is thus efficient to increase Cr incorporation into muscle. Thus, in most studies showing improvements of bird performance, these effects were ascribed in part to increased Cr and cellular energy status. However, a novel function of Cr has also been reported in the attenuation of acute stress responses by quenching superoxide anions and other aqueous reactive species (Lawler et al., 2002; Deminice and Jordao, 2012). It is well known that excessive production of reactive species is harmful to normal metabolism, which can cause cellular damage resulting from lipid peroxidation, protein oxidation and DNA modification. Sestili et al. (2006) showed that added Cr, at concentrations comparable to those attainable in plasma upon oral supplementation in human, exerted direct antioxidant activity in cultured mammalian cells exposed to various oxidizing agents. Investigations showed protective effects of Cr exposure on oxidatively injured mitochondrial DNA (Guidi et al., 2008) and against RNA-damaging agents (Fimognari et al., 2009). Similar reports are available *in vivo*: in rats, it was reported that short-term Cr supplementation (5 g/d for 6 days) decreases reactive oxygen species content in skeletal muscle, possibly due to the direct action of Cr on scavenging superoxide radicals (Guimarães-Ferreira, 2014). Also, Deminice and Jordao (2012) have demonstrated the protective effects of Cr against OS induced by a single bout of moderate aerobic exercise in rats, evidenced by increase in total plasma antioxidant capacity and muscle GSH. The latter corroborates with Young et al. (2010) who reported the capacity of Cr exposure to up-regulate the thiol redox system, of which GSH is an important component, similar to findings by Kang et al. (2006). These systems catalyse thiol-disulfide exchange reactions and hereby control the redox state of cytoplasmic cysteine residues, thus protecting e.g. radical sensitive enzymes from oxidative damage (Young et al., 2010). Cr may also exert antioxidant outcomes through its primary action on cellular energy status. The putative benefits of Cr in a number of muscular, neurological, and cardiovascular diseases have been generally attributed and not surprisingly to the Cr-induced buffering of cellular ATP levels, whose fall would lead to the accumulation of intracellular Ca^{2+}, and stimulation of formation of reactive species leading to tissue oxidative damage (Persky and Brazeau, 2001). Contrary, Zugno et al. (2006, 2008) showed that GAA administration to the rat brain led to a decrease of the non-enzymatic antioxidant capacity likely due to oxidation of sulfhydryl groups, leading to lower GSH levels. Similarly, a detrimental effect on oxidative status was also described in healthy men, where dietary GAA did not impact markers of oxidative status, apart from an elevation of fasting plasma SOD activity (Ostojic, 2015). Furthermore, Aksentijevic et al. (2014) questioned any physiologically relevant antioxidant activity of Cr in oxidatively challenged mice heart and guanidine compounds, like GAA, are also discussed to induce the formation of free radicals (review by Hiramatsu, 2003). It is thus equivocal what the effect would be of dietary GAA on the oxidative status of animals. Therefore, in the current study, we applied a heat challenge to birds in order to induce OS and investigate the interaction with GAA feeding and Cr loading. Indeed, the

involvement of HS as an inducer of OS has been widely acknowledged (Akbarian et al. 2016). It is well-known that production of reactive species largely depends on the animal's metabolic rate. It appears that in normal physiological conditions animals can cope well with pro-oxidative agents whilst when they are facing stressful situations such as HS, OS might prevail (Akbarian et al., 2016). In Chapter 4 we showed that dietary GAA improved animal performance and reduced mortality when broilers were subject to chronic HS. It remains to be established whether this protective effect of GAA is also related to the antioxidant activity from elevated cellular Cr.

Altogether, the purpose of this study was to evaluate the effect of GAA supplementation with GAA to broilers subjected to acute and chronic HS during the finisher period, on different vital organs' oxidative status, such as MDA and GPx and SOD activity in plasma, breast muscle, liver, kidney and heart, and deepening insights in GAA and Cr metabolism in key organs. Therefore, broilers were fed increasing levels of GAA and organs were sampled on the 2nd and 14th day of HS (i.e. d26 and 39 of age, respectively). Data were subjected to analysis of variance, and additionally data were used in a PCA to explore the correlation structure across variables.

5.3. MATERIALS AND METHODS

5.3.1. Animals, housing and diets

To test the current hypotheses, additional samples were taken from the same sampled broilers mentioned in Chapter 4. Hence, experimental procedures including HS protocol and treatments are accordingly. In brief, 720 male Ross 308 broilers were allocated to 3 treatments: 0, 0.6 or 1.2 g/kg GAA (CreAMINO®, GAA, feed grade >96.0%, made available by Evonik Nutrition & Care GmbH, Hanau-Wolfgang, Germany, at the time of the study) added to corn-SBM diets and fed for 39d, with 12 replicates (20 birds each). A chronic cyclic HS model (T increase to 34°C with 50-60% RH for 7h daily) was applied in the finisher phase (d25-39).

5.3.2. Sampling during acute and chronic heat stress

On d26 (acute HS) and d39 (chronic HS), one animal per pen with weight close to average weight of the pen was selected. It is important to notify that sampling started >4h after inducing HS on that day, which means that broilers experienced HS at the moment of sampling. The latter was verified by measuring rectal T and reported in Chapter 4. After euthanasia as described in Chapter 4, blood was taken by puncture from heart and plasma was harvested from K2EDTA tubes after centrifugation (3000 × g, 15 min), and was stored at -80 °C, pending analysis. Subsequently the animal was bled and immediately, within seconds, the skin was removed from the breast, and a 2 cm flat piece of the right breast (*pectoralis major*), middle of right breast in length and 2 cm away from median, from 0.5 cm in depth, was transferred to liquid N_2. Snap frozen tissues were broken, collected in precooled cryovials and submerged in liquid N_2 and stored at

-80°C pending analysis. Next, the abdomen was opened and liver, heart and kidney were collected. The middle lob of the liver was sampled. Prior to processing, adhering fat and large vessels were removed from the heart. Liver and heart tissue were processed similarly as breast muscle. In case of kidney, the complete organ was sampled, wrapped in aluminum foil, and snap frozen in liquid N_2. All samples were rinsed with saline prior to processing and freezing.

5.3.3. Protein and amino acids in plasma

GAA, HCy and AA in plasma were determined by means of LC-MS/MS (Chapter 4). Sample preparation included protein precipitation and addition of the suitable internal standards (stable isotopic labeled) prior to analysis. Cr was quantified photometrically according to the Barrit reaction. CrN was analyzed photometrically using the Jaffé reaction.

5.3.4. Energy metabolites in breast muscle, kidney and liver

ATP, PCr, free Cr and glycogen were determined as described by DeGroot et al. (2018, 2019) in freeze dried muscle biopsies. The analytical method was based on enzymatic determinations, which ultimately resulted in either reduction of NADP to NADPH (for ATP and PCr) or oxidation of NADH to NAD (for free Cr). Final data included the absolute concentration of each Cr-related metabolite (ATP, PCr, and free Cr and glycogen) on a DM basis, along with calculation of the absolute concentration of TCr and the PCr:ATP ratio. As muscle ATP is an indicator for the muscle tissue content in the sample, the PCr:ATP ratio is more appropriate than absolute PCr values since it cancels out variance due to differences in biopsy samples in their contents of blood and connective tissue (Harris et al., 1992). The analysis of GAA, Cr and CrN in liver and kidney was performed in original substance by separation using reverse phase chromatography followed by detection with triple stage quadrupole MS/MS in the selected monitoring mode. CK, lactate, cholesterol, triglyderides and glucose in plasma were assayed using an AU 5800 Beckman Coulter.

5.3.5. Oxidative status parameters

MDA, GPx activity, and SOD activity was determined in plasma, breast muscle, heart, liver, and kidney samples. After thawing of organ samples, all samples were kept on ice during the procedure. An 1 g sample of heart, liver and kidney and 5 g sample of breast muscle were homogenized with Turax T2S in 10 ml of 0.05 mol/l ice-cold phosphate buffer (pH=7.0) during 45 s and centrifuged at 4°C for 15 min at 10,000 × g. The supernatant fraction was filtered through glass wool before determining MDA and enzyme activities. The TBARS method was used to quantify MDA as a marker for lipid peroxidation (Grotto et al., 2007). MDA was allowed to react with 2-thiobarbituric acid in an acid environment. Absorbance of the coloured complex was measured at 532 nm after 1-butanol extraction. A standard curve with 1,1,3,3-tetramethoxypropane was used.

The activity of GPx was determined by measuring the oxidation of NADPH according to Hernandez et al. (2004). One unit of GPx activity was defined as the amount of plasma or extract needed to oxidize 1 µmol of NADPH per min at 25°C. The SOD activity assay for breast muscle, heart, liver, and kidney was performed as described by Marklund and Marklund (1974) by measuring the inhibition of pyrogallol autoxidation. One unit of enzyme activity was defined as the amount of extract needed to inhibit the rate of oxidation by the control (no SOD) by 50%. SOD in plasma was determined using the commercial SOD assay kit according the manufacturer's instructions (19160 SOD determination kit, Sigma-Aldrich, St. Louis, USA).

5.3.6. Calculations and statistical analysis

Statistical analytical techniques appropriate to a completely randomized design were used (SAS Enterprise Guide 7, SAS Institute, Cary, USA). As one individual animal per pen was taken, the bird was considered the experimental unit. Data were checked for normality by Kolmogorov-Smirnov and Shapiro-Wilk tests and for homogeneity of variances by Levene's test. Analysis was run for each sampling day separately. All parameters were analysed by one-way ANOVA or the non-parametric Kruskall-Wallis test, if appropriate, with GAA supplementation being the fixed effect. Linear and quadratic contrasts were included as well. Post-hoc Tukey test was used to compare means. Level of $P<0.05$ was considered significant, $0.05<P< 0.10$ was considered a trend.

Initially it was aimed to deduce correlations between all physiological variables by employing a PCA. However, as minimal treatment effects on oxidative status parameters were found and because of the absence of correlations between variables representing oxidative status on one side and Cr and energy metabolism on the other during a preliminary PCA it was decided to restrict the PCA to variables of Cr and energy metabolism in all tissues. Hence, 20 variables were included, and the PCA was carried for each sampling day separately. The methodology as outlined by Montagne et al. (2007) and Michiels et al. (2013) was applied. Briefly, the data of continuous variables were standardized, i.e. the data were diminished by the mean and divided by the standard deviation of that variable. A first PCA, including a scree plot was done to determine the number of principal components to be retained. Five and six principal components at d26 and 39, respectively, appeared before a clear break where the eigenvalues leveled off. The eigenvalues of these 5 and 6 principal components were greater than 1. Then, variables that did not load on any principal component retained (correlation coefficient between variable and principal components ≤ 0.4) were excluded. This was the case for ATP in breast muscle on d26 only. Only loadings ≥ 0.4 are shown in the results, and finally an one-way ANOVA was done on scores for all principal components for the broilers. Here, all calculations were carried out using the IBM SPSS Statistics Version 24.0 program for Windows (SPSS Inc., Chicago IL, USA).

5.4. RESULTS

5.4.1. Creatine metabolism, energy metabolites, and antioxidant enzyme activities in plasma

GAA and total Cr in plasma were highly and linearly increased by feeding GAA to broilers, on either sampling day (all $P<0.05$, Table 5.1). CrN was below the detection limit in all samples and timepoints. Interestingly, while both GAA and Cr for control birds did not differ between sampling days, the elevation of both metabolites by feeding GAA was smaller on d39 *vs*. d26. HCy was not affected, thus ruling out hyperhomocysteinemia by feeding GAA. Next, linear increases in plasma Arg with supplemental GAA were seen on d26, which was different from control in 1.2 g/kg GAA-fed broilers ($P<0.05$), whilst only a trend for linear increase could be perceived for d39 ($P<0.10$). Neither CK activity, lactate, cholesterol, or triglycerides were different across treatments on any day. Also, a linear decrease in plasma cholesterol was found by feeding GAA ($P<0.05$ on d26, $P<0.10$ on d39). A linear reduction in plasma glucose by feeding GAA was noticed on d26 ($P<0.05$), but not on d39. The lipid peroxidation marker MDA, nor the antioxidant enzymes SOD and GPx showed alterations by dietary GAA.

Table 5.1. Effect of guanidinoacetic acid (GAA) supplementation on plasma creatine metabolism, energy metabolites, MDA concentration and antioxidant enzyme activities of male broilers subjected to heat stress in the finisher phase at day 26 and 39[1]. (n=12)

Item	Dietary treatment [2]			*SEM*	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
Guanidinoacetic acid, nmol/mL							
d26	0.5^{c}	6.3^{b}	14.3^{a}	1.15	<0.001	<0.001	0.458
d39	0.5^{c}	3.8^{b}	7.4^{a}	0.50	<0.001	<0.001	0.620
Total creatine, nmol/ml							
d26	78^{b}	127^{ab}	170^{a}	11.3	0.002	<0.001	0.906
d39	72^{b}	84^{b}	121^{a}	5.0	<0.001	<0.001	0.122
Homocysteine, nmol/ml							
d26	57	57	61	1.7	0.558	0.324	0.688
d39	48	45	50	1.5	0.427	0.661	0.222
Arginine, nmol/ml							
d26	263^{b}	311^{ab}	344^{a}	12.8	0.029	0.009	0.752
d39	226	271	302	16.9	0.185	0.070	0.847
Creatine kinase, U/ml							
d26	17	16	13	1.5	0.543	0.277	0.878
d39	20	17	25	1.8	0.229	0.268	0.187
Lactate, µmol/ml							
d26	3.1	3.4	3.3	0.33	0.943	0.817	0.803
d39	2.2	2.0	2.0	0.12	0.720	0.477	0.704
Cholesterol, µmol/ml							
d26	3.9	3.8	3.6	0.07	0.111	0.048	0.498
d39	3.9	3.5	3.4	0.10	0.116	0.055	0.421
Triglycerides, µmol/ml							
d26	1.6	1.3	1.7	0.08	0.108	0.745	0.038
d39	1.0	1.2	1.0	0.09	0.708	0.923	0.413
Glucose, µmol/ml							
d26	18	18	16	0.4	0.060	0.041	0.219
d39	17	17	16	0.2	0.133	0.360	0.073
MDA, nmol/ml							
d26	14	14	13	0.2	0.261	0.225	0.267
d39	14	14	13	0.2	0.876	0.615	0.927
SOD, U/ml							
d26	42	41	49	4.4	0.739	0.510	0.689
d39	49	72	67	5.1	0.472	0.378	0.353
GPx, U/ml							
d26	1.4	1.6	1.4	0.05	0.318	0.970	0.133
d39	1.3	1.2	1.2	0.03	0.256	0.187	0.318

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a row are significantly different at P<0.05.

5.4.2. Creatine metabolism, energy metabolites, and antioxidant enzyme activities in breast muscle, liver, kidney, and heart

Breast muscle samples revealed at both sampling days increases of PCr ($P<0.05$, linear effect at d26; $P<0.05$ at d39), free Cr ($P<0.10$ at d26, $P<0.05$ at d39), TCr ($P<0.05$, both days), and, PCr:ATP ($P<0.05$, linear effect at d26; $P<0.05$ at d39) with increasing dietary GAA (Table 5.2). For instance, PCr:ATP in 1.2 g/kg GAA-fed broilers was 27 and 23% higher than control on d26 and 39, respectively, underlining a higher energy load. Strikingly, muscle free Cr levels appeared to be dramatically lower at d39 as compared to d26, whereas the opposite was seen for PCr and hence PCr:ATP. Muscle energy reserve as glycogen was also higher in GAA-fed birds on d26, but no increase was detected on d 39. SOD activity was lower when feeding GAA on d26 (linear trend, $P<0.10$) and d39 (linear effect, $P<0.05$). In liver, intra-treatment variation of Cr and metabolites in liver, and even more in kidney was very large, as seen by high SEM values in these cases which might have been caused by inhomogeneity in the organ. In liver, the organ for conversion of GAA to Cr by means of methylation, Cr was almost two-fold in GAA-fed broilers *vs*. control on d26 ($P<0.05$), however this was not the case on d39 (Table 5.3). Data showed a linear increase in liver CrN, and levels were overall low. GAA in liver was not affected by graded GAA in the diet suggesting rapid turnover of GAA to Cr. MDA and antioxidant enzyme activities were not affected by treatment. No significant effects on Cr metabolism and antioxidant enzyme activities in kidney were found, apart from a quadratic and linear effect on TCr on d26 and 39, respectively, and a linear elevation in CrN on d26 (all $P<0.05$, Table 5.4). Yet, large differences were found in GAA concentration in kidney between d26 and d39. Heart tissue did not reveal any treatment effect on antioxidant enzyme activities (Table 5.5).

Table 5.2. Effect of guanidinoacetic acid (GAA) supplementation on breast muscle creatine metabolism, energy metabolites, MDA concentration and antioxidant enzyme activities of male broilers subjected to heat stress in the finisher phase at day 26 and 39[1]. (n=12)

Item	Dietary treatment [2]			*SEM*	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
ATP, µmol/g DM							
d26	26	27	26	0.6	0.703	0.898	0.410
d39	29	29	28	0.5	0.472	0.387	0.388
Phosphocreatine, µmol/g DM							
d26	49	55	65	3.0	0.085	0.029	0.784
d39	85^{b}	95^{ab}	99^{a}	2.2	0.016	0.005	0.532
Free creatine, µmol/g DM							
d26	111	128	129	3.9	0.097	0.056	0.310
d39	45^{b}	57^{a}	61^{a}	1.9	0.001	<0.001	0.245
Total creatine, µmol/g DM							
d26	160^{b}	183^{a}	194^{a}	3.9	<0.001	<0.001	0.357
d39	130^{b}	151^{a}	160^{a}	3.4	<0.001	<0.001	0.274
Phosphocreatine/ATP							
d26	1.9	2.1	2.6	0.13	0.080	0.033	0.488
d39	3.0^{b}	3.3^{ab}	3.7^{a}	0.09	0.008	0.002	0.785
Glycogen, µmol/g							
d26	239^{b}	291^{ab}	306^{a}	10.2	0.016	0.006	0.373
d39	277	305	305	7.1	0.172	0.102	0.353
MDA, nmol/g							
d26	7.1	5.9	7.0	0.40	0.433	0.920	0.202
d39	7.0	6.9	7.8	0.36	0.561	0.384	0.517
SOD, U/g							
d26	29	28	27	0.5	0.156	0.057	0.803
d39	30	28	26	0.6	0.049	0.015	0.781
GPx, U/g							
d26	0.36	0.33	0.35	0.01	0.608	0.688	0.365
d39	0.52	0.51	0.50	0.01	0.837	0.562	0.906

[1] Broilers were fed a corn-soybean starter diet from d 0 to 10, a grower diet from d 10 to 25 and a finisher diet from d 25 to 39; [2] Mean values with different superscripts per sampling day are significantly different at P<0.05.

Table 5.3. Effect of guanidinoacetic acid (GAA) supplementation on liver creatine metabolism, MDA concentration and antioxidant enzyme activities of male broilers subjected to heat stress in the finisher phase at day 26 and 39[1]. (n=12)

Item	Dietary treatment [2]			*SEM*	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
Total creatine, mg/kg							
d26	2.3[b]	4.1[a]	4.3[a]	0.26	0.001	0.001	0.095
d39	3.2	3.6	3.9	0.23	0.497	0.243	0.951
Creatinine, mg/kg							
d26	0.21[b]	0.25[ab]	0.27[a]	0.09	0.010	0.004	0.383
d39	0.21[b]	0.24[ab]	0.30[a]	0.01	0.009	0.003	0.417
GAA, mg/kg							
d26	6.4	7.4	5.7	0.40	0.190	0.457	0.104
d39	3.7	3.6	2.5	0.28	0.147	0.093	0.353
MDA, nmol/g							
d26	96	87	94	2.7	0.396	0.707	0.194
d39	77	77	82	1.8	0.418	0.247	0.532
SOD, U/g							
d26	172	164	159	5.3	0.591	0.312	0.909
d39	283	260	257	11.7	0.631	0.386	0.691
GPx, U/g							
d26	3.9	4.2	3.8	0.09	0.237	0.807	0.095
d39	3.7	3.6	3.6	0.08	0.727	0.435	0.889

[1] Broilers were fed a corn-soybean starter diet from d 0 to 10, a grower diet from d 10 to 25 and a finisher diet from d 25 to 39; [2] Mean values with different superscripts per sampling day are significantly different at P<0.05.

Table 5.4. Effect of guanidinoacetic acid (GAA) supplementation on kidney creatine metabolism, MDA concentration and antioxidant enzyme activities of male broilers subjected to heat stress in the finisher phase at day 26 and 39[1]. (n=12)

Item	Dietary treatment[2]			*SEM*	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
Total creatine, mg/kg							
d26	4.7	11.9	6.4	1.5	0.120	0.678	0.042
d39	8.7	9.1	10.8	4.2	0.102	0.042	0.476
Creatinine, mg/kg							
d26	1.2	1.2	1.6	0.07	0.073	0.038	0.320
d39	1.1	1.2	1.1	0.06	0.843	0.976	0.565
GAA, mg/kg							
d26	33.7	65.4	62.0	12.9	0.583	0.394	0.527
d39	15.1	14.3	15.5	1.75	0.957	0.913	0.789
MDA, nmol/g							
d26	33	34	34	1.1	0.871	0.807	0.644
d39	31	32	32	0.27	0.344	0.366	0.251
SOD, U/g							
d26	209	203	204	5.2	0.898	0.727	0.764
d39	218	210	223	3.9	0.446	0.613	0.246
GPx, U/g							
d26	3.8	4.0	4.0	0.06	0.249	0.159	0.370
d39	4.7	4.7	4.6	0.08	0.983	0.869	0.939

[1] Broilers were fed a corn-soybean starter diet from d 0 to 10, a grower diet from d 10 to 25 and a finisher diet from d 25 to 39; [2] Mean values with different superscripts per sampling day are significantly different at P<0.05.

Table 5.5. Effect of guanidinoacetic acid (GAA) supplementation on heart MDA concentration and antioxidant enzyme activities of male broilers subjected to heat stress in the finisher phase at day 26 and 39[1]. (n=12)

Item	Dietary treatment [2]			*SEM*	*P-value*		
Supplemental GAA, g/kg	0.0	0.6	1.2		Model	Linear	Quadratic
MDA, nmol/g							
d26	85	84	103	5.6	0.317	0.209	0.397
d39	73	72	71	3.2	0.972	0.813	0.992
SOD, U/g							
d26	52	52	49	3.0	0.872	0.658	0.787
d39	56	52	59	1.8	0.285	0.540	0.145
GPx, U/g							
d26	1.2	1.2	1.2	0.03	0.692	0.651	0.470
d39	1.3	1.2	1.3	0.04	0.687	0.640	0.469

[1] Broilers were fed a corn-soybean starter diet from d 0 to 10, a grower diet from d 10 to 25 and a finisher diet from d 25 to 39; [2] Mean values with different superscripts per sampling day are significantly different at $P<0.05$.

5.4.3. Principal component analysis of variables describing creatine metabolism and energy metabolites

A PCA was run for each sampling day separately, including 19 or 20 variables, for d26 and 39, respectively, representing Cr and energy metabolism in all tissues. For d26, 5 principal components were retained, for which 3 were affected by treatment (all $P<0.05$, Table 5.6). Representation of the most affected principal components, i.e. principal component 1 and 2, allowed to discriminate treatments visually (Fig. 5.1). Principal component 1, containing 27.3% variance explained, showed high positive loadings for GAA, Cr, Arg and lactate in plasma, PCr and glycogen in breast muscle, Cr and CrN in liver, and CrN in kidney. Not surprisingly, this was associated with higher dietary GAA inclusion, as means for treatments 0.0, 0.6, and 1.2 g/kg GAA were -0.78, 0.03, and 0.76, respectively ($P<0.05$). Interestingly, the negative loading of -0.43 for GAA in liver suggests that feeding GAA reduces this metabolite in liver. Principal component 2 groups negatively free Cr and TCr in breast (-0.67 and -0.78 loadings, respectively) and positively lactate, cholesterol and glucose in plasma and Cr in kidney. Here, negative loadings are correlated with higher dietary GAA as 1.2 g/kg GAA-fed broilers had a mean of -0.69 for the scores of principal component 2, *vs*. 0.59 for control birds. Principal component 3 suggests control and 0.6 g/kg GAA-fed broilers can be discriminated by plasma triglycerides and glucose, PCr and Cr in breast, and liver CrN; whereas 1.2 g/kg GAA-fed broilers are intermediate. On d39, only principal component 1 showed differences between treatments (Table 5.7). Means and loadings for the variables of this principal component seem to be very similar to principal component 1 on d26, and underlines consistent differences in Cr metabolic pathways as affected by feeding GAA to birds. However, worth mentioning is that on d26, Cr in liver was included in this principal component and not on d39; and further on d26 CrN in kidney was seen while on d39 it was Cr in kidney. This may highlight notable Cr synthesis in liver on either days, but probable higher Cr losses by urinary excretion on d39, after 2-weeks of HS, which is congruent to the almost doubled Cr levels in kidney on d39 as compared to d26 as yet shown in Table 5.4, at least for some treatments.

Table 5.6. Description of the major principal components obtained by principal component analysis (PCA) of 19 variables characterising creatine metabolism and energy metabolites in male broilers subjected to heat stress in the finisher phase at day 26[1].

Principal component	1	2	3	4	5
% variance explained	27.3	13.6	9.5	9.3	8.4
% cumulative	27.3	40.9	50.4	59.6	68.0
Means for treatments[2], *supplemental GAA, g/kg*					
0.0	-0.78^{c}	0.59^{a}	-0.46^{b}	-0.02	-0.22
0.6	0.03^{b}	0.10^{ab}	0.59^{a}	-0.12	0.25
1.2	0.76^{a}	-0.69^{b}	-0.12^{ab}	0.14	-0.03
SEM	0.17	0.17	0.17	0.17	0.17
P-value	<0.001	0.004	0.027	0.824	0.527
Guanidinoacetic acid, plasma	0.78				
Creatine, plasma	0.79				
Homocysteine, plasma					0.65
Arginine, plasma	0.81				
Creatine kinase, plasma				-0.68	
Lactate, plasma	0.45	0.40			-0.47
Cholesterol, plasma		0.56			0.46
Triglycerides, plasma			-0.54	0.45	
Glucose, plasma		0.76	0.42		
Phosphocreatine, breast muscle	0.65		-0.44		
Free creatine, breast muscle		-0.67	0.56		
Total creatine, breast muscle		-0.78			
Glycogen, breast muscle	0.69				
Creatine, liver	0.70				
Creatinine, liver	0.66		0.51		
GAA, liver	-0.43				
Creatine, kidney		0.42			
Creatinine, kidney	0.78				
GAA, kidney				0.59	

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a column are significantly different at P<0.05.

Table 5.7. Description of the major principal components obtained by principal component analysis (PCA) of 20 variables characterising creatine metabolism and energy metabolites in male broilers subjected to heat stress in the finisher phase at day 39[1].

Principal component	1	2	3	4	5	6
% variance explained	27.2	10.2	9.4	8.7	8.3	6.8
% cumulative	27.2	37.4	46.9	55.6	63.8	70.6
Means for treatments[2], *supplemental GAA, g/kg*						
0.0	-0.85[c]	0.14	-0.16	0.12	-0.14	0.13
0.6	0.01[b]	-0.11	-0.08	0.10	-0.34	-0.32
1.2	0.84[a]	-0.04	0.23	-0.21	0.48	0.19
SEM	0.17	0.17	0.17	0.17	0.17	0.17
P-value	<0.001	0.826	0.619	0.679	0.107	0.399
Guanidinoacetic acid, plasma	0.74					
Creatine, plasma	0.89					
Homocysteine, plasma		0.77				
Arginine, plasma	0.74					
Creatine kinase, plasma			0.64			0.47
Lactate, plasma				0.50	0.46	
Cholesterol, plasma			-0.58			0.44
Triglycerides, plasma		0.76	-0.40			
Glucose, plasma				0.70		
ATP, breast muscle					-0.72	
Phosphocreatine, breast muscle	0.74					
Free creatine, breast muscle	0.75					
Total creatine, breast muscle	0.87					
Glycogen, breast muscle	0.52		-0.40			
Creatine, liver						0.45
Creatinine, liver	0.74					
GAA, liver	-0.43	-0.46				
Creatine, kidney	0.61					
Creatinine, kidney			0.42			0.48
GAA, kidney				0.58		

[1] Broilers were fed a corn-soybean starter diet from d0 to 10, a grower diet from d10 to 25 and a finisher diet from d25 to 39; [2] Values with different superscripts within a column are significantly different at P<0.05.

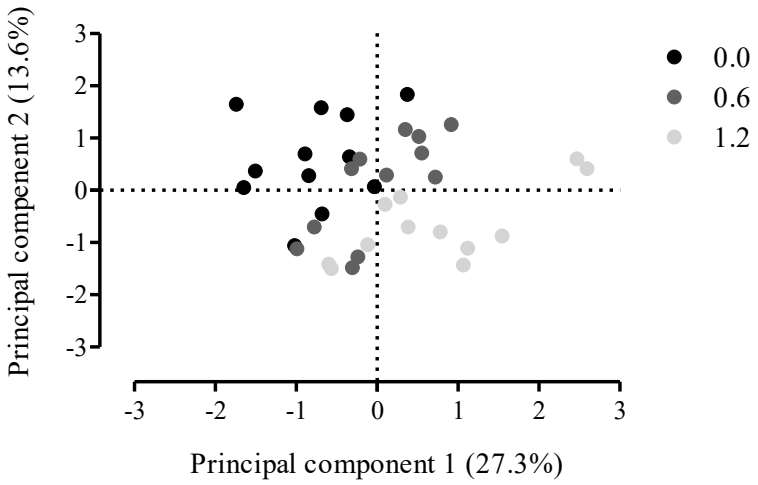

Fig 5.1. Representation of broilers supplemented with guanidinoacetic acid (GAA) at 0.0, 0.6, and 1.2 g/kg and sampled at d26 according to their principal component scores for principal component 1 and 2 from the principal component analysis. Broilers can be discriminated visually according to treatment; principal component 1 (27.3%), P<0.001, and principal component 2 (13.6%), P=0.004.

5.5. DISCUSSION

5.5.1. Dietary guanidinoacetic acid has limited effect on oxidative status, but demonstrates benefits for energy metabolism

The present study aimed to study the effects of dietary GAA on the oxidative status of broiler chickens. Hence, a model of chronic cyclic HS was adapted and a cohort of different endpoints in various organs was assessed. However, the study failed to demonstrate convincing effects from GAA feeding apart from reductions in SOD activity in breast muscle on d26 (linear trend) and d39 (linear effect). These effects were also notably small, but as breast muscle is a major source of reactive species, the observed reduced SOD activity may still be highly relevant. HS reduces the efficiency of electron transfer along the electron transport chain which in turn leads to aberrant leakage of electrons and eventually results in higher superoxide anion formation (Mujahid et al., 2009). Various isoforms of SOD, i.e. CuZnSOD in the intermembrane space (also present in cytosol) and MnSOD in the matrix of mitochondria, reduce superoxide anion to hydrogen peroxide (H_2O_2) to prevent chain reactions in forming other radicals such as $ONOO^-$. H_2O_2 in turn can act as signalling molecule, or is further metabolized to harmless water by peroxidase activity (Akbarian et al., 2016). A lower SOD activity may point at a lower need for this catalysis in line with postulations that Cr has a direct action on scavenging superoxide anion (Guimarães-Ferreira, 2014), or at a lower production of superoxide anion through the Cr-induced buffering of cellular ATP levels (Persky and

Brazeau, 2001). Mitochondria are equipped with uncoupling proteins (UCP) that assist in the control of superoxide anion production (Pamplona and Costantini, 2011). Mild uncoupling by UCP, i.e. transferring protons back into the matrix, slightly stimulates electron transport and reduces superoxide anion production, but redox energy is dissipated as heat instead of being used for ATP synthesis. It was shown that chronic HS enhanced avian UCP transcript levels in breast muscle mitochondria by not less than 71 % (Dridi et al., 2008) and 100 % (Toyomizu et al., 2011). Therefore, higher Cr load and PCr:ATP may act as buffer for this loss of ATP generation and support efficiency of electron transfer. Indeed, in poultry, Wang et al. (2015) who studied the effect of CMH on muscle lipid peroxidation and antioxidant capacity in transported broilers in summer reported that transport stress accelerated muscle lipid peroxidation by increasing TBARS production. The SOD and GPx activities increased and the upregulation of avian UCP, avian peroxisome proliferator-activated receptor γ coactivator-1α and heat shock protein 70 were insufficient to reduce muscle TBARS and prevent muscle from transport-induced OS. Despite CMH elevated muscle Cr load, it did not demonstrate potential for scavenging free radicals and activation of antioxidant enzymes. These authors supposed that the protective effect of CMH for maintaining meat quality during transport stress, possibly is correlated to loading more PCr to generate ATP and consequently reduce muscle glycolysis instead of any direct antioxidant activity. Notwithstanding this, Wang et al. (2016) found further supporting evidence for antioxidant outcomes by feeding GAA to Cherry valley ducks. Ducks fed 0.5 g/kg GAA exhibited reduced MDA and increased serum GPx activity and GSH in blood, whereas in liver the activity of catalase, SOD, GPx and GSH increased. They concluded that GAA can improve the body's antioxidative capacity to some extent due to the ability of GAA to increase the level of Cr in the body. Though this is somewhat surprising, as Cr is mainly present in skeletal muscle and thus antioxidant effects are expected in that tissue as we found, whereas Wang et al. (2016) found evidence in serum and liver. In our study the effects of GAA on SOD activity were more evident on d39 than on d26 and thus only referring to breast muscle, and it may be concluded that antioxidant effects may rather be indirect.

The Cr loading potential in breast muscle by feeding GAA to broilers, and concomitant higher PCr:ATP ratio were already reported in Chapter 4 and are well established in literature. Here, we were able to correlate this to other energy metabolites in plasma, liver and breast muscle. First, muscle energy reserves as glycogen were higher in GAA-fed birds on d26, while numerical increases were seen on d39. Also DeGroot et al. (2018) reported elevation of muscle glycogen from GAA feeding to broilers under TN conditions. Apart from improving energy status as such, higher glycogen stores may prevent substantial pH drop in muscle post-mortem, which is particularly relevant for heat stressed birds as birds in HS consume their glycogen reserves faster than non-stressed birds post-mortem (Song et al., 2015). Together with higher energy stores by Cr loading it may affect meat quality characteristics, though we were not able to demonstrate this (see Chapter 4). On this note, for both d26 and 39, glycogen in breast muscle showed positive loadings for principal component 1, albeit higher on d26 *vs*. d39, thus indicating that GAA feeding fosters carbohydrate stores as a result of Cr loading

and ATP buffering. On d26, GAA did not affect plasma lactate or triglycerides, and minimal linear decreases were found for plasma cholesterol and glucose. However, principal component 2 highlights an interesting observation. This principal component groups negatively free Cr and TCr in breast and positively lactate, cholesterol and glucose in plasma. Here, positive loadings are associated with lower dietary GAA. This suggest that dietary GAA dose-dependently reduces blood lactate, cholesterol and glucose for this sampling. This supports the assumption that dietary GAA shifts energy generation towards the use of readily available PCr instead of anaerobic metabolism (lower lactate) and oxidative phosphorylation (lower glucose). Also, lower lactate would simply indicate higher tolerance to stress, as lactate is widely used for the evaluation of stress. Peculiar is principal component 3 on d26, with a negative loading for plasma triglycerides, which actually corroborates with the quadratic effect (Table 5.1). Further, reports in humans are in agreement with our findings for reduced plasma cholesterol by GAA, which occurred on both sampling days. In fact, Kreider et al. (1998) studying Cr supplementation in human demonstrated high density lipoprotein (HDL) concentrations were increased (+13%), while there was some evidence very low density lipoprotein levels (VLDL) (-13%) and the ratio of total cholesterol to HDL levels (-7%) decreased in the Cr group; corroborating with earlier results by Earnest et al. (1996). In this latter study, Cr supplementation to humans decreased total cholesterol, triglycerides, and VLDL in moderately hyperlipidemic, physically active male and female subjects. In Chapter 4 we yet alluded to the role of AMPK herein. AMPK is the main sensor of cellular energy status, and is activated in response to energy stress to restore energy balance by inhibiting ATP-consuming processes and promoting ATP-generating pathways; driven by fluctuations in the AMP:ATP ratio (Corton et al., 1994, 1995). In Chapter 4 we postulated that GAA feeding might enhance cellular energy in liver and thus lower AMPK stimulation. This in turn would alter energy metabolism and potentially increase liver cholesterol 7-alpha-hydroxylase activity (Hu et al., 2019). This enzyme converts cholesterol to 7-alpha-hydroxycholesterol, the first and rate limiting step in bile acid synthesis. Higher activity of this enzyme might thus cause lower circulating cholesterol, congruent to our observations. On the other hand, AMPK phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-controlling enzyme of the mevalonate pathway that produces cholesterol, amongst others. However, Fouad et al. (2013) showed cholesterol-lowering effect in plasma by supplementing Arg in broiler chicken diet via modulating lipid metabolism. Arg inclusion reduced HMG-CoA reductase mRNA expression. Liver is the most important vital organ for fatty acid synthesis in avian species, in which almost 85% of the fat accumulated by growing birds is produced in the liver (Molette et al., 2012). As GAA is able to spare Arg in broilers (Dilger et al., 2013; DeGroot et al., 2018), it could be speculated how GAA can affect lipid metabolism. The effects of feeding GAA to subjects on liver energy status and AMPK and lipid metabolism therefore remains enigmatic even more so as we did not measure PCr, ATP or AMPK in hepatic tissue. In our study, liver TCr increased linearly with increasing GAA dose on d26, but only numerically on d39. Similar reports are documented in EFSA (2009) in broilers under TN conditions, however, increases in liver GAA were observed at higher dose levels

documentation presents studies in which graded GAA has no (up to 0.6 g/kg GAA) or elevating effects on liver Cr (up to 1.5 g/kg GAA) in poultry.

5.5.2. Effects of dietary guanidinoacetic acid on creatine metabolism in key organs

Increases in GAA and TCr in plasma by feeding GAA to broilers suggests efficient absorption in the gastro-intestinal tract and methylation of GAA, respectively. Interestingly, for both plasma GAA and TCr elevations were markedly higher on d26 as compared to d39. It is fair to speculate that differences between these days are primarily related to the sustained HS broilers faced. For example, it could be hypothesized that intestinal absorption of GAA might be hampered by chronic HS, although true fecal digestibility is believed to be close to 100% in TN conditions (Tossenberger et al., 2016). To the best of our knowledge, a full description of transport mechanisms for GAA in the intestine is not available. However, it is well described that GAA is taken up by a variety of cell types through several transporters such as SCL6A8, and protein carriers for taurine (SLC6A6) and γ-aminobutyric acid (GAT2) (Ostojic, 2017). SLC6A6 was also shown to be highly expressed in the human intestine; but likely GAA might have affinity for other intestinal AA transporters as well. Habashy et al. (2017) demonstrated that HS down-regulated the expression of several ileal AA transporters. It is thus plausible that lower increases in plasma GAA on d39 are caused by reduced absorption. Next, lower augment of plasma Cr may indicate limited GAMT activity, similar to a slow down of other enzymatic reactions. Notably, Chamruspollert et al. (2004) reported that higher temperatures slowed Arg metabolism negatively affecting Cr synthesis pathways. However, lower plasma Cr is more likely a simple consequence of lower GAA availability due to lower GAA absorption. Plasma, HCy was not affected. It means that methylation of GAA did not cause accumulation of HCy.

Following increased plasma Cr levels in GAA-fed birds, muscle Cr load increased, possibly facilitated by SLC6A8, which is highly expressed in brain, liver, intestine and thus also skeletal muscle (Ostojic, 2017). For all treatments, thus including control birds, breast TCr levels appeared to be substantially lower on d39 *vs*. d26. This might be a consequence of lower plasma Cr on d39 but it cannot be completely ruled out that there is also an influence of age. Actually, there is paucity of data on Cr content in breast muscle of chickens affected by age. Fisher et al. (1956) found that Cr in muscle of chickens steadily increased from hatching (≈2400 mg/kg) to 57 days of age (≈4300 mg/kg), however, done with so different genetics back in the ’50 (White Rock male x New Hampshire female). Later, Ngo et al. (1977) came to similar conclusion. Contrary to that, in Chapter 2 (taking d10 and d42) and Chapter 3 (d10 and d25), no marked differences upon age were seen. This warrants more attention. Various reports elucidate that GAA concentration in breast is extremely low (e.g. 0.039 µmol/g in control chickens of Tossenberger et al., 2016). Opposite to breast, in liver, the major organ for GAMT activity, TCr is more comparable across days, which then may reject the hypothesis that the supply via *de-novo* synthesis is downsized. Only on d26 Cr in liver was affected by treatment. In literature, Cr concentration in liver is unchanged or increased as dietary

GAA is higher, whereas GAA concentration in liver is decreased (e.g. Tossenberger et al., 2016; EFSA, 2009). The latter could not be clearly demonstrated here, but was confirmed in the PCA on both days (loading for principal component 1 for GAA in liver). This could be caused by downregulation of the SCL6A8. Also it should be noted that Cr and GAA concentrations we found for liver are a multitude lower than in reports of Tossenberger et al. (2016) and EFSA (2009), for unknown reasons. Surprisingly, relatively high GAA concentrations were found in kidney, differing immensely between days but not affected by treatment. From the PCA statistics, it was derived that Cr losses by urinary excretion on d39 might be elevated. This could also in part explain lower Cr breast levels. Again, it should be noted that Cr and GAA concentrations we found for kidney are a multitude lower than in the same reports, for unknown reasons, and particularly for kidney exhibited large variations across subjects. Thus together, lower absorption of GAA most likely leads to lower conversion of GAA to Cr and lower uptake of circulatory Cr into muscle cells. We speculate that this is followed by higher Cr losses in urine that may have contributed to lower Cr concentrations in breast muscle. Data from PCr and PCr:ATP do not show a similar trend, however, this might be due to sampling difficulties on d26 and should be confirmed.

5.6. CONCLUSION

In this work we found that feeding GAA to heat stressed finisher broilers had limited effects on the oxidative status as solely evidenced by reduced muscle SOD activity. Even if other OS markers were not affected, this finding may support indirect effects due to Cr loading protecting muscle function, in particular after chronic HS. In addition, energy metabolism in muscle was greatly supported: Cr, PCr:ATP and glycogen were improved and blood markers revealed an improved capacity of readily available Cr and PCr which can be of particular relevance when oxidative phosphorylation is limited, such as during HS.

5.7. ACKNOWLEDGEMENTS

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CHAPTER 6

GENERAL DISCUSSION AND FUTURE PROSPECTS

CHAPTER 6

GENERAL DISCUSSION AND FUTURE PROSPECTS

This thesis aimed to increase our understanding of how GAA interacts with other nutrients in the diet of broiler chickens on performances and if differential effects are to be expected when used in specific conditions such as HS. Further, this thesis wanted to generate additional insights in the mode of action by exploring the metabolism of GAA and Cr, and its potential antioxidant action in the chicken. Therefore, three elaborate broiler experiments in which GAA supplemented to vegetable diets and fed to broilers, were conducted and results were detailed in Chapter 2, 3, 4 and 5.

The highlights of our findings are:

[1] the effects of GAA are independent of ND,

[2] the effects of GAA are influenced by dietary Met, however, only in the finisher period, which urges for proper sulfur AA formulation and consideration of methylation potential of diets when feeding GAA,

[3] GAA supplementation improved feed efficiency and survival during chronic cyclic HS to an extent beyond commonly seen in TN conditions,

[4] GAA consistently enhanced breast muscle energy status, that indirectly may improve oxidative status and was associated with higher glycogen levels, and an Arg sparing effect.

It remains puzzling why in only a few cases in our experiments growth was improved by feeding GAA, whereas mostly feed conversion was improved related to lower feed intake. Probable causes for reduced feed intake have been discussed in the experimental Chapters. In general, most studies show improvements in growth (Fig. 1.6), but variation is large. Extremely high responses were found by Dilger et al. (2013) because of using Arg deficient diets. Other sources of variation might be differences in duration of the study, ingredient and nutrient composition of basal diet, sex, breed, growth rates, and environmental conditions. This warrants further attention.

6.1. GUANIDINOACETIC ACID ENHANCES CREATINE LOADING OF MUSCLE AND SPARES ARGININE BUT PARTITIONING TO THESE EFFECTS MAY DEPEND ON DOSAGE, AGE AND ENVIRONMENT

In line with ample literature we found that the benefits for GAA-fed broilers are to a major part due to Cr loading of muscle and Arg sparing. However, the contribution of these two benefits to the overall mode of action may depend on dosage, age of bird, and environment. In an attempt to quantitate these separate effects following steps were taken. First, it was necessary to know the *de-novo* synthesis, defined as the percentage of Cr synthesis from endogenously formed GAA, in GAA-fed chickens. Therefore, a factorial approach to determine chickens' Cr synthesis was adopted from Tossenberger et al. (2016) with modifications. Key figures are taken from our studies or alternatively from literature (Table 6.1). For full understanding, we recall that Cr synthesis is necessary to replenish muscle Cr that is irreversibly converted to CrN (maintenance) and to supply new muscle tissue (retention), totalling to the net requirement for Cr. Equations to calculate Cr for maintenance and retention are given in Table 6.1. To estimate Cr for maintenance, the daily degradation rate of Cr of 1.7%/d (Walker, 1979) was used. In part, synthesized Cr will come from *de-novo* GAA synthesis that is subsequently methylated. The remainder is furnished by dietary GAA. For dietary GAA, digestibility of GAA and utilization of digested GAA are applicable (definitions, Table 6.1). Important to say is that these digestibility and utilization coefficients are taken from one publication as this is the sole report that established these figures in a comprehensive way (Tossenberger et al., 2016). Dietary GAA intake can be calculated from daily feed intake and intented or analyzed, if available, GAA dosage. Thus, dietary GAA should match the Cr synthesis that is not derived from endogenously synthesized GAA, whilst correcting for digestibility of GAA and utilization of digested GAA and for differences in molar weight between GAA and Cr. This leads to equation [1] in Table 6.1. Hence, equation [2] can be made (Table 6.1). From this equation, *de-novo* synthesis can be calculated for each case, it means for each treatment within each experiment, thus based on reported values in Chapters 2, 3 and 4. However, it was not possible to do the calcutions for all ages within each case because many assumptions are not applicable or could not be derived for young broilers (e.g. 10 day-old chickens) such as proportion of muscle in the body, digestibility of dietary GAA and utilization of digested GAA. For the latter two items, these were established in finisher broilers (Tossenberger et al., 2016), and thus possibly not transferable to young broilers. Therefore, only for cases whereby sampled birds had BW over 1.3 kg, the calculations were made. Also, similar procedures were applied to data from Michiels et al. (2012), i.e. birds were fed GAA at 0.6 and 1.2 g/kg and sampled at d26 under TN conditions, as for comparison. Then, from the above, the TCr synthesis and the portion from *de-novo* synthesis can be calculated and is presented in Fig. 6.1. Note that in this graph, Cr synthesis is expressed per metabolic BW and on daily basis, as for the respective day. Thus, assuming 99% digestibility of dietary GAA, 76.2% utilization of digested GAA and 1.7%/d daily degradation rate of Cr, then *de-novo* synthesis accounts for 66.8, 76.4, 69.9 and 72.7%

for our data from Chapter 2 at d42, Chapter 4 at d26 and d39, and Michiels et al. (2012) in case of 0.6 g/kg GAA, respectively, and 52.7, 54.6, 43.9 and 46.8% in case of 1.2 g/kg GAA, respectively. As dietary GAA increases, total daily Cr synthesis augments, but the proportion from *de-novo* synthesis drops in line with negative feedback mechanism of circulating Cr on AGAT. Clearly, Cr synthesis expressed on metabolic BW on d39 and d42 is lower as compared to the 2 cases reported for d26, which has been discussed in previous Chapters, and again difficult to say whether this is solely an age effect and/or due to chronic HS. Next, using the CON treatments as reference, the Cr muscle loading effect of dietary GAA can be derived. Therefore, Cr synthesis in GAA-fed birds was diminished by the level of Cr synthesis in respective CON treatment. Hence, in Fig. 6.1 light grey represents *de-novo* synthesis; grey, synthesis by methylation of GAA from the diet and hitherto corresponding to Arg sparing; and dark grey, synthesis by methylation of GAA from the diet and adding surplus Cr in body as compared to CON. The latter two then correspond to the effect of Cr loading of muscle and Arg sparing, respectively, of dietary GAA. It appears that the ratio between these two effects is mainly dependent on GAA dosage. At 0.6 g/kg, the effect of Cr loading of muscle exceeds the Arg sparing effect, with the exception for the case of Chapter 2, d42 whereby at 0.6 g/kg no increased muscle Cr was found. Peculiar is that for Chapter 2, d42 adding the highest level of GAA to the diet did increase Cr loading but not the Arg sparing effect as compared to GAA at 0.6 g/kg. The opposite can be said for the other cases, here clearly, increasing GAA in diet provokes mostly a larger Arg sparing effect and not Cr loading boost. Then, it can appear that GAA at 1.2 g/kg may be a less cost-effective strategy to boost Arg availability. However, this may be profitable to reduce mortality, as in Table 4.2 it is clear that the major difference in performance indices between these two levels of GAA is further improvement of survival. Interestingly, TCr synthesis at d26 for our thesis and Michiels et al. (2012) are very comparable, however, the proportion of *de-novo* synthesis is much smaller and thus Arg sparing effects much larger in the latter study. It is hard to speculate on these differences. Related to the Arg sparing capacity, further calculations were made. Arg sparing was defined as the proportion of digested Arg that is not being used for GAA synthesis as compared to CON, and is given in Fig. 6.1. by the black line. It ranges between 2.2 to 5.7% and between 5.9 to 8.3% for groups with 0.6 and 1.2 g/kg GAA, respectively; with minor differences between cases per dietary GAA level. On the one hand these numbers may look small, but extending this to the nutrition of the broiler these are very relevant. Digestible Arg makes up a large proportion of dietary protein (e.g. 1.37 to 1.10% in total diet as recommended for Ross 308 broilers) and various studies have shown potential benefits of dosing Arg beyond recommended levels, either or not in challenge conditions (e.g. Khajali and Wideman, 2010; Khajali et al. 2014). No similar data were found in literature, however as outlined in Chapter 1, section 1.3.4.1, both Dilger et al. (2013) and Lemme et al. (2018b) provided GAA equivalencies relative to dietary Arg based on performance data.

However, to the above presentation some comments should be made. The daily degradation, i.e. 1.7%/d was taken from Walker (1979), which can be debated, despite the fact that conversion of Cr into CrN is spontaneous and non-enzymatic and thus may

be universal. Keshavarz and Fuller (1971) in their early work on the interaction between Arg and Met in broilers found Cr+CrN excretions of approximately 26 mg/kg BW for 14 day-old chickens fed replete diets. Using their data, an approximate 1.7%/d degradation of Cr can be calculated, identical to Walker (1979). However, recently, doubts have been postulated on daily degradation rate of Cr in modern fast-growing broilers (Tossenberger et al., 2016). These authors reported 3.395 mg/kg $BW^{0.75}$ CrN excretion in control birds at d38-42, which suggests a 10-fold lower degradation rate of Cr than found by Keshavarz and Fuller (1971). Differences between these studies may be related to age of birds, and improvement of genetics and inherent muscle accretion capacity, and quality of diets ever since. Also, using data from the EFSA reports (EFSA, 2009, 2016) may support that discrepancies in daily degradation of Cr across species exist. Although in these documents daily degradation of Cr was not assessed *per se*, EFSA (2009) reports Cr and CrN levels in breast muscle of broiler (control) chickens varying between 3896 to 4741 mg/kg and 6.7 to 11.8 mg/kg, respectively, whereas in piglets' muscle this was 5759 mg/kg and 41.0 mg/kg, respectively (EFSA, 2016). Even considering the fact that normally CrN diffuses out of the muscle and that only Cr is retained, the ratio CrN to Cr in muscle is very different between these species and might be indicative for degradation rate of Cr. For the piglet, this is then 0.71%, and much higher than chicken (0.15-0.27%), likely suggesting that modern fast-growing chickens have lower daily degradation rates then assumed. However, our data, Tables 2.6 and 3.5 also report Cr and CrN in breast muscle, and there CrN was varying between 30 and 65 mg/kg, depending on age, thus in contrast to EFSA (2016). On the other hand, in fast-growing animals the requirement of Cr for maintenance is far smaller that the requirement for retention, thus the daily degradation rate should not affect markedly Cr synthesis. Nonetheleless, scientifically the universal daily degradation rate can be questioned, this warrants more investigation.

Another point of controversy in these calculations may come from the figure taken for utilization of digested GAA. Tossenberger et al. (2016) reported an 'apparent' post-absorptive utilization of 76.2% for GAA at 0.6 g/kg, but at the 'tolerance test' dosage of 6.0 g/kg it was 45.6%; whereas also Lemme et al. (2018a) showed that utilization of digested GAA may depend on Arg in diet and GAA dosage, here different between 0.6 and 1.2 g/kg GAA. Thus, also utilization of digested GAA may be different for the cases taken above. The value taken for utilization of digested GAA has a large impact on the outcome of the calculations. For example, if a lower utilization of digested GAA would be taken then the calculated *de-novo* synthesis will increase accordingly. It means that without knowing exactly this figure, computed data on Cr synthesis remain questionable, and thus this excersise is in some way artificial. Nonetheless, from all the above we may conclude that the *de-novo* synthesis in subjects fed GAA or Cr is likely not a fixed value, as opposed to the generally accepted 66% taken from Delanghe et al. (1989).

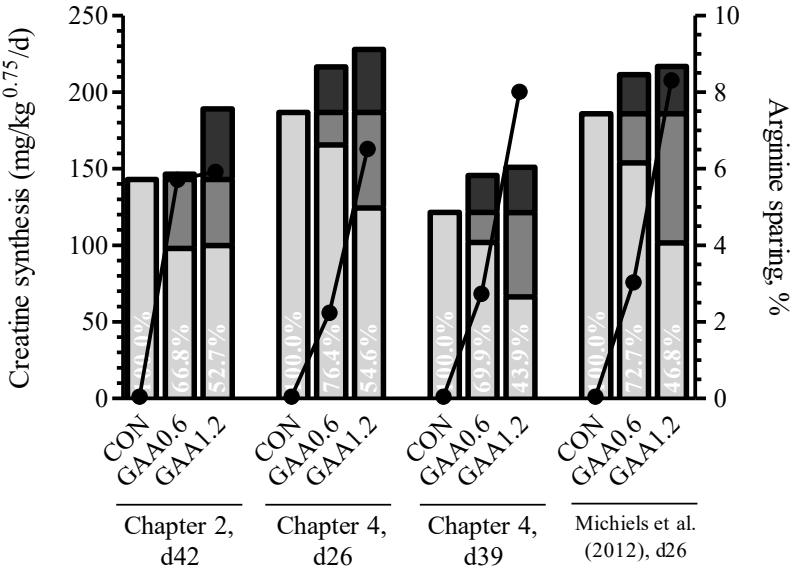

Fig. 6.1. Calculated Cr synthesis (bars) on left Y-axis and Arg sparing (lines) on right Y-axis for broilers taken from Chapter 2 and 4 and Michiels et al. (2012) (for calculation and definitions, see text and Table 6.1). Light grey, *de-novo* synthesis (percentages given in white are relative to total Cr synthesis); grey, synthesis by methylation of GAA from the diet and corresponding to Arg sparing; dark grey, synthesis by methylation of GAA from the diet and adding surplus Cr in body as compared to CON.

Table 6.1. Factorial approach to the calculation of creatine synthesis in broilers, modified from Tossenberger et al. (2016).

Item	Unit	Description	Reference
Body weight	kg	Taken from Chapter 2 and 4, mean BW of sampled birds (data not shown in Chapter 2 and 4)	
Key figures			
Daily feed intake	kg/d	Taken from Chapter 2, Table 2.2 and Chapter 4, Table 4.2; by interpolation from performance data of pen	
Daily gain	kg/d	Taken from Chapter 2, Table 2.2 and Chapter 4, Table 4.2; by interpolation from performance data of pen	
Proportion of muscle tissue in body	%	Set between 44 to 47% along 1.4 to 3.0 kg BW, taken from references and own data and adjusted	Aviagen (2014) (Ross 308: performance objectives) and own data
Creatine content in muscle	mg/kg	Taken from Chapter 2, Table 2.6 and Chapter 4, Table 4.6; analyzed values	
Creatine content in total body	mg/kg	95% is present in muscle, Cr for total body is recalculated based on content in muscle and this figure $$creatine\ content\ in\ total\ body = \frac{creatine\ content\ in\ muscle}{0.95}$$	Li et al. (2018)
Daily degradation rate	%/d	1.7%/d, irreversible daily degradation of creatine into creatinine	Walker (1979)
De-novo synthesis	%	***To be calculated***, percentage of creatine synthesis from endogenously formed GAA	
Digestibility of dietary GAA	%	99%	Tossenberger et al. (2016)
Utilization of digested GAA	%	76.2%, percentage of digested GAA that will support creatine requirement	Tossenberger et al. (2016)
Maintenance			
Muscle tissue	kg	$$muscle\ tissue = body\ weight * propertion\ of\ muscle\ tissue\ in\ body$$	
Creatine in body	mg	$$creatine\ in\ body = \ creatine\ content\ in\ total\ body * muscle\ tissue$$	
Requirement for maintenance	mg/d	$$requirement\ for\ maintenance = creatine\ in\ body * daily\ degradation\ rate$$	
Retention			
Muscle gain	kg/d	$$muscle\ gain = daily\ gain * proportion\ of\ muscle\ tissue\ in\ body$$	
Requirement for retention	mg/d	$$requirement\ for\ retention = muscle\ gain * creatine\ content\ in\ total\ body$$	
GAA intake			
GAA of diet	mg/kg	Taken from Chapter 2, Table 2.1., intended values and Chapter 4, Table 4.1, analyzed values	
GAA intake	mg/d	$$GAA\ intake = GAA\ of\ diet * daily\ feed\ intake$$	

$$GAA\ intake * \left(\frac{digestibility\ of\ dietary\ GAA}{100}\right) * \left(\frac{utilization\ of\ digested\ GAA}{100}\right) * \left(\frac{131.1}{117.1}\right) = (requirement\ for\ maintenance\ +\ requirement\ for\ retention) * \frac{(100 - \boldsymbol{de\ novo\ synthesis})}{100} \quad [1]$$

with 117.1 and 131.1 g/mol being the molar weight of GAA and Cr, respectively

$$\boldsymbol{de\ novo\ synthesis} = 100 - \left(\frac{84.46 * GAA\ intake}{(requirement\ for\ maintenance\ +\ requirement\ for\ retention)}\right) \quad [2]$$

6.2. GUANIDINOACETIC ACID HAS NO OR MINOR EFFECTS ON CARCASS YIELD AND COMPOSITION AND BREAST MEAT QUALITY

Our literature review suggests that dietary GAA may offer potential to increase breast meat yield, but has no or reduces ultimate pH slightly associated with lower WHC. Next, meat colour turns more light by feeding GAA (Chapter 1). In Chapter 2, 3 and 4 we studied these endpoints as well. In line with literature, Chapter 4 showed that GAA had no effect on pH, thawing loss and oxidative stability during simulated retail display. However, here we did not see any effect on colour indices. Also in Chapter 2 (42d-old birds) we found no effect on the breast meat characteristics pH, press loss, cooking loss, shear force, and colour. Further, in Chapter 2 (42d-old birds) and Chapter 3 (25d-old birds) we could not observe any effect on proximate analysis of breast muscle. Also, in these chapters we did not see an effect on portions of carcass, apart from increase of relative wing percentage on d25 in Chapter 2. Then, it is fair to conclude that literature as summarized in Chapter 1 are pertinent, and that also increased breast meat yield is not necessarily a result of GAA feeding.

6.3. THE EFFICACY OF GUANIDINOACETIC ACID MAY DEPEND ON OTHER NUTRIENTS IN THE DIET

Chapter 2 and 3 were designed based on the assumption that the effects of supplemental GAA may depend on several nutritional factors in the diet such as different levels of ND and provision of Met, which delivers SAM for methylation of GAA to synthesise Cr. Accordingly, Arg and Gly due to their contributions for the endogenous synthesis of Cr could be the factors that manipulate the efficacy of GAA.

As only changing ME in diets may cause imbalances between energy and protein, in Chapter 2 we examined the interaction between GAA and ND in the diet. Contrary to our expectations, no two-way interactions were found for performance indices, which suggests that the effects of GAA were independent of dietary ND. In all phases of feeding, when ND increased in the diet, BW and ADG increased substantially, in line with the similar study using wheat-SBM diets (Yazdi et al., 2017). The improvement in growth performances seen by increasing ND in the diet was in agreement with Brickett et al. (2007), Li et al. (2010a) and Yazdi et al. (2017). In general, broilers have the ability to regulate feed intake based on the dilution of ND but it is possibly affected by other nutritional factors also (Brickett et al., 2007). FI increases with dilution of ND, but birds may have difficulty maintaining energy intake with high levels of dilution (Nielsen, 2004). Concerning FI, which in the starter period was not affected and in the grower and finisher period was increased, our finding in Chapter 2 was opposite to the reported data of Brickett et al. (2007) who found a larger reduction in FI when birds were older upon higher ND in the diet, corroborating the assertion that birds increase feed consumption to compensate for lower dietary energy to meet requirements. It might be interpreted that

on the one hand gut capacity will not allow the consumption of sufficient amounts of a conventional starter feed in low ND diets. On the other hand, it could be interpreted that the efficacy of energy utilization in broilers is increased or there was wastage of feed during this period for the high ND group which was considerd as a FI. Furthermore providing mash rations (Chapter 2), seems to make regulation of bird's feed consumption more difficult (Brickett et al., 2007). But again no interaction with GAA was found, which may be similarly explained as the conclusions made by Mousavi et al. (2013) with regard to ME. These authors suggested that with higher energy levels more rapid BWG and muscle growth are achieved and that in this respect a higher Cr provision may contribute to a more efficient utilization of dietary nutrients and energy, resulting in improved ADG and particularly F:G, thus excluding any potential to spare ME. Overall, from all our studies it may appeal that GAA is most efficacious when birds achieve highest growth rates (e.g. finisher periods in all Chapters).

In Chapter 3 the interaction of GAA and Met on performance and Cr loading in broiler chickens was studied. In fact, no interactive effects between dietary Met and GAA were found in the starter and grower phases. In line with the hypothesis, there was an interaction between dietary Met and GAA for growth in the finisher phase, showing that, at deficient and excess Met, GAA at 1.2 g/kg negatively affected growth. Lemme et al. (2010b) concluded that 0.8 g/kg supplemental GAA was not effective in improving F:G in situations of deficient Met, whereas it enhanced feed efficiency at adequate Met levels. In our study and in the case of deficient Met, this could be explained by a decreased Met availability for growth. However, when Met is limiting, it is unclear whether protein synthesis or transmethylation is primarily affected and whether certain re-methylation pathways are spared. In contrast to the current findings, Robinson et al. (2016b) demonstrated that the synthesis of Cr was sacrificed in the liver of Met deficient piglets, which is of clinical significance as Cr accretion has been estimated to consume ~30% of dietary Met during suckling (Brasnon et al., 2009, 2011). In other words, methyl donors contribute an equivalent of 9-12% of dietary Met, for Cr synthesis and during methyl restriction, dietary Met is preferentially partitioned to protein synthesis at the expense of transmethylation reactions. The results with 1.2 g/kg GAA and high Met were more difficult to interpret. Nonetheless, several assumptions were made suggesting that: 1/ the balance among various methylation pathways might be disturbed, rather than methylation potential *per se*, 2/ stimulation of SAM conversion to sarcosine by consuming Gly catalysed by GNMT could effect FI, 3/ polyamine synthesis may be affected. Altogether, these remain speculations, and also confirmation of the interactive effect between GAA and Met is warranted.

It is surfeit to say that GAA efficacy is influenced by dietary Arg level, which may be correlated with increasing requirements of Arg as an essential AA in modern broilers. NRC requirements for total Arg in broilers was established at 1.25% of the diet until 3 weeks, 1.10% from 3 to 6 weeks, and 1.00% from 6 to 8 weeks of age (NRC, 1994). The NRC (1994) recommendation for the Arg:Lys ratio is 104:100. Jahanian (2009) indicated that the Arg requirements of starting broiler chicks grown in TN condition for maximum

growth performance, feed efficiency, and optimal immune functions were 101, 103 and 107% of NRC values and these were dependent on dietary protein concentration. In Chapter 4, linear increases in plasma Arg with supplemental GAA were seen on d26, which was different from control in 1.2 g/kg GAA-fed broilers, whilst only a trend for linear increase could be perceived for d39. Arg requirement during the finisher phase has been seldom studied, data obtained from this period showed larger variation than the observations from the starter and grower phases where Arg supplementation has shown to increase breast meat yield (Corzo et al., 2003). In terms of gender, some studies have suggested that males require greater quantities of AA than females at a similar age. It has been indicated that Arg requirements are higher for male broilers than for females (Kessler and Thomas, 1976). This was attributed to the higher protein and less fat deposition in male BWG (Han and Baker, 1994). The Arg requirement is influenced by several factors including rates of protein synthesis and degradation, and synthesis of metabolic compounds (Ball et al., 2007). It is now obvious that GAA may spare Arg, and this leads to an interesting advantage of GAA over Arg as 'dietary source of Arg'. Indeed, high GAA in the diet will have no antagonistic effect with Lys as opposed to Arg, which may become critical when designing high Arg diets for specific purposes. Further, our findings from Chapter 4 indicate that 0.6 g/kg GAA increased plasma Gly at d39. This increase amounted to 15%, and interestingly, Ser was also increased by 10%. Since Gly and Ser are interconvertible, this suggests a potential Gly_{equiv} sparing effect for this treatment, although it does not appear to be the case on d26, nor for 1.2 g/kg GAA at d39 (Siegert and Rodehustcord, 2019). Considering the trend for lower CP diets, this is intriguing.

6.4. THE SUPPLEMENTATION OF GUANIDINOACETIC ACID MAY BE MORE APPEALING IN CERTAIN CONDITIONS

In agreement with our hypotheses in Chapter 4 and 5, dietary supplementation with GAA was beneficial to heat stressed finisher broilers because of muscle Cr loading, antioxidant action (though limited to breast muscle) and Arg sparing. In that experiment, supplementation with GAA improved F:G in grower and particularly in finisher period, with 10 and 11 points in 0.6 and 1.2 g/kg GAA fed birds, respectively, the highest improvements shown in this PhD thesis. Amiri et al. (2019) showed 12 points reduction in F:G at 1.2 g/kg GAA in low CP diet, in finisher period under HS conditions. To recall, in Chapter 1, section 1.4.2.1 we showed that on average (and most studies were in TN conditions) F:G can be reduced with 2.9 and 5.8 points at 0.6 and 1.2 g/kg GAA, respectively. HS is believed to alter the balance between muscle anabolic and catabolic reactions. In fact, it was shown that the expression of IGF-I, phosphatidyl-inositol 3-kinase, and p70S6 kinase associated with protein synthesis was lower, whereas the expression of muscle ring-finger protein-1 and MAFbx associated with protein degradation were higher in HS condition (Zou et al., 2015). Likely this can lead to higher UA production, and thus Gly need; possibly here GAA's potential to spare Gly_{equi} might interfere. Next to this, HS may induce OS resulting in higher needs for the endogenous

antioxidant GSH, that is composed of Gly, glutamate, and Cys, the latter also needing Ser for its endogenous synthesis (Akbarian et al., 2016). After all, Gly_{equiv} is supposed to be the first limiting non-essential AA in broiler chickens (Siegert and Rodehustcord, 2019). Nonetheless, it remains to be confirmed whether GAA is able to promote Gly metabolic functions, possibly advantaging broilers under HS. In other challenge conditions such as HA; Ahmadipour et al. (2018c) found 14 and 15 points improvements in F:G compared to control group at 1.0 and 1.5 g/kg GAA, respectively. Next to this, they reported that ascites mortality was significantly prevented by GAA when added at 1.5 g/kg and serum NO levels in the blood increased as a consequence of GAA supplementation. This suggests that Arg sparing effects are prominent. On the other hand, Faraji et al. (2019) and Khodambashi et al. (2018) who studied the effect of GAA in CS condition did not find positive effects of feeding GAA. Altogether, this may manifest that using GAA in challenge models as HA and HS appears to elicit responses slightly beyond average, but not for CS.

Above, we already mentioned that GAA might be interesting in low CP diets because of its presumed effect on Gly metabolism. Recently, Awad et al. (2019) reviewed the responses of broilers to low CP diets under HS conditions. This leads to some interesting remarks. Decreasing heat increment through lowering CP level via supplemental AA have long been suggested as a nutritional practice to alleviate negative HS effects on broiler performance. Awad et al. (2019) summarized that dietary CP could be reduced safely by 2.3% via essential AA supplementation during later ages, when birds are exposed to an average daily ambient T of ≤27.3°C. When Gly was added, the margin of CP reduction could be increased to 5.1% without compromising the growth of broilers subjected to this T limit. Important to understand is that these authors determined the T for HS conditions in case of a cyclic HS model as the average of the high and low T. For example, for our cyclic HS model (T to 34°C with 50-60% RH for 7h daily) used in Chapter 4 and 5, this would be (22+34)/2 = 28°C. This approach is similar to the one suggested by Flécher et al. (2019). It means that in particular for models with cyclic HS, CP can be lowered without affecting performances. Models with constant high T turned out to have a different outcome. Indeed, under constant HS conditions, feeding the reduced CP diets resulted in lower FI, lower BWG and higher F:G in comparison with those fed the control diet. Findings from two recent studies by Law et al. (2018) and Law et al. (2019) provided similar evidence that the response of heat stressed broilers to reduced CP diets mainly depends on the severity of heat exposure. While the change in CP from 19.0 to 16.7% had no negative effect on growth performance under cyclic HS (average daily T of 26.2°C) conditions (Law et al., 2019), feeding a similarly reduced-CP diet negatively affected weight gain and F:G of broilers in a hot and humid tropical environment, where the estimated average daily T was at least 28.5°C (Law et al., 2018). In our model of cyclic HS, GAA in diets with 'normal (recommended by strain specifications)' CP levels showed thus remarkable improvements, but also Amiri et al. (2019) showed huge improvements in F:G under cyclic HS but then in low CP diet supplemented by GAA. From this we cannot conclude whether GAA would be more efficacious in low CP diets when birds are heat stressed. The paucity of data regarding

the effect of HS and low CP diet supplemented by GAA makes the interpretation of this difficult.

6.5. PRACTICAL USE OF GUANIDINOACETIC ACID IN FORMULA FOR BROILERS WILL DEPEND ON THE CONTEXT

GAA as feed additive can be used in practical diets, but the nutritionist may consider various aspects prior to decide to have GAA in the formula. First, the use of GAA in broiler diets should be discussed when matrix values are allocated to the additive. For example, Lemme et al. (2018a) reported Arg equivalencies of 77% for F:G. As F:G is the major driver for economic profit in broiler production, it may be elegant to use this equivalency as matrix value, thus 1 kg GAA could provide 770 g of dArg. This figure has been taken for commercial purposes as well. In addition, an energy value beyond its ME *per se*, should be considered. Though, in 1.4.4.5, it was concluded that it remains elusive whether and how much ME dietary GAA could spare, and possibly GAA is most efficient at recommended ME. Nonetheless, in this scenario, and when offered to the least cost diet formulation, GAA will be taken when it is cost competitive for the marginal supply of dArg and/or competitive for the marginal supply of ME. Concerning the former, it needs to compete mainly with feed-grade Arg but of course also with feed raw materials providing dArg. In cases were basal ingredients do not fullfil digestible Arg, the least cost formula may be obliged to use GAA or feed-grade Arg. For example, if we target a level of dLys of 12.0 g/kg and a ratio dArg:dLys of 105%, thus following the ideal amino acid concept and a value that is commonly accepted in practice for a broiler starter diet, then the formula warrants 12.6 g/kg dArg. In case 0.6 g/kg GAA would be used in this diet, this represents 0.46 g/kg dArg, which amounts to 3.7% of total dArg. However, it is questionable if in common formulation excercises such a high amount of GAA, and thus a proportional amount of dArg by the feed additive, would be taken (personal communication Jan Van Ginderachter). The reason for that is that higher inclusion of GAA may shift the choice of basal ingredients so that the need for some (or all) supplemental AA increases, which may render the formula more expensive, or some AA may be needed not routinely added to broiler diets. Also, the need for supplemental Arg for a broiler diet is not a general thing, in many cases basal ingredients will provide sufficient dArg. But again, GAA's contribution to ME may change the least cost formulation outcome. From all the above it is clear that inclusion of GAA in a least cost formula will largely depend on the context.

First, it will depend on the matrix values applied to GAA. In particular, if ME beyond its ME *per se* is allocated to GAA it may have a large impact on the likelihood to be within a formula. However, the latter makes it more challenging for the nutritionist, since more ingredients in the formula contribute significantly to ME of the diet. Also, in 1.3.4.1. various numbers are given for Arg equivalency, but nutritionists may prefer a conservative value. Second, what about the benefits that GAA feeding may provide that are not translated into matrix values? Essentially, here is the question how that can be

valorised (see below). Next, nutrient constraints or targets in feed formulation are key, and here in particular dArg:dLys. Common recommendations can be followed (e.g. breed nutrition guidelines) or lower for economical reasons, but higher ratios can be chosen by the nutritionist for example in summer periods (high T) or when rearing males separately (not done in EU). By rearing slow(er)-growing broilers it may be fair to state that dArg:dLys will be set at the lower end of the range, and equally feeding mash diets that are known to result in lower FI and growth, the nutritionist may lower dArg:dLys requirements. Next, if opted for lower CP formulations, then Arg may become limiting more easily, and fortification is needed. Together with overall higher inclusion of crystalline AA, this may increase the cost of the formula. Also, Gly_{equi} should be taken into account, but here no data are available how much Gly_{equi} GAA could spare. Finally, the major driver may be the price and availability of all ingredients, including raw materials and additives. For example, using SFM having a high dArg:dLys, may omit the need for supplemental Arg.

Another situation presents itself when GAA is used at levels beyond dictated by least cost formulation, or simply 'on top'. Here, the feed cost will increase. This is justified if there is appropriate return on investment. To summarize, the benefits for the broiler (meat) producer may be at best: lower feed conversion, thus lower feed consumption for the same slaughter weight, lower mortality, higher breast meat yield, and lower incidence of WB. It it clear that a vertical integrator in the poultry business may collect all these benefits, whereas an independent broiler farmer will likely only valorise lower feed usage and reduced mortality. The application of GAA 'on top' is thus an interesting approach for a vertical integrator, however 'on top' addition relates to an increase of feed cost.

6.6. FUTURE PERSPECTIVES

This thesis has found new evidence for the efficacy of GAA as feed additive and explored new modes of action. Though, the thesis also addressed items which are not resolved yet, and deserve attention in future research. Therefore following perspectives are given:

[1] Doubts remain about the parameters that quantitatively characterise body's Cr metabolism when chickens either or not are fed GAA, such as daily degradation rate of Cr, utilization of digested GAA and *de-novo* synthesis of GAA, and moreover what is the impact of age and strain herein. This warrants further investigation, and moreover, studies addressing species differences for GAA, Cr and CrN metabolism may be necessary. The fate of these metabolites in birds may differ from mammals, and very limited information is available on the specificities in chicken's organs. Furture research may focus on this scientific gap. Related to daily degradation of Cr, elegant work may be done by combination of measuring bird growth, CT-scans, breast and thigh muscle analysis for Cr, and excreta collection for CrN to determine BWG, body composition, Cr content herein, and daily urinary CrN excretion; or alternatively non-invasive methods using ^{13}C (guanidino C-4 position) enriched Cr

by uploading muscles and following exponential signal decreases (Kang et al., 2006).

[2] Following to the above, measuring AGAT and GAMT activity in key organs (e.g. Zhang et al., 2019), eventually enzymes involved in remethylation and transsulfuration, would significantly contribute to our understanding of GAA and Cr metabolism, and should be included in future studies. Eventually, enzyme activities can be used to construct models that allow balance calculations for GAA and Cr more elegantly, as are available for fatty acids (e.g. Poureslami et al., 2010).

[3] In our studies we measured Cr in breast muscle, liver, kidney and plasma, but failed to determine Cr in heart tissue. Nonetheless, and because the regeneration of ATP from the Cr and PCr system appears to be of paramount importance in the cardiac energy management of fast-growing broilers (Nain et al., 2008), methods needs to be implemented. Can dietary GAA equally upload Cr and PCr in heart tissue as in breast muscle? One argument in favour of this hypothesis is the finding in Chapter 4 that dietary GAA reduced mortality in particular towards the end of the HS period, and we also found that most mortality cases were 'flip-overs' and thus likely caused by cardio-vascular insuffiency.

[4] Refinement of matrix value information as various Arg equivalencies are seen in literature with vast variation, and since it appears from several studies that it remains elusive whether and how much ME dietary GAA could spare, and possibly GAA is most efficient at recommended ME. Also, in the context of lowering CP in diets, more attention can be given to potential Gly_{equi} sparing effect, although this is experimentally hard to establish.

[5] It is urged to explore the full potential of dietary GAA in a wider variety of basal diets, employing low CP diets and rations with high levels of other ingredients than commonly used such as DDGS and RSM. Minimal work ongoing this is available in literature. In this respect, it would be interesting to perform a meta-analysis of available literature data (see Figurs 1.6, 1.7, 1.8, and 1.10) and to discern the variables that declare most of the variation between studies.

SUMMARY – SAMENVATTING – چکیده

SUMMARY

Guanidinoacetic acid as feed supplement to broiler chickens

Poultry meat consumption has become increasingly popular, and hence broiler production is yet to grow in the time to come (Mottet and Tempio, 2016). However, broiler production faces many challenges (Porter, 2016). The use of animal protein such as poultry by-product meal (PBM) and meat and bone meal (MBM) in diets has being prohibited in EU (Regulation (EC) No 999/2001) or is decreasing, depending on the region, which means that some nutrients are less present in the diet. For example, creatine (Cr), essential for energy conservation in muscle cells by supporting the adenosine diphosphate (ADP) to adenosine triphosphate (ATP) shuttle, can only be found in animal protein sources. Also, it is well established that Cr is degraded into creatinine (CrN) on a continuous basis and excreted via urine. Cr therefore needs daily replenishment. The body can synthesize Cr by a two-step pathway (Wyss and Kaddurah-Daouk, 2000). This synthesis requires three amino acids (AA): L-arginine (Arg), glycine (Gly), and L-methionine (Met) and two enzymes: L-arginine:glycine amidinotransferase (AGAT) and N-guanidinoacetate methyltransferase (GAMT). In the first step, the amidino group from Arg is transferred to the amino group of Gly, yielding L-ornithine and guanidinoacetic acid (GAA), a reaction catalyzed by AGAT and occurring mainly in the kidney (Tormanen, 1990). In the second step, GAMT induces the GAA methylation, on the original Gly nitrogen, using S-adenosyl methionine (SAM) as the methyl donor to form S-adenosyl homocysteine (SAH) and Cr (Da Silva et al., 2009; Walker, 1979; Stead et al., 2006). This step occurs essentially in the liver. Yet, decades ago it was established that human vegetarians have lower Cr levels than non-vegetarians (Delanghe et al., 1989). Hence, it is believed that fast-growing broiler chickens fed vegetable diets may benefit from dietary supplementation of Cr, or its direct precursor GAA. Indeed, it was shown that dietary GAA may load Cr of breast muscle, and hence enhance growth and feed efficiency in broilers fed vegetable diets (e.g. Lemme et al., 2007b; Michiels et al., 2012). In addition, current trends to lower crude protein (CP) in broiler feeds and increases in the use of by-product ingredients such as distillers' dried grains with solubles (DDGS) or rapeseed meal (RSM) (Parsons and Baker, 1983) all result in decreased inherent Arg concentrations in the diet of broilers. In contrast, increased growth rate of modern broilers (Havenstein et al., 2003) mandate higher dietary Arg requirements. Therefore, dietary strategies fuelling the non-protein functions of Arg might be promising solutions to spare Arg, thereby allowing a greater proportion of this AA to be used for muscle protein synthesis. Feeding GAA to broiler chickens by-passes the first step of Cr synthesis and hence may spare Arg, and is thus appealing in this respect. Finally, in view of world-wide efforts to reduce greenhouse gas emissions, improving energy utilization in producing animal protein for human is paramount. As improvement of energy efficiency by genetic selection may have reached its limits in broiler chickens (Tallentire et al., 2018), nutritionists may look for novel approaches to improve

availability of energy for broiler chickens. One mechanism is to store the flow of energy in the body within the cells mediated through the formation of high-energy phosphate bonds (Lemme et al., 2007a). On this note, GAA, as precursor of Cr may increase energy utilization in broilers.

Altogether, GAA, a feed additive for broilers that is available in many parts of the world, may contribute to sustainable broiler meat production in various ways. However, seeing the many interactions with other nutrients, many questions regarding its optimal use and efficacy remain. The efficacy of the feed additive may depend on the context, which needs further elucidation in order to use the additive in the best way. Therefore, in general, the thesis aims to increase our understanding of how GAA interacts with other nutrients in the diet of broiler chickens on their performances and if larger benefits are to be expected when used in specific conditions such as heat stress (HS). Further, this thesis wants to generate additional insights in the mode of action by exploring the metabolism of GAA and Cr, and its potential antioxidant action in the chicken.

Feed intake (FI) by broilers increases with dilution of nutrient density (ND) of the diet, but birds may have difficulty maintaining energy intake with high levels of dilution (Nielsen, 2004), which may negatively affect growth rate. Hence, it is appealing to study the interaction between ND and GAA. The aim of the first experiment therefore was to investigate the main and interactive effects of ND and GAA supplementation in corn-soybean meal (SBM) diets on animal performance and carcass characteristics and organ weights, and traits of the energy metabolism in breast muscle of broiler chickens. A total of 540 day-old male Ross 308 broilers were allocated to 9 dietary treatments with 6 replicates (10 birds each) in a 3 × 3 factorial arrangement with 3 levels of ND (low, 2800; medium, 2950 and high, 3100 kcal metabolizable energy (ME)/kg); and with the other nutrients being constant relative to ME and with 3 levels of GAA (0, 0.6 and 1.2 g/kg) for 42 days. In the starter and grower period, increasing levels of ND improved body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed to gain ratio (F:G), with the exception of ADFI in the starter period. GAA supplementation did not change these performance characteristics. All performance indicators responded markedly to increasing ND in the finisher period, whereas the highest GAA level reduced ADFI compared to control (156 *vs*. 162 g/d) and concomitantly F:G (1.81 *vs*. 1.93). No interactive effects were noted for any performance trait. The high ND level resulted in a higher breast yield at d42, associated with higher fat content and darker colour compared to the other ND levels, whereas GAA supplementation did not affect carcass and breast traits. At the end of the experiment, Cr was elevated when feeding GAA at 1.2 g/kg (5455 *vs*. 4338 mg/kg fresh muscle). To conclude, ND had a substantial effect on performance and carcass traits, whereas the effect of GAA was limited to F:G in the finisher period and independent of diet ND level.

Dietary GAA is endogenously converted to Cr by methylation, therefore Met is converted to SAM that is used to methylate GAA rendering Cr. It can thus be hypothesized that the effects of dietary GAA supplementation might depend on Met

provision in corn-SBM diets. A total of 540 day-old male Ross 308 broilers were allocated to 9 dietary treatments with 6 replicates (10 birds each) in a 3 × 3 factorial arrangement with 3 graded levels of supplementary Met (+0.4 g/kg per level) whilst cysteine (Cys) was equal across groups, resulting in a low, medium and high level of total sulfur AA (TSAA), and with 3 levels of GAA (0, 0.6 and 1.2 g/kg), for 42 days. Increasing levels of supplemental Met enhanced performance indices in all rearing periods, apart from no effect on F:G in the grower and ADFI in the finisher period. Final BW was 8.8 and 14.6% higher in medium and high Met diets, respectively, as compared to the low level. Relative breast weight and protein content in muscle on d25 linearly increased with increasing levels of Met. At low and high Met levels, growth in the finisher phase was negatively affected by supplementing GAA at 1.2 g/kg. It is suggested that disturbances in methylation homeostasis and/or changes in Arg metabolism might explain these findings. At the end of the grower phase, muscle Cr content was higher when feeding GAA at 0.6 and 1.2 g/kg (4464 and 4472, respectively, *vs.* 4054 mg/kg fresh muscle, in control). To conclude, the effects of dietary GAA supplementation were influenced by the dietary Met level only in the finisher period, which urges for proper sulfur AA formulation of diets and consideration of methylation potential of diets when feeding GAA.

Importantly, world-wide broiler production is most growing in (sub)tropical regions, and concomitantly the impact of climate change increases the likelihood for HS in poultry production (Mottet and Tempio, 2016). Heat exposure has a significant impact on well-being and production (Lara and Rostagno, 2013). Dietary solutions have been generally proposed as being effective and relatively cheap (Renaudeau et al., 2012). Potentially, GAA may improve tolerance of broilers to HS. Indeed, it was hypothesized that dietary supplementation with GAA, as precursor of Cr, would be beneficial to heat stressed finishing broilers because of: 1/ improved energy status, due to Cr's pivotal role in energy homeostasis since FI in heat stressed birds is reduced and cellular energy consumption may increase; and 2/ improved Arg metabolism, due to Arg sparing effects; Arg that could be used for other beneficial functions such as nitric oxide and polyamine synthesis. These hypotheses were tested in heat stressed finisher broilers in a model of chronic cyclic HS (Akbarian et al., 2014). A total of 720 day-old male Ross 308 broilers were allocated to 3 treatments: 0, 0.6 or 1.2 g/kg GAA added to complete corn-SBM diets and fed for 39d, with 12 replicates (20 birds each) per treatment. A chronic cyclic HS model (temperature to 34°C with 50-60% relative humidity for 7h daily) was applied in finisher phase (d25-39). Samples were taken on d26 and 39 to determine thrombocytes, white blood cells, corticosterone, protein and AA in blood and Cr, phosphocreatine (PCr) and ATP in breast muscle. In addition, meat quality was assessed on d40, after overnight fasting. GAA at 1.2 g/kg decreased F:G compared to control in grower phase (1.32 *vs*. 1.35) due to slightly reduced FI. In finisher period, 0.6 and 1.2 g/kg GAA reduced FI more pronounced (1.1 and 3.3%, respectively), whilst F:G improved substantially (1.76, 1.66 and 1.67 for control, 0.6 and 1.2 g/kg GAA, respectively). Mortality outcomes highlight that GAA-feeding improved survival during HS, in particular towards the end, supported by lower panting frequency (linear effect). Plasma Arg was higher with

increasing dietary GAA (+18.3 and +30.8% for 0.6 and 1.2 g/kg GAA, respectively) on d26 and a trend for linear increase was detected on d39. This suggests enhanced availability of Arg for other metabolic purposes than *de-novo* GAA formation. Examination of breast muscle revealed at both sampling days increases of PCr (linear effect at d26 and effect at d39), free Cr (at d39), total creatine (TCr) (both days), and, PCr:ATP ratio (linear effect at d26 and effect at d39) with increasing dietary GAA. GAA supplementation improved feed conversion and survival during chronic cyclic HS; associated with enhanced breast muscle energy status and Arg sparing effect.

Further, various authors demonstrated the ability of Cr to quench superoxide anions and other aqueous reactive species, and thus present antioxidant capacity (Lawler et al., 2002; Deminice and Jordao, 2012). Cr may also exert indirect antioxidant outcomes through its primary action on cellular energy status (Persky and Brazeau, 2001). It is well known that fast-growing broilers need antioxidant protection (Aurousseau, 2002), and that some circumstances like HS can induce oxidative stress (OS) in poultry (Akbarian et al., 2016). It is therefore appealing to know whether dietary GAA through Cr loading may protect broiler's tissues from oxidative damage. Hence, in this experiment, the effect of supplementing GAA to broilers subjected to HS during the finisher period on oxidative status and GAA and Cr metabolism in key organs was studied. A total of 720 day-old male Ross 308 broilers were allocated to 3 treatments: 0, 0.6 or 1.2 g/kg GAA was added to complete corn-SBM diets and fed for 39d, with 12 replicates (20 birds each) per treatment. A chronic cyclic HS model (T to 34°C with 50-60% RH for 7h daily) was applied in the finisher phase (d25-39). Samples from one bird per pen were taken on d26 and 39 to assess energy metabolites and parameters of oxidative status in blood, breast muscle, liver, kidney and heart. GAA and Cr in plasma were linearly increased by feeding GAA on either sampling day, illustrating efficient absorption and methylation. Energy metabolism in muscle was greatly supported as visible by increased Cr, PCr:ATP and glycogen stores providing an improved capacity for readily available PCr which may be of particular relevance when oxidative phosphorylation can be limited, e.g. HS. Blood cholesterol levels linearly decreased by graded GAA (effect on d26, trend on d39), which may suggest that GAA affects adenosine monophosphate-activated protein kinase in liver. The lipid peroxidation marker MDA, and the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase showed no alterations by dietary GAA in plasma. Opposite to that, SOD activity in breast was linearly lowered when feeding GAA (trend on d26, effect on d39). It may highlight an indirect antioxidant effect of dietary GAA due to Cr loading protecting muscle function, in particular after chronic HS. To conclude, in addition to previous findings, beneficial performance in heat stressed broilers by GAA may be associated with enhanced energy metabolism resulting in improved oxidative status.

Altogether, we conclude that: [1] the effects of GAA are independent of ND, [2] the effects of GAA are influenced by dietary Met, however, only in the finisher period, which urges for proper sulfur AA formulation and consideration of the methylation potential of diets when feeding GAA, [3] GAA supplementation improved feed efficiency and

survival during chronic cyclic HS to an extent beyond commonly seen in thermo-neutral conditions, and [4] GAA consistently enhanced breast muscle energy status, which indirectly may improve oxidative status and was associated with higher glycogen levels, and an Arg sparing effect.

SAMENVATTING

Guanidinoazijnzuur als voedersupplement voor vleeskippen

Pluimveevlees consumptie neemt steeds toe en verwacht wordt dat de productie van vleeskuikens de komende tijd nog gestaag zal groeien (Mottet en Tempio, 2016). De productie van vleeskuikens staat echter voor veel uitdagingen (Porter, 2016). Het gebruik van dierlijke eiwitten zoals bij-producten van pluimvee slachtafval en vlees- en beendermeel in voeders is verboden in EU (Verordening (EG) nr. 999/2001) of neemt af, afhankelijk van de regio, wat betekent dat sommige voedingsstoffen minder aanwezig zijn in het dieet. Creatine (Cr) bijvoorbeeld, essentieel voor energiemetabolisme in spiercellen door de adenosinedifosfaat (ADP) / adenosine trifosfaat (ATP) shuttle te ondersteunen, kan alleen worden gevonden in dierlijke eiwitbronnen. Het is ook goed gekend dat Cr continu wordt afgebroken tot creatinine (CrN) wat op zijn beurt wordt uitgescheiden via urine. Cr pools in het lichaam moeten dus continu aangevuld worden. Het lichaam kan Cr synthetiseren via een twee-staps-synthese (Wyss en Kaddurah-Daouk, 2000). Deze synthese vereist drie aminozuren: L-arginine (Arg), glycine (Gly) en L-methionine (Met) en twee enzymen: L-arginine: glycine amidinotransferase (AGAT) en N-guanidinoacetaat methyltransferase (GAMT). In de eerste stap wordt de amidinogroep van Arg overgebracht naar de aminogroep van Gly, wat L-ornithine en guanidinoazijnzuur (GAA) oplevert, een reactie die wordt gekatalyseerd door AGAT en voornamelijk voorkomt in de nieren (Tormanen, 1990). In de tweede stap induceert GAMT de GAA-methylering, op de oorspronkelijke Gly-stikstof, met behulp van S-adenosylmethionine (SAM) als de methyldonor om S-adenosyl homocysteïne (SAH) en Cr te vormen (Da Silva et al., 2009; Walker, 1979; Stead et al., 2006). Deze stap vindt hoofdzakelijk plaats in de lever. Reeds decennia geleden werd bij mens vastgesteld dat vegetariërs lagere Cr-niveaus hebben dan niet-vegetariërs (Delanghe et al., 1989). Er kan aangenomen dat snelgroeiende vleeskuikens die plantaardig voeder krijgen, baat kunnen hebben bij supplementatie met Cr of de directe voorloper GAA. Er werd inderdaad aangetoond dat bij GAA supplementatie in het voeder, Cr gehalte van de borstspier sterk verhoogde en daarmee de groei en voederefficiëntie in vleeskuikens die gevoed worden met plantaardige diëten kan verbeteren (o.a. Lemme et al., 2007b; Michiels et al., 2012). Bovendien zijn er de huidige trends om ruw eiwit in het voeder van vleeskuikens te verlagen en het gebruik van bijproducten zoals destillers' dried grains with solubles (DDGS) of raapzaadmeel (Parsons en Baker, 1983) die allen leiden tot verlaagde inherente Arg-concentraties in het voeder van vleeskuikens. In tegenstelling daarmee vereist de verhoogde groeisnelheid van moderne vleeskuikens (Havenstein et al., 2003) hogere behoeften aan Arg. Daarom kunnen strategieën die de niet-eiwitfuncties van Arg ondersteunen, veelbelovende oplossingen zijn om Arg te sparen, waardoor een groter deel van dit aminozuur kan worden gebruikt voor spiereiwitsynthese. Het supplementeren van GAA aan vleeskuikens omzeilt de eerste stap van de Cr-synthese en kan daarom Arg sparen, en is dus in dit opzicht aantrekkelijk. Ten slotte is het, gezien de wereldwijde inspanningen om de uitstoot van broeikasgassen te verminderen, van cruciaal belang om het energieverbruik bij de productie van dierlijke eiwitten voor de

mens te verbeteren. Omdat de verbetering van de energie-efficiëntie door genetische selectie bij vleeskuikens mogelijks zijn grenzen heeft bereikt (Tallentire et al., 2018), gaan nutrionisten zoeken naar nieuwe benaderingen om de beschikbaarheid van energie voor vleeskuikens te verbeteren. Een mechanisme is om de energiestroom in het lichaam op te slaan in de cellen gemedieerd door de vorming van energierijke fosfaatbindingen (Lemme et al., 2007a). GAA, als voorloper van Cr, kan op die manier de energiefficiëntie bij vleeskuikens verhogen. Samengevat, GAA, een toevoegingsmiddel voor vleeskuikens dat in vele delen van de wereld beschikbaar is, kan dus op verschillende manieren bijdragen tot duurzame vleeskuikenproductie. Gezien de vele interacties met andere voedingsstoffen, blijven er echter veel vragen over het optimale gebruik en de werkzaamheid. De werkzaamheid van het toevoegingsmiddel kan afhankelijk zijn van de context, die verdere toelichting behoeft om het toevoegingsmiddel op de beste manier te gebruiken. Daarom beoogt het proefschrift in het algemeen om ons inzicht te vergroten in de interactie van GAA met andere voedingsstoffen in het voeder van vleeskuikens en of er meer voordelen te verwachten zijn bij gebruik in specifieke omstandigheden zoals hitte stress (HS). Verder wil dit proefschrift extra inzichten genereren in het werkingsmechanisme door het metabolisme van GAA en Cr en mogelijke antioxidantwerking in de kip te onderzoeken.

Voederropname door vleeskuikens neemt toe met verdunning van de nutriëntendichtheid (ND) van het voeder, maar vleeskuikens kunnen moeite hebben met het handhaven van de energie-inname bij hoge verdunningsniveaus (Nielsen, 2004), wat de groeisnelheid negatief kan beïnvloeden. Het is daarom aantrekkelijk om de interactie tussen ND en GAA te bestuderen. Het doel van het eerste experiment was daarom om de hoofd- en interactieve effecten van ND en GAA supplementatie in maïs-sojaschroot (SBM) voeders te onderzoeken op dierprestaties en karkaskenmerken en orgaangewichten, en eigenschappen van het energiemetabolisme in borstspier van vleeskuikens. In totaal werden 540 mannelijke Ross 308 ééndagskuikens toegewezen aan 9 voederbehandelingen met 6 herhalingen (elk 10 kuikens) in een 3 x 3 factoriële opstelling met 3 ND niveaus (laag, 2800; gemiddeld, 2950 en hoog, 3100 kcal metaboliseerbare energie (ME)/kg); en met de andere voedingsstoffen constant ten opzichte van ME en met 3 niveaus van GAA (0, 0,6 en 1,2 g/kg) gedurende 42 dagen. In de starter- en groeiperiode verbeterden de toenemende ND niveaus het lichaamsgewicht, gemiddelde dagelijkse groei, gemiddelde dagelijkse voederopname en voederbenutting, met uitzondering van voederopname in de starterperiode. GAA supplementatie veranderde deze prestatiekenmerken niet. Alle prestatie-indicatoren reageerden duidelijk op toenemende ND in de finisher periode, terwijl het hoogste GAA niveau de voederopname verminderde in vergelijking met controle (156 *vs.* 162 g/d) en tegelijkertijd de voederbenutting verhoogde (1,81 *vs.* 1,93). Er werden geen interactieve effecten genoteerd voor geen enkel prestatiekenmerk. Het hoge ND niveau resulteerde in een hogere borstopbrengst op d42, geassocieerd met een hoger vetgehalte en een donkerdere kleur in vergelijking met de andere ND niveaus, terwijl GAA supplementatie geen invloed had op karkas- en borstkenmerken. Aan het einde van het experiment was Cr verhoogd wanneer GAA werd gevoederd aan 1,2 g/kg (5455 *vs.* 4338 mg/kg verse

spier). Concluderend had ND een aanzienlijk effect op de prestaties en karkaskenmerken, terwijl het effect van GAA beperkt was tot voederbenutting in de finisher fase en onafhankelijk was van het ND niveau van het voeder.

GAA in de voeding wordt endogeen omgezet in Cr door methylering, daarvoor wordt Met omgezet in SAM dat wordt gebruikt om GAA te methyleren waardoor Cr wordt verkregen. Er kan dus worden verondersteld dat de effecten van GAA supplementatie in het voeder mogelijks afhankelijk zijn van het Met gehalte in maïs-SBM voeders. In totaal werden 540 mannelijke Ross 308 ééndagskuikens toegewezen aan 9 voederbehandelingen met 6 herhalingen (elk 10 kuikens) in een 3 x 3 factoriële opstelling met 3 niveaus van toegevoegde Met (+0,4 g/kg per niveau) terwijl cysteïne (Cys) gelijk was voor alle groepen, resulterend in een laag, gemiddeld en hoog niveau van totaal zwavel aminozuren (TSAA), en met 3 niveaus GAA (0, 0,6 en 1,2 g/kg), gedurende 42 dagen. Toenemende niveaus van toegevoegde Met verbeterde prestatiekenmerken in alle opfokperioden, afgezien van geen effect op voederbenutting in de groei- en voederopname in de finisher-periode. Het eindgewicht was respectievelijk 8,8 en 14,6% hoger in gemiddelde en hoge Met voeders in vergelijking met het lage niveau. Relatief borstgewicht en eiwitgehalte in spieren op d25 nam lineair toe met toenemende niveaus van Met. Bij lage en hoge Met-niveaus werd de groei in de finisher fase negatief beïnvloed door GAA aan 1,2 g/kg. Er wordt gesuggereerd dat verstoringen in methylatie-homeostase en/of veranderingen in het Arg-metabolisme deze bevindingen kunnen verklaren. Aan het einde van de groeifase was het Cr gehalte in borstpsier hoger bij het voederen van GAA aan 0,6 en 1,2 g/kg (respectievelijk 4464 en 4472 *vs*. 4054 mg/kg verse spier bij controle). Concluderend werden de effecten van GAA supplementatie in de voeder alleen beïnvloed door het Met-voedingsniveau in de finisher-periode, wat aangeeft dat een juiste zwavel-aminozuur voorziening in het voeder en voldoende methylatie potentieel uitermate belangrijk zijn wanneer GAA wordt gesupplementeeerd.

Belangrijk is dat de wereldwijde vleeskuikenproductie het meest groeit in (sub)tropische regio's, en op dezelfde manier verhoogt de impact van klimaatverandering de kans op HS bij pluimveeproductie (Mottet en Tempio, 2016). Blootstelling aan hitte heeft een significante impact op welzijn en productie (Lara en Rostagno, 2013). Voederoplossingen worden in het algemeen voorgesteld als effectief en relatief goedkoop (Renaudeau et al., 2012). Mogelijks kan GAA de tolerantie van vleeskuikens voor HS verbeteren. Er werd inderdaad verondersteld dat GAA, als voorloper van Cr, gunstig zou zijn bij vleeskuikens onder HS vanwege: 1/ verbeterde energiestatus, vanwege de centrale rol van Cr in energiehomeostase, sinds voeropname bij vleeskuikens onder HS vermindert en het cellulair energieverbruik kan toenemen; en 2/ verbeterd Arg-metabolisme, vanwege de sparende effecten van GAA voor Arg; Arg die dan zou kunnen worden gebruikt voor andere nuttige functies zoals stikstofmonoxide en polyamine-synthese. Deze hypothesen werden getest in een model van chronisch cyclisch HS bij vleeskuikens (Akbarian et al., 2014). Een totaal van 720 mannelijke Ross 308 ééndagskuikens werden toegewezen aan 3 behandelingen: 0, 0,6 of 1,2 g / kg GAA toegevoegd aan een maïs-SBM voeder en gevoed voor 39d, met 12 herhalingen (elk 20

kuikens) per behandeling. Een chronisch cyclisch HS model (temperatuur tot 34°C met 50-60% relatieve vochtigheid gedurende 7 uur per dag) werd in de finisher fase (d25-39) geïmplementeerd. Monsters werden genomen op d26 en 39 om trombocyten, witte bloedcellen, corticosterone, eiwit en aminozuren in bloed en Cr, fosfocreatine (PCr) en ATP in borstspier te bepalen. Bovendien werd de vleeskwaliteit beoordeeld op d40, na een nacht vasten. GAA bij 1,2 g/kg verbeterde de voederbenutting vergeleken met controle in groei fase (1,32 *vs.* 1,35) als gevolg van enigszins verminderde voederopname. In de finisher periode verminderde 0,6 en 1,2 g/kg GAA de voederopname meer uitgesproken (respectievelijk 1,1 en 3,3%), terwijl de voederbenutting aanzienlijk verbeterde (1,76, 1,66 en 1,67 voor respectievelijk controle, 0,6 en 1,2 g/kg GAA). Cijfers over uitval benadrukken dat GAA supplementatie de overleving tijdens HS verbeterde, met name tegen het einde van de proef, samenhangend met een lagere hijg-frequentie (lineair effect). Plasma Arg was hoger met toenemend gehalte GAA in voeder (+18,3 en + 30,8% voor respectievelijk 0,6 en 1,2 g/kg GAA) op d26 en een trend voor lineaire toename werd gedetecteerd op d39. Dit suggereert een verbeterde beschikbaarheid van Arg voor andere metabolische doeleinden dan *de-novo* GAA vorming. Onderzoek van borstspier onthulde op beide bemonsteringsdagen toenames van PCr (lineair effect op d26 en effect op d39), vrije Cr op d39, totaal creatine (TCr) (beide dagen) en PCr:ATP-verhouding (lineair effect op d26 en effect op d39) met toenemende GAA in het voeder. GAA supplementatie verbeterde voederbenutting en overleving tijdens chronisch cyclisch HS; geassocieerd met verbeterde borstspier energiestatus en een Arg sparend effect.

Verschillende auteurs hebben aangetoond dat Cr in staat is om superoxide-anionen en andere reactieve species onschadelijk te maken en dus antioxidantcapaciteit te vertonen (Lawler et al., 2002, Deminice and Jordao, 2012). Cr kan ook indirecte antioxidant werking uitoefenen door zijn primaire werking in verhoging van cellulaire energiestatus (Persky and Brazeau, 2001). Het is bekend dat snelgroeiende vleeskuikens bescherming tegen antioxidanten nodig hebben (Aurousseau, 2002), en dat sommige omstandigheden zoals HS oxidatieve stress (OS) bij pluimvee kunnen veroorzaken (Akbarian et al., 2016). Het is daarom aantrekkelijk om te weten of GAA via de voeding door verhogen van Cr in spieren, de vleeskuikens kan beschermen tegen oxidatieve schade. Daarom werd in dit experiment het effect bestudeerd van supplementatie van GAA aan vleeskuikens die tijdens de finisher-periode aan HS waren onderworpen op de oxidatieve status en GAA en Cr metabolisme in sleutelorganen. Een totaal van 720 mannelijke Ross 308 ééndagskuikens werden toegewezen aan 3 behandelingen: 0, 0,6 of 1,2 g/kg GAA toegevoegd aan een maïs-SBM voeder en gevoed voor 39d, met 12 herhalingen (elk 20 vleeskuikens) per behandeling. Een chronisch cyclisch HS model (T tot 34°C met 50-60% relatieve vochtigheid gedurende 7 uur per dag) werd in de finisher fase (d25-39) geïmplementeerd. Op d26 en 39 werden monsters genomen van één kip per pen om energiemetabolieten en parameters van oxidatieve status in bloed, borstspier, lever, nier en hart te beoordelen. GAA en Cr in plasma werden lineair verhoogd door GAA te voederen op elke bemonsteringsdag, wat een efficiënte absorptie en methylatie van GAA illustreert. Het energiemetabolisme in spieren werd enorm ondersteund: Cr, PCr:ATP en

glycogeen werden gestimuleerd, dat dus een verhoogde capaciteit voor gemakkelijk PCr betekent die van bijzonder belang kan zijn wanneer oxidatieve fosforylering geremd is zoals in HS. Cholesterol nam lineair af met toeneemde GAA, wat erop kan duiden dat GAA adenosine-monofosfaat-geactiveerd proteïnekinase in de lever beïnvloedt. De lipide peroxidatiemarker malondialdehyde (MDA), noch de antioxidant enzymen superoxide dismutase (SOD) en glutathion peroxidase (GPx) vertoonden veranderingen door GAA in plasma. Daar tegenover was de SOD activiteit in de borst lineair verlaagd bij supplementatie met GAA. Het kan een indirect antioxidant effect van GAA benadrukken vanwege de hogere Cr gehalten die de spierfunctie beschermt, met name na chronische HS. Concluderend, in aanvulling op eerdere bevindingen, kunnen gunstige prestaties in vleeskuikens onder hitte stress door GAA worden geassocieerd met een verbeterd energiemetabolisme resulterend in een verbeterde oxidatieve status.

Samengevat kan geconcludeerd worden dat: [1] de effecten van GAA onafhankelijk zijn van ND, [2] de effecten van GAA worden beïnvloed door Met in de voeding, echter alleen in de finisher periode, wat aangeeft dat een juiste zwavel-aminozuur voorziening in het voeder en voldoende methylatie potentieel uitermate belangrijk zijn wanneer GAA wordt gesupplementeeerd, [3] GAA supplementatie een verbeterde voederbenutting en overleving tijdens chronisch cyclisch HS geeft in een mate die veel groter is dan in thermo-neutrale omstandigheden, en [4] GAA verbeterde op consistente wijze de borstspier energiestatus, die indirect de oxidatieve status kan verbeteren en dit was geassocieerd met hogere glycogeengehaltes en een Arg-sparend effect.

چکیده

گوانیدینواستیک اسید به عنوان مکمل خوراکی در تغذیه جوجه‌های گوشتی

امروزه مصرف گوشت طیوربه طور قابل توجهی با اقبال مواجه شده است، از این رو تولید گوشت طیور در آینده روبه افزایش می‌باشد (موتت و تمپیو، ۲۰۱۶). با این حال، تولیدات گوشتی با چالش‌های بسیاری روبرو هستند (پورتر، ۲۰۱۶). استفاده از پروتئین‌های حیوانی مانند پودر طیور، پودر گوشت و استخوان در جیره‌های غذایی در اتحادیه اروپا ممنوع (۲۰۰۱/۹۹۹ EC) یا بسته به کشور، در حال کاهش می‌باشد، بدین معنی که برخی ازمواد مغذی در جیره کمتر وجود خواهند داشت. به عنوان مثال، کراتین (Cr)، مولکول ضروری جهت تأمین انرژی در سلول‌های ماهیچه‌ای در تبدیل آدنوزین دی فسفات (ADP) به آدنوزین تری فسفات (ATP)، فقط درمنابع پروتئینی حیوانی یافت می‌شود. همچنین، مشخص شده است که Cr به طور مداوم به کراتینین تجزیه و از طریق ادرار دفع می گردد. بنابراین Cr باید به صورت روزانه تامین گردد. بدن قادر است Cr را طی یک مسیر دو مرحله ای سنتز کند (وایس و کادوراک، ۲۰۰۰). این سنتز به سه اسید آمینه آرژنین (Arg)، گلیسین (Gly)، متیونین (Met) و دو آنزیم آرژنین- گلیسین آمیدینو ترانسفراز (AGAT) و ان- گوانیدینو استات متیل ترانسفراز (GAMT) نیاز دارد. در مرحله اول ، گروه آمیدینو از Arg به گروه آمین Gly منتقل شده، اورنیتین و گوانیدینواستیک اسید (GAA) را تولید می‌کند، واکنشی که توسط AGAT کاتالیز و عمدتا در کلیه اتفاق می‌افتد (ترومنن، ۱۹۹۰). در مرحله دوم، GAMT متیلاسیون را بر روی نیتروژن اصلی Gly با استفاده از اس-آدنوزیل متیونین (SAM) به عنوان دهنده گروه متیل القا و اس- آدنوزیل هموسیستئین (SAH) وCr را تولید می‌کند (داسیلوا و همکاران، ۲۰۰۹؛ والکر، ۱۹۷۹و اشتد و همکاران، ۲۰۰۶). این مرحله در کبد انجام می‌شود. ده‌ها سال پیش مشخص شد که افراد گیاهخوار نسبت به غیرگیاهخواران سطح Cr پایین‌تری دارند (دلانگه و همکاران، ۱۹۸۹). از این رو، اعتقاد بر این است که استفاده از مکمل Cr، یا پیش ساز مستقیم آن GAA، برای جوجه‌های گوشتی با سرعت رشد بالا که صرفا ازجیره غذایی گیاهی تغذیه می‌شوند، می‌تواند سودمند باشد. بعلاوه نشان داده شده است که تغذیه GAA سبب افزایش Cr در عضله سینه جوجه‌های گوشتی می‌شود، و از این رو رشد و راندمان خوراك را در جوجه‌های گوشتی افزایش می‌دهد (لمه و همکاران ، ۲۰۰۷b ؛ میخیلز و همکاران، ۲۰۱۲). علاوه بر این، روند فعلی جهت کاهش پروتئین خام جیره جوجه‌های گوشتی و افزایش استفاده ازمحصولات فرعی مانند تفاله تقطیری گندم خشک (DDGS) یا کنجاله کلزا (RSM) (پارسونز و بیکر ، ۱۹۸۳) منجر به کاهش غلظت Arg درجیره جوجه‌های گوشتی می‌شود. از طرف دیگر، افزایش نرخ رشد جوجه‌های گوشتی جدید (هاونشتاین و همکاران ، ۲۰۰۳) سبب افزایش احتیاجات Arg جیره می‌گردد. بنابراین، راهکارهای تغذیه‌ای که سایر وظایف غیرپروتیینی Arg را پوشش دهد می‌تواند راه حلی جهت صرفه‌جویی در میزان Arg جیره باشد و اجازه دهد تا بخش بیشتری از این اسید آمینه برای سنتز پروتئین ماهیچه مورد استفاده واقع شود. تغذیه GAA در جوجه‌های گوشتی سبب گذر از مرحله اول سنتز Cr شده و از این منظر که سبب اثرصرفه‌جویی در مصرف Arg (arginine sparing effect) می‌شود قابل توجه است. این موضوع با توجه به تلاش‌های جهانی برای کاهش انتشار گازهای گلخانه‌ای و بهبود مصرف انرژی در تولید پروتئین حیوانی قابل توجه می‌باشد. از آنجا که بهبود راندمان انرژی با انتخاب ژنتیکی ممکن است در جوجه‌های گوشتی به بیشینه حد خود رسیده باشد (تالنتیره و همکاران، ۲۰۱۸)، متخصصان تغذیه به دنبال یافتن روش‌های جدید برای بهبود انرژی قابل دسترس جوجه‌های گوشتی می‌باشند. یکی از این راهکارها، ذخیره جریان انرژی موجود در سلولهای بدن از طریق ایجاد پیوندهای فسفات پرانرژی می‌باشد (لمه و همکاران، ۲۰۰۷a)، که در این ارتباط GAA به عنوان پیش‌ساز Cr سبب افزایش کارایی انرژی در جوجه‌های گوشتی می‌شود.

GAA ماده افزودنی خوراکی در جیره جوجه‌های گوشتی است که در بسیاری از نقاط جهان موجود و با مکانیسم‌های مختلف در تولید پایدار گوشت مرغ می‌تواند موثرباشد. با این حال، با مشاهده اثرات متقابل آن با سایر مواد مغذی، سؤالات بسیاری را در مورد استفاده بهینه و اثربخشی آن باقی می‌گذارد. بنابراین، به طور کلی، این پژوهش با هدف افزایش درک ما از نحوه تعامل GAA با سایر مواد مغذی در جیره غذایی جوجه‌های گوشتی، اثر آن برعملکرد و مزیت استفاده از به کارگیری آن در شرایط خاصی مانند تنش گرمایی (HS) انجام پذیرفت.

بعلاوه، این پژوهش بر این است تا با بررسی متابولیسم GAA و Cr و اثرات آنتی‌اکسیدانی بالقوه آن در جوجه‌های گوشتی به درک بهتری از نحوه عملکرد GAA دست یابد.

مصرف خوراک (FI) جوجه‌های گوشتی با رقیق‌سازی (ND) مواد مغذی جیره افزایش می‌یابد، اما ممکن است پرندگان در دریافت انرژی با جیره‌های به شدت رقیق شده مشکل داشته باشند (نیلسن ، ۲۰۰٤) ، که این موضوع بر سرعت رشد آنها تأثیر منفی خواهد داشت. از این رو، مطالعه اثر متقابل بین سطح رقت جیره و GAA قابل توجه می‌باشد. هدف از آزمایش اول، بررسی اثرات اصلی و متقابل ND و GAA در جیره‌های غذایی ذرت- سویا بر عملکرد، خصوصیات و کیفیت لاشه وپارامترهای متابولیسم انرژی در عضله سینه جوجه‌های گوشتی بود. بدین منظور ٥٤٠ جوجه نر یک روزه سویه راس ۳۰۸، با ۹ جیره آزمایشی حاصل از روش فاکتوریل ۳×۳ (سه سطح انرژی قابل متابولیسم(ME) ۲۸۰۰، ۲۹٥۰ و ۳۱۰۰ کیلوکالری در کیلو گرم) با ٦ تکرار (۱۰ پرنده در هر تکرار)؛ سایر مواد مغذی جیره نسبت به سطح ME جیره رقیق شدند؛ وسه سطح GAA (۰، ۰/٦ و ۱/۲ گرم بر کیلو گرم GAA در جیره) در طول دوره آزمایشی ٤۲ روزه اختصاص یافت. اثر متقابل برای شاخص های عملکرد معنی دار نبود، به استثنای ADFI در دوره آغازین، با افزایش سطوح ND (کاهش رقیق سازی) وزن بدن (BW)، میانگین افزایش وزن روزانه (ADG)، متوسط مصرف خوراک روزانه (ADFI) و ظریب تبدیل غذایی (F:G) در دوره آغازین و رشد بهبود یافت. مکمل GAA تاثیری بر عملکرد نداشت. در دوره پایانی اثرافزایش سطح ND به طور قابل توجهی برتمام شاخص‌های عملکرد معنی دار بود، در حالی که بالاترین سطح GAA باعث کاهش ADFI (۱٥٦ در مقابل ۱٦۲ گرم برای هر پرنده در روز) و سبب بهبود F: G (۱/۸۱ در مقابل ۱/۹۳) در مقایسه با گروه شاهد (بدون GAA) شد. افزایش سطح ND در سن ٤۲ روزگی سبب افزایش وزن، چربی و رنگ تیره تر گوشت سینه جوجه‌های گوشتی شد. در حالی که مکمل GAA بر خصوصیات لاشه و گوشت سینه بی تاثیر بود. میزان Cr عضله سینه با جیره حاوی ۱/۲ گرم در کیلوگرم GAA (٥٤٥٥ در مقابل ٤۳۳۸ میلی گرم در کیلوگرم گوشت سینه) افزایش یافت. نتایج این آزمایش نشان داد، افزایش سطح ND تأثیر معنی‌داری بر شاخص‌های عملکرد و خصوصیات لاشه داشته، در حالی که اثر GAA در دوره پایانی بر F: Gمعنی‌دار و فاقد اثر متقابل با سطح ND جیره غذایی بود.

GAA دربدن با استفاده از متیلاسیون به Cr تبدیل می‌شود، Met به SAM تبدیل و جهت متیلاسیون GAA در تولید Cr مورد استفاده قرار می‌گیرد. بنابراین آزمایش دوم به منظور بررسی تاثیر سطوح مکمل GAA و Met در جیره‌های ذرت- سویا انجام شد. بدین منظور ٥٤٠ جوجه نر یک روزه سویه راس ۳۰۸، با ۹ جیره آزمایشی حاصل از روش فاکتوریل ۳×۳ با ٦ تکرار (۱۰ پرنده در هر تکرار)؛ حاوی سه سطح افزایشی Met (۰/٤ گرم در کیلوگرم در هر سطح) با سطح سیستئین (Cys) ثابت در بین گروههای آزمایشی (TSAA، پایین، احتیاج و بالاتر از احتیاجات حیوان) و سه سطح GAA (۰، ۰/٦ و ۱/۲ گرم در کیلو گرم جیره) در طول دوره آزمایشی ٤۲ روزه اختصاص یافت. به استثنای F: G در دوره رشد و ADFI در دوره پایانی، در تمام دوره‌های آزمایشی افزایش سطح Met جیره سبب بهبود شاخص‌های عملکرد شد. GAA تاثیر معنی‌داری بر عملکرد نداشت. وزن نهایی جوجه در سطوح مورد احتیاج و بالای Met در مقایسه با سطح پایین Met به ترتیب ۸/۸٪ و۱٤/٦٪ بیشتر بود. وزن نسبی و درصد پروتئین عضله سینه جوجه‌های گوشتی با افزایش سطح Met جیره در ۲٥ روزگی به صورت خطی افزایش یافت. اضافه وزن در دوره پایانی در سطوح پایین و بالای Met، در جیره حاوی ۱/۲ گرم در کیلوگرم GAA به طور معنی داری کاهش یافت. به نظر می‌رسد که این کاهش تحت تاثیر تداخل در روند متیلاسیون و یا تغییر در متابولیسم Arg باشد. در پایان دوره رشد میزان Cr عضله سینه در جیره‌های حاوی ۰/٦ و ۱/۲ گرم GAA در کیلوگرم جیره به طور معنی‌داری بیشتر بود (به ترتیب، ٤٤٦٤ و ٤٤۷۲ در مقابل ٤۰٥٤ میلی گرم در کیلوگرم عضله سینه در گروه شاهد). نتایج این آزمایش نشان داد، اثرمتقابل GAA و سطوح Met جیره در دوره پایانی معنی دار بوده، که این امر فرمولاسیون مناسب اسیدهای آمینه گوگرد دار با در نظر گرفتن میزان متیلاسیون در جیره‌های غذایی هنگام تغذیه GAA را مورد تاکید قرار می‌دهد.

تولید جهانی گوشت طیور در مناطق گرمسیری و استوایی در حال افزایش است و به طور همزمان تأثیر تغییرات آب و هوایی، احتمال HS را در زمان پرورش جوجه های گوشتی افزایش می‌دهد (موتت و تمپیو، ۲۰۱٦). قرار

گرفتن در معرض تنش گرمایی تأثیر قابل توجهی بر رفاه و تولید دارد (لارا و رستاگنو، ۲۰۱۳). به طور کلی راهکارهای تغذیه‌ای به عنوان روشی موثر و نسبتاً ارزان قابل توجه‌اند (رنودو و همکاران، ۲۰۱۲). به طور بالقوه، GAA می‌تواند سطح تحمل جوجه‌های گوشتی در شرایط HS را بهبود بخشد. GAA، به عنوان پیش ساز Cr، در جیره جوجه‌های گوشتی تحت تاثیر تنش گرمایی به این دلایل می‌تواند مفید واقع شود: ۱ - بهبود وضعیت انرژی، به دلیل نقش محوری Cr در هموستاز انرژی؛ FI در پرندگان تحت تأثیر تنش گرمایی کاهش و مصرف انرژی توسط سلول افزایش یابد. ۲ - بهبود متابولیسم Arg، به دلیل صرفه‌جویی در مصرف Arg؛ یکی از وظایف Arg سنتز نیتریک اکسید و پلی آمین می‌باشد. بنابراین آزمایش سوم با ۷۲۰ جوجه گوشتی نر یک روزه سویه راس ۳۰۸ و ۳ تیمار ۰، ۰/٦ و ۱/۲ گرم GAA در کیلو گرم جیره‌های غذایی ذرت - سویا در دوره آزمایشی ۳۹روزه با ۱۲ تکرار (۲۰ پرنده در هر تکرار) انجام پذیرفت و مدل تنش گرمایی دوره‌ای پیوسته (درجه حرارت ۳٤ درجه سانتیگراد همراه با رطوبت نسبی ٥۰-٦۰ ٪ به مدت ۷ ساعت به صورت روزانه) در دوره پایانی پرورش اعمال شد (اکبریان و همکاران، ۲۰۱٤). نمونه گیری در روزهای ۲٦ و ۳۹ آزمایش جهت اندازه‌گیری فاکتورهای خونی، ترومبوسیت‌ها، گلبول‌های سفید، کورتیکواسترون، پروتئین و اسیدهای‌آمینه پلاسما، Cr ، فسفوکراتین (PCr) و ATP در عضله سینه صورت پذیرفت. بعلاوه ، کیفیت گوشت در روز ٤۰ آزمایش، بعد از گرسنگی در طول شب مورد ارزیابی قرار گرفت. در دوره رشد، F: G در جیره حاوی ۱/۲ گرم GAA در کیلوگرم جیره در مقایسه با گروه شاهد (۱/۳۲ در مقابل ۱/۳٥) همراه با کاهش FI، کاهش یافت. در دوره پایانی، جیره حاوی ۰/٦ و ۱/۲ گرم GAA در کیلوگرم ،FI را به طور معنی‌داری (بترتیب ۱/۱ و ۳/۳) کاهش وF:G به طور قابل توجهی بهبود یافت (به ترتیب ۱/۷٦، ۱/٦٦ و ۱/٦۷ در گروه شاهد به ترتیب برای ۰/٦ و ۱/۲ گرم GAA در کیلوگرم). بررسی تلفات در طول دوره آزمایش نشان می‌دهد که مکمل GAA در طول تنش گرمایی به ویژه در پایان دوره سبب بهبود زنده‌مانی شده که این نتایج با بررسی اندازه گیری تعداد و شدت له له زدن (panting) مورد تایید قرار گرفت (اثر خطی). در ۲٦ روزگی با افزایش سطح مکمل GAA از صفر به ۰/٦ و ۱/۲ گرم در کیلو گرم جیره، Arg پلاسما به ترتیب ۱۸/۳ ٪ و ۳۰/۸ ٪ افزایش یافت. در ۳۹ روزگی روند خطی این افزایش قابل مشاهده بود، که این موضوع می‌تواند تاییدی بر افزایش قابلیت دسترسی Arg در سایر واکنش های متابولیکی نسبت به سنتز *de-novo* در بدن باشد. بررسی عضله سینه در هر دو روز نمونه برداری افزایش PCr (اثر خطی در ۲٦ و ۳۹ روزگی) Cr آزاد (۳۹ روزگی) Cr کل (۲٦ و ۳۹ روزگی) و نسبت PCr:ATP (اثر خطی در ۲٦ روزگی و روند افزایشی در ۳۹ روزگی)، در اثر مکمل سازی با GAA را نشان داد. مکمل GAA سبب بهبود ضریب تبدیل غذایی و شاخص زنده مانی در طول دوره تنش گرمایی شد که برآیند افزایش سطح انرژی عضله سینه واثر صرفه جویی Arg می باشد.

پژوهشگران زیادی نشان داده اند که Cr توانایی خنثی‌سازی آنیونهای سوپراکسید و سایر گونه‌های واکنش پذیر (رادیکال های آزاد) را دارد، بنابراین Cr می‌تواند دارای ظرفیت آنتی‌اکسیدانی باشد (لاولر و همکاران ، ۲۰۰۲ ؛ دمینیچ و جوردائو ، ۲۰۱۲). همچنین به دلیل تاثیر بر وضعیت انرژی سلولی، قادر است به صورت غیرمستقیم اثرات آنتی اکسیدانی خود را اعمال کند (پرسکی و برازوآ، ۲۰۰۱). مشخص شده است که جوجه‌های گوشتی با رشد سریع نیاز به محافظت آنتی‌اکسیدان‌ها داشته (اوروسو، ۲۰۰۲) و برخی شرایط مانند HS می‌تواند سبب استرس اکسیداتیو (OS) در آنها شود (اکبریان و همکاران، ۲۰۱٦). بنابراین این سوال مطرح است که آیا جیره غذایی حاوی GAA از طریق افزایش سطح Cr بافت بدن طیورقادر است جوجه‌های گوشتی را در مقابل آسیب‌های اکسیداتیو محافظت نماید. از این رو، در آزمایش چهارم، تأثیر مکمل GAA در جیره جوجه‌های گوشتی که در طول دوره پایانی در معرض HS قرار گرفته‌اند به لحاظ وضعیت اکسیداتیو و متابولیسم GAA و Cr در اندامهای کلیدی مورد مطالعه قرار گرفت. ۷۲۰ جوجه گوشتی نر یک روزه سویه راس ۳۰۸ و ۳ تیمار ۰، ۰/٦ و ۱/۲ گرم GAA در کیلو گرم جیره های غذایی ذرت - سویا در دوره آزمایشی ۳۹روزه با ۱۲ تکرار (۲۰ پرنده در هر تکرار) اختصاص یافت و مدل تنش گرمایی دوره‌ای پیوسته (درجه حرارت ۳٤ درجه سانتیگراد همراه با رطوبت نسبی ٥۰-٦۰٪ به مدت ۷ ساعت به صورت روزانه) در دوره پایانی پرورش اعمال شد. برای ارزیابی متابولیت‌های انرژی و پارامترهای اکسیداتیو در خون، عضله سینه، کبد، کلیه و قلب، نمونه گیری از یک پرنده به ازای هر پن در روزهای ۲٦ و ۳۹ آزمایش انجام شد. با تغذیه GAA در هر دو روز نمونه برداری، میزان GAA و Cr پلاسما افزایش یافت، که نشان دهنده کارایی مرحله جذب و متیلاسیون می‌باشد. وضعیت

متابولیسم انرژی در عضله ماهیچه تا حد قابل توجهی با افزایش ذخایر Cr، PCr:ATP و گلیکوژن مورد تایید و به طور مثال قابلیت دسترسی PCr که مرتبط با محدودیت فسفوریلاسیون اکسیداتیو در شرایط HS می باشد، بهبود یافت. مکمل GAA سطح کلسترول خون را بصورت خطی کاهش داد (اثرمعنی دار در ۲۶ روزگی و روند افزایشی در ۳۹ روزگی) که ممکن است در نتیجه تاثیر GAA بر آدنوزین مونوفسفات پروتئین کیناز فعال در کبد باشد. GAA بر میزان مالون دی آلدهاید، سوپراکسید دیسموتاز (SOD) و گلوتاتیون پراکسیداز پلاسما اثر معنی داری نداشت. در مقابل فعالیت SOD در عضله سینه به صورت خطی کاهش یافت (روند خطی در ۲۶ و ۳۹ روزگی). این یافته می‌تواند نشانگر اثر آنتی اکسیدانی غیرمستقیم GAA در نتیجه افزایش Cr عضله ماهیچه به ویژه در شرایط HS باشد. نتایج این آزمایش نشان داد، GAA سبب عملکرد بهتر جوجه‌های گوشتی در شرایط HS می‌شود که مرتبط با افزایش سطح انرژی و در نتیجه بهبود شرایط اکسیداتیو پرنده می‌باشد.

به طور کلی این پژوهش نشان می دهد که [۱] اثر مکمل GAA در جیره‌های غذایی طیورمستقل از ND است، [۲] اثر مکمل GAA در جیره‌های غذایی تحت تاثیر سطح Met در دوره پایانی می‌باشد، که این امر فرمولاسیون مناسب اسیدهای آمینه گوگرد دار با در نظر گرفتن میزان متیلاسیون در جیره‌های غذایی هنگام تغذیه GAA را مورد تاکید قرار می‌دهد، [۳] مکمل GAA سبب بهبود راندمان غذایی و شاخص زنده مانی در شرایط HS دوره‌ای پیوسته شده در حالی که هنوز راندمان و شاخص‌های عملکرد پایین تر از شرایط معمول دمایی پرورش طیور است و [٤] GAA سبب بهبود وضعیت انرژی عضله سینه شده، به نحوی .که به طور غیرمستقیم شرایط اکسیداتیو سلول بهبود یافته که این در ارتباط با افزایش سطح گلیکوژن و اثر صرفه جویی Arg می باشد.

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CURRICULUM VITAE

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Maryam Majdeddin (Iran, Shiraz, 30 May 1981) obtained her Bachelor degree in Agricultural Engineering: Animal Sciences at Shiraz University in Iran, 2004. Thereafter, she worked as dairy cow nutrition adviser for local dairy operators. From 2006 to 2008 she successfully completed the Master degree in Poultry Nutrition at Tehran University in Iran. The years after, she was teaching at various universities, courses related to animal production such as Animal Husbandry, Poultry Nutrition, and Poultry Management. In 2011, she was selected for a PhD scholarship at Ferdowsi University of Mashhad, Mashhad, Iran. Her PhD research was entitled 'Guanidinoacetic acid as feed supplement to broiler chickens'. She first conducted broiler chicken experiments at her home university, before pursuing a sabbatical at Ghent University, initiated in 2015. Her doctoral research at Ghent University, together with the experimental work at her home university is part of her joint PhD project. Maryam also carried out multiple research and service projects on monogastric nutrition at the Laboratory for Animal Nutrition and Animal Product Quality (LANUPRO) at Ghent University. She supported exchange students and MSc thesis students. She first-authored 2 international peer-reviewed papers, and presented her research on international conferences. She also translated several English textbooks to Persian.

PUBLICATIONS

First authored A1 publications

Majdeddin, M., A. Golian, H. Kermanshahi, S. De Smet, and J. Michiels. 2018. Guanidinoacetic acid supplementation in broiler chickens fed on corn-soybean diets affects performance in the finisher period and energy metabolites in breast muscle independent of diet nutrient density. Br. Poult. Sci. 59:443-451.

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