Efficacy and safety of dietary *N,N*-dimethylglycine in broiler production

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(Henri Martin Didon)

ABSTRACT

N,*N*-dimethylglycine (DMG), the dimethyl derivative of the amino acid glycine, is a naturally occurring intermediary metabolite in the choline to glycine metabolism. The molecule was first reported in 1943 and is currently used for a variety of applications, including the enhancement of athletic performances in both man and racing animals. With respect to its biological activities, DMG is for instance suggested to enhance oxygen utilisation and to posses non-enzymatic anti-oxidant properties. The studies described in this thesis aimed to evaluate DMG as a feed additive in chickens for fattening.

In a pilot study, broilers were challenged with both cold stress and a high energy feed in order to incite broiler ascites syndrome. This metabolic disease results from an imbalance between oxygen requirement and supply, and is an important cause of financial losses and a major welfare issue in the modern broiler industry. A low dosage of dietary DMG effectively attenuated progression towards ascites. We hypothesize that this effect results from reduction in endothelial damage and dysfunction caused by plasma free fatty acids, which were substantially lowered by DMG supplementation. Furthermore, DMG improved nutrient digestibility and reduced nitrogen emission, which can be attributed to an emulsifying effect of DMG at the gut level. A subsequent trial revealed dose-dependent effects of dietary DMG on technical performance, carcass yield, oxidative stress parameters and broiler ascites syndrome. However, the nature and magnitude of the effects depended on fatty acid profile of the basal ration. Herein, effects were most pronounced when fed a diet rich in poly-unsaturated fatty acids. Generally, effects showed a linear relationship with dose, except for progression towards broiler ascites syndrome, which showed a quadratic relationship with dietary DMG content. Next, a series of efficacy trials were performed on farms at different European locations, using broiler strains and rearing conditions common to each region. Basal flock efficiency showed a wide range between trials; still, DMG systematically improved broiler performance. Finally, a safety and tolerance trial demonstrated a wide safety range of DMG in the target species. Moreover, DMG did not accumulate in consumer parts of broilers when included in diets at the recommended dosage, and bioaccumulation in meat at tenfold dosage did not exceed DMG content in for instance spinach.

In conclusion, current investigations clearly demonstrate a wide applicability of DMG as a new feed additive in broiler production, in which both economic efficiency and environmental load as well as animal welfare is enhanced without compromising consumer safety.

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ACRBC	Athens Canadian randombred control
ADFI	average daily feed intake
ADG	average daily growth
AFD	apparent faecal digestibility
AHI	ascites heart index
AIA	acid insoluble ash
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BHMT	betaine homocysteine methyltransferase
BW	bodyweight
CA	crude ash
CF	crude fibre
CfG	carcass for grilling
CP	crude protein
DM	dry matter
DMG	*N*,*N*-dimethylglycine
EE	ether extract
FA	fulminant ascites
Fat_{IM}	intramuscular fat
FCR	feed conversion ratio
FM	fresh matter
GGT	gamma-glutamyl transferase
Glu	glucose
Hct	haematocrit
LW	live weight
ME_n	metabolizable energy corrected for zero N-balance
MS	methionine synthase
MUFA	monounsaturated fatty acids
N	nitrogen
Na	sodium
NEFA	non-esterified fatty acids
NfE	N-free extract
PH	pulmonary hypertension
PUFA	polyunsaturated fatty acids
PV	production value
SAM	S-adenosylmethionine
SFA	saturated fatty acids
T_3	triiodothyronine
T_4	thyroxine
TBARS	thiobarbituric acid reactive species
TG	triglycerides
THF	tetrahydrofolate
UA	uric acid

CHAPTER 1

GENERAL INTRODUCTION

BACKGROUND

Chickens have been raised as food for human consumption since at least 2,000 years BC. For this reason, they are continuously selected for certain desirable characteristics by active selection of parent birds to produce birds which fit our perceived needs (Wheeler and Campion, 1993). Notwithstanding an already long history of selective breeding and improvements in rearing techniques, the efficiency in the production of broiler meat has shown a remarkable leap over the past few decades. First, persistent selection towards rapid growth, high feed utilisation efficiency and large cut yield has led to meat-type poultry lines with superior genetic potential with regard to productivity. The short generation interval of poultry, the relatively high heredity of production traits and industrial-scaled selection have contributed to the success of such breeding programs. Second, the increase in knowledge of nutritional physiology has led to adjustment of diets to enable rapid growth. Also knowledge about the response of chickens to climatic conditions has led to housing which provides the most optimal conditions for growth. And finally, enhanced general management has further improved production efficiency and supported maximal exploitation of improved genetic traits (Rauw *et al.*, 1998; Wheeler and Campion, 1993).

The extent of recent advances achieved in broiler production has been provided by performance trials using 2x2 factorial designs comparing broiler lines and diets representative for the time of trial run with those of a few decades earlier. Havenstein and colleagues (2003a), for instance, measured technical performance of original 1957 Athens Canadian Randombred Control (ACRBC) strain and modern 2001 Ross 308 strain birds fed either a 1957 or a 2001 representative diet. Body weight at 42 days of age was only 539 and 578 g in the ACRBC strain when fed the 1957 and 2001 diet, respectively. At the same age, the modern Ross breed reached 2,126 and 2,672 g when fed these respective diets. Diet optimisation resulted in a decrease in feed intake relative to body weight gain or feed conversion ratio in the ACRBC and Ross strains from 2.34 to 2.14 and from 1.92 to 1.63, respectively (Havenstein *et al.*, 2003a). In conjunction with technical performance, carcass yield expressed as a percentage of body weight was also more profoundly increased by genetics rather than dietary improvements. It is clear that dietary modifications showed greater response in the modern compared to the older strain birds. Herein, carcass yield at the age of 43 days in the ACRBC strain was 60.0 and 61.0% compared to 68.3 and 72.3% in the Ross strain when fed the 1957 and 2001 diet, respectively. In addition, this superior carcass yield in the modern broiler strain was almost entirely attributable to a near doubling of breast

meat yield, which is the most valuable cut yield in broilers (Havenstein *et al.*, 2003b). Moreover, Havenstein *et al.* (2003a) estimate the sum of all improvements in broiler production achieved between 1957 and 2001 to approximate a reduction in rearing period from 101 to 32 days, and a reduction in feed conversion ratio from 4.42 to 1.47 to yield 1,815 g broilers. The significance hereof is shown by the fact that a 5-day reduction in rearing period yields an additional raising round per year (Emmerson, 1997). Furthermore, the economic advantage of maximum product yield per kg feed intake becomes obvious when bearing in mind that feed cost is the principal variable cost in broiler production (Rauw *et al.*, 1998).

Intense selection pressure and modern rearing techniques have thus enabled enhanced production rate and efficiency. However, this coincided with undesirable side-effects such as an increase in incidence of heart failure and leg problems (Rauw *et al.*, 1998). Mortality also increased in parallel to growth rate (Havenstein *et al.*, 2003a). One of the aims of the trials reported here was to study nutritional means of reducing broiler ascites syndrome, which is a syndrome that is intrinsic to modern broiler production. The central aetiology of this metabolic disorder is a hypoxaemic condition resulting from an imbalance between oxygen requirement and supply. A cascade of compensatory mechanisms eventually leads to increased pulmonary pressure, which is initially overcome by right ventricular heart hypertrophy. If persistent, this leads to right ventricular valve insufficiency, volume overload, dilatation and right ventricular failure. As a result, venal blood pressure increases and fluid leaks out of the veins and accumulates in peritoneal cavities and pericardium, resulting in ascites and further impairment of cardiac function (Currie, 1999; Julian, 1998). Ascites is defined as the accumulation of transudate fluid within the abdominal cavity. The resulting pressure on the air sacs causes respiratory signs, which together with congestive failure, ultimately leads to death (Bottje *et al.*, 1998; Julian, 1998).

Previously, broiler ascites syndrome was essentially observed in birds raised at high altitude, which entails low partial oxygen pressure and thus diminished oxygen supply. However, since 1980, the incidence of ascites has steadily increased and, more importantly, its occurrence has extended to lower altitudes and even to sea level (Julian, 1993). De Smit *et al.* (2005) estimated broiler ascites syndrome to account for over 25% of overall broiler mortality on a worldwide basis, which means that this metabolic disorder has become the most important non-infectious cause of loss in the modern broiler industry. Death occurs generally late in the

rearing period or even during transport to the slaughter house (Neidam *et al.*, 2006). In addition, it is a severe welfare problem as the symptoms, such as breathing difficulties, are progressive and distressing to the animal (Aksit *et al.*, 2008). Moreover, carcasses of ascitic birds are rejected for consumption (Haslam *et al.*, 2008). Hence, although these birds have been fed for up to the entire rearing cycle, they have no economical value at all. The economical cost is thus considerable, both by nature and by incidence of the condition.

The causes of the high incidence of insufficient oxygen supply and subsequent development of broiler ascites syndrome in modern broiler lines are threefold. First, a superior growth rate inherently implies a high oxygen demand to sustain the metabolic needs. Second, achieved progress in profitable growth negatively affects relative heart and lung size, and thus diminished cardiopulmonary capacity. This has been suggested and evidenced by Havenstein *et al.* (2003b), who demonstrated a 10.3 and 9.0% decline in relative heart and lung size, respectively in 43-day-old birds when comparing the ACBRC strain fed a 1957 representative diet with the 2001 Ross 308 strain fed a 2001 representative diet. In addition, compared to other birds, the respiratory membrane is thicker in poultry and in meat-type chickens in particular. Consequently, broilers presumably display a lower rate of oxygen diffusion from the lungs into red blood cell haemoglobin compared to other birds (Banghbanzadesh and Decuypere, 2008). A high growth rate, however, implies a faster blood flow through the capillary bed of the lungs, which possibly further impairs oxygenation of blood (Wideman and Kirby, 1995a and 1995b). Third, an increase in metabolic rate not only augments oxygen requirement at tissue level, but also elevates mitochondrial production of reactive oxygen species. Oxidative stress, in turn, causes lipid peroxidation mediated damage to the pulmonary vasculature, which further deteriorates oxygenation and aggravates hypoxaemia (Aksit *et al.*, 2008; Bottje and Wideman, 1995; Diaz-Cruz *et al.*, 2003). Augmented free radical release caused by hypoxic lung injury, in turn, feeds a viscous circle that reinforces itself towards progressive hypoxaemia (Herget *et al.*, 2000). Given these additional impairments, it is not surprising that fast growth, which implies increased oxygen requirement, in broiler strains that are already faced with a relatively undersized cardiopulmonary capacity, may result in hypoxaemia.

Other contributing factors in the development towards broiler ascites or pulmonary hypertension syndrome include environmental, dietary or animal factors that additionally augment the physiological oxygen requirement or decrease oxygen supply. To maintain

normal body temperature in conditions of cold stress, for instance, endogenous heat production is upregulated through an increase in metabolic rate and concomitantly both oxygen requirement and blood flow are elevated (Julian *et al.*, 1989; Julian, 2000; Lubritz and McPherson, 1994). Respiratory disease and poor air quality comprise examples of conditions that additionally may impair blood oxygenation and thus lower oxygen supply (Julian, 2002). Poor air quality is typically attributable to insufficient ventilation, through which dust and gaseous pollutants, such as ammonia and carbon monoxide build up in the air (Bottje *et al.*, 1998). Moreover, hypertension exciting factors, such as excessive dietary salt, appear to be additive to other conditions that incite ascites (Julian, 1987; Julian *et al.*, 1992).

Balog *et al.* (2003) suggest genetic selection for ascites resistant broiler lines to be a permanent solution to the ascites problem. However, until selection programs successfully develop such broiler lines and implementation of these new strains reveals to be satisfactory, alternative preventive measures have to be considered. This has been mainly addressed by development of feed and lighting regimes that slow down early growth (Özkan *et al.*, 2010). Limitation of early growth rate can be achieved by qualitative feed restriction, which implies dilution of energy density of the ration, or by quantitative feed restriction through offering a diminished quantity of feed each day, periodical feed withdrawal or a lighting schedule with increased dark hours. However, these methods entail some concerns. First, the effectiveness of limiting early growth rate to diminish incidence of ascites is debated in literature, as reviewed by Madrigal *et al.* (2002). Second, several trials indicate inadequate compensatory growth in later growth phases, resulting in depressed body weight or yield at market age (e.g. Acar *et al.*, 1995; Lee and Leeson, 2001). Third, Lee and Leeson (2010) point at the extra cost of non-nutritive fillers, and in addition, extra transport costs per unit of feed to attain qualitative feed restriction in form of diet dilution. Therefore, they suggest that quantitative feed restriction is preferred at an industrial scale (Lee and Leeson, 2010). Fourth, feed restriction in broiler lines that were selected for high feed intake may impair welfare as demonstrated both by physiological stress parameters and behavioural criteria indicative of frustration to hunger (Savory and Lariviere, 2000). With respect to the above, quantitative feed restriction through limiting daily amounts of feed seems not to diminish welfare further compared to qualitative feed restriction (Savory *et al.*, 1996; Savory and Lariviere 2000). Finally, Banghbanzadeh and Decuypere (2008) draw attention to adverse secondary effects of imprudent feed restriction on adequate intake of, for instance, anticoccidial products on control of coccidiosis, or on intake of pigmentation precursors on product quality.

Optimisation of the diet to ameliorate the ascites syndrome has been investigated to a minor extent. The multi-factorial aetiology of the syndrome, however, renders pulmonary hypertension also responsive to dietary strategies other than limitation of growth rate in an attempt to attenuate progression towards fulminant ascites. First, an increment in fluidity of the red blood cell membrane through a dietary shift in fatty acid profile towards a higher ratio of unsaturated to saturated fatty acids has been demonstrated to improve erythrocyte deformability. This way, the resistance of blood flow through the lung capillaries is diminished and pulmonary hypertension abated (Bond *et al.*, 1996; Walton *et al.*, 1999). Furthermore, as suggested by Bond *et al.* (1996) and Walton *et al.* (1999), those dietary fat sources which contain a high *n*-3 to *n*-6 ratio may additionally slow down progression towards broiler ascites syndrome. This is reached by release of endogenous coronary relaxants and attenuated inflammatory compounds derived from cellular membrane lipids. A reduction in reactive oxygen mediated damage to the cardiopulmonary system forms yet another approach that could alleviate the predisposition of fast-growing birds to ascites mortality (Herget *et al.*, 2000). Dietary supplementation with anti-oxidant vitamins sometimes, but not always, reduced oxidative damage and improved pulmonary vascular performance (e.g. Bautista-Ortega and Ruiz-Feria, 2010; Lorenzoni and Ruiz-Feria, 2006; Nain *et al.*, 2008). Also, forthcoming effects on incidence of ascites are inconsistent in the literature (Baghbandzadeh and Decuypere, 2008; Nain *et al.*, 2008; Xiang *et al.*, 2002). Further investigation of dietary supplements reducing broiler ascites syndrome is thus warranted.

Apart from prevention of metabolic disorders associated with fast growth, the pressure to enhance feed efficiency in animal production remains important. Feed cost will continue to rise as a result of increasing competition for cereal grains between agriculture and other industries (Gohin, 2008). Livestock production is hence challenged to develop new strategies that further optimise feed conversion. In addition, there is a growing pressure of society to decrease nitrogen pollution of surface water caused by livestock production. This also stimulates developments towards improved feed utilisation, *in casu* protein utilisation (Nahm, 2007). Moreover, new feed additives should replace both the formerly widely used feed antibiotics and also the hormonal growth promoters. Both are now strictly regulated to safeguard consumer and environmental safety (Lević *et al.*, 2007; Steinfeld, 2003). Evaluation of the efficacy of a potential new feed additive to improve feed efficiency and

protein utilisation comprises a second aim in the current thesis.

N,*N*-DIMETHYLGLYCINE (DMG) AND RELATED COMPOUNDS

DMG, the test compound investigated in the studies reported here, is a naturally occurring intermediary metabolite in the choline to glycine metabolism (Tonda and Hart, 1992). In short, choline is converted into betaine, which donates one of its three methyl groups to homocysteine. This reaction is one of two pathways that remethylizes homocysteine to methionine and is catalyzed by the enzyme betaine homocysteine methyltransferase (BHMT). Next to methionine, this reaction also renders DMG, which in turn donates its two methyl groups to tetrahydrofolate through which DMG is catabolised into sarcosine and subsequently glycine. Transmethylation between methylated tetrahydrofolate and homocysteine, which is catalysed by the enzyme methionine synthase (MS), forms the second pathway to restore methionine (**Figure 1**; Slow *et al.*, 2004). Methionine, as S-adenosylmethionine (SAM), is the common methyl donor in most biological reactions, resulting in its conversion to homocysteine (Klasing, 2000; Lever and Slow, 2010). This transfer of methyl groups from SAM is essential for both synthesis reactions and for DNA methylation, which controls gene expression (Jacob, 2000; Lever and Slow, 2010). The importance of the BHMT and MS pathways is twofold: first, maintenance of methylation capacity, and second, reduction of the homocysteine level, which is put forward as an independent risk factor of cardiovascular disease (Lever and Slow, 2010; Welch and Loscalzo, 1998). As homocysteine is oxydised when it is not remethylated, insufficient remethylation of homocysteine will cause a marked increase in methionine requirement, which is often the first limiting amino acid in avian diets (Klasing, 2000).

Choline, although it can be synthesised in the liver in limited amounts, is generally considered a vitamin (NRC, 1994). However, unlike other vitamins, it is not involved as a cofactor in enzymatic reactions and moreover, its requirements fall in the range and magnitude of essential amino acids or fatty acids rather than trace amounts (Church and Pond, 1974; Klasing, 2000). The three main biological functions of choline include: a structural compound in cellular membranes and lipoproteins**,** e.g. phosphatidylcholine, a neurotransmitter in the parasympatic system as acetylcholine and the above mentioned methyl donor function through betaine and DMG (Church and Pond, 1974; NRC, 1994). Slow growth and perosis - slippage of the Achilles tendon from its condyles - are the primary deficiency symptoms (Church and Pond, 1974; Fritz et al, 1967). Other symptoms include hepatic steatosis as a

consequence of failure in packaging of hepatic triglycerides into very low density lipoproteins; and excitability, curled toes and paralysis due to impaired acetylcholine level (Klasing, 2000). Moreover, Dodson and Sachan (1996) report a nutrient-nutrient interaction between choline and its structural analogue carnitine, in which choline conserves carnitine through increased tissue uptake and decreased urinary losses of carnitine. As carnitine is imperative for translocation of long chained fatty acids across the inner mitochondrial membrane, choline hence indirectly improves fatty acid oxidation (Daily and Sachan, 1995; Dodson and Sachan, 1996). However, although birds tolerate high levels of phosphatidylcholine, diets excessively supplemented with cholinechloride show depressed growth rate (Klasing, 2000). Also, the gut flora may metabolise choline into trimethylamine if dietary choline is excessive, which has been shown to result in a fishy flavour of eggs (March and MacMillan, 1979).

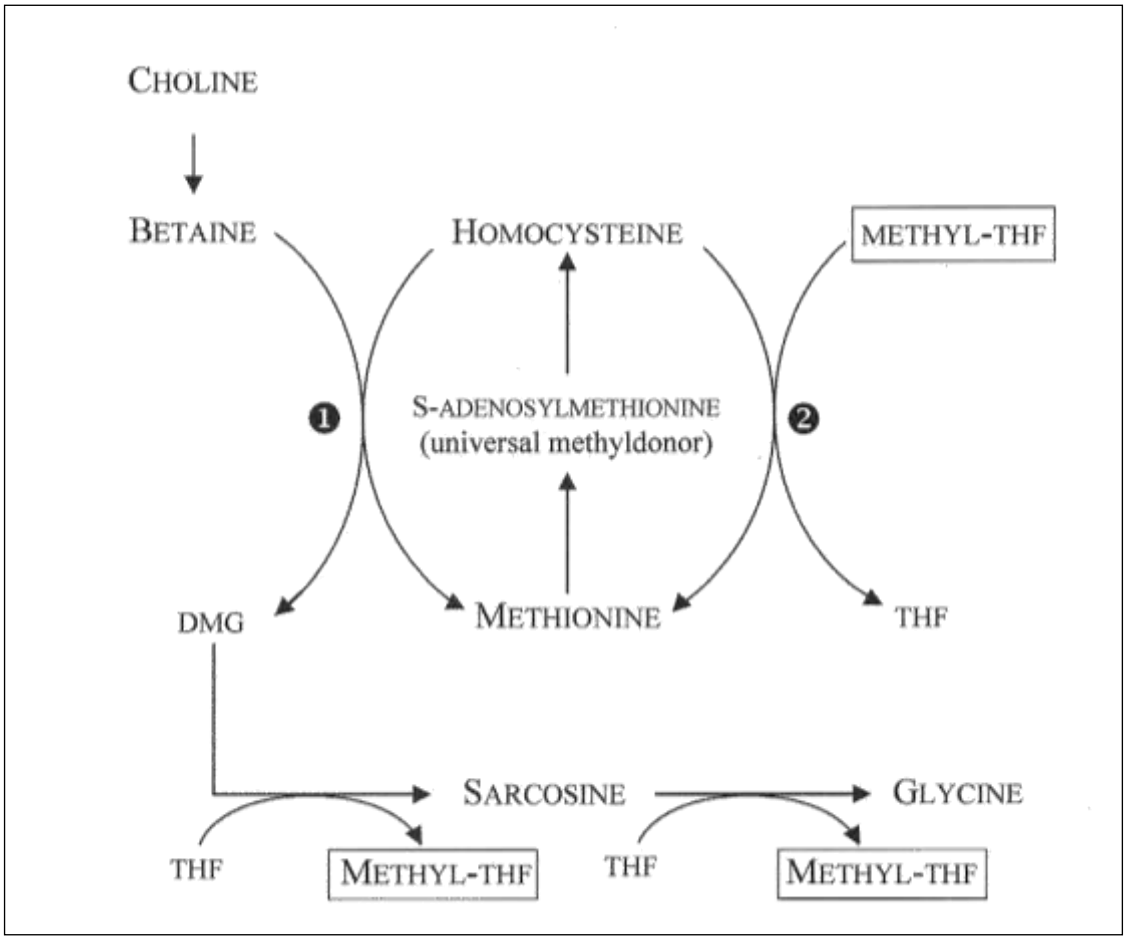

Figure 1. Choline to glycine metabolism.
Reactions ❶ and ❷ represent the two pathways that restore methionine:
❶ BHMT: betaine homocysteine methyltransferase pathway
❷ MS: methyl synthase pathway
(DMG: *N*,*N*-dimethylglycine, THF: tetrahydrofolate)
(based on Slow *et al*., 2004)

Betaine is a natural by-product of the sugar beet (*Beta vulgaris*) industry, and has next to its key function in methyl donation also an important osmoprotectant function (Häussinger, 1996; Lever and Slow, 2010). Importantly, high tonicity reduces the expression of BHMT (pathway 1 in **Figure 1**), through which betaine is spared from oxidative demethylation and mainly used as an osmolyte to assist cell volume regulation to preserve normal cell function (Delgado-Rayes and Garrow, 2005). In common with most osmolytes, betaine also enhances stability of proteins and hereby provides for protection against denaturation (Gilles, 1997; Yancey and Burg, 1990). With respect to its methyl donation function, betaine has been reported to affect lipid metabolism by which it results in decreased tissue lipid. On the one hand, it decreases the expression of enzymes involved in lipogenesis (Huang *et al.*, 2008), on the other hand, it increases hepatic apolipoprotein B synthesis, which is needed to export tissue triglycerides (Sparks *et al.*, 2006). A beneficial effect of dietary betaine to improve weight gain or feed conversion in broilers is contested in the literature. However, several trials demonstrate improved carcass traits (Konca *et al.*, 2008; Maghoul *et al.*, 2009).

DMG is the compound of interest in the current investigation. Firstly, DMG esters have been demonstrated to possess surfactant properties (Clapes and Infante, 2002). The emulsifying effect of DMG can easily be demonstrated by addition of DMG to a water and oil mixture, which results in formation of an emulsion. Therefore, DMG might improve nutrient absorption through diminished encapsulation of nutrients by fat droplets, which enhances accessibility to digestive enzymes and the absorptive brush border of the small intestine. Second, DMG is a small, water-soluble molecule that is sufficiently lipophilic to cross cellular membranes and hence, it is likely to be well-absorbed through the gut wall when orally administered (Cupp and Tracy, 2003). Therefore, as DMG offers a second route, next to the BHMT pathway, for remethylation of homocysteine, dietary supplementation with DMG may either directly or indirectly support the highly challenged metabolism of fast-growing broilers. Direct support of the metabolism by DMG may arise from its methyl donor function, whereas a decreased catabolisation of choline and betaine may indirectly support the metabolism by sparing these compounds for their other respective functions. Third, results of Hariganesh and Prathiba (2000) suggest free-radical scavenging potential of DMG in rats, through which incidence of stress-induced gastric ulcers is diminished. In addition, DMG is reputed to enhance oxygen utilization and is therefore used to improve athletic performance in both man and racing animals. The latter application of oral DMG supplementation is primarily based on anecdotal reports, as are many other suggested beneficial properties of

dietary DMG. The few randomized, controlled human and animal studies, however have failed to support these anecdotal claims (Currell *et al.*, 2010; Warren *et al.*, 1999). Nevertheless, as concluded by Currell *et al.* (2010), lack of evidence of effects is not equivalent to evidence of lack of effects. As broiler ascites syndrome is characterised by an imbalance between oxygen need and oxygen supply (Julian, 1998), and as oxidative stress is involved in its pathogenesis (Bottje and Wideman, 1995; Nain *et al.*, 2008), DMG seems a valuable candidate feed additive to attenuate this syndrome.

In order to study DMG in broiler nutrition, the following research questions were investigated in this thesis:

1. Does dietary supplementation with DMG exhibit anti-oxidative properties and does this result in attenuation of broiler ascites syndrome? **Chapter 2**

2. If DMG has emulsifying properties, can dietary DMG improve nutrient digestibility? As a consequence, more dietary nutrients, including protein, may become available for utilisation, with additionally a possible reduction in nitrogen emission. **Chapter 2**

3. Are health or productivity related traits in broilers influenced by DMG in a dose-dependent matter and are effects dependent on fatty acid profile of test diets? **Chapter 3**

4. If DMG shows beneficial effects on broiler performance, do such traits show persistence over a range of broiler strains and rearing conditions? **Chapter 4**

5. Does DMG show a wide tolerance and safety range in broilers and does DMG accumulate in consumer parts of the target species? **Chapter 5**

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CHAPTER 2

Dietary *N,N*-dimethylglycine supplementation improves nutrient digestibility and attenuates pulmonary hypertension syndrome in broilers

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ABSTRACT

N,N-dimethylglycine (DMG) is an intermediary metabolite in cellular choline and betaine metabolism. The present trial aimed to evaluate the effect of dietary DMG on nutrient digestibility and development of pulmonary hypertension syndrome in broilers. A total of 64 14-day-old broiler hens (Ross-308) were raised until age 40 days under cold environmental temperature conditions (15°C) and were fed a high energy feed in order to incite pulmonary hypertension. Birds were randomly assigned to two groups of which each group had eight replicate pens of four birds each. Test diets contained 0 or 167 mg Na-DMG (Taminizer® D; Taminco N.V., Ghent, Belgium) per kg feed. N,N-dimethylglycine supplementation resulted in a significant improvement in apparent faecal digestibility of crude protein and nitrogen-free extract. Further, fulminant ascites was numerically lowered by DMG and incidence of pulmonary hypertension decreased significantly from 44.8% in the control group to 14.6% in the DMG group. Finally, fasted plasma level of non-esterified fatty acids (NEFA) was twofold in the control group in relation to the DMG group. In conclusion, these data demonstrate beneficial effects of DMG on digestibility of non-fat fractions, on fat metabolism and on progression towards broiler ascites syndrome.

INTRODUCTION

Important objectives in the meat-type broiler industry remain to improve feed conversion ratio (FCR), mortality rate and carcass yield. Moreover, alleviating welfare problems is currently considered essential in enhancing animal production systems. Metabolic diseases often give rise to compromised welfare next to financial losses because of deterioration of production value on account of increased mortality rate or less efficient utilisation of nutrients, or to condemnation of carcasses. Potential means to achieve above mentioned goals comprise genetic improvement and refinements in rearing conditions, next to optimising nutrition, including development of new feed additives.

Both intensive, quantitative selection and improvements in diet and management have led to a tremendous increase in overall performance of broilers over the past 50 years. Havenstein *et al.* (2003a), for instance, compared performance traits of the Athens Canadian Randombred Control (ACBRC), a strain established in 1957 that is still maintained, and Ross 308, when fed either a diet representative for 1957 or a modern diet, representative for 2001. These authors demonstrated an almost fourfold increase in bodyweight at age 42 days on account of significant improvements in both genetics and nutrition, of which over 90% could be attributed to selection efforts. Further, FCR significantly improved with 30%, of which 80% on account of improved genetics. However, mortality increased numerically with 100%, feed and genetics being equally involved. An important unfavourable consequence of persistent selection towards fast growing, high yielding broiler strains is a significant decrease in relative cardiopulmonary capacity. This has been demonstrated by Havenstein *et al*. (2003b), who showed a significant improvement in relative carcass yield by 20%, but a significant decrease in relative heart and lung size by 10% and 9% in 43-day-old broilers of Ross 308 and ACBRC, respectively. The cardiopulmonary capacity in modern, fast growing broiler strains being inadequate to sustain physiological homeostasis, is a major contributing factor to the observed increase in mortality and particularly in pulmonary hypertension syndrome, also referred to as broiler ascites syndrome (Emmerson, 1997; Havenstein *et al*., 2003b).

The primary occurrence of pulmonary hypertension syndrome is in most cases a hypoxaemic condition. Physiologically, hypoxia occurs when the cardiopulmonary capacity is inadequate relative to the physiological needs or in response to environmental challenges (Julian, 1998; Baghbanzadesh and Decuypere, 2008). Modern broiler strains are highly susceptible to hypoxia because of a combination of high oxygen demand due to rapid growth rate and a

relatively underdeveloped cardiopulmonary system (Havenstein *et al.*, 2003a and 2003b). Further, an increase in mitochondrial reactive oxygen species, as a result of an increase in metabolic rate, causes lipid peroxidation mediated damage to the pulmonary vasculature through which oxygenation is further deteriorated and hypoxaemia aggravated (Aksit *et al.*, 2008; Bottje and Wideman, 1995; Diaz-Cruz *et al.*, 2003). At first, the hypoxaemic state is compensated through an increase in haematocrit (Hct) level, which eventually leads to an elevated blood viscosity that on its turn gives rise to pulmonary hypertension followed by right heart failure. As a consequence, the venal pressure increases and liquid leaks from the blood vessels and accumulates in the peritoneal spaces (Currie, 1999). Next to rapid growth, additional factors that further increase incidence of ascites include for instance high altitude, cold or hot ambient temperature, certain chemicals, rickets and respiratory disease (Julian, 1998).

On a worldwide basis, broiler ascites syndrome is estimated to account for over 25% of overall broiler mortality and has become the most important non-infectious cause of loss in the modern broiler industry (De Smit *et al.*, 2005). Next to this financial cost, broiler ascites syndrome is considered an important welfare concern (Aksit *et al.*, 2008). Balog *et al.* (2003) suggest selection of ascites resistant broiler lines to be a permanent solution to the ascites problem. However, optimising environmental conditions and diet might be a more feasible method to ameliorate the problem, especially with regard to sustaining currently achieved improvements in overall performance.

The main objectives of the present trial were to investigate the effect of dietary supplementation with *N*,*N*-dimethylglycine (DMG) on apparent faecal digestibility (AFD) and on development of pulmonary hypertension in broilers. Firstly, DMG-esters have been demonstrated to possess surfactant properties (Clapes and Infante, 2002), through which beneficial effects of DMG might be present on nutrient digestibility. Secondly, DMG is reputed to be involved in a variety of biological processes as it is an intermediary metabolite in the cellular choline and betaine metabolism. With respective to the current trial, Friesen *et al.* (2007), for instance, describe a role of DMG as a source of glycine for glutathione synthesis. Next, data of Hariganesh and Prathiba (2000) suggest free radical scavenging potential of orally administered DMG. Because oxidative stress is a well-recognized physiological factor in the pathogenesis of pulmonary hypertension in broilers (Bottje and Wideman, 1995), above mentioned non-enzymatic anti-oxidant properties of DMG might possibly attenuate progression towards this metabolic disorder.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC 2005/46).

Experimental design

Sixty-four 14-day-old broiler hens (Ross-308) were randomly allocated in 16 pens (0.72 m^2 per pen) of four birds each until the age of 40 days. The floor pens had a layer of peat, topped with wood shavings as bedding. Individual identification of chicks was performed by means of leg rings. After 1 day feeding their accustomed diet, one of two test diets was assigned to eight replicate pens per treatment.

Feed and drinking water were provided *ad libitum* and a lighting schedule of 23 h light:1 h dark was applied. Ambient temperature was kept below the thermoneutral zone (at 15°C), forcing the birds to increase metabolic rate and thus oxygen consumption, as a challenge to increase the occurrence of pulmonary hypertension syndrome (ascites) (Shlosberg *et al.*, 1992; Korte *et al.*, 1999; Buyse *et al.*, 2001). The control diet consisted of 95% commercial broiler crumble (47.5% Vitaki N59 and 47.5% Vitaki N83; AVEVE N.V., Leuven, Belgium), 4% corn oil and 1% Celite® (VWR International, Leuven, Belgium) (source of acid insoluble ash, AIA). The second diet was additionally supplemented with 167 mg *N*,*N*-dimethylglycine sodium salt (Na-DMG) (Taminizer® D; Taminco, Belgium)/kg feed. This dosage is equivalent to 138 mg DMG/kg feed. Celite was added to the feed as an external marker to allow determination of AFD, whereas corn oil was added to increase energy density, as a second challenge to incite broiler ascites syndrome. Nutrient composition of the feed was as follows: 18.05% crude protein (CP), 9.13% ether extract (EE), 50.01% N-free extract (NfE), 4.32% crude fiber (CF) and 6.13% crude ash on an as fed basis and dry matter (DM) content was 87.56%. The ingredient composition of Vitaki N59 was in decreasing order: corn, wheat bran, soybean meal (toasted), sunflower seed meal, calcium carbonate, molasses, corn gluten feed, monocalcium phosphate, palm oil and sodium chloride; whereas the ingredient list of Vitaki N83 was wheat, soybeans, peas, sunflower seed meal, rapeseed meal, soybean meal (toasted), calcium carbonate, monocalcium phosphate and sodium chloride.

Apparent faecal digestibility and N-excretion

Excreta samples were collected at the pen level after an adaptation period of 15 days. Proximate analysis was performed on homogenized feed and lyophilized excreta samples according to standard methods of the Association of Official Analytical Chemists (AOAC, 1984) and AIA content was determined using the procedure of Van Keulen and Young (1977), as adapted by Atkinson *et al.* (1984). As birds void faeces and urine together, uric acid (UA) content in excreta samples was determined in order to calculate protein content in faeces as described by Kalmar *et al.* (2007) (equation [1]). This was carried out spectrophotometrically according to the method of Terpstra and De Hart (1974). The external marker method with AIA as external marker was used to calculate AFD (AFD_X, with X = DM, EE, NfE or CP) as performed by Sales and Janssens (2003a and 2003b) (equation [2]).

$$CP = (\text{total nitrogen} - \text{UA nitrogen}) \times 6.25 \quad [1]$$

$$\text{AFD\_X (\%)} = 100 - 100 \times \frac{X_{excreta} \times AIA_{feed}}{AIA_{excreta} \times X_{feed}} \quad [2]$$

Contents in above equations are expressed in percentage. Nitrogen to AIA ratio was used as a measure of nitrogen excretion.

Performance

Broilers were individually weighed at age 15, 30 and 40 days and feed intake was measured per pen between age 15 and 30 days (phase 1) and between age 30 and 40 days (phase 2). Average daily gain in phase 1 and phase 2 was calculated at pen level. Feed conversion ratio was calculated by ratio of feed consumption to weight gain (g:g).

Carcass yield

At the age of 40 days, all broilers were euthanized through injection of 0.5 ml T61® (Intervet, Mechelen, Belgium) in the brachial vein. Confirmation of death was performed by exsanguination through decapitation. Carcass weight was determined by dissection of the head and feet at hock joint, evisceration and deduction of average feather weight. Next, abdominal fat pad and the weights of the breast and leg meat (buttock and thigh muscle) were determined and expressed as percentage of live weight.

Fulminant ascites and pulmonary hypertension

Death broilers were removed daily, necropsied and visually inspected for macroscopic lesions related to pulmonary hypertension syndrome: massive accumulation of fluid in the peritoneal spaces, right heart dilatation and hydropericard (Scheele *et al.*, 2003). **Figure 1** illustrates the image of right heart dilatation in fulminant ascites. Further, progression towards pulmonary hypertension induced heart failure was quantified on all euthanized birds. Hereto, the ascites heart index (AHI) was calculated by ratio of the weights of the right heart ventricle to both heart ventricles. This was performed both on fresh matter basis (AHI_{FM}) as well as on freeze-dried basis (AHI_{DM}). A value of AHI_{FM} above 0.27 was considered an objective and accurate measure of right ventricular hypertrophy and thus pulmonary hypertension, which is regarded as the onset of ascites, as described by Huchzermeyer and De Ruyck (1986) and Paecock *et al.* (1988).

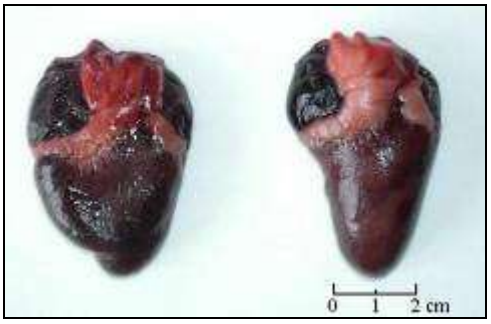

Figure 1. Morphology of right heart dilatation (left) and a normal chicken heart (right)

Blood parameters

Three consecutive fasted blood samples (2 ml) were obtained from each bird. At the ages of 15 and 30 days, blood was sampled from the right jugular vein, whereas at the age of 40 days from the brachial vein. All samples were obtained using heparinised syringes, collected in heparinised tubes and immediately submerged in iced water (4°C). Haematocrit was determined by centrifugation of subsamples, collected into heparinised microcapillary tubes, at 16,099 g for 5 min (Sigma® 1-15; Sigma Laboratory Centrifuges, Osterode, Germany). The remaining part was centrifuged at 2,205 g for 5 min at 4°C (Jouan® B4i; Thermo Scientific, Paisley, UK) and the plasma stored at -20°C until further analysis. The thyroid hormones triiodothyroxine (T_3) and thyroxine (T_4) were determined by radioimmunoassay as described by Darras *et al*. (1992). Commercial colorimetric diagnostic kits were used to measure glucose (Glu) (IL Test® kit No. 182508-00; Instrumentation Laboratories, Zaventem, Belgium), lactate (Sigma kit®, No. 826-UV, Sigma Diagnostics, Steinheim, Germany), non-esterified fatty acids (NEFA) (Wako® NEFA C test kit, Wako Chemicals GmbH, Neuss, Germany) and triglycerides (TG) (IL Test® kit, No. 181610-60; Instrumentation Laboratories) using the Monarch® 2000 Chemiesystem Model 760 (Monarch Chemistry System, Instrumentation Laboratories). Lipid peroxidation was measured using a thiobarbituric acid reactive species (TBARS) assay, as described by Yagi (1984).

Statistics

All data were pooled per pen and statistically analysed with pen as experimental unit. Normality and homogeneity were tested with the Kolmogorov-Smirnov and modified Levine test, respectively. Apparent faecal digestibility, technical performance (except for mortality), carcass traits and indices of pulmonary hypertension were analysed using one-way ANOVA, whereas plasma metabolites were analysed using the general linear model repeated measures with age as within-subject variable and diet as between-subject variable. Mortality, fulminant ascites and incidence of right ventricular hypertrophy were not normally distributed, hence these data were analysed with the two-way Wilcoxon test (non-parametric). Regression analysis between AHI_{FM} and AHI_{DM} was carried out using unpooled data. All statistics were performed in S-PLUS 8.0 (TIBCO Software, Palo Alto, CA, USA) and SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and significance was set at $p < 0.05$.

RESULTS

Apparent faecal digestibility and N-excretion

Apparent faecal digestibility of DM, NfE and CP were significantly higher in the DMG group in relation to the control group, whereas AFD of EE was not influenced by the diet (**Table 1**). Nitrogen to AIA ratio was lowered by 14% when fed the DMG supplemented diet in relation to the control diet ($p = 0.043$).

Table 1. Effect of *N*,*N*-dimethylglycine (DMG) on apparent faecal digestibility (AFD, %) in broiler hens ($N = 8$ pens of 4 birds per treatment).

	Control	DMG	sem	*p*-value
AFD_DM	61.8	69.1	1.4	*0.003*
AFD_EE	92.7	92.9	0.7	0.908
AFD_NfE	62.5	70.6	1.7	*0.013*
AFD_CP	81.4	86.5	1.0	*0.009*

DM: dry matter, EE: ether extract, NfE: N-free extract, CP: crude protein

Performance and carcass yield

Overall performance traits were not significantly influenced by diet; still, DMG-supplemented groups showed an improved FCR in the second growth phase with a statistical trend ($p < 0.1$). Further, starting bodyweight was not significantly different between test groups, but was noticeably lower in relation to Ross 308 objectives. Growth rate, in contrast, was much higher and finishing bodyweight only slightly below the top 25% of Ross 308 broilers worldwide.

Then again, FCR was higher in both test groups in relation to Ross 308 objectives (Aviagen, 2007). Next, although the DMG supplemented chicks weighed numerically less at the start of the trial, their finishing bodyweight was almost identical compared to this of the control chicks. Mortality was numerically highest in the control groups. Carcass yield was near Ross 308 objectives and both treatment groups showed similar yield (**Table 2**).

Table 2. Effect of *N*,*N*-dimethylglycine (DMG) on technical performance and carcass yield in broiler hens (*N* = 8 pens of 4 birds per treatment).

			Ross 308*	Control	DMG	sem	*p*-value
TECHNICAL	Bodyweight	(g)					
	▪ day 15		487	422	399	9	0.224
	▪ day 30		1471	1451	1384	26	0.118
	▪ day 40		2274	2251	2249	28	0.843
	Feed intake	(g/d)					
	▪ day 15-30		112	162	157	5	0.568
	▪ day 30-40		176	186	188	4	0.798
	▪ day 15-40		138	172	170	4	0.755
	Daily Growth	(g/d)					
	▪ day 15-30		66	73	70	2	0.274
	▪ day 30-40		80	80	87	2	0.177
	▪ day 15-40		71	76	77	1	0.691
	Feed Conversion						
	▪ day 15-30		1.71	2.23	2.23	0.08	0.981
	▪ day 30-40		2.19	2.33	2.17	0.04	0.058
	▪ day 15-40		1.93	2.26	2.19	0.04	0.445
	Mortality	(%)	NA	9.4	6.3	3.0	0.445
CARCASS	Carcass	($\%_{LW}$)	71.08	71.20	71.31	0.49	0.912
	Breast	($\%_{LW}$)	19.13	17.85	18.07	0.14	0.802
	Leg meat	($\%_{LW}$)	15.15	14.87	14.74	0.43	0.672
	Abdominal fat	($\%_{LW}$)	NA	1.39	1.28	0.08	0.548

*performance objectives for Ross 308 and objectives for carcass yield for 2200 g birds (top 25% worldwide; Aviagen, 2007)

$\%_{LW}$: percentage of live weight, NA: not available

Fulminant ascites and pulmonary hypertension

Cause of death of all birds that died before the end of the trial was fulminant ascites, as was indicated by marked macroscopic lesions. Further, all surviving birds were apparently healthy at the end of the trial. The AHI_{FM} ranged from 0.149 to 0.416, whereas AHI_{DM} ranged from 0.166 to 0.531. The AHI_{FM} of euthanized birds was numerically lower in the DMG supplemented group in relation to the control group. The observed improvement in AHI_{FM} became statistically significant when ventricle weights were expressed on dry matter basis.

Moreover, the onset of ascites was present in 44.8% of surviving animals of the control group, whereas in only 14.6% in surviving animals in the treated group ($p < 0.05$). Nevertheless, Hct was similar between treatments at all tested ages, but significantly increased over time (**Tables 3 and 4**).

Table 3. Effect of *N*,*N*-dimethylglycine (DMG) on indices of broiler ascites syndrome and pulmonary hypertension ($N = 8$ pens of 4 birds per treatment).

		Control	DMG	sem	*p*-value	Power (%)
FA	(%)	9.4	6.3	3.0	0.445	
PH	(%)	44.8	14.6	6.6	*0.036*	
Hct_{d40}	(%)	30	31	0.4	0.285	17
AHI						
▪ FM		0.265	0.229	0.012	0.155	34
▪ DM		0.304	0.242	0.013	*0.009*	85

FA: fulminant ascites, PH: pulmonary hypertension, Hct: haematocrit
AHI: ascites heart index, FM: fresh matter, FD: dry matter

Finally, statistical power of AHI_{FM} and Hct was fairly low, but freeze-drying improved the power from 34% for AHI_{FM} to 85% for AHI_{DM} (**Table 4**). The cut-off value of 0.27 as measured by AHI_{FM} for pulmonary hypertension or onset of ascites equals the value of 0.30 as measured by AHI_{DM} (**Figure 2**).

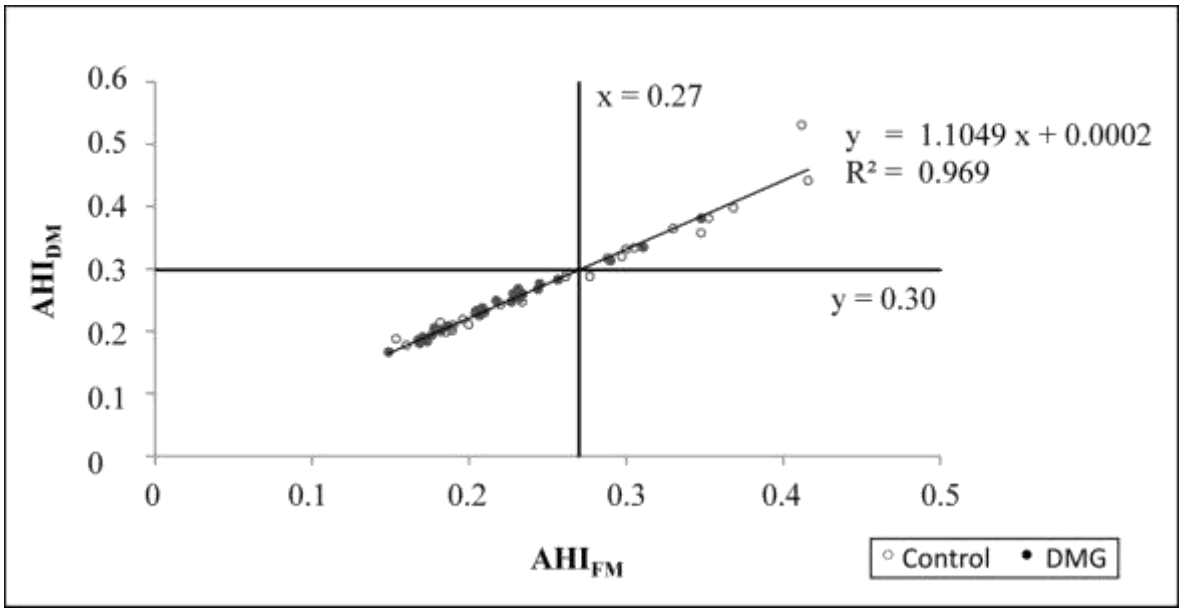

Figure 2. Linear regression between ascites heart index calculated on fresh matter basis (AHI_{FM}) and on dry matter basis (AHI_{DM}). Empty and filled circles indicate data points of broilers fed a control diet and a DMG-supplemented diet, respectively. Data points located above the horizontal line or to the right of the vertical line indicate pulmonary hypertension.
(DMG: *N*,*N*-dimethylglycine)

Blood parameters

Non-esterified fatty acids significantly increased with age but was at slaughter age significantly lower in the DMG groups in relation to the control groups. No other dietary effects where present, but significant age effects were seen in all blood parameters except for TBARS (**Table 4**).

Table 4. Effect of age (A) and dietary supplementation (D) of *N*,*N*-dimethylglycine (DMG) on selected plasma metabolites and PCV in broiler hens (*N* = 8 pens of 4 birds per treatment).

		Control	DMG	Statistics			
				sem	D	A	D x A
T_3/T_4							
▪ day 15		0.44^{a}	0.29^{a}	0.06	0.756	*< 0.001*	0.399
▪ day 30		0.98^{b}	1.09^{b}				
▪ day 40		0.40^{a}	0.36^{a}				
TBARS	(nmol/ml)						
▪ day 15		4.92	4.06	0.24	0.614	0.288	0.158
▪ day 30		3.22	4.76				
▪ day 40		3.56	3.43				
Hct	(%)						
▪ day 15		27^{a}	28^{a}	< 1	0.092	*< 0.001*	0.995
▪ day 30		31^{ab}	32^{ab}				
▪ day 40		30^{b}	31^{b}				
Glucose	(mg/dl)						
▪ day 15		266	260^{ab}	3	0.763	*0.033*	0.215
▪ day 30		268	282^{b}				
▪ day 40		260	256^{a}				
Lactate	(mg/dl)						
▪ day 15		24.3^{a}	21.7^{a}	0.6	0.593	*< 0.001*	*0.028*
▪ day 30		17.5^{b}	17.8^{b}				
▪ day 40		16.6^{ab}	20.4^{bc}				
TG	(mg/dl)						
▪ day 15		90	73^{ab}	4	0.898	*< 0.001*	0.130
▪ day 30		95	101^{b}				
▪ day 40		38	51^{a}				
NEFA	(mmol/l)						
▪ day 15		0.25^{a}	0.29^{a}	0.07	*0.002*	*< 0.001*	*< 0.001*
▪ day 30		0.48^{b}	0.49^{b}				
▪ day 40		$1.49^{c,x}$	$0.60^{ab,y}$				

T_3: triiodothyronine, T_4: thyroxine, TBARS: thiobarbituric acid reactive species, Hct: haematocrit, TG: triglycerides, NEFA: non-esterified fatty acids

^{a,b,c} different superscripts within columns indicate significant age (A) effects ($p < 0.005$)

^{x,y} different superscripts within rows indicate significant diet (D) effects ($p < 0.005$)

DISCUSSION

The main objectives of the current study were to evaluate the effect of dietary supplementation with DMG on nutrient digestibility and pulmonary hypertension syndrome in broilers. Apparent faecal digestibility of non-fat fractions was significantly improved by dietary supplementation with DMG. This beneficial, intestinal effect of DMG is presumably the result of an emulsifying action of DMG. The latter can be visualized by adding DMG to a water and oil mixture, which results in emulsification of the oil phase. In addition, the observed effects on digestibility are in agreement with data of Dierick and Decuypere (2004), in which addition of an emulsifier to the diet of growing pigs showed a significant improvement on AFD of non-fat fractions, while AFD of fat was not affected. Seen the great governmental compulsion to diminish nitrogen pollution of surface water caused by livestock production, which led to high costs for disposal of broiler manure, a reduction in faecal and urinary nitrogen excretion offers, besides a decrease in feed cost (Nahm, 2007), an additional environmental benefit. This is demonstrated by the observed significant decrease of 14% in nitrogen to AIA ratio when supplementing DMG in relation to the control. Nitrogen to AIA ratio was preferred above apparent protein digestibility as a measure of nitrogen excretion, because it includes the UA fraction.

The relatively high overall FCR in relation to Ross 308 performance objectives is concordant with previous investigations in which poor FCR was observed when broilers are reared in conditions of low environmental temperature (Aksit *et al.*, 2008; Leenstra and Cahaner, 1991; Pan *et al.*, 2005). This can be explained by the energetic cost for additional heat production to maintain body temperature, resulting in a lower proportion of energy available for growth (Malan *et al.*, 2003). Current data clearly demonstrate that the negative effect of cold stress on growth efficiency, as indicated by higher FCR, is most pronounced in young chickens. The latter is likely because of lower basal heat production in these young chicks that consequently need more additional heat production in relation to older and thus heavier chickens. Moreover, pen density increases with age, through which the birds provide more exogenous heat to each other, which further lowers the need for additional endogenous heat production. Our observations on basal metabolic rate further substantiate this hypothesis, as T_3:T_4 ratio more than doubled during the first growth phase, but returned to initial levels at the end of the second growth phase. In addition, cold stress resulted predominantly in the first growth phase in relative high feed intakes.

Notwithstanding inefficient feed utilisation in both treatment groups over the whole trial period, FCR of the DMG supplemented group showed to be slightly below Ross 308 performance objectives during the second growth phase, whereas FCR of the control group was as well in the second growth phase far above Ross 308 objectives. This difference showed, however, only a statistical trend.

Occurrence of fulminant ascites was numerically higher in the control group. In contrast, dietary supplementation with DMG significantly decreased both mean AHI_{DM} as well as the incidence of pulmonary hypertension in apparently healthy broilers. Hence, an important protective effect of DMG on progression towards pulmonary hypertension syndrome is demonstrated. Insufficiency or dysfunction of either lung or heart results in an imbalance between oxygen need and oxygen supply, which eventually leads to pulmonary hypertension syndrome. As previously mentioned, the cardiopulmonary capacity in modern broiler lines is often insufficient to cope with additional work load. Additionally, lipid peroxidation of subcellular membranes of heart and lung tissue, which consequently results in cardiopulmonary failure, is considered the molecular basis of progression towards pulmonary hypertension syndrome. Herein, either primary hypoxia or an imbalance between oxidants and anti-oxidant capacity leads to lipid peroxidation.

The plasma level of TBARS was not altered by dietary treatment, so current data do not substantiate a systemic anti-oxidant effect of DMG. Yet, it is of interest to mention that free radicals are generated in tissues as a result of the metabolism, having a very short lifespan and hereby causing local oxidative damage in form of lipid peroxidation (Nain *et al.*, 2008). Lipid peroxidation was not directly assessed in heart and lung tissue in the current trial. However, NEFA level in plasma was significantly higher in broilers fed the control diet in relation to the DMG-supplemented diet. Non-esterified fatty acids, in turn, have been demonstrated to induce important vascular effects and dysfunctions, which lead to an increase in arterial pressure (Avogaro *et al.*, 2003; Sarafadis and Bakris, 2007). Moreover, endothelial dysfunction of the extra-pulmonary conduit arteries results in hypoxia, which also triggers progression towards pulmonary hypertension (Zoer *et al.*, 2009). Hence, the protective mechanism of dietary DMG on development of pulmonary hypertension can, at least partly, be attributed to its effect on fat metabolism. Further, Sarafadis and Bakris (2007) attribute an increase in oxidative stress, next to increased arterial stiffness and vasoconstriction, and decreased endothelium-dependent vasodilatation, being associated with an increase in plasma

NEFA level. In addition, in vivo data of Sainsbury *et al.* (2004) presume oxidative stress to be at the basis of the detrimental effect of increased plasma NEFA level on endothelial dysfunction. Concordantly, the observed effect of DMG on NEFA suggests an indirect anti-oxidant effect of DMG.

Sarafadis and Bakris (2007) describe an elevated release of NEFA with increased abdominal fat. However, relative abdominal fat pad in the current trial was only modestly and numerically higher when provided the control diet in relation to the DMG-supplemented diet. Hence, the observed over twofold NEFA level when fed the control diet is likely only partly attributable to higher abdominal fat mass. Therefore, DMG might have affected fat metabolism by assisting or promoting metabolisation of fatty acids, as a decrease in fat mobilisation rate is unlikely because the relative abdominal fat mass was lower in DMG supplemented broilers, while apparent fat digestibility was comparable.

Finally, AHI_{FM} and AHI_{DM} were highly correlated. Moreover, freeze drying heart ventricles before calculation of AHI substantially improved statistical power to evaluate mean AHI between treatment groups, which implies a methodological improvement. The former is likely because of the increase in range of AHI through freeze-drying. Regression analysis determined a value of 0.30 for AHI_{DM} to be equivalent to the cut-off of value of 0.27 for AHI_{FM} in the assessment of onset of pulmonary hypertension.

In conclusion, the present trial demonstrated a considerable beneficial effect of dietary supplementation with 167 mg Na-DMG/kg feed on AFD of non-fat nutrients. Further, this study proved a potent protective effect of DMG against progression towards pulmonary hypertension or broiler ascites syndrome in broilers challenged with cold stress and a high energy diet. Further research with large scale trials is necessary to assess the magnitude of beneficial effects of dietary DMG on overall performance of broilers raised in common management conditions.

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CHAPTER 3

Dietary supplementation with *N*,*N*-dimethylglycine affects broiler performance and plasma metabolites depending on dose and dietary fatty acid profile

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ABSTRACT

The effect of dietary supplementation with N,N-dimethylglycine sodium salt (Na-DMG) was evaluated in a feeding trial with 1500 1-day-old broiler chicks (Cobb 500). DMG was supplemented at 0, 100, 200, 500 or 1,000 mg Na-DMG/kg feed to a ration with either animal fat (chicken fat) or vegetal fat (soy oil) as the main fat source. In the vegetal fat diets, production value was linearly improved by supplementation with DMG by up to +11% relative to the control (%$_{rel\ \Delta}$). Irrespective of dietary fat source, abdominal fat percentage was linearly reduced by up to -24%$_{rel\ \Delta}$ and meat yield tended to linearly increase with DMG dose by up to +4%$_{rel\ \Delta}$. Moreover, in the vegetal fat groups, DMG significantly lowered abdominal fat pad by up to -38%$_{rel\ \Delta}$ and tended to increase meat yield up to +6%$_{rel\ \Delta}$ at the highest dose. Fasted non-esterified fatty acid level significantly decreased with increasing DMG dose by up to -36%$_{rel\ \Delta}$ and thiobarbituric acid reactive species (TBARS) tended to decrease by up to -46%$_{rel\ \Delta}$ at the highest dose. In vegetal fat diets, addition of DMG resulted in significant lower TBARS level by -56%$_{rel\ \Delta}$ at the highest dose. Finally, a quadratic effect on ascites heart index was present in the vegetal fat diets, with a minimal value at 500 mg Na-DMG/kg. In conclusion, dietary supplementation with DMG may improve technical and slaughter performance, and may reduce oxidative stress and pulmonary hypertension, but the degree of effects is modulated by fatty acid profile of the diet. Herein, effects are more pronounced in a diet rich in polyunsaturated fatty acids compared to a diet rich in saturated and monounsaturated fatty acids.

INTRODUCTION

Dimethylglycine (DMG) is a tertiary amino acid that is an intermediary metabolite in the choline pathway, as it is formed in liver mitochondria by removal of one methyl group from betaine (Friesen *et al.*, 2007). It can be metabolized in liver mitochondria, rendering both its methyl groups through transmethylation of tetrahydrofolate, and free glycine (Mackenzie and Frisell, 1958; Slow *et al.*, 2004). Being a small, water-soluble molecule that is lipophilic enough to cross cellular membranes, it is likely to be absorbed rapidly and completely when orally administered (Cupp and Tracy, 2003). Hariganesh and Prathiba (2000) suggest free-radical scavenging potential of DMG, as demonstrated by a significant reduction in plasma and tissue level of thiobarbituric acid reactive species (TBARS) and by a significant decline in stress-induced gastric ulcers in rats by oral administration of DMG at a dose of both 25 and 35 mg/kg. Amongst other biological functions, DMG is reputed to enhance oxygen use in muscle tissue and is sold as a nutritional supplement to enhance athletic performance in human athletes and racing horses; however, the concerned scientific data are inconsistent, as reviewed by Cupp and Tracy (2003).

Previous data in broilers revealed a significant improvement in apparent digestibility of carbohydrate and protein when a control diet was supplemented with 167 mg Na-DMG/kg (Kalmar *et al.*, 2010). Also in sows, an increase in digestibility was demonstrated for most proximate components with dietary supplementation of 500 mg Na-DMG/kg (Cools *et al.*, 2010). The effect on digestibility can be explained by the emulsifying effect of DMG, which can be easily demonstrated by adding DMG to a water and oil mixture, resulting in a stable emulsion. The improvement of digestibility coefficients by addition of an emulsifier to the diet occurs because non-fat nutrients become less insulated by fat droplets, hence the availability to digestive enzymes and the absorptive brush border of the small intestine increases. Furthermore, Kalmar *et al.* (2010) evidenced the protective effect of dietary DMG against pulmonary hypertension or broiler ascites syndrome. This study demonstrated a considerably reduction in plasma level of non-esterified fatty acids (NEFA). In short, an increase in plasma NEFA level inflicts vascular dysfunction initiated by oxidative stress, which leads to hypoxia and hence development towards pulmonary hypertension (Avogaro *et al.*, 2003; Sainsbury *et al.*, 2004; Sarafidis and Bakris, 2007; Zoer *et al.*, 2009). Thus by abating a rise in NEFA, DMG lowers oxidative stress at the level of the pulmonary arteries, through which progression towards pulmonary hypertension is decelerated.

The objectives of the current study were to assess dose-response effects of dietary DMG on technical and slaughter performance in broilers, on their fat metabolism, oxidative stress-related plasma metabolites and on development of broiler ascites syndrome. Seen a presumably higher oxidative stress level with more unsaturated fat sources, a further objective was to evaluate whether effects of DMG are modulated by dietary fatty acid profile.

MATERIALS AND METHODS

Animals and housing

Fifteen hundred 1-day-old broiler chicks (Cobb 500) were randomly allocated in 50 pens (2.2 m² per pen) of 30 birds each (15 males and 15 females). The floor pens had a layer of peat, topped with wood shavings as bedding. Lighting schedule was 23 h light:1 h dark during the first 3 days, followed by 20 h light:4 h dark until slaughter age. All animals were vaccinated against bronchitis (Hitchner spray, Poulvac IB H120, Fort Dodge Animal Health Holland, Weesp, The Netherlands) at age 1 day, and against Newcastle disease at ages 1 and 16 days (La Sota clone 30, Nobilis ND Clone 30, Intervet Belgium NV, Mechelen, Belgium).

Diets

A total of 10 dietary treatments were tested in a complete block design with five replicates per treatment. Herein, five different doses of Na-DMG (Taminizer® D, Taminco, Belgium) were tested in broiler diets with one of two fat sources. Tested dosages of Na-DMG were 0 (control), 100, 200, 500 and 1,000 mg/kg feed. The added fat source was either mainly animal fat (chicken fat) or mainly vegetal fat (soybean oil). Depending on the fat source, the diets are further labelled as animal fat diets and vegetal fat diets, respectively.

A three-phase feeding schedule was applied, beginning with a starter diet from day 1 until day 14, followed by a grower diet from day 15 until day 28 and a finisher diet from day 29 until day 42. Both feed and drinking water were provided *ad libitum*. Proximate analysis was performed on homogenized feed samples according to standard methods of the Association of Official Analytical Chemists (AOAC, 1984), whereas fatty acid profile was determined as described by Raes *et al.* (2001). The animal fat diets were higher in saturated (SFA) and monounsaturated fatty acids (MUFA), whereas vegetal fat diets were higher in polyunsaturated fatty acids (PUFA). All test diets complied to dietary requirements for linoleic acid, which is the only essential fatty acid for which a dietary requirement has been demonstrated in poultry (NRC, 1994). The ratio of n-3 PUFA to n-6 PUFA was similar in all diets.

Ingredient and nutrient composition of the test diets are presented in **Tables 1** and **2** and the profile of the main fatty acids is provided in **Table 3**.

Table 1. Ingredient composition (%) of experimental diets.

Feedstuff	Starter	Grower	Finisher
Wheat	40.73	54.20	60.56
Soybean meal-48	27.71	24.10	22.48
Yellow corn	15.00	5.00	0.00
Added fat (a or b)	7.50	7.31	8.42
a. mainly animal fat			
- chicken lard	*6.50*	*6.31*	*7.42*
- soy oil	*1.00*	*1.00*	*1.00*
b. mainly vegetal oil			
- chicken lard	*1.00*	*1.00*	*1.00*
- soy oil	*6.50*	*6.31*	*7.42*
Soybean meal-44	5.00	5.00	5.00
Dicalciumphosphate	1.37	1.17	0.82
Vitamin and trace mineral premix[1]	1.00	1.00	1.00
CaCO3	0.64	0.71	0.75
NaCl	0.29	0.29	0.30
L-lysine.HCl	0.25	0.26	0.25
DL-methionine	0.25	0.24	0.22
NaHCO3	0.12	0.06	0.04
R phytase	0.02	0.02	0.02
L threonine	0.10	0.11	0.11
NSP-degrading enzymes[2]	0.02	0.03	0.03

[1] contents in mg per kg diet:
Vitamins: A: 4.05, B_1: 2.2, B_2: 7.5, B_3: 38, B_5: 13, B_6: 5.5, B_8: 0.2, B_{12}: 0.035,B_p: 650, D_3: 0.05, E: 30 and K_1: 2.5.
Minerals: I: 2.1, Co: 1.1, Se: 0.43, Cu: 25, Mn: 60, Zn: 70, Fe: 45 and Mg: 110
[2] NSP: non-starch polysaccharides

Table 2. Proximate analysis (%) of test diets as fed.

	Starter		Grower		Finisher	
	Animal	Vegetal	Animal	Vegetal	Animal	Vegetal
Dry Matter	89.88	89.68	89.82	89.67	90.13	90.02
N-free Extract	50.64	51.44	51.38	50.72	51.31	50.93
Crude Protein	20.05	19.94	19.66	20.20	20.08	20.17
Ether Extract	9.23	8.86	9.42	9.24	9.29	9.50
Crude Ash	5.73	5.38	5.11	5.15	5.21	5.20
Crude Fibre	4.23	4.06	4.25	4.36	4.24	4.22

Table 3. Analyzed profile of main fatty acids (g/100 g fatty acids) of test diets.

		Starter		Grower		Finisher	
		Animal	Vegetal	Animal	Vegetal	Animal	Vegetal
SFA		**27**	**20**	**28**	**20**	**28**	**20**
Palmitic acid	C16:0	19	14	19	14	19	14
Stearic acid	C18:0	7	5	7	5	7	5
UFA		**70**	**77**	**69**	**77**	**69**	**77**
Oleic acid	C18:n1-9	34	24	35	25	35	25
Linoleic acid	C18:2n-6	26	45	24	44	25	44
α-linolenic acid	C18:3n-3	3	5	2	5	2	5
Undetermined		**3**	**3**	**3**	**3**	**3**	**3**
n3:n6		0.11	0.12	0.11	0.12	0.11	0.12

SFA and UFA: saturated and unsaturated fatty acids

Measurements

Technical performance

Bodyweight (BW) and feed intake were recorded weekly at pen level. Dead and culled broilers were removed daily and recorded per pen. Average daily feed intake (ADFI), average daily growth (ADG), feed conversion ratio (FCR, equation [1]) and production value (PV, equation [2]) were calculated.

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Feed Intake (g)}}{\text{Growth (g)}} \quad [1]$$

$$\text{Production Value (PV)} = \frac{\text{Survival Rate (\%) x Finishing Weight (g)}}{\text{Rearing Period (d) x FCR x 10}} \quad [2]$$

Slaughter performance

At the end of the trial, a total of 50 birds (one male per pen) were individually killed after an overnight fast, through injection of 250 mg Na-pentobarbital/kg BW in the brachial vein. Confirmation of death was done by exsanguination through decapitation. Carcass weight was determined by removal of feet at the hock joint, evisceration and by deduction of average feather weight, the heads already being removed. Next, abdominal fat pad and breast, drumstick and thigh meat were determined and expressed as percentage of live weight ($\%_{LW}$) and meat yield to abdominal fat pad ratio calculated as an extended measure of fatty tissue deposition. Finally, samples of breast meat were analysed for intramuscular fat content (fat_{IM} %) by means of the Soxhlet method (AOAC, 1984).

Blood parameters

Fasted blood (5 ml) was sampled by exsanguination and collected in heparinised tubes, which were immediately submerged in iced water (4°C). Haematocrit (Hct), an indicator of broiler ascites syndrome, was determined by centrifugation at 16,099 g for 5 min (Sigma® 1-15, Sigma Laboratory Centrifuges, Osterode, Germany) of subsamples collected into heparinised microcapillary tubes. The remaining part was centrifuged at 2205 g for 5 min at 4°C (Jouan® B4i, Thermo Scientific, Paisley, UK) and the plasma stored at -20°C until further analysis. Commercial colorimetric diagnostic kits were used to measure glucose (Glu) (IL Test® kit No. 182508-00, Instrumentation Laboratories, Zaventem, Belgium) and lactate (Sigma kit, No. 826-UV, Sigma Diagnostics, Steinheim, Germany) as indicators for aerobic and anaerobic glucose metabolism, respectively, and NEFA (Wako Chemicals GmbH test kit, Neuss, Germany) and triglycerides (TG) (IL Test® kit, No. 181610-60, Instrumentation Laboratories, Zaventem, Belgium), as indicators of the lipid metabolism, using the Monarch® 2000 Chemiesystem Model 760 (Monarch Chemistry System, Instrumentation Laboratories, B-1930, Zaventem, Belgium). A TBARS assay, as described by Yagi (1984), was used to determine plasma malondialdehyde level, which is a reliable indicator of lipid peroxidation and free radical activity (Holley and Cheeseman, 1993).

Pulmonary hypertension

Progression towards broiler ascites syndrome as indicated by right ventricular hypertrophy was quantified by means of the ascites heart index (AHI), which is calculated by ratio of the weight of the right heart ventricle to both heart ventricle weights. This was done on both fresh matter basis (AHI_{FM}) as well as after freeze-drying of ventricles (AHI_{DM}). Values of AHI_{FM} above 0.27 (Huchzermeyer and De Ruyck, 1986; Paecock *et al.*, 1988) or AHI_{DM} above 0.30 (Kalmar *et al.*, 2010) were considered an objective and accurate measure of pulmonary hypertension induced heart failure.

Statistics

Normality and homogeneity were tested with the Kolmogorov-Smirnov and modified Levine test, respectively. Technical performance, slaughter performance, ascites indices and plasma metabolites were analysed using one-way ANOVA with dietary fat source (dummy variable: animal fat = 0; vegetal fat = 1) and Na-DMG dose as independents. There were no significant interactions between dietary fat source and Na-DMG dose, except for partitioning of energy; hence the models were generated without interaction term, except for meat yield to abdominal

fat ratio. Mortality and incidence of pulmonary hypertension were not normally distributed and were analysed with the Kruskal-Wallis rank sum test and Friedman rank sum test, respectively (non-parametric). Linear and quadratic effects of Na-DMG on split-plots per dietary fat source are presented when significant ($p < 0.05$) or when showing a statistical tendency ($0.100 < p < 0.050$). Correlations between technical performance, slaughter performance, plasma metabolites, dietary fat source and Na-DMG dose were calculated using Pearson and Spearman correlation tests for normally distributed and non-normally distributed variables (mortality), respectively. Average values are expressed as means ± standard error of means (SEM) and effects of DMG are expressed as percentual difference relative to the control ($\%_{rel\,\Delta}$). All statistics were carried out in S-PLUS 8.0 (TIBCO Software Inc., Palo Alto, CA, USA) and SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Technical performance

Starting weight was 44.6 ± 0.1 g and was not significantly different between treatment groups. Supplementation of DMG resulted in a significant, linear improvement of PV in the vegetal fat groups, in which PV was increased by $11\%_{rel\,\Delta}$ in the 1,000 mg Na-DMG/kg group compared with the control (**Figures 1 and 4**). Yet, irrespectively to dietary fat source, or within the animal fat groups, finishing BW, ADFI, ADG, FCR and PV were not significantly affected by DMG. Technical performance was also not significantly influenced by dietary fat source (**Table 4**). Finally, mortality was neither affected by dietary DMG dose, nor by dietary fat source, and averaged 3.4 ± 0.9% and 3.7 ± 1.0% in animal fat and vegetal fat diets, respectively.

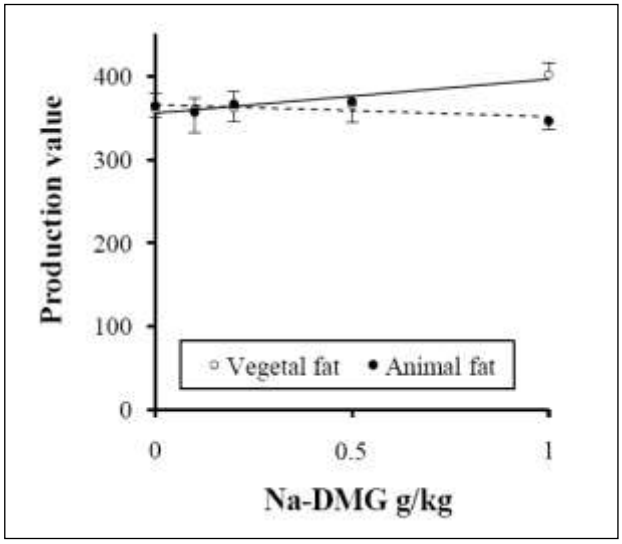

Figure 1. linear regression of production value in broilers fed vegetal or animal fat diets containing 0 to 1,000 mg *N*,*N*-dimethylglycine sodium salt (Na-DMG) per kg (mean ± SEM, line: $p < 0.05$, dashed line: $p > 0.05$)

Table 4. Technical performance of broilers depending on dose level of dietary supplementation with *N*,*N*-dimethylglycine sodium salt (Na-DMG) (0, 0.1, 0.2, 0.5 or 1 g/kg) and dietary fat source (animal fat: 0 or vegetal oil: 1).

		Intercept		Fat		DMG	p_{Fat}	p_{DMG}
Starting BW	(g)	45	+	0	-	0	*0.055*	0.723
Finishing BW	(g)	2555	+	67	+	61	0.411	0.586
ADFI	(g/d)	103	+	3	+	0	0.276	0.963
ADG	(g/d)	60	+	2	+	1	0.413	0.585
FCR		1.66	-	0.01	-	0.01	0.678	0.560
PV		356	+	10	+	14	0.363	0.374

BW: bodyweight, ADFI: average daily feed intake, ADG: average daily growth, FCR: feed conversion ratio, PV: production value

Slaughter performance

Abdominal fat pad tended to be 12%$_{rel\ \Delta}$ lower in the vegetal fat groups compared to the animal fat groups. Further, independently to dietary fat source, supplementation with DMG tended to increase overall meat yield by 4%$_{rel\ \Delta}$ and significantly lowered abdominal fat pad by -24%$_{rel\ \Delta}$ at 1,000 mg Na-DMG/kg (**Table 5**). Moreover, in vegetal fat diets, DMG supplementation showed a significant, linear decrease in abdominal fat and a tendency to a linear increase in meat yield, resulting in a -38%$_{rel\ \Delta}$ decrease in abdominal fat and a +6%$_{rel\ \Delta}$ increase in meat yield when 1,000 mg Na-DMG/kg was supplemented to the diet (**Figures 2 and 4**). The meat yield to abdominal fat ratio (%:%) showed a significant interaction between DMG dose and dietary fat source [3]. In the vegetal fat groups, meat yield to abdominal fat ratio (%:%) increased significantly with DMG dose [4].

Overall:
Meat:Fat = 17.85 - 2.42 Fat (0 = animal; 1 = vegetal) + 0.49 Na-DMG (g/kg) + 12.97 (Fat x Na-DMG) [3]
(p_{Fat} = 0.323; $p_{Na\text{-}DMG}$ = 0.886; $p_{Fat\ x\ Na\text{-}DMG}$ = 0.009)

Vegetal fat:
Meat:Fat = 15.43 + 13.45 Na-DMG (g/kg) [4]
(p < 0.001).

Table 5. Slaughter performance of broilers depending on dose level of dietary supplementation with *N*,*N*-dimethylglycine sodium salt (Na-DMG) (0, 0.1, 0.2, 0.5 or 1 g/kg) and dietary fat source (animal fat: 0 or vegetal oil: 1).

		Intercept		Fat		DMG	p_{Fat}	p_{DMG}
Carcass	(%)	75.91	-	0.27	+	0.07	0.558	0.916
Meat	(%)	35.97	-	0.71	+	1.48	0.256	0.090
Abd. fat	(%)	2.43	-	0.29	-	0.58	*0.065*	*0.010*
Fat_{IM}	(%)	2.73	+	0.41	+	0.01	0.107	0.978

Abd. fat: abdominal fat, Fat_{IM}: intra-muscular fat

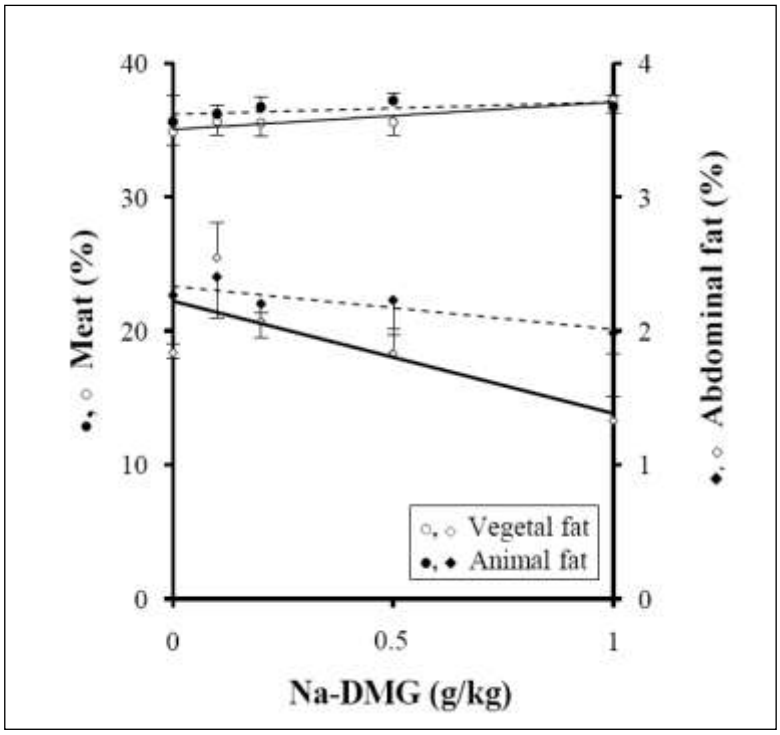

Figure 2. linear regression of slaughter performance in broilers fed vegetal or animal fat diets containing 0 to 1,000 mg *N*,*N*-dimethylglycine sodium salt (Na-DMG) per kg (mean ± SEM, fat line: $p < 0.05$, line: $p < 0.1$, dashed line: $p > 0.1$).

Blood parameters

Overall, TBARS were numerically higher (+22%) when fed the vegetal fat diets compared to the animal fat diet. Next, TBARS were significantly, linearly reduced by DMG in vegetal fat diets, with a reduction of -56%$_{rel\,\Delta}$ when supplemented at the highest DMG dose [6]. Fasted glycaemia significantly decreased with increasing DMG dose in animal fat diets [7], although glycaemia already tended to be lower in the animal fat groups compared to the vegetal fat groups. Independently to dietary fat source, dietary DMG significantly reduced plasma NEFA level by -36%$_{rel\,\Delta}$ at 1,000 mg Na-DMG/kg and tended to decrease plasma TBARS by -46%$_{rel\,\Delta}$ at 1,000 mg Na-DMG/kg (**Table 6**).

Vegetal fat: TBARS (nmol/ ml) = 1.50 - 0.84 Na-DMG (g/kg) ($p = 0.021$) [6]

Animal fat : glu (mg/dl) = 315.4 - 82.2 Na-DMG (g/kg) ($p = 0.019$) [7]

Table 6. Plasma metabolites in broilers depending on dose level of dietary supplementation with *N*,*N*-dimethylglycine sodium salt (Na-DMG) (0, 0.1, 0.2, 0.5 or 1 g/kg) and dietary fat source (animal fat: 0 or vegetal oil: 1).

		Intercept		Fat		DMG	p_{Fat}	p_{DMG}
TBARS	(nmol/ml)	1.13	+	0.25	-	0.52	0.197	*0.059*
NEFA	(mmol/l)	0.50	+	0.01	-	0.18	0.818	*0.048*
TG	(mg/dl)	62.22	+	2.72	+	1.51	0.790	0.915
Lactate	(mg/dl)	14.74	-	0.24	-	1.01	0.840	0.536
Hct	(mg/dl)	31.28	-	0.60	-	0.01	0.424	0.995
Glucose	(mg/dl)	297	+	33	-	32	*0.095*	0.251

TBARS: thiobarbituric acid reactive species, NEFA: non-esterified fatty acids, TG: triglycerides, Hct: haematocrit

Pulmonary hypertension

Dietary fat source did not significantly affect AHI_{FM} or AHI_{DM} and DMG did not affect AHI in the animal fat group. However, in the vegetal fat group, supplementation with DMG had a significant, quadratic effect on AHI_{DM} (tendency in case of AHI_{FM}), as seen in **Figure 3 - left**. This image is confirmed when looking at the distribution of birds suffering from pulmonary hypertension across the treatment groups within the vegetal fat diet (**Figure 3 - right**).

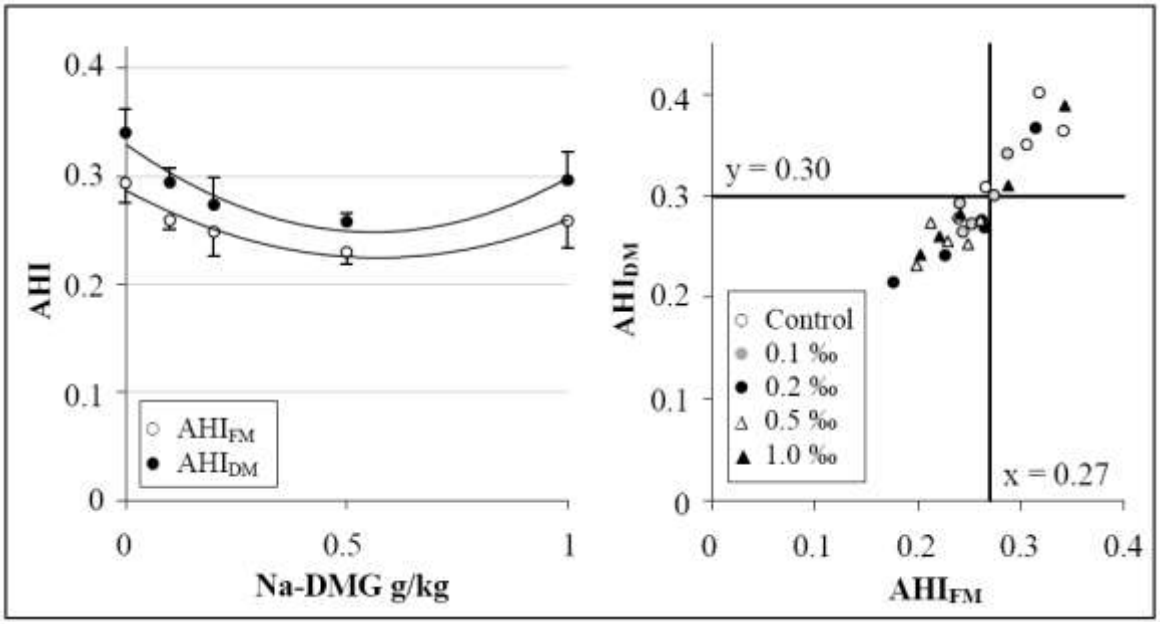

Figure 3. Quadratic regression between ascites heart index (AHI_{FM} and AHI_{DM}) in broilers fed vegetal fat diets containing 0 to 1 g *N*,*N*-dimethylglycine sodium salt (Na-DMG)/kg (AHI_{FM}: $p < 0.1$ and AHI_{DM}: $p < 0.05$) (left). Occurrence of pulmonary hypertension in broilers fed vegetal fat diets, depending on dietary DMG level (right). Data points situated above the horizontal line or to the right of the vertical line indicate pulmonary hypertension (AHI_{FM} and AHI_{DM}: AHI based on fresh matter and dry matter, respectively).

Interconnections between tested variables

Apart from some obvious correlations, finishing BW showed a positive correlation with meat yield and a negative tendency ($p < 0.1$) with fat_{IM} content. Meat yield was further positively correlated with Hct, and negatively correlated with TG, TBARS and abdominal fat pad. Abdominal fat pad was also negatively correlated with fat_{IM} content, but positively correlated with TBARS (**Figure 4**).

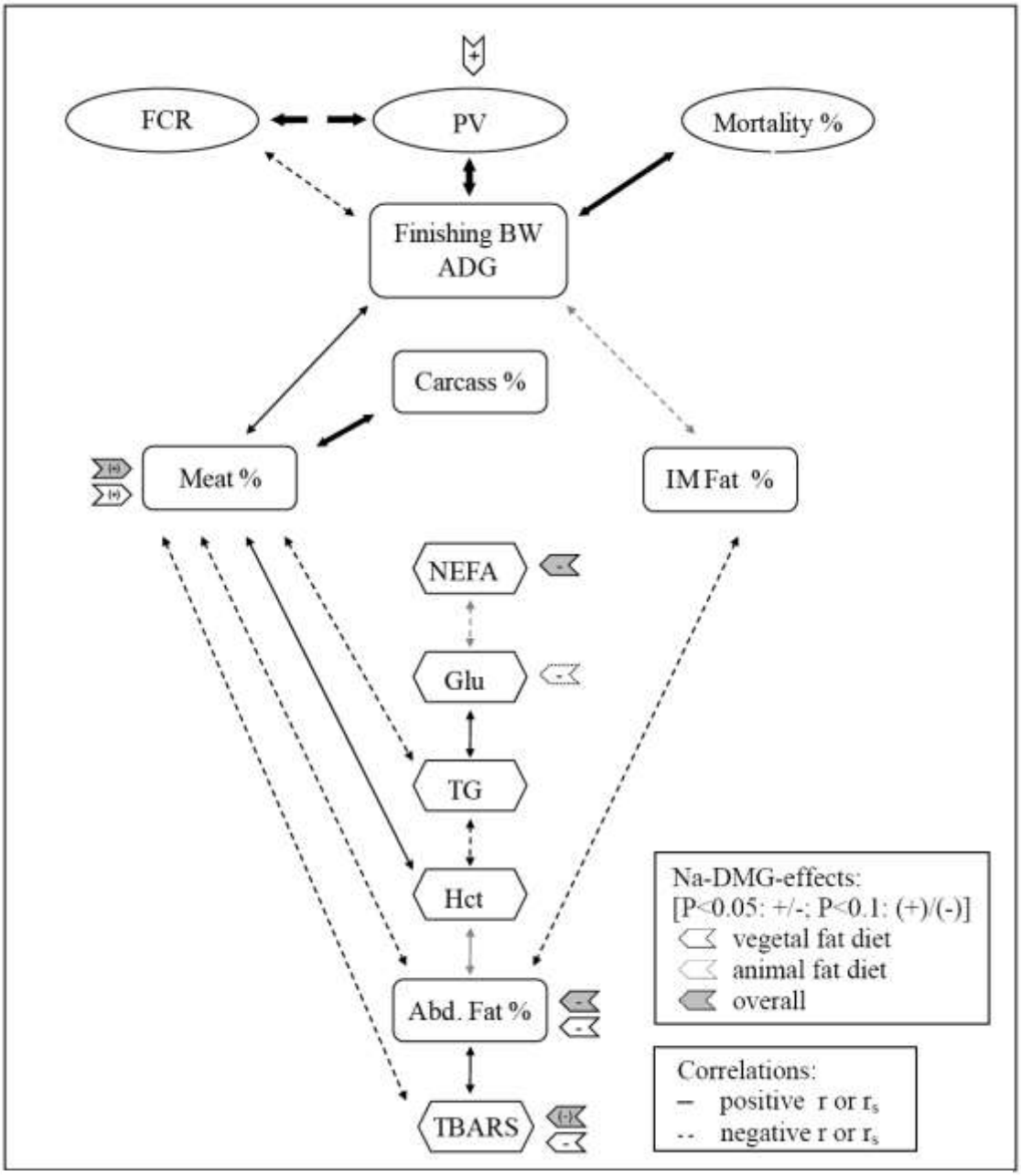

Figure 4. Correlations between technical performance, slaughter characteristics and plasma metabolites in broilers: **—** strong correlation ($p < 0.05$; $|r|$ or $|r_s| > 0.70$), **—** relatively strong correlation ($p < 0.05$; $|r|$ or $|r_s| > 0.50$), **—** moderate correlation ($p < 0.05$; $|r|$ or $|r_s| > 0.30$), **—** statistical trend or weak correlation ($0.1 < p < 0.05$ or $p < 0.05$ and $|r|$ or $|r_s| < 0.30$). Linear *N*,*N*-dimethylglycine sodium salt (Na-DMG) effects are indicated with arrows. (FCR: feed conversion ratio, PV: production value, IM and Abd. Fat: intra-muscular and abdominal fat, NEFA: non-esterified fatty acids, Glu: glucose, TG: triglycerides, Hct: haematocrit, TBARS: thiobarbituric acid reactive species, PUFA: poly unsaturated fatty acids).

DISCUSSION

In agreement with Newman *et al.* (2002), dietary fat source did not significantly affect finishing BW but abdominal fat pad tended to be 23% lower when fed the PUFA-rich diet compared to the SFA and MUFA rich diets. The current trial also demonstrated a numerical increase in meat to fat ratio by increasing PUFA level in the diet, which is also consistent with data of Newman *et al.* (2002). Furthermore, independently to dietary fat source, supplementation with DMG tended to linearly increase meat yield and significantly, linearly lowered both abdominal adipose tissue and fasted NEFA level. In contrast to mammals, lipogenesis in birds occurs mainly in the liver, rather than in adipose tissue. This makes fat deposition into adipose tissue dependent on availability of plasma lipoproteins originating from either diet or liver (Leveille, 1969; Yuan *et al.*, 2008). Because feed and thus fat intake were similar between diets, the obvious effect of DMG on depot fat storage is likely the result of enhanced hepatic fatty acid metabolism, through which fatty acids were rather used as an energy source instead of being stored in abdominal depot fat. This effect on hepatic fat metabolism was more pronounced in the PUFA-rich, vegetal fat diet compared to the SFA-and MUFA-rich, animal fat diet. Considering slaughter performance traits in the vegetal fat diets, DMG supplementation resulted in significantly higher meat: fat ratio. The significant dietary fat source x DMG interaction in meat:fat ratio further substantiates the influence of both dietary fatty acid profile and DMG on nutrient partitioning in broiler chickens. Thus, as fatty acid profile of test diets was not extremely different, comparison between diets with for instance pork lard as saturated fat source and fish oil as unsaturated fat source, might have elucidated even a greater modulating effect of dietary fatty acid profile on DMG effects.

Although only numerical, plasma TBARS levels were markedly higher in vegetal fat diets compared to animal fat diets. This is in accordance with human trials, in which next to favourable effects on cholesterol profile, it was demonstrated that high levels of dietary PUFA also resulted in an undesired increase in plasma indices of oxidative stress and lipid peroxidation (Brown and Wahle, 1990; Nair *et al.*, 1993; Jenkinson *et al.*, 1999). The protective effect of DMG against development of pulmonary hypertension or broiler ascites syndrome in the vegetal fat diets is likely the result of the decrease in both TBARS and NEFA. These findings are the first data reported on DMG supplementation under standard rearing conditions, but are in agreement with Kalmar *et al.* (2010), reporting similar effects of DMG during a challenge trial in which broiler hens were fed a vegetal fat diet with corn oil as main dietary fat source and raised under cold ambient temperature in order to increase feed intake and thus to incite pulmonary hypertension.

CONCLUSION

The current trial demonstrated a linear improvement in PV in the vegetal fat, PUFA-rich diet with increasing DMG supplementation. In diets of either fat source, DMG supplementation resulted in a significant decrease in deposition of dietary fat into abdominal depot tissue, and a tendency towards increased lean tissue. However, the extent of these effects depended on dietary fatty acid profile, in which effects were more pronounced when broilers were fed a vegetal fat, PUFA-rich diet compared to an animal fat, SFA-and MUFA-rich diet. Finally, these data demonstrated dose-dependent anti-oxidative properties of dietary DMG in broilers fed a PUFA-rich diet, which is reflected in the degree of pulmonary hypertension as measured by AHI_{FD}. Previously, Hariganesh and Prathiba (2000) also suggested anti-oxidative capacity of DMG when orally administered to rats. Further investigation is necessary to elucidate the exact working mechanism of DMG on both gastrointestinal and metabolic level.

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CHAPTER 4

Efficacy of N,N-dimethylglycine as a feed additive to improve broiler production.

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Animal

ABSTRACT

N,N-dimethylglycine (DMG), a naturally occurring glycine derivative, possesses valuable characteristics which makes it useful as a new feed additive in broiler diets. Firstly, it improves nutrient digestibility and secondly, it reduces the progression towards broiler ascites syndrome. The objective of the current trial was to evaluate the efficacy of dietary DMG to enhance performance in chickens for fattening. Three trials were conducted in which technical and slaughter performances were evaluated in broilers fed a control diet or the same diet supplemented with 1,000 mg Na-DMG/kg feed for the whole rearing period. Trial 1 (Germany), 2 (Austria) and 3 (Italy) consisted of 480 Cobb Germany Avimex GmbH chicks, 360 Ross 308 chicks and 352 Ross 508 chicks, respectively. Finishing bodyweight (BW_{fin}), feed conversion ratio (FCR) and production value (PV) were significantly improved in trial 2, trial 1, 2 and 3, and trial 1 and 2, respectively. Moreover, irrespective of trial site, these technical performance traits were significantly improved by +2.3% (BWf_{in}), -2.8% (FCR) and + 4.3% (PV), relative to the control ($\%_{rel\ \Delta}$). In all three trials, the yield of carcass for grilling and meat yield were similar between control and DMG groups, but breast meat yield and meat to fat ratio were significantly improved by dietary DMG in trial 1 and trial 3, respectively. Furthermore, meta-analysis showed a significant overall increase in breast meat yield by 2.2%$_{rel\ \Delta}$ and meat to fat ratio by +12.6%$_{rel\ \Delta}$. In conclusion, dietary supplementation with DMG at a dose of 1,000 mg Na-DMG/kg considerably improves technical performance in broilers, although degree and mode of improvement seems to be regulated by rearing conditions and broiler strain. In addition, slaughter traits are either similar or improved by DMG supplementation.

INTRODUCTION

N,*N*-dimethylglycine (DMG) is a naturally occurring tertiary amino acid in the intermediary metabolism of betaine in living organisms. It possesses valuable properties which makes it useful as a feed additive. Firstly, dietary supplementation with DMG does improve apparent faecal digestibility of protein and carbohydrate. This is likely the result of an emulsifying effect of DMG in the intestinal tract through which non-fat nutrients are more efficiently absorbed (Kalmar *et al*., 2010a). Consequently, the non-fat nutrients become more available for utilization. Hence, dietary DMG renders both a reduction in feed cost as well as less environmental load. This benefit comes from improved protein utilization as this diminishes nitrogen excretion into manure and results in less pollution of surface water (Kalmar *et al.*, 2010a). With respect to the above, data of Kalmar *et al.* (2010b) showed a significant, positive linear relation between production value and dietary DMG supplementation within the range between 0 and 1,000 mg Na-DMG/kg.

Furthermore, dietary DMG gave less pulmonary hypertension or broiler ascites syndrome (Kalmar *et al*., 2010a). This metabolic disease, for which fast growing broiler strains are particularly susceptible, accounts for major financial losses and severe welfare issues in the broiler industry (Aksit *et al*., 2008; De Smit, 2005). The hypothesis of the mode of action of DMG on preventing broiler ascites syndrome is that it reduces oxidative stress at the level of the pulmonary arteries. Firstly, dietary DMG linearly decreased fasted plasma level of malondialdehyde in broilers fed a polyunsaturated fatty acid (PUFA) rich diet (Kalmar *et al*., 2010b). This may indicate a reduction in lipid peroxidation and in free radical activity (Holley and Cheeseman, 1993). In addition, independent of dietary fatty acid profile, DMG reduced fasted plasma level of non-esterified fatty acids (NEFA) linearly with level of dietary inclusion (Kalmar *et al*., 2010b). This will most probably also result in a decline in NEFA inflicted vascular dysfunction and in turn in less hypoxia, less increased arterial pressure and hence less progression towards pulmonary hypertension (Avogaro *et al.*, 2003; Kalmar *et al.*, 2010a; Sainsbury *et al.*, 2004; Sarafidis and Bakris, 2007; Zoer *et al.*, 2009).

In addition, DMG improved slaughter traits by a decline in depot fat weight and a tendency to linearly increase meat yield. These changes were linear in the range between 0 and 1,000 mg Na-DMG/kg feed (Kalmar *et al.*, 2010b). Kalmar *et al.* (2010b) suggested enhanced hepatic fatty acid metabolism. This effect is more pronounced with increased dietary PUFA level and results in increased utilisation of dietary fat as an energy source. As a consequence, less body

fat is deposited. Hence, protein is less used for energy and this promotes growth of lean tissue. Possibly DMG also influences hepatic gene expression by affecting DNA-methylation, as has been demonstrated for other methylamine derivates (Emmert *et al.*, 1996; Niculescu *et al.*, 2006). Effects of dietary DMG on hepatic gene expression is currently under investigation, in which it is studied by means of microarray analysis and validated by reverse-transcription quantitative real-time PCR (Erkens *et al.*, unpublished data).

Finally, a tolerance and safety trial on DMG indicated that there is a wide safety range of dietary DMG in broilers (Kalmar *et al.*, submitted). Also, dietary supplementation with DMG at a dose of 1,000 mg Na-DMG/kg feed, which is the recommended level based on a dose-response trial (Kalmar *et al.*, 2010b), would not increase DMG intake of humans through consumption of chicken meat or liver. Moreover, the DMG content in chicken meat was only twofold compared to the control when a tenfold dose (10,000 mg Na-DMG/kg) had been fed to the chickens. Furthermore, these contents are comparable with wheat bran and are considerably lower when compared to for instance spinach (Kalmar *et al.*, submitted).

The current study includes three broiler trials which were conducted at different European locations at which broilers were raised and fed according to standard practice of the region. The aim of the study is to assess the efficacy of dietary supplementation with DMG at a level of 1,000 mg Na-DMG/kg feed to improve broiler performance.

MATERIALS AND METHODS

In all trials, 1-day-old chicks of broiler strains commonly reared at each respective region were randomly divided over several pens to which one of two diets was fed for the whole rearing period. All test sites were equipped with a thermostat and automatic ventilation, and housing conditions were in compliance with the minimal space restrictions according to the revised European Treaties series No. 123 (ETS 123). The diets tested were either a control diet or the control diet supplemented with 1,000 mg Na-DMG/kg feed. In all trials feed was offered as a mash and was fed *ad libitum*. Rearing conditions and control diets were formulated according to standard practice, common to the region. Following technical performance traits were assessed at pen level: average daily gain (ADG), average daily feed intake (ADFI), finishing bodyweight (BW_{fin}), mortality, feed conversion ratio (FCR) and production value (PV).

FCR and PV were calculated as ([1] and [2]):

$$\text{FCR (g:g)} = \text{ADFI (g/d)} / \text{ADG (g/d)} \quad [1]$$

$$\text{PV} = [(100 - \text{mortality (\%)}) \times BW_{fin}\text{ (g)}] / [\text{rearing period (d)} \times \text{FCR} \times 10] \quad [2]$$

A number of replicate birds per treatment, which depended on the trial (see further), were humanely euthanatized at slaughter age after an 8-hour fasting period. Then, carcass yield, meat yield and abdominal depot fat were determined. Live weight (LW) with empty crop was determined immediately prior to slaughter. Then, birds were mechanically plucked after immersion in hot water, manually eviscerated, and weight of abdominal depot fat measured. The remaining carcass was chilled for 24 hours at 3°C in a cooling chamber. Head, neck and feet at hock joint were removed from chilled carcasses to weigh carcass for grilling (CfG). Finally, breast meat, legs and wings were manually dissected to determine meat yield.

Trial 1 (Germany)

Trial 1 was conducted at the Free University of Berlin in Germany. A total of 480 one-day old Cobb Germany Avimex GmbH broilers were randomly assigned to 24 pens with each 20 same-sex chicks. Hence, there were 12 pens with female chicks and 12 pens with male chicks. Control and DMG group consisted each of 6 replicate male pens and 6 replicate female pens. Rearing period was until 39 days of age, according to standard practice of the region.

The floor pens each had dimensions of 2.2 m x 1.8 m (length x width) and surface area ± 4 m²/pen and had softwood shaving litter as bedding. Lighting schedule was 24 hours light during the first 3 days, followed by 23 hours light: 1 hour darkness until day 7 and 18 h light: 6 hours darkness until slaughter age. Ambient temperature was kept at 28°C during the first two weeks, and was from day 15 onwards reduced by 0.5°C per day until 22°C was reached. Additionally, the temperature at the surface of the bedding was monitored and maintained at about 34°C by infra-red heaters until day 21. Relative humidity was 60.0 ± 3.5%. A three-phase feeding schedule was applied, beginning with a starter diet from day 1 until day 14, followed by a grower diet from day 15 until day 28 and a finisher diet from day 29 until day 39. All birds were vaccinated against coccidiosis with Paracox (Essex Pharma GmbH, München) at age 9 days by individual oral application at the dose level of 0.1 ml per bird. Feed was offered in flat, plastic feeders during the first 8 days, and afterwards in hanging feeders which were refilled with pre-weighed amounts when required. Fresh drinking water

was provided *ad libitum* by 6 drinking nipples per pen. The control diets were formulated to meet energy and nutrient requirements according to GfE (1999) and the breeder (Cobb) in order to optimize ADG and FCR. Proximate analysis was performed on feed samples according to VDLUFA (1988) by the Institut für Tierernärhung of the Free university Berlin, Germany. The ingredient composition of starter, grower and finisher diets were in decreasing order: corn, soybean meal, soy oil, sunflower oil, calcium carbonate (limestone), $Ca(H_2PO_4)_2$, premix, DL-methionine, L-lysine HCl, L-threonine and L-tryptophan. Nutrient composition of test diets is presented in **Table 1.**

Table 1. Nutrient composition (g/kg) and metabolizable energy content (ME_n, MJ/kg) of test diets as fed.

	Trial 1 (Germany)			Trial 2 (Austria)			Trial 3 (Italy)	
	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter/Grower	Finisher
DM	912	915	924	889	886	884	902	906
NfE	485	500	520	512	531	542	508	479
CP	222	209	187	211	189	178	205	201
EE	117	118	129	74	82	76	96	111
CA	57	57	55	56	56	51	56	76
CF	31	31	33	37	38	38	37	39
ME_n	12.6	13.1	13.3	13.0	13.0	12.9	13.4	13.8

DM: dry matter, NfE: N-free extract, CP: crude protein, EE: ether extract, CA: crude ash, CF: crude fibre, ME_n: metabolisable energy corrected at zero N-balance

Technical performance traits, which were obtained as described above, were calculated based on weekly weighing of broilers and weekly determination of feed intake at pen level. Mortality was recorded daily. Slaughter performance was assessed on one animal per pen and included 12 replicate birds (6 females and 6 males) per treatment. Slaughtering was done by concussion followed by exsanguinations through neck-cutting.

Trial 2 (Austria)

Trial 2 was conducted at the poultry trial station in Äussere Wimitz, Kraig, Austria. A total of 360 one-day old Ross 308 broilers were randomly allocated in 24 pens of 15 mixed-sex chicks each until the age of 36 days. The floor pens had dimensions of 2 m x 1.5 m (length x width) with a surface area 3 m²/pen, and had wood shavings as litter. Lighting schedule was 24 hours light during the first 3 days, followed by 22 hours light: 2 hours darkness until slaughter age. Ambient temperature was initially kept at 28°C and gradually reduced to 20°C. A three-phase feeding schedule was applied, beginning with a starter diet from day 1 until day

14, followed by a grower diet from day 15 until day 28 and a finisher diet from day 29 until day 36. Feed and drinking water were provided *ad libitum* in feeding troughs of three different sizes and automatic water dispensers. Feeding trays were regularly refilled with pre-weighed amounts. Proximate analysis of feed samples was done according to standard methods of the AOAC (1980) and was carried out by the Futtermittel-Labor Rosenau, Austria. The ingredient composition of starter, grower and finisher diets were: corn, soybean meal, wheat, dried distiller's grains and solubles of wheat, fat (50% animal fat and 50% vegetal fat), grass meal, corn gluten meal, calcium carbonate, dicalcium phosphate, L-lysine HCl, DL-methionine, sodium chloride, L-threonine, choline chloride, trace element premix, vitamin premix, ZY-phytase and Sanor endox™. Monensin sodium (100 ppm, Elancoban™) was used as coccidiostat in starter and grower feeds. Nutrient composition of test diets is presented in **Table 1.**

Birds were weighed at pen level at days 1, 14, 28 and at the end of the trial. Feed consumption per pen was measured during the starter (day 1-14), grower (day 15-28) and finisher (day 29-36) periods. At the end of the trial 2 birds per pen, 1 male and 1 female, were humanely euthanized by concussion followed by decapitation through neck-cutting. Meat quality was assessed in addition to above mentioned technical and slaughter performance traits. The organoleptic test was done at slaughter age on breast meat of 12 replicate male and 12 replicate female animals per treatment group. Pieces of breast meat (3 cm x 3 cm x 1 cm) were roasted on both sides for 6 minutes at 180°C and then graded by a test-panel consisting of 4 independent, trained persons. The meat was subjectively graded for tenderness, juiciness and taste using a score range between 1 and 6 (**Table 2**).

Table 2. Organoleptic scoring test to assess meat quality.

Score	Tenderness	Juiciness	Taste
1	very tough	very dry	untypical
2	tough	dry	tasteless
3	below average	below average	below average
4	above average	above average	above average
5	tender	juicy	tasty
6	very tender	very juicy	very tasty

Trial 3 (Italy)

Trial 3 was conducted at the certified (ISO 9001) poultry farm "Luca Fornello" in Settimo Torinese, Italy. A total of 352 one-day old Ross 508 broilers were randomly allocated in 22 pens of 16 mixed-sex chicks (8 males and 8 females) per pen. At hatching, chicks were vaccinated against coccidiosis, Newcastle disease and infectious bronchitis (Izovac I.B. H120 by Izo S.p.A.-Brescia). The vaccine against coccidiosis was administered in the drinking water while those for Newcastle disease and infectious bronchitis were administered by inhalation. Rearing period was 35 days. Dimensions of floor pens were 1.5 m length x 1 m width, with a surface area of 1.5 m². Rice hulls were used as litter. Lighting schedule was 23 hours light: 1 hour dark during the whole rearing period. Infrared lamps were used for heating during the first three weeks. Minimum and maximum temperatures were 21.9 and 30.4°C in starter/grower period and 22.4 and 26.3°C in finisher period. A two-phase feeding schedule was applied, beginning with a starter/grower diet from day 1 until day 21, followed by a finisher diet from day 22 until day 35. In the region both 1,4-beta-xylanase (E-1613) and 3-phytase (E-300) are commonly used in commercial diets, hence these additives were included in the test diets. Feed and drinking water were provided *ad libitum* in feeding trays and by 3 drinking nipples per pen. Feeding trays were refilled with pre-weighed amounts when required. Proximate analysis of feed samples was done according to standard methods of the AOAC (2000) and was carried out by the Dipartimento di Produzioni Animali, Epidemiologia ad Ecologia of the University of Torino, Italy. Ingredient composition of test diets were: soybean (kernels without hulls), wheat, corn meal, soybean, soy oil, dicalciumphosphate, calcium carbonate, premix, DL-methionine, sodium chloride, L-lysine, 1.4-beta-xylanase, sodium bicarbonate, L-threonine, choline chloride and 3-phytase. Nutrient composition of test diets is presented in **Table 1**.

Live weight and feed consumption were measured weekly at pen level and technical performance traits calculated as mentioned above. At the end of the trial, one male and one female animal per pen were humanely euthanized by individual CO_2 gassing followed by exsanguination through neck-cutting for determination of slaughter performance traits as described above.

Statistics

Data on technical performance traits were statistically analysed with pen as experimental unit, whereas replicate slaughtered birds were used as experimental unit for slaughter traits. Normality and homogeneity were tested with the Kolmogorov-Smirnov and modified Levine test, respectively. All parameters except for mortality were analysed using one-way ANOVA. Technical performance traits were in trial 1 analysed with diet, sex and interactions as independents, whereas in trial 2 and 3 with diet as independent. Slaughter traits were in all trials analysed with diet, sex and interactions as independents. Meta-analysis of technical and slaughter traits were analysed with diet, x trial and diet x trial x sex, respectively, as independents. Statistical analysis of technical performances was done per trial for the whole rearing period as well as for the different feeding phases as described above, whereas its meta-analysis was done for the rearing period until age 35d or 36d and per growth phase: starting period (d1-d14), growing period (d15-28) and finisher period (d29-d35 or d29-d36). Results of the organoleptic test were subject to the general linear model repeated measures analysis of variance with test person as within-subject variable and diet as between-subject variable. Mortality was not normally distributed, hence these data were analysed with the non-parametrical two-way Wilcoxon test with diet as grouping variable and for meta-analysis with the Friedman rank sum test with diet as grouping variable and trial as blocking variable. Average values are expressed as means ± standard error of the means (SEM), and effects of DMG are expressed as percentual difference relative to the control ($\%_{rel\ \Delta}$). All statistics were done in S-PLUS 8.0 (TIBCO Software Inc., Palo Alto, California) SPSS 16.0 (SPSS Inc., Chicago, Illinois). Significance was set at $p < 0.05$. Statistical trends were set at $p < 0.10$. All differences discussed in the following are significant unless stated otherwise.

RESULTS

In trial 1, both PV and total FCR for the whole rearing period were improved when diets were supplemented with DMG compared to the control (**Table 3**). FCR per feeding phase showed only an improvement in the starter phase and tended to improve in the finisher phase (data not shown). The other technical performance traits were not affected by dietary DMG. Starting BW was higher in male broiler chicks compared to females. Both ADG and ADFI were higher in male broilers and FCR was lower compared to females. Although significant over the whole rearing period, these gender effects were only significant in grower and finisher phase, but not in the starter phase. PV was not affected by gender. Finally, there were no diet x sex interactions (**Table 3**).

Table 3. Technical performance in broiler chickens fed a control diet or the same diet supplemented with DMG at 1,000 mg Na-DMG/kg feed.

		Trial 1 (Germany, *n* =12)		**Trial 2** (Austria, *n* = 12)		**Trial 3** (Italy, *n* = 11)		Statistics							
								Trial 1				Trial 2		Trial 3	
		Control	**DMG**	**Control**	**DMG**	**Control**	**DMG**	sem	D	S	DxS	sem	D	sem	D
BW_{d1}	(g)	43.6	43.6	41.9	41.9	39.9	40.4	0.3	NS	***	NS	0.2	NS	0.2	NS
BWf_{in}	(g)	2215	2272	2105	2221	1736	1750	23	NS	***	NS	17.2	***	11	NS
ADG	(g/d)	55.7	57.1	57.3	60.5	48.4	48.8	0.6	NS	*	NS	0.5	***	0.3	NS
ADFI	(g/d)	85.5	84.4	103.4	107.0	81.7	79.2	0.9	NS	***	NS	0.7	*	0.6	*
FCR		1.54	1.48	1.80	1.77	1.69	1.62	0.01	*	NS	NS	0.01	*	0.01	***
Mortality	(%)	0.8	0.8	1.7	3.3	1.14	1.70	0.4	NS	NS	NS	0.9	NS	0.6	NS
PV		367	392	318	337	291	304	5	*	NSD	NS	4.0	*	3.9	NS

DMG: *N*,*N*-dimethylglycine, BW_{d1}: starting bodyweight (age 1 d), BW_{fin}: finishing bodyweight (age 39 d in trial 1, age 36 d in trial 2 and age 35 d in trial 3), ADG: average daily gain, ADFI: average daily feed intake, FCR: feed conversion ratio, PV: production value, D: diet, S: Sex
Significance: ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; NS: not significant

Supplementation with DMG resulted in trial 2 in an increase in finishing bodyweight and total ADG. Total ADFI was higher in DMG groups compared to the control, but total FCR was lower when fed the DMG supplemented diet. Mortality was not affected by dietary DMG, but PV was improved (**Table 3**). Technical performance per feeding phase showed an increase in ADG and ADFI in starter and grower but not in finisher phase when supplemented with DMG. Yet, a tendency for higher ADFI was also present in the finisher phase when supplemented with DMG. Finally, FCR per feeding phase showed an improvement by DMG in the starter phase, but not in the grower and finisher phase (data not shown).

Technical performance traits in trial 3 showed similar finishing BW, total ADG, mortality rate and PV between dietary treatments, but a decrease in total ADFI as well as in total FCR in the DMG groups compared to the control (**Table 3**). ADG per feeding phase was not different between dietary treatments in both starter/grower and finisher phase. However, ADFI tended to decrease in the starter/grower phase and significantly decreased in the finisher phase when broiler diets were supplemented with DMG. Furthermore, FCR was in both the starter/grower phase and in the finisher phase lower in DMG groups compared to the control (data not shown).

Meta-analysis of technical performance traits showed a significant increase in finishing BW at age 35 d or age 36 d, irrespective of trial site, when diets were added with DMG. The effect on ADG was significant and most pronounced in the starter period and this improvement tended to continue in the grower period, whereas ADG in the finisher period was similar between dietary treatments. Nevertheless, total ADG over the whole rearing period was improved by dietary DMG. ADFI was not different between dietary treatments, but total FCR over the whole rearing period was lowered in DMG groups compared to the control. FCR was also most pronouncedly affected in the starter period, but was reduced by DMG in the grower period as well. Mortality showed no differences between diets. Furthermore, overall performance showed a higher PV in DMG groups compared to the control. Finally, all technical performance traits except for mortality rate were different between test sites. Finishing BW and PV, but also starting BW, were highest in trial 1 and lowest in trial 3, whereas FCR was lowest in trial 1 and highest in trial 2. Results also showed several diet x trial interactions (**Table 4**).

Table 4. Meta-analysis of effects of diet (control and DMG) and trial (trial 1, 2 and 3) on technical performance in broiler chickens (n = 35).

		Control	DMG	sem	D	T	DxT
BW_{d1}	(g)	41.9	42.0	0.2	NS	***	NS
$BW_{d35\ or\ d36}$	(g)	1916	1959	22	*	***	*
ADG	(g/d)	53.0	54.2	0.6	*	***	*
• Starter	(g/d)	26.6	28.2	0.4	***	***	NS
• Grower	(g/d)	63.7	65.1	0.8	°	***	*
• Finisher	(g/d)	83.0	82.9	1.9	NS	***	NS
ADFI	(g/d)	88.1	87.7	1.6	NS	***	***
• Starter	(g/d)	35.8	35.6	0.3	NS	***	*
• Grower	(g/d)	100.1	100.9	1.6	NS	***	***
• Finisher	(g/d)	163.9	160.9	3.7	NS	***	*
FCR		1.66	1.61	0.02	***	***	NS
• Starter		1.36	1.27	0.01	***	***	NS
• Grower		1.57	1.55	0.01	*	***	NS
• Finisher		2.02	1.97	0.04	NS	***	NS
Mortality	(%)	1.2	2.0	0.4	NS	NS	NS
PV		324	338	4	*	***	NS

DMG: *N*,*N*-dimethylglycine, BW: bodyweight, ADG: average daily gain, ADFI: average daily feed intake, FCR: feed conversion ratio, PV: production value, D: diet, T: trial, Starter = day 1-14; Grower = day 15-28; Finisher = day 29-35 or day 29-36 Significance: ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, °: $p < 0.1$, NS: not significant

Slaughter performances in trial 1 were similar between dietary treatments, except for breast meat yield which was higher in DMG groups compared to the control. Breast meat yield also showed an interaction between gender and diet, and the dietary effect was higher in females ($p < 0.01$) than in males ($p > 0.1$) (**Table 5**). Moreover, breast meat yield, irrespective of diet, was significantly lower and total meat yield tended to be lower in females compared to males (data not shown).

Trial 2 showed no dietary effects on slaughter traits (**Table 5**). Both carcass for grilling and abdominal depot fat, irrespective to diet, were higher in females compared to males. Yet, meat yield to abdominal fat ratio was higher in males. The leg parts of CfG were also higher in males compared to females (data not shown).

Table 5. Carcass traits in broiler chickens fed a control diet or the same diet supplemented with DMG at 1,000 mg Na-DMG/kg feed.

		Trial 1 (Germany, *n* =12)		**Trial 2** (Austria, *n* = 12)		**Trial 3** (Italy, *n* = 11)		Statistics											
								Trial 1				Trial 2				Trial 3			
		Control	**DMG**	**Control**	**DMG**	**Control**	**DMG**	sem	D	S	DxS	sem	D	S	DxS	sem	D	S	DxS
CfG	(%$_{LW}$)	80.83	81.03	69.25	69.88	74.60	74.44	0.44	NS	NS	NS	0.23	NS	*	NS	0.25	NS	***	NS
Meat parts	(%$_{CfG}$)	60.99	61.93	69.21	69.07	60.65	61.32	0.32	NS	°	NS	0.30	NS	NS	NS	0.19	°	*	°
◦ **Breast**	(%$_{CfG}$)	22.82	23.58	29.08	29.56	23.54	24.08	0.08	*	***	*	0.28	NS	NS	NS	0.14	°	*	*
◦ **Legs**	(%$_{CfG}$)	27.88	28.47	28.80	28.41	27.35	27.60	0.60	NS	NS	NS	0.22	NS	*	NS	0.18	NS	*	*
◦ **Wings**	(%$_{CfG}$)	10.29	9.88	11.33	11.10	9.76	9.65	0.65	NS	NS	NS	0.11	NS	NS	NS	0.08	NS	NS	NS
Depot fat	(%$_{LW}$)	1.93	1.80	2.00	2.05	1.63	1.29	0.29	NS	NS	NS	0.06	NS	*	NS	0.06	*	NS	NS
Meat:fat		26.54	29.86	24.93	24.48	30.00	3742	1.42	NS	NS	NS	0.72	NS	*	*	1.51	*	NS	NS

DMG: *N*,*N*-dimethylglycine, CfG: carcass for grilling, LW: live weight (trial 1: 39 days, trial 2: 36 days and trial 3: 35 days), D: diet, S: sex

Total meat yield, and breast meat yield tended to increase when DMG was added to the diet in trial 3. These slaughter traits showed a tendency and significant diet x gender interaction, respectively. The effect on total meat yield was highest in females ($p < 0.01$), whereas the effect on breast meat yield was highest in males ($p < 0.1$). Next, abdominal depot fat was lower and meat yield to abdominal fat ratio higher in DMG groups compared to the control (**Table 5**). The leg parts of CfG showed a diet x gender interaction, and its yield was higher in females fed the DMG supplemented diet compared to females fed the control diet (data not shown).

Meta-analysis of slaughter performance revealed similar CfG and total meat yield between diets, but both meat yield to abdominal fat ratio and breast meat yield were higher in DMG groups compared to the control. Next, CfG was highest in trial 1 and lowest in trial 3. But meat yield, including all different meat parts, was highest in trial 2 compared to trial 1 and 3. Total meat yield as well as legs expressed as percentage of CfG was higher in male chickens compared to females. Abdominal depot fat tended to decrease when fed the diet supplemented with DMG. Next to a dietary effect, abdominal depot fat also showed a diet X gender interaction in which the dietary effect was most pronounced in males ($p < 0.05$) compared to females ($p > 0.1$). Finally, abdominal depot fat was lower in trial 3 compared to trial 1 and trial 2, whereas meat yield to fat ratio was higher in trial 3 compared to both other trials (**Table 5 and 6**).

Table 6. Meta-analysis of effects of diet (control and DMG), sex (male and female), and trial (trial 1, 2 and 3) on carcass traits in broiler chickens ($n = 60$).

		Control	DMG	sem	D	T	S	DxT	DxS	TxS	DxTxS
CfG	($\%_{LW}$)	73.70	73.93	0.41	NS	***	NS	NS	NS	*	NS
Meat parts	($\%_{CfG}$)	64.14	64.54	0.42	°	***	***	NS	NS	*	NS
• Breast	($\%_{CfG}$)	25.61	26.17	0.29	*	***	NS	NS	NS	*	NS
• Legs	($\%_{CfG}$)	28.03	28.10	0.16	NS	*	***	NS	NS	NS	*
• Wings	($\%_{CfG}$)	10.49	10.27	0.10	°	***	NS	NS	NS	NS	NS
Depot fat	($\%_{LW}$)	1.83	1.70	0.04	°	***	NS	°	*	NS	NS
Meat: fat		27.28	30.73	0.82	*	***	NS	*	NS	NS	NS

DMG: *N*,*N*-dimethylglycine, CfG: carcass for grilling, LW: live weight at slaughter age (trial 1: 39 days, trial 1: 36 days, trial 3: 35 days), D: diet, T: Trial, S: Sex
Significance: ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, °: $p < 0.1$, NS: not significant

A test panel scored tenderness, juiciness as well as taste of roasted breast meat for trial 2 and evaluated these traits above average in both dietary treatment groups. Organoleptic scoring showed a taster effect, but none of the meat quality traits was differently appraised between control and DMG groups (**Table 7**).

Table 7. Organoleptic quality on a score between 1 to 6 (≤ 3 means below average and ≥ 4 means above average) of breast meat in chickens fed a control diet or the same diet supplemented with DMG at 1,000 mg Na-DMG/kg feed (trial 2; $n = 24$).

	Control	DMG	sem	D	Tp	DxTp
Tenderness	4.95	4.85	0.07	NS	*	NS
Juiciness	4.56	4.38	0.07	NS	*	NS
Taste	4.69	4.53	0.07	NS	*	NS

DMG, *N*,*N*-dimethylglycine; D, diet, Tp, test person
Significance: *: $p < 0.05$ and NS: not significant

Summarised results show, irrespective to trial site, an overall improvement in finishing BW, FCR and PV by +2.3%$_{rel\ \Delta}$, -2.8%$_{rel\ \Delta}$ and +4.3%$_{rel\ \Delta}$, respectively, when diets were supplemented with DMG. In trial 1, FCR was lowered by 3.8%$_{rel\ \Delta}$ and PV increased by 6.8%$_{rel\ \Delta}$. In trial 2, finishing BW was increased by 5.5%$_{rel\ \Delta}$, FCR lowered by 2.0%$_{rel\ \Delta}$ % and PV increased by 5.9%$_{rel\ \Delta}$. In trial 3, FCR was lowered by 3.9%$_{rel\ \Delta}$. Mortality was similar between dietary treatments. Slaughter performance showed, irrespective to trial site, an overall increase in meat yield to abdominal fat ratio and breast meat yield by 12.6%$_{rel\ \Delta}$ and 2.2%$_{rel\ \Delta}$, respectively, and an overall tendency of lowered abdominal depot fat by 7.5%$_{rel\ \Delta}$. In trial 1, breast meat yield was improved by +3.3%$_{rel\ \Delta}$ and tended to improve by +2.3%$_{rel\ \Delta}$ in trial 3. Furthermore, abdominal depot fat was lowered by 20.5%$_{rel\ \Delta}$ and meat yield to abdominal fat ratio increased by 24.7%$_{rel\ \Delta}$ in trial 3 (**Table 8**). Organoleptic assessment of roasted breast meat showed no dietary effects (**Table 7**).

Table 8. Main significant effects ($p < 0.05$) of dietary supplementation with DMG on technical and slaughter performance in broiler chickens, expressed as % compared to the control. Statistical tendencies at $p < 0.1$ are indicated between brackets.

		Trial 1	Trial 2	Trial 3	Overall
Rearing period	(d-d)	1-39	1-36	1-35	1-35 or 1-36
BW_{fin}	(g)	NS	+5.5	NS	+2.3
FCR	(g:g)	-3.8	-2.0	-3.9	-2.8
PV		+6.8	+5.9	NS	+4.3
Mortality	(%)	NS	NS	NS	NS
CfG	(%$_{LW}$)	NS	NS	NS	NS
Breast meat	(%$_{CfG}$)	+3.3	NS	(+2.3)	+2.2
Abdominal fat	(%$_{LW}$)	NS	NS	-20.5	(-7.5)
Meat: fat		NS	NS	+24.7	+12.6

DMG: *N*,*N*-dimethylglycine, BW_{fin}: finishing bodyweight, FCR: feed conversion ratio, PV: production value, CfG: carcass for grilling, LW: live weight, NS: not significant

DISCUSSION

Gain to feed ratio or FCR in the control groups varied between trial sites, in which feed efficiency was outstanding in trial 1 (FRC = 1.54), satisfactory in trial 3 (FCR = 1.69) and rather inefficient in trial 2 (FCR = 1.80). Yet, FCR of the starter diet was improved by DMG in all three locations, but the magnitude of DMG effects on FCR in grower and finisher diets depended on the trial. Nevertheless, feed efficiency over the whole rearing period was significantly improved by DMG in all trials by 2.0 to 3.9%$_{rel\ \Delta}$ and as a mean by 2.8%$_{rel\ \Delta}$. Total feed efficiency and feed efficiency in grower and finisher diets were higher in males compared to females, with a similar effect of DMG supplementation among sexes (trial 1, D x S: $p > 0.05$). Hence, these data suggest a beneficial effect of dietary DMG on FCR in both genders and over a wide range of flock efficiencies. Flock performance being influenced by broiler strain, basal ration and rearing conditions.

The underlying mechanism of improved feed efficiency is likely to be, at least partly, the result of improved digestibility of protein and N-free extract due to the emulsifying action of DMG at the intestinal tract (Kalmar *et al.*, 2010a). The indirect effect of increased fat emulsification on improved digestibility of non-fat fractions can be explained by increased availability for digestive enzymes as a result of a better accessibility (Kalmar *et al.*, 2010b). The fact that highest effects of DMG on FCR are consistently noticed in the starter period further supports an emulsifying effect of dietary fats to be one of the mechanisms to improve FCR by DMG. As apart from yolk utilization, for which the importance of pancreatic and biliary secretions seems to be negligible, it is widely demonstrated that the digestive capacity of fat in broilers increases with age (Freeman *et al.*, 1976; Krogdahl, 1985). In particular, digestion of vegetal oils, which were depending to the trial site the sole or main fat source in current trials, is underdeveloped in broiler chicks until the first two weeks of age (Freeman *et al.*, 1976). In consequence, an emulsifying agent is indeed expected to be most efficient in improving digestibility in the starter phase age group.

An important increase in the ratio between meat yield and abdominal fat occurred in trial 3 as well as overall. This indicates leaner growth. Fat accretion has a higher energetic cost per mass unit compared to lean accretion (protein plus water). Thus an increase in meat to fat ratio also contributes to a more efficient feed conversion. Furthermore, irrespective of trial site, abdominal depot fat expressed as a percentage of live weight tended to be lower in DMG groups compared to the control. These results are concordant to previous data in which a

linear inverse relation was demonstrated between abdominal fat pad and dietary DMG supplementation within a range of 0 to 1,000 mg Na-DMG/kg feed (Kalmar *et al.*, 2010b). A plausible cause of lower fat deposition relative to lean growth in DMG supplemented broilers is an increase in protein supply as a result of its increased digestibility. This agrees with results of e.g. Namroud *et al.* (2008). These authors showed a decrease in abdominal fat deposition and a concomitant lower FCR in broilers when increasing dietary protein content from 17% to 21%. This is within the range of protein content of current finisher diets. Abdominal depot fat in control groups of current investigation was also inversely related to protein content of finisher diets. In contrast to Namroud *et al.* (2008), in which the degree of improvement in FCR was highest when increasing dietary protein content from 17% to 19% compared to an increase from 19% to 21%, in the current trials the lowest improvement in FCR on account of DMG was noticed at lowest dietary protein content of finisher diet. Hence, additional factors are likely to be involved in the working mechanism of DMG.

Although FCR was improved in all trials, ADFI was differently influenced by dietary DMG, being either increased, decreased or similar compared to the control. However, finishing BW was either similar or increased when diets were supplemented with DMG. Overall, irrespective of trial site, finishing BW was increased by 2.3%$_{rel\ \Delta}$ in the DMG groups. Production value, the overall technical performance measure, was improved in trial 1 and trial 3 by 6.8%$_{rel\ \Delta}$ and 5.9%$_{rel\ \Delta}$, respectively, and irrespective of trial site by 4.3%$_{rel\ \Delta}$ or 14 units. These results indicate consistency of the positive effects of DMG on overall technical performance.

In contrast to PV, CfG was not different between dietary treatments, indicating a true improvement of productivity. Moreover, notwithstanding similar CfG, the proportion of breast meat yield was irrespectively of trial site increased by 2.2%$_{rel\ \Delta}$. In trial 1 breast meat yield was significantly increased by DMG, whereas in trial 3 this effect showed only a tendency. A significant interaction was present between diet and gender in both trials. Herein, breast meat yield was only significantly improved in females in trial 1 and tended to increase only in males in trial 3. In parallel, gender effects in both trials showed highest breast meat yield in males and females in trial 1 and 3, respectively. In trial 2, on the other hand, in which breast meat yield was neither affected by gender nor by DMG, was this cut yield overall considerably higher compared to trial 1 and 3. This means that the positive effect of DMG on breast meat yield was largest in those chickens which had the lowest breast meat yield to start

with. Then again, breast meat yield irrespective of trial site was similar between both genders, as was the effect of DMG. However, abdominal fat pad, irrespective of trial site, showed a diet x gender interaction but no gender effect. Herein, the tendency of DMG to decrease abdominal fat in both genders was significant for males but not for females. The latter is in contrast with results of trial 3, in which abdominal fat was significantly increased in both genders and in which diet x gender interaction was not significant.

An increase in breast meat yield, although not significant in all separate trials but significant after meta-analysis of all data, together with a tendency for lower abdominal depot fat suggests superior slaughter performance as a result of dietary supplementation with DMG. Breast meat yield is the most valuable cut yield, whereas abdominal fat can be considered a high energetic offal tissue in terms of slaughter performance. Importantly, meat quality, as assessed by tenderness, juiciness and taste, was comparable between control and DMG groups and was evaluated above average for all tested traits. Hence, supplementation of broiler diets with Na-DMG did not compromise meat quality. Furthermore, as demonstrated in a previous tolerance and safety trial, DMG does not accumulate in consumer parts of broiler chickens when supplemented at a dosage of 1,000 mg Na-DMG/kg feed and therefore does not pose a consumer risk of involuntary intake of DMG intended as a broiler feed additive (Kalmar *et al.*, submitted).

In conclusion, 3 trials were performed in which different broiler strains were reared under conditions common to the region of each trial location. Although FCR widely varied between trial sites, supplementation with DMG at a dose of 1,000 mg Na-DMG/kg feed resulted in all trials in an improvement in feed efficiency. Moreover, finishing BW and PV were increased in 1 of 3 and 2 of 3 trials, respectively. Irrespective of trial site, FCR, finishing BW and PV were improved by -2.8%$_{rel\ \Delta}$, +2.3%$_{rel\ \Delta}$ and +4.3%$_{rel\ \Delta}$, respectively. Although CfG was similar between DMG and control groups, breast meat yield and meat yield to abdominal fat ratio in the combined data were increased by DMG by 2.2%$_{rel\ \Delta}$ and 12.6%$_{rel\ \Delta}$ respectively, whereas abdominal fat percentage tended to decrease by 7.5%$_{rel\ \Delta}$. However, these effects on slaughter performance were not consistently present in each of the trials. Meat quality of roasted breast meat, as determined in trial 2 by tenderness, juiciness and taste, was similar between control and DMG groups. On the whole, this investigation firstly demonstrated an improvement in feed efficiency, consistent in occurrence but varying in degree likely depending on broiler strain and rearing conditions. Secondly, other beneficial effects of

dietary DMG on technical and slaughter performances were clearly present considering the overall results, although not consistently significant in each trial. Thirdly, appraisal of meat quality was not altered by DMG.

IMPLICATIONS

Dietary supplementation with *N,N*-dimethylglycine (DMG) at a dosage of 1,000 mg Na-DMG/kg feed during the whole rearing period of chickens for fattening consistently resulted in improved productivity. This was demonstrated in three distinct trials, which were performed at different European locations using broiler strains, basal rations and rearing conditions common to the region. Current data clearly demonstrated beneficial effects of supplementary DMG over a wide range of flock efficiency, but magnitude and mode (*e.g.* feed efficiency, production value, finishing bodyweight and breast meat yield) of effects seems to depend on broiler strain and rearing practice.

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CHAPTER 5

Tolerance and safety evaluation of *N*,*N*-dimethylglycine, a naturally occurring organic compound, as a feed additive in broiler diets.

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ASTRACT

N,N-dimethylglycine (DMG) is a tertiary amino acid which naturally occurs as an intermediate metabolite in the choline to glycine metabolism. Recently it has been found that when supplemented to the diet it may enhance broiler performance. The objective of the current trial was to evaluate tolerance, safety and bioaccumulation of dietary Na-DMG in broilers when supplemented at the recommended (1,000 mg/kg) and the tenfold dose (10,000 mg/kg). A feeding trial was performed on 480 one-day-old broiler chicks that were randomly allocated in 24 pens and fed one of three test diets added with 0, 1,000 or 10,000 mg Na-DMG/kg during a 39-day growth period. Production performance was recorded at pen level (n = 8) to assess tolerance and efficacy of the supplement. At the end of the trial, toxicity was evaluated by means of haematology, plasma biochemistry and histopathology of liver, kidney and heart (n = 12) whereas bioaccumulation was assessed on meat, liver, blood, kidney and adipose tissue (n = 8). Finishing bodyweight, feed-to-gain ratio and production value were significantly improved at recommended dose ($p < 0.05$) without adverse effects on carcass traits ($p > 0.05$). At tenfold dose, technical performance was in between control and recommended dose groups. Histological examinations did not show pathological effects and results of haematology and plasma biochemistry revealed similar values at the three treatments. Bioaccumulation occurred in blood at both doses, and in meat, liver and kidney at the tenfold dose. The resulting DMG content in raw meat at tenfold dose was comparable with, for instance, wheat bran and much lower than uncooked spinach. In conclusion, the experimental results showed dietary DMG to be well-tolerated and safe in broilers.

INTRODUCTION

N,*N*-dimethylglycine (DMG), a methylated derivative of the amino acid glycine with the chemical formula $(CH_3)_2NCH_2COOH$, has been used for various human and animal applications. The molecule was first reported in 1943, and is a naturally occurring intermediate metabolite in the choline to glycine metabolism (Tonda and Hart, 1992). In short, choline is oxydised into betaine. Next, DMG is formed within the mitochondria from betaine (*N*,*N*,*N*-trimethylglycine) by removal of one methyl group and after this it is further demethylated to sarcosine (*N*-methylglycine) and finally to glycine (Craig; 2004; Garrow, 2001). Currently, DMG is used as an enhancer of athletic performance in human athletes as well as in racing dogs and horses, for which it is claimed to enhance oxygen utilization and diminish muscle acidification. This application is however primarily based on anecdotal reports. To date, only few randomized, controlled studies have been performed and these have failed to demonstrate the above claims related to athletic performance (Cupp and Tracy, 2003).

A new application of DMG is to use it as a dietary supplement in poultry diets. Dietary supplementation with 167 mg Na-DMG/kg has been demonstrated to improve nutrient digestibility (Kalmar *et al.*, 2010a). This beneficial effect of dietary DMG has also been described in sows and has been attributed to an emulsifying action at the gut level through which nutrients become more available for digestion and absorption (Cools *et al.*, 2010; Kalmar *et al.*, 2010a and 2010b). The emulsifying properties of DMG can be demonstrated by visual emulsification of the oil phase when DMG is added to a water and oil mixture (Kalmar *et al.*, 2010a). Concomitantly to improved protein digestibility, dietary DMG at a dose of 167 mg Na-DMG resulted in diminished nitrogen excretion (Kalmar *et al.*, 2010a). The latter effect is of particular interest as it diminishes nitrogen pollution of surface water through livestock manure and hence provides for an environmental benefit next to the economic advantage of a reduction in feed cost (Kalmar *et al.*, 2010a; Nahm, 2007). A recommended dosage of 1,000 mg Na-DMG was suggested based on the results of a dose-response trial (Kalmar *et al.*, 2010b). This dosage was applied in a series of efficacy trials, which consistently demonstrated enhanced technical performances in broilers. The degree of improvement depended on rearing conditions and on broiler strain. However, supplementation generally resulted in a decline in feed to gain ratio and an incline in finishing bodyweight and production value without compromising carcass traits or organoleptic scoring of meat quality.

Moreover, some trials even demonstrated a significant decrease in adipose tissue depots and an increase in meat to fat ratio (Kalmar *et al.*, submitted).

The aims of the current trial were to evaluate the tolerance and safety of this new application for DMG in the target species. To this end, dietary supplementation with DMG at the recommended dose of 1,000 mg Na-DMG per kg feed and at a tenfold higher dose was applied during a production period of 39 days and compared to chickens fed a control diet during the same period. Technical performance, haematology and clinical biochemistry, as well as histopathology of liver, kidney and muscle are evaluated and compared to the control. Carcass traits in the control and recommended DMG dose groups are assessed. Finally, the accumulation of DMG and glycine - the metabolite of DMG after complete demethylation - in meat and organs of the target species is evaluated. To facilitate interpretation of the broiler tissue results, DMG content was also determined in several unrelated food items for human consumption.

MATERIALS AND METHODS

The safety study was conducted at the trial facility of the Institution for Animal Nutrition at the Free University of Berlin in Germany. The implementation of the trial and experimental design complied with Good Clinical Practice criteria based on the consensus guidelines of the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (EMEA, 2001) and lasted 4 days longer than required, as stipulated in Commission Regulation (EC) No 429/2008 (EC, 2008).

Experimental design

A total of 480 one-day-old Cobb Germany Avimex GmbH broiler chicks were randomly allocated to 24 pens (20 male or 20 female chicks/pen). The pens were then assigned to one of three dietary treatment groups (8 replicate pens consisting of 4 male and 4 female pens /diet group) for the entire production period of 39 days. The test diets were a common control diet, the control diet supplemented with the recommended dose of 1,000 mg Na-DMG *per* kg feed and the control diet with a Na-DMG dose that was tenfold the recommended dose (10,000 mg Na-DMG *per* kg feed). Feed was offered as a mash and was available *ad libitum*. The floor pens with dimensions 2.2 m x 1.8 m (length x width) and surface area ± 4 m²/pen were strewed with softwood shaving litter. The lighting schedule was 24 hours of light during the first 3 days, followed by 23 hours of light (1 hour of darkness) until day 7 and 18 hours of

light (6 hours of darkness) until slaughter age. Ambient temperature was maintained between 32 and 32.5°C during the first week of the trial, was gradually reduced to 31°C during the second week, and from day 15 onwards it was reduced by about 0.4°C per day until 22°C was reached at day 39. Surface temperature of the bedding was maintained at 34°C by infra-red heaters until day 21. Relative humidity started at 50% on day 1 and reached 60% at the end of the trial. All birds were vaccinated against coccidiosis with Paracox (Essex Pharma GmbH, Munich) by individual oral application at the dose level of 0.1 ml per bird at 9 days of age.

Diets

A three-phase feeding schedule was applied to all pens, beginning with a starter feed formulation from day 1 until day 14, followed by a grower feed from day 15 until day 28 and a finisher feed from day 29 until day 39. Treatments for each growth phase comprised the unsupplemented control diet, or this diet supplemented with 1,000 or 10,000 mg Na-DMG per kg feed. Broiler diets were offered in flat, plastic feeders during the first 8 days, and afterwards in automatic feeders that were refilled with pre-weighed amounts when required. Fresh tap water was provided *ad libitum* from drinking nipples. The control diets were formulated to meet energy and nutrient requirements according to the Gesellschaft für Ernährungsphysiologie (GfE) and the breeder (Cobb) (GfE, 1999). Proximate analysis was performed on feed samples according to the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsantalen (VDLUFA) by the Institut für Tierernährung of the Free University Berlin, Germany (VDLUFA, 1988). Calculated metabolizable energy corrected at zero N-balance (ME_n) of test diets were 12.62 MJ ME_n/kg, 13.05 MJ ME_n/kg and 13.34 MJ ME_n/kg in starter, grower and finisher diets, respectively. Nutrient and ingredient composition of the starter, grower and finisher control feeds, to which DMG was added, is presented in **Tables 1** and **2**, respectively.

Table 1. Analyzed nutrient composition (g/kg) of starter, grower and finisher control feeds.

Ingredients	Starter	Grower	Finisher
Dry matter	912	915	924
N-free extract	485	500	520
• Starch	358	393	415
• Sugars	40	38	36
Crude protein	222	209	187
Crude fat	117	118	129
Crude ash	57	57	55
• Calcium	8.7	8.6	8.1
• Phosphorus	6.9	6.3	6.0
• Sodium	1.9	2.0	2.0
Crude fibre	31	31	33

Table 2. Ingredient composition (%) of starter, grower and finisher control feeds.

Ingredients	Starter	Grower	Finisher
Corn	56.49	59.26	62.21
Soybean meal	33.80	29.80	26.10
Soy oil	3.70	5.00	5.70
Sunflower oil	1.50	1.50	1.50
$CaCO_3$	1.48	1.48	1.46
$Ca(H_2PO_4)_2$	1.42	1.34	1.32
Premix*	1.20	1.20	1.20
DL-methionine	0.26	0.26	0.28
L-lysine-HCl	0.13	0.12	0.18
L-threonine	0.02	0.02	0.04
L-tryptophan	0.00	0.02	0.01

* kg^{-1} diet : 4800 IE vitamin A; 480 IE cholecalciferol; 50.4 mg vitamin E; 2.4 mg menadione; 2.4 mg vitamin B_1; 3.0 mg vitamin B_2; 42 mg niacin; 4.8 mg vitamin B_6; 0.04 mg vitamin B_{12}; 240 mg biotin; 18 mg pantothenic acid; 1.2 mg folic acid; 60 mg Zn, 90 mg Fe; 60 mg Mn; 14.4 mg Cu; 0.60 mg I; 0.48 mg Co; 0.42 mg Se; 1.6 g Na; 2.0 g Mg; choline: starter 1300 mg, grower: 1000 mg, finisher: 700 mg.

Health status and performance in target species

All broilers were observed daily for status of health. Monitored parameters included signs of disturbed behaviour and the presence of deviant excreta consistency. Mortality and culled birds were recorded daily and macroscopic necropsies were performed on dead or culled birds. Technical performance traits were assessed at pen level with bodyweight and feed intake determined weekly. At slaughter age (40 days of age), all birds were weighed individually (BW_{fin}). Cumulative average daily gain (ADG) and cumulative average daily feed intake (ADFI) were determined from measured values.

Cumulative feed-to-gain ratio or feed conversion ratio (FCR, equation [1]) and overall production value (PV, equation [2]) were calculated, as:

$$FCR = ADFI\ (g/d) / ADG\ (g/d) \quad [1]$$

$$PV = [(100 - mortality\ (\%)) \times BW_{fin}\ (g)] / [rearing\ period\ (d) \times FCR \times 10] \quad [2]$$

In addition to technical performance, carcass traits were evaluated in 8 replicate birds (1 per pen) from the control and recommended DMG dose groups. At slaughter age, 4 replicate female birds and 4 replicate male birds were fasted for 8 hours. Live bodyweight (LW) with empty crop was determined. Then birds were humanely euthanatized by concussion followed by neck-cutting. After slaughtering, the birds were immersed in hot water, mechanically plucked and manually eviscerated. The weight of abdominal depot fat was measured and the remaining carcass was chilled for 24 hours at 3°C in a cooling chamber. The heads, necks and feet at the hock joint were removed from the chilled carcasses before obtaining carcass for grilling (CfG) weights. Finally, the breast meat, legs (drumsticks and thighs) and wings were manually dissected and weighed to determine meat parts.

Haematology, clinical biochemistry and histopathology in target species

At slaughter age, fasted blood samples (2 ml) were collected to determine haematology and clinical biochemistry. Blood from 12 replicate broilers (6 males and 6 females) per dietary treatment (1 or 2 per pen) was sampled from the ulnar vein and immediately submerged in iced water (4°C). Samples were collected in 5 ml plastic tubes (Sarstedt, Nümbrecht) either without anticoagulant for serum separation or containing ethylene diamine tetra acetic acid (EDTA) for determination of haematological parameters. To obtain plasma, the tubes were centrifuged by 3,000 rpm for 10 minutes. Haemoglobin, erythrocyte and differential leukocyte count were analyzed using systemic K 4500 (Sysmex, Norderstedt, Germany). Next, plasma was analyzed for electrolytes (sodium, potassium, chloride, calcium and phosphorus) and the activities of alanine aminotransferase (ALT), asparatate aminotransferase (AST), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) were analyzed by flame photometry and spectrophotometric methods using AAS Vario 6 (Analytik Jena AG, Jena, Germany) and AU 800 Olympus (Olympus Diagnostica GmhH, Hamburg, Germany), respectively. Finally, the plasma metabolites cholesterol, triglycerides, uric acid, glucose, total protein and albumin were analyzed spectrophotometrically using AU 800 Olympus.

After blood collection, these 36 birds (12 per treatment) were humanely euthanized, as described previously and representative samples of liver, kidney and heart were taken. Immediately after excision, tissue samples were fixed in 4% buffered formalin, embedded in paraffin and cut into 5-7 µm slices. After conventional haematoxylin–eosin staining with an automatic Leica ST 4040 (Leica Microsystems Nussloch GmbH, Nussloch, Germany), tissue sections were evaluated using light microscopy. Samples were first screened for pathological changes. Liver sections were further examined for histopathological lesions which were classified according to the degree of hepatocellular vacuolisation, inflammatory cell infiltration and extra-medullar haematopoiesis.

Assessment of potential bioaccumulation of DMG in target species

At slaughter age, 8 broilers (1 per pen) from each of the three dietary treatment groups were humanely euthanized, as described above, after an 8-hour fasting period. Samples of full blood, kidney, liver, abdominal adipose tissue and breast muscle from each bird, as well as fresh excreta from each pen at study termination and subsamples of all test diets were analyzed for DMG content and for the content of its metabolite glycine. DMG and glycine were isolated from the freeze-dried samples by aqueous extraction. After drying, the DMG in the extracted residues was derivatised with N,O-Bis(trimethylsilyl)trifluoro-acetamide (BSTFA) to $(CH_3)_2NCH_2COO\text{-}Si(CH_3)_3$ and analyzed *via* gas chromatography using flame ionization detection (RDM0890). Glycine content was determined by derivation with 9-fluorenylmethoxycarbonyl chloride (FMOc) and analysis *via* liquid chromatography using UV detection (RDM0900). Both analyses were conducted by the Research & Development laboratory at Taminco NV, Belgium. To put DMG content in broiler tissue in perspective with common food items for human consumption, DMG content was also determined in: beef (sirloin steak), salmon (steak), shelled whole egg, whole milk, corn, wheat, wheat bran and spinach. Because these food items were raw when analysed, homogenates of three fully prepared customary meals for human consumption were also analyzed for DMG content. These meals included: (1) hamburger, beans and potatoes, (2) roast beef, peas, carrots and potatoes, and (3) chicken, rice and curry.

Statistics

Data on technical performance were statistically analysed with data per pen as experimental unit, whereas sampled birds were used as experimental unit for carcass traits and analysis on blood and tissue samples. Finishing bodyweight was measured per bird, and was thus also analysed with bird as experimental unit. Normality and homogeneity were tested with the Kolmogorov-Smirnov and modified Levine test, respectively. All parameters (excluding mortality) were analysed using one-way ANOVA, and if significant followed by Tukey post hoc tests to determine the significance of differences between groups. Mortality was not normally distributed, hence these data were analysed with the non-parametrical Kruskal-Wallis rank sum test. Average values are expressed as mean ± standard error of the means (SEM). All statistics were performed using S-PLUS 8.0 (TIBCO Software Inc., Palo Alto, California) and SPSS 16.0 (SPSS Inc., Chicago, Illinois). Significance was set at $p < 0.05$.

RESULTS

Health status and performance in target species

Mean starting weight was 39.4 ± 0.4 g and did not differ significantly between dietary treatment groups. Neither excreta consistency nor behaviour or general health showed visually noticeable aberrations in either treatment group. Overall mortality was only 0.83 ± 0.39 % and was not significantly different between treatments (**Table 3**). The number of dead and culled birds per dietary treatment and the reasons for culling are summarized in **Table 4**.

Table 3. Effect of dietary supplementation with DMG on overall performance in broilers at recommended (1,000 mg Na-DMG/kg feed) or tenfold dose (values are means of 8 replicates).

	Control	1,000 mg Na-DMG/kg	10,000 mg Na-DMG/kg	sem	*p*-value
BW_{fin}, g	2211[a]	2290[b]	2230[a]	11	0.011
Cumulative FCR					
• day 1-7	1.44	1.34	1.32	0.02	0.103
• day 1-14	1.50[a]	1.36[b]	1.43[ab]	0.02	*0.005*
• day 1-21	1.49	1.40	1.42	0.02	0.050
• day 1-28	1.50	1.45	1.47	0.01	0.286
• day 1-35	1.58	1.46	1.50	0.02	0.075
• day 1-39	1.62[a]	1.50[b]	1.54[ab]	0.02	*0.007*
Mortality, %	1.25	0.63	0.63	0.39	0.750
Production value	346[a]	391[b]	368[ab]	5	*0.003*

[a,b] different superscripts within a row indicate a significant effect at $p < 0.05$

Table 4. Mortality and culling of broilers fed a control diet, or the same diet supplemented with the recommended (1,000 mg Na-DMG/kg) or tenfold (10,000 mg Na-DMG/kg) DMG dose.

	Control		1,000 mg Na-DMG/kg	10,000 mg Na-DMG/kg
Number of dead birds	0		0	0
Number of culled birds	2		1	1
• age (d)	8	8	32	24
• bodyweight (g)	97	128	1289	588
• gender	female	male	male	male
• reason for culling	cachexia	cachexia	enlarged crop	enlarged crop
• post mortem findings	cachexia	cachexia	enlarged crop	enlarged crop

DMG: *N,N* dimethylglycine

The BW_{fin} and overall FCR of birds fed the diet supplemented with DMG at the recommended dose were significantly improved compared to birds fed the control diet. Herein, 1,000 mg Na-DMG resulted in a 4% increase in BW_{fin} and an 8% decrease in FCR relative to the control. The tenfold dose resulted in values which were in between those of control and recommended dose groups ($p > 0.05$). Moreover, except for the cumulative FCR during the first week, which was lowest when fed the diet supplemented with the tenfold dose, all cumulative feed-to-gain ratios were either numerically or significantly lowest in broilers fed the 1,000 mg Na-DMG/kg diet and highest when fed the control diet (**Table 3**). The effect of dietary DMG on FCR was most pronounced during the starter and finisher phases ($p < 0.05$) and resulted from a combined effect of lower feed intake and higher weight gain ($p > 0.05$) (**Table 5**). Finally, PV, the overall technical performance criterion, was significantly increased by 45 units or by 13% in broilers fed a diet supplemented with 1,000 mg Na-DMG/kg compared to the control diet group ($p < 0.01$). Feeding a diet supplemented with the tenfold DMG dose resulted in an intermediate PV (**table 3**). Carcass traits did not reveal significant differences between the control and recommended DMG dose groups (**Table 6**).

Table 5. ADFI, ADG and FCR of starter, grower and finisher phase in broilers fed a control diet, or the same diet supplemented with the recommended (1,000 mg Na-DMG/kg) or tenfold DMG supplementation (values are means of 8 replicates).

		Control	1,000 mg Na-DMG/kg	10,000 mg Na-DMG/kg	sem	*p*-value
Starter						
• ADFI	(g/d)	35	33	32	< 0	0.058
• ADG	(g/d)	23	25	23	< 0	0.110
• FCR		1.50[a]	1.36[b]	1.43[ab]	0.02	*0.005*
Grower						
• ADFI	(g/d)	92	92	91	1	0.867
• ADG	(g/d)	61	62	61	1	0.893
• FCR		1.51	1.49	1.49	0.02	0.845
Finisher						
• ADFI	(g/d)	159	147	151	2	0.118
• ADG	(g/d)	90	95	92	2	0.489
• FCR		1.76[a]	1.55[b]	1.64[ab]	0.03	*0.011*

DMG: *N*,*N*-dimethylglycine, ADFI: average daily feed intake, ADG: average daily growth, FCR: feed conversion ratio

[a,b] different superscripts within a row indicate a significant effect ($p < 0.050$)

Table 6. Carcass traits in broilers fed a control diet or the same diet supplemented with DMG at a recommended dose of 1,000 mg Na-DMG/kg feed (values are means of 4 replicates).

		Control	DMG	sem	*p*-value
Female					
Live weight (LW)	(g)	2135	2172	52	0.747
Abdominal depot fat	(%$_{LW}$)	2.0	1.5	0.1	0.052
Carcass for grilling (CfG)	(%$_{LW}$)	80.0	80.5	0.6	0.682
• Meat parts	(%$_{CfG}$)	63.2	63.4	1.7	0.961
▫ Breast meat	(%$_{CfG}$)	24.4	24.0	0.2	0.500
▫ Wings	(%$_{CfG}$)	10.9	9.8	0.6	0.406
▫ Legs	(%$_{CfG}$)	27.9	29.6	1.1	0.476
• Remainder	(%$_{CfG}$)	36.8	36.6	1.7	0.961
Male					
Live weight	(g)	2175	2240	36	0.412
Abdominal depot fat	(%$_{LW}$)	2.3	2.3	0.1	0.756
Carcass for grilling	(%$_{LW}$)	81.5	81.4	0.5	0.941
• Meat parts	(%$_{CfG}$)	61.4	62.0	0.7	0.683
▫ Breast meat	(%$_{CfG}$)	22.9	23.4	0.3	0.400
▫ Wings	(%$_{CfG}$)	10.2	9.8	0.2	0.501
▫ Legs	(%$_{CfG}$)	28.3	28.8	0.7	0.783
• Remainder	(%$_{CfG}$)	38.6	38.0	0.7	0.683

DMG: *N*,*N*-dimethylglycine

Haematology, clinical biochemistry and histopathology in target species

Erythrocyte and leukocyte counts, differential leukocyte counts and haemoglobin were similar between dietary treatments. Heterophil to lymphocyte ratio was 0.10, 0.10 and 0.14 in control, recommended and tenfold DMG dose, respectively (SEM 0.01, p = 0.253). Likewise, electrolytes, enzyme activities and metabolites in plasma of fasted birds (n = 12/group) were not significantly altered by dietary supplementation with DMG at either test dose (**Table 7**).

Table 7. Effects of dietary supplementation with DMG on blood cell numbers and plasma chemistry in broilers (values are means of 12 replicates).

			Control	1,000 mg Na-DMG/kg	10,000 mg Na-DMG/kg	sem	*p*-value
HAEMATOLOGY	Erythrocyte count	(10^6/mm^3)	2.32	2.37	2.34	0.01	0.384
	Leukocyte count	(10^3/mm^3)	29.8	23.1	27.0	2.7	0.055
	▫ Heterophils	(%)	8.83	8.50	10.83	0.81	0.460
	▫ Eosinophils	(%)	1.50	1.33	1.83	0.25	0.714
	▫ Monocytes	(%)	3.08	3.58	3.92	0.35	0.626
	▫ Lymphocytes	(%)	86.58	86.50	83.42	1.09	0.412
	▫ Basophils	(%)	< 0.00	< 0.00	< 0.00	< 0.00	-
	Haemoglobin	(mmol/L)	5.73	5.68	5.60	0.04	0.490
ELECTROLYTES	Sodium	(mmol/L)	141.92	142.17	142.75	0.44	0.740
	Potasium	(mmol/L)	12.99	12.93	12.29	0.25	0.480
	Chloride	(mmol/L)	111.17	111.17	111.33	0.32	0.972
	Calcium	(mmol/L)	2.83	2.80	2.83	0.02	0.738
	Phosphorus	(mmol/L)	2.56	2.66	2.65	0.04	0.449
ENZYMES	ALT	(µkat/L)	0.03	0.04	0.03	0.00	0.461
	AST	(µkat/L)	3.63	3.84	3.52	0.13	0.585
	GGT	(µkat/L)	0.34	0.34	0.39	0.02	0.487
	ALP	(µkat/L)	0.66	0.89	0.98	0.07	0.162
METABOLITES	Cholesterol	(mmol/L)	3.72	3.48	3.40	0.06	0.083
	Triglyceride	(mmol/L)	1.24	1.51	1.20	0.12	0.521
	Uric acid	(µmol/L)	299.50	310.92	268.17	11.21	0.280
	Glucose	(mmol/L)	13.47	13.11	12.58	0.17	0.083
	Total protein	(g/l)	29.91	28.21	28.26	0.43	0.187
	Albumin	(g/l)	12.65	12.28	12.16	0.21	0.627

DMG: *N*,*N*-dimethylglycine, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma-glutamyl transferase, ALP: alkaline phosphatase

Histological examination of kidney and heart sections showed normal tissue structures and evidence of mild extra-medullary granulopoiesis in all 36 chickens (n = 12/group) but no pathological changes were noted. Independent of dietary treatment group, most liver samples showed a discrete to marked hepatocellular vacuolization and about half the samples showed evidence of discrete to moderate extra-medullary granulopoiesis. In the control group, liver sections showed occasional heterophilic granulocyte (heterophil) infiltration in the sinusoidal spaces (n=5) and mild perivascular lymphocytic cell infiltration (n = 1). In four control birds, multifocal lymphocyte aggregations were seen. None of the control samples showed an increase in fibrous tissue, but one sample showed a focal area with extensive haemorrhage and parenchymal necrosis. In chickens fed a diet supplemented with 1,000 mg Na-DMG/kg, histopathological examination revealed occasional sinusoidal heterophils (n = 3) and multifocal lymphocyte aggregations (n = 6). No additional changes were noted in the samples of birds fed the recommended DMG dosage. Liver sections of birds fed the diet supplemented with the tenfold DMG dose showed occasional sinusoidal heterophils (n = 5) and multifocal portal lymphocyte infiltrations (n = 1). Three samples in this group showed mild bile duct proliferation. There was no evidence of hepatocellular damage or increase in fibrous tissue in liver sections of birds fed a diet supplemented with DMG at recommended or at tenfold dose (**Table 8**).

Assessment of potential bioaccumulation of DMG in target species

Overall DMG content was highest in fasted whole blood, followed by muscle tissue, whereas free glycine was predominantly present in liver and kidney tissue. DMG and glycine contents in excreta were similar between broilers fed the control diet and broilers fed the same diet supplemented with 1,000 mg Na-DMG/kg. But DMG content was significantly increased in excreta from birds fed a diet supplemented with the tenfold DMG dose ($p < 0.001$). Similarly, the DMG and glycine concentrations measured in muscle, liver, kidney and adipose tissue were similar between control and 1,000 mg Na-DMG/kg diet groups. The concentration of DMG in fasted whole blood showed a numerical increase when fed the diet supplemented with 1,000 mg Na-DMG/kg compared to the control. In comparison, dietary supplementation with DMG at a tenfold dose resulted in a significant increase in DMG content in fasted whole blood, meat, liver and kidney ($p < 0.001$ for all), whereas glycine content in hepatic tissue was significantly decreased ($p < 0.05$) (**Table 9**).

Table 8. Histopathological degree of hepatocellular vacuolization, extra-medullary haematopoiesis and inflammatory cell infiltration in liver samples of 39-day old broilers fed a control diet or the same diet supplemented with DMG at recommended (1,000 m g Na- DMG/kg feed) or tenfold dose (10,000 mg Na-DMG/kg feed) (n = 12).

	Control	1,000 mg Na-DMG/kg	10,000 mg Na-DMG/kg
• Hepatocellular vacuolisation[1]			
▫ none	0/12	0/12	0/12
▫ discrete	2/12	3/12	4/12
▫ mild	1/12	3/12	3/12
▫ moderate	7/12	3/12	4/12
▫ marked	1/12	2/12	0/12
total	11/12	11/12	11/12
total score[2]	29	26	22
• Heterophils in sinusoidal spaces	5/12	3/12	5/12
• Extra-medullar granulopoiesis			
▫ discrete	2/12	1/12	0/12
▫ mild	1/12	2/12	5/12
▫ moderate	2/12	3/12	0/12
total	5/12	6/12	5/12
total score[2]	10	14	10
• Lymphocyte infiltration			
▫ mild perivascular infiltration	1/12	0/12	0/12
▫ multi-focal periportal infiltration	0/12	0/12	1/12
▫ multi-focal aggregation	4/12	6/12	0/12

DMG: *N*,*N*-dimethylglycine
[1]diffuse, hepatocellular micro and macrovesicular infiltration
[2]sum of grading scores multiplied by number of birds graded with respective scores (grading scores: none = 0, discrete = 1, mild = 2, moderate = 3, marked = 4)

The DMG concentrations quantified in prepared meals for human consumption (30 - 40 µg/g) were comparable to the DMG concentration measured in the control broiler diet (40 µg/g) for this trial. Of all analysed individual raw food items, including the meat of broilers fed the 10,000 mg Na-DMG/kg diet (302 µg/g), spinach (380 µg/g) showed the highest DMG content. In comparison, salmon and beef had much lower DMG contents (20 and 50 µg/g, respectively) compared to broiler meat from the control group (156 µg/g) in the current trial. The content of DMG in the test diets was very close to the intended level (**Table 10**).

Table 9. Assessment of eventual bioaccumulation of DMG or its metabolite glycine in excreta of broilers and in tissue of fasted broilers when fed a control diet or the same diet supplemented with a recommended DMG dose of 1,000 mg Na-DMG/kg feed or a tenfold dose (10,000 mg DMG/kg) (values are means of 8 replicates).

		Control	1,000 mg Na-DMG/kg	10,000 mg Na-DMG/kg	sem	*p*-value
Excreta						
DMG	(µg/g)	30 [a]	37 [a]	620 [b]	63	*< 0.001*
glycine	(µg/g)	265	232	209	11	0.118
Full blood						
DMG	(µg/g)	278 [a]	408 [a]	663 [b]	40	*< 0.001*
glycine	(µg/g)	105	115	105	4	0.436
Meat						
DMG	(µg/g)	156 [a]	155 [a]	302 [b]	15	*< 0.001*
glycine	(µg/g)	302	269	299	9	0.225
Adipose tissue						
DMG	(µg/g)	0	5	9	3	0.361
glycine	(µg/g)	378	363	373	8	0.768
Liver						
DMG	(µg/g)	61 [a]	55 [a]	136 [b]	8	*< 0.001*
glycine	(µg/g)	1147 [a]	998 [a]	877 [b]	43	*0.029*
Kidney						
DMG	(µg/g)	47 [a]	55 [a]	124 [b]	8	*< 0.001*
glycine	(µg/g)	710	700	726	31	0.942

DMG: *N*,*N*-dimethylglycine, DM: dry matter basis
[a,b,c] different superscripts within a row indicate a significant effect ($p < 0.050$)

Table 10. DMG content in raw food items, prepared meals for human consumption and dietary treatments in the current broiler trial.

	DMG (µg/g)
Raw food items	
Beef (steak)	50
Salmon (steak)	20
Full egg (without shell)	not detected
Full-cream milk	not detected
Corn	not detected
Wheat	50
Wheat bran	300
Spinach	380
Prepared meals	
Hamburger, beans, potatoes	40
Roast beef, peas, carrots, potatoes	30
Chicken, rice, curry	40
Broiler diets	
Starter	
• control	40
• 1,000 mg Na-DMG/kg	1030
• 10,000 mg Na-DMG/kg	10140
Grower	
• control	40
• 1,000 mg Na-DMG/kg	1130
• 10,000 mg Na-DMG/kg	9850
Finisher	
• control	40
• 1,000 mg Na-DMG/kg	1040
• 10,000 mg Na-DMG/kg	11570

DMG: *N*,*N*- dimethylglycine

DISCUSSION

Dietary supplementation with DMG did not result in apparent health or behavioural problems at the tested doses, observations that are in agreement with those of other trials where DMG was added at 1000 mg Na-DMG/kg feed or less to broiler diets (Kalmar *et al*. 2010a,b; Kalmar *et al*., submitted). This is consistent with a clinical trial with human subjects in which DMG was orally administered at 300 mg per day for 14 days followed by 600 mg per day for another 14 days and a study using New Zealand white rabbits that were force fed DMG at 20 mg DMG per kg BW per day for 44 days (Gascon *et al*., 1989; Reap and Lawson, 1990). In the highest DMG dose group of the current trial, total daily intake of DMG was much higher compared to these previous studies, namely 329, 892 and 1745 mg DMG per day in starter, grower and finisher phase, respectively.

At the recommended DMG dose, in agreement with previous trials, significant improvements on technical performance traits concurred with preservation of carcass yield and dressing percentage. This denotes a true beneficial effect on production efficiency as compared to an increase in BW_{fin} or improved FCR as a result of water retention (e.g. ascites), increased fat deposition or increased organ weights (Kalmar *et al*., Submitted). Technical performance was not significantly influenced by the addition of DMG at the tenfold dose compared to the control. This indicates a high tolerance range for DMG in broilers. Moreover, the tenfold recommended DMG dose still showed numerically higher performance results compared to the control.

Leukocyte counts were numerically higher in control birds and DMG-supplemented birds at tenfold dose compared to DMG-supplemented birds at recommended dose, but values remained in all treatment groups well within the normal reference range of 19.8-32.6 10^3/ml (Apo, 2008). Differential leukocyte counts showed high similarity between treatments. Erythrocyte and haemoglobin concentration were also highly similar and remained within normal reference ranges of 2.2-3.3 10^6/ml and 5.52-8.39 mmol/l (Apo, 2008). Toxicologically, altered enzyme activity in plasma is of high importance because it not only reflects organ function, it also indicates leakage from cells resulting from damaged cellular membrane integrity (Danishefski, 1980; Hoffmann *et al*., 1989). Plasma enzymology is of special interest because increased activity in plasma may occur even without morphologic evidence of necrosis or cellular damage (Hoffmann *et al*. 1989). In avian species, plasma AST activity is considered a very sensitive, but not specific, indicator of hepatocellular damage

(Jaensch *et al*., 2000). In the current trial AST activity was similar between treatment groups. GGT activity was also not affected by dietary DMG; increased GGT activity in birds indicates cholestasis or biliary epithelial disorders (Harr, 2002). Non-specific cellular damage as indicated by increased plasmatic ALT activity was also absent in the current trial (Samour, 2008). Plasma ALP activity, on the other hand, was numerically increased when birds were supplemented with DMG, an effect that was likely the result of the numerically increased growth rate during the finisher phase (Meluzzi *et al*., 1991). Plasma electrolytes and metabolites were analysed to investigate eventual detrimental effects on functional organ capacity (Samour, 2008). Although most plasma electrolytes and metabolites were similar between treatment groups, glucose and cholesterol concentration in fasted plasma showed a non-significant ($p < 0.100$) decline when diets were supplemented with DMG at either test dose. A tendency for a higher glucose concentration in fasted plasma of control birds might possibly indicate a higher stress level (Carpenter *et al*., 2001). However, heterophil to lymphocyte ratio, a reliable indicator of chronic stress in birds, was similar in all treatment groups (Gross and Siegel, 1983; Maxwell, 1993). Moreover, heterophil to lymphocyte ratio and fasted glucose in control birds were only 0.10 and 13.5 mmol/l, respectively, whereas stress in birds is generally considered at respective ranges of 0.6-1.2 and 22.2-33.3 mmol/l (Carpenter *et al*., 2001; Siegel and Gross, 2000). Conversely, a fasted glucose concentration below 5.55 mmol/l might indicate hepatic dysfunction or neoplasia, but glucose concentration in DMG supplemented birds remained far above this very low glucose concentration (Carpenter *et al*., 2001). A numerically higher plasma cholesterol level in control birds might indicate a higher level of hepatic steatosis, which is in agreement with the slightly higher hepatic vacuolisation score in birds fed the control diet (Carpenter *et al*., 2001). Nonetheless, mean plasma glucose and cholesterol in all treatment groups remained within the normal reference ranges of 2.23-5.46 mmol cholesterol/l and 12.60-16.65 mmol glucose/l (Johnson-Delaney and Harrison, 1996).

Concordant to plasma metabolite and enzyme activity results, histological examination of liver, kidney and heart tissue did not reveal pathological changes. The degree of hepatocellular degeneration or necrosis and oval cell proliferation at the limiting plate are important parameters in toxicity studies. In the current trial, a discrete to marked degree of hepatocellular vacuolization, without evidence of apoptotic or necrotic hepatocytes was observed in the livers of 11 out of 12 chickens of each group. The presence of these intrahepatocytic micro- and macrovesicles are considered normal in broiler chickens (Hodges,

1974). Although foci of heterophils and lymphocytes in the liver can be associated with disease conditions such as ascites, they are a common and non-specific histological finding in normal broiler chicken livers (Maxwell *et al*., 1986; Crespo and Shivaprasad, 2003). In one control animal, a focal area with haemorrhage and parenchymal necrosis was detected in the liver, which is an incidental finding. Mild bile duct proliferation as seen in three liver samples at the tenfold dose, without concurrent evidence of hepatocellular necrosis or deviant plasma biochemistry can also be considered an incidental finding (Doupnik and Peckham, 1970).

As a result of growing governmental compulsion to decrease nitrogen pollution of surface water caused by animal production and in view of the increased competition for cereal grains between livestock and humans, the livestock industry is now more than ever driven to search for new feed additives (Nahm, 2007; Wheeler and Campion, 1993). Yet, increased public concerns towards possible deleterious effects of residues of feed additives in foods of animal origin require investigation of eventual bioaccumulation of new feed additives that may result in involuntary intake of those additives through consumption of animal products (Bird, 1961). Although DMG is a natural metabolite in human cells and oral treatment in human subjects does not show toxic effects, bioaccumulation in broilers as a result of dietary supplementation with DMG was investigated in the current trial (Gascon *et al*., 1989). The DMG content in excreta was found to be similar between control and recommended DMG groups, which indicates a high efficiency of absorption, as is described in literature (Cupp and Tracy 2003; Anonymous, 1996). Next, as DMG was vastly increased in excreta when dietary supplementation was tenfold the recommended dose, absorption was either incomplete at such high dosage or excess DMG was excreted to a certain degree. Further, although DMG tended to increase in whole blood when supplemented at the recommended dose, its concentration in meat and liver, as well as in adipose tissue and kidney, were similar to the control. Hence, consumption of chicken meat or liver from broilers fed diets added with DMG at the recommended dose does not imply increased DMG intake by consumers. In support of this assertion, the DMG contents of adipose tissue and kidney in control and recommended DMG dose groups show metabolisation of DMG instead of bioaccumulation. However, when supplemented at tenfold dose, DMG significantly increased in all tested tissues and its content in meat was almost two-fold compared to the control or recommended DMG dose groups. Nevertheless, even raw meat of broilers fed a diet supplemented with a tenfold DMG dose during the whole trial period contained an amount of DMG similar to the amount measured in uncooked wheat bran and far less than the amount present in uncooked spinach.

In conclusion, in agreement with previous studies, supplementation of a broiler ration with DMG at the recommended dose resulted in improved technical performances without compromising carcass traits. In female broilers, abdominal depot fat even tended to decline when supplemented with DMG. A tenfold recommended dose resulted in intermediate technical performances compared to controls and DMG supplementation at the recommended dose. The results of both haematology and plasma chemistry, as well as histological examination of liver, kidney and heart tissue did not reveal pathological changes or indications of a toxic effect of dietary supplementation with DMG at the recommended or tenfold dietary DMG dose. Moreover, DMG supplementation at the recommended dose did not accumulate in consumer parts of the target species, thus consumption of chicken meat or liver from these broilers will not increase DMG intake by the consumer. Additionally, chicken meat or liver from broilers supplemented at the tenfold dose did not contain a higher amount of DMG than other common food items, e.g. spinach. The current trial confirms previously reported favourable effects on the rearing efficacy of broilers, reveals a high level of tolerance and safety for DMG in the target species and demonstrates no consumer risk of unintentionally increased DMG intake through consumption of chicken meat or liver from poultry supplemented with DMG at the recommended dose of 1,000 mg Na-DMG/kg feed.

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CHAPTER 6

GENERAL DISCUSSION

A high incidence of broiler ascites syndrome remains a global problem in the modern broiler industry. This metabolic disorder results in a large economic loss, and is considered a severe welfare problem as its course progressively inflicts discomfort to the animal (Aksit *et al*., 2008; De Smit *et al*., 2005). A strong increase in the occurrence of this syndrome has resulted as a direct effect of improved rearing techniques and selective breeding towards fast growing, high meat yielding broiler strains (Julian, 1993; Rauw *et al*., 1998). On the one hand, these advances have resulted in a high metabolic rate, concomitant with a fast growth, implying a high oxygen requirement. On the other hand, selective breeding for high meat yield, led to birds with a disproportionately low heart and lung size, which consequently have a relatively underdeveloped capacity for blood oxygenation (Havenstein *et al*., 2003). Moreover, a high metabolic rate implies increased free radical mediated damage to the pulmonary vasculature, as well as a faster blood flow through the lungs, which both further diminish oxygenation of blood (Bottje and Wideman, 1995; Diaz-Cruz *et al*., 2003; Wideman and Kirby, 1995a and 1995b). As a result, an imbalance between oxygen demand and supply arises, which incites pulmonary hypertension and subsequently right heart failure, followed by transudation of fluid into the abdominal cavity, which is defined as ascites (Currie, 1999; Julian, 1998). (**Chapter 1**)

Selective breeding for ascites resistant strains is considered a permanent solution to the problem. Until resistant strains are successfully developed and implemented on a global scale, alternative measures remain imperative (Balog *et al*., 2003; Özkan *et al.*, 2010). Optimisation of the diet and rearing conditions, and development of new feed additives that ameliorate the ascites syndrome form such alternative measures. Important prerequisites herein include, next to a low economical cost, preservation of high growth rate, animal health and welfare, as well as product quality (**Chapter 1**). The first overall aim of this thesis was directed towards investigation of the usefullness of dietary supplementation with *N,N*-dimethylglycine (DMG) to attenuate broiler ascites syndrome. A study of Hariganesh and Prathiba (2000) in rats showed the free radical scavenging potential of DMG. Moreover, anecdotal reports claim enhanced oxygenation by DMG (Currell *et al.*, 2010). Therefore, this modified amino acid might form a valuable feed additive that attenuates broiler ascites syndrome.

As a result of increasing feed prices, the livestock industry is driven to develop new strategies to further improve feed utilisation (Gohin, 2008). Moreover, a growing pressure of society to decrease pollution of surface water caused by livestock manure additionally requires this industry to lower protein wastage in the form of faecal and urinary nitrogen losses (Nahm, 2007). In addition, hormonal growth promoters and feed antibiotics, which were formerly widely used to improve growth efficiency, have become increasingly regulated in view of consumer and environmental safety (Steinfeld, 2003; Lević *et al.*, 2007). Hence, alternative measures ought to be developed to achieve improved feed utilisation and in particular protein efficiency. The second main goal of the current work included the investigation, if at all, and to which dose-response relationship, dietary DMG can contribute to meet these challenges. As DMG esters possess surfactant properties (Clapes and Infante, 2002), DMG might enhance emulsification of nutrients at the gut level. This way, nutrients can be rendered more available for digestive enzymes and the absorptive brush border of the small intestine, which would improve nutrient digestibility. Also, demethylation of DMG offers an alternative route, next to catabolisation of choline and betaine, for remethylation of homocysteine to methionine (Slow *et al.*, 2004). Therefore, DMG might either directly, through improved nutrient digestibility or by its methyl donation function, or indirectly, through a sparing effect on choline and betaine, support the intense metabolism of fast growing broilers and hence improve their performance.

In case desirable traits of DMG can be demonstrated once, the forthcoming aim arises to assess repeatability of these results when using different broiler strains, basal diets and general rearing conditions. As a final aim, as mentioned before, animal and consumer safety, as well as product quality, have to be investigated in order to evaluate the appropriateness of the application of DMG as a feed additive to broiler rations.

1. DIETARY DMG ATTENUATES BROILER ASCITES SYNDROME

Cold environmental temperature conditions of 15°C from day 14 until slaughter age at 40 days, with concomitantly a high energy diet through 4% addition of corn oil to a standard broiler ration, successfully induced broiler ascites syndrome in the challenge trial (**Chapter 2:** Kalmar *et al*., 2010a). Herein, fulminant ascites caused death to 9.6% and 6.3% of birds in the control (0 mg Na-DMG/kg feed) and DMG (167 mg Na-DMG/kg feed) groups (standard error of the means [SEM] = 3.0%), respectively. In addition, pulmonary hypertension was present in 44.8 and 14.6% of apparently healthy control and DMG-supplemented birds (SEM = 6.6%), respectively.

As ascites and growth rate are positively interconnected, it is important to note that the significant decrease in pulmonary hypertension on account of a low dose of DMG did not result from slower growth rate. In contrast, the protective effect of dietary DMG against broiler ascites syndrome likely resulted from the vast decrease in plasma level of non-esterified fatty acid (NEFA). This is hypothesised based on the fact that NEFA induce vascular effects and inflict endothelial dysfunction through which arterial pressure is increased and oxygenation impaired, which both trigger pulmonary hypertension (Avagaro *et al*., 2003; Sarafidis and Bakris, 2007; Zoer *et al*., 2009). Hence, the over 50% reduction in plasma NEFA level in DMG-supplemented birds, compared to the control, may have resulted in a concomitant decrease in NEFA-inflicted vascular effects, through which progression towards broiler ascites syndrome was abated. Moreover, *in vivo* data of Sainsbury *et al.* (2004) presume oxidative stress to be at the basis of the detrimental effect of increased plasma NEFA levels on endothelial dysfunction. The observed effect of DMG on NEFA suggests an indirect, local anti-oxidant effect of DMG. The current trial could however not substantiate a systemic anti-oxidant effect of DMG at this low dosage of 167 mg Na-DMG per kg feed.

The underlying mechanism of the pronounced decrease in plasma NEFA level through supplementation with DMG remains to be elucidated. However, a decrease in fat mobilisation rate is unlikely because the relative abdominal fat mass was not higher in DMG supplemented broilers, while both feed intake and apparent fat digestibility were comparable between dietary treatments. In addition, the over two fold higher NEFA level in the plasma of control birds cannot be explained by an elevated release of NEFA from, and in conjunction with, an increase in abdominal fat mass (Sarafidis and Bakris, 2007). As the latter was only slightly and moreover, not significantly, higher in control compared to DMG supplemented birds.

Hence, dietary DMG must either have enhanced fatty acid oxidation or must have lowered lipogenesis in order to reduce plasma NEFA level.

Enhanced fatty acid oxidation may have resulted from a choline sparing effect of DMG, through which the choline mediated increase in tissue carnitine uptake was augmented, and in turn, the intra-mitochondrial transport and hence oxidation of long chain fatty acids facilitated (Daily and Sachan, 1995; Dodson and Sachan, 1996). Attenuated lipogenesis, on the other hand, is also plausible. Betaine, the direct precursor of DMG, has been demonstrated to reduce the expression of lipogenic enzymes through altered DNA-methylation (Huang *et al.*, 2008). A betaine sparing effect of DMG might thus have promoted this betaine effect. In addition, as demonstrated for other methylamine derivatives, DMG might also have directly affected gene expression through DNA-methylation (Emmert *et al.*, 1996; Niculescu *et al.*, 2006).

A sparing effect of DMG on choline and betaine is highly likely. Firstly, oxidative demethylation of DMG provides for an alternative source of methyl-groups, needed for remethylation of homocyteine into methionine, next to the catabolisation of choline into betaine and further on into DMG (Slow *et al.*, 2004). Moreover, a negative feed-back mechanism strongly inhibits betaine homocysteine methyltransferase activity. In other words, oxidative demethylation of betaine is inhibited by its reaction product DMG (Finkelstein *et al.*, 1972). Betaine itself, in turn, effectively spares choline in broilers on a 1 to 1 molar basis (Klasing, 2000). Therefore, dietary supplementation of DMG might well have spared betaine and choline from being catabolised to serve as a methyl donor, and hence increase the availability of these metabolites for above mentioned metabolic functions.

In the dose-response trial reported in **Chapter 3** (Kalmar *et al.*, 2010b), broilers were raised under thermo-neutral environmental conditions from day 1 until slaughter at the age of 42 days. DMG was supplemented at a dose of 0, 100, 200, 500 or 1,000 mg Na-DMG per kg feed to a basal ration with either animal fat (chicken fat) or vegetal oil (soy oil) as the main fat source. In agreement with the challenge trial, DMG significantly lowered plasma NEFA levels. The dose-response relationship showed to be linear, and was independent of dietary fat source. At the highest DMG dose, the effect on plasma NEFA was -36% relative to the control ($\%_{rel\ \Delta}$). The extent of the effect was thus not as high as compared to the challenge trial, but this coincided with an already lower plasma NEFA level in the control birds of the

current dose-response trial. Mortality averaged only 3.6% (SEM = 0.7%), which was also much lower compared to the challenge trial, and was neither influenced by dietary fat source nor by DMG. Progression towards pulmonary hypertension, as indicated by ascites heart index (AHI), was also not influenced by dietary fat source. In contrast, DMG dose showed a quadratic effect on AHI in the vegetal fat diets. Herein, the minimal AHI, as indicated by the least right ventricular hypertrophy, was reached at a DMG dose of 500 mg Na-DMG/kg feed.

In the vegetal fat diets, DMG linearly reduced plasma thiobarbituric acid reactive species (TBARS), which is an indicator of the extent of systemic lipid peroxidation and free radical activity (Holley and Cheeseman; Yagi, 1984). Plasma TBARS were reduced by up to 56 $\%_{rel\ \Delta}$ at the highest DMG dose. As oxidative stress is involved in the pathogenesis of pulmonary hypertension (Bottje and Wideman, 1995; Nain *et al.*, 2008), and DMG linearly reduced TBARS, it would be expected that DMG would as well have linearly reduced pulmonary hypertension. The quadratic, instead of linear effect of DMG on this syndrome likely resulted from an ascites-inciting side-effect from a concomitant positive linear effect of DMG on broiler productivity in the vegetal fat groups. Improved productivity is expected to increase oxygen requirement, and thus elicits pulmonary hypertension (Julian, 1998). Thus, the higher the DMG dosage, the higher the productivity and consequently the higher the oxygen requirement will be, and in turn the more pulmonary hypertension is elicited. Nevertheless, although the minimal AHI was reached at a dose of 500 mg Na-DMG/kg feed, a dose of 1,000 mg Na-DMG/kg feed showed a lower AHI compared to the control.

The reason why DMG significantly lowered both TBARS and AHI in the vegetal fat groups, but not in the animal fat groups remains unclear. However, the overall, 22% higher plasma TBARS level in the vegetal compared to the animal fat groups ($p > 0.05$), might have contributed. Anti-oxidant contents, e.g. vitamins A and E, in the fat sources were not assessed. Hence, interactions with dietary anti-oxidants can not be excluded. The numerical difference in plasma TBARS level between vegetal and animal fat diets did not coincide with differences in AHI. This can be explained by two counteracting effects inherent to the higher polyunsaturated fatty acid (PUFA) content in the vegetal compared to the animal fat diets. As is demonstrated in human trials, and in agreement with the current data, high dietary PUFA levels result in an increase in plasma indices of oxidative stress (Brown and Wahle, 1990; Nair *et al.*, 1993; Jenkinson *et al.*, 1999), which can trigger pulmonary hypertension (Bottje and Wideman, 1995; Nain *et al.*, 2008). Second, a higher ratio of dietary unsaturated to

saturated fatty acids increases erythrocyte fluidity, through which blood flow resistance and hence pulmonary hypertension is diminished (Bond *et al.*, 1996; Walton *et al.*, 1999). These two effects of dietary PUFA counteract each other in the progression towards right ventricular hypertrophy. Important to note is that the *n*-3 to *n*-6 ratio was highly similar between the vegetal and the animal fat diets. This is imperative with respect to the former reasoning, as a high dietary *n*-3 to *n*-6 ratio is suggested to abate broiler ascites syndrome through augmented release of endogenous coronary relaxants and diminished production of inflammatory compounds from cellular membrane lipids (Bond *et al.*, 1996; Walton *et al.*, 1999).

Comparison of diets with a more extreme fatty acid profile might have elucidated a modulating effect of dietary fat source on the DMG effect. However, as dietary fatty acid profile is reflected in poultry meat, health and sensory traits inherent to the fatty acid profile of various fat sources contribute to their suitability as feed ingredient (Özpinar *et al*., 2003; Wood *et al*., 2002). Beef tallow and to a lesser extent pork lard, for instance, are much more saturated compared to chicken fat (Bureau *et al*., 2002). However, ingestion of high amounts of saturated fatty acids through meat consumption is considered to augment the risk of coronary heart disease and development of various cancers to man, rendering these fat sources less appropriate to be incorporated in poultry rations (Wood *et al*., 2002). Although soy oil is a superior source of total PUFA, fish oil provides a more favourable *n*-3 PUFA profile and *n*-3 to *n*-6 ratio, which reduce the risk of inflammatory diseases (Özpinar *et al*., 2003; Rymer and Givens, 2005). Then again, very long chain *n*-3 PUFA enrichment of poultry meat through the use of fish oil may unfavourably affect shelf life and organoleptic quality as a result of a more rapid formation of rancid flavours and off-odour by-products of lipid peroxidation, compared to vegetal oil derived PUFA's (Bou *et al*., 2005; López-Ferrer *et al*., 1999; Özpinar *et al*., 2003; Rymer and Givens, 2005). Moreover, dioxins and polychlorinated biphenols (PCBs), which pose human health risks associated with carcinogenesis, are lipophilic and bioaccumulate in animal fat and fish oil (Eljarrat *et al.*, 2002; WHO, 1999). Hence, dioxin and PCB contamination in fat suitable for inclusion in animal feeds is much lower in vegetal oil as compared to animal fat, but highest in fish oil, especially European fish oil (Ábalos *et al*., 2008; EC, 2000).

The work described in the current thesis has, besides the finding of a considerable attenuating effect of DMG on development of broiler ascites syndrome, also advanced the methodology to study this syndrome. By calculation of an AHI on a dry matter (AHI_{DM}) instead of a fresh

matter basis (AHI_{FM}), the statistical power to detect differences in pulmonary hypertension was greatly improved. The challenge trial revealed a correlation coefficient (r) of 0.98 between AHI_{DM} and AHI_{FM}, however, statistical power in this trial was only 34% for AHI_{FM} but 85% for AHI_{DM}. The higher statistical power is likely due to an increase in the range of AHI after freeze-drying, which increases its sensitivity. Regression analysis determined an AHI_{DM} cut-off value of 0.30 (Kalmar *et al*., 2010a) to be equivalent to the AHI_{FM} cut-off value of 0.27 (Huchzermeyer and De Ruyck, 1986; Peacock *et al*., 1988) in the diagnosis of right ventricular hypertrophy.

2. DIETARY DMG IMPROVES BROILER PERFORMANCE

In the challenge trial (**Chapter 2**), it was confirmed that dietary DMG improves apparent faecal digestibility (AFD) of crude protein and N-free extract by $+6.3\%_{rel\ \Delta}$ and $+13.0\%_{rel\ \Delta}$, respectively. AFD of crude fat was already high in the control group (92.7%), and was not affected by dietary DMG. As DMG is known to possess surfactant properties (Clapes and Infante, 2002), the positive effect on AFD of non-fat fractions was likely attributable to an emulsifying action of DMG at the gut level. As reviewed and experimentally confirmed by Dierick and Decuypere (2004), the available literature on pigs also in general describes more positive effects of emulsifiers on AFD of non-fat fractions than of the fat fraction of the diet. Likewise, addition of 1,000 mg Na-DMG per kg feed resulted in sows in a $+8.3\%_{rel\ \Delta}$ increase in AFD of crude fat, which averaged 84% in the control group, but this supplementation dosage resulted in a more profound increase in AFD of crude protein and N-free extract, namely +20.9 and $+16.0\%_{rel\ \Delta}$, respectively (Cools *et al*., 2010). An enhanced, or rather more proximal emulsification of dietary fat through the action of a surfactant, such as DMG, likely diminishes fatty insulation of non-fat nutrients. In turn, proximal liberation of these non-fat nutrients from a fatty coating renders them available for enzymatic digestion and absorption through the intestinal brush border sooner, through which AFD is improved. As lipid digestion increases with age, and in particular, digestion of vegetal fat sources is known to be impaired in chickens until two weeks of age (Freeman *et al.*, 1976; Krogdahl, 1985), assessment of AFD in the starter instead of the finisher age group would possibly have revealed an even greater DMG response.

The improvement in AFD of crude protein in the DMG supplemented group did not result in an increase in uric acid excretion. Therefore, instead of being catabolised for energy supply, the augmented supply of amino acids to the body must have supported lean tissue gain.

Enhanced anabolic metabolisation of amino acids, on top of improved AFD of crude protein, is demonstrated by a -14%$_{rel\,\Delta}$ decrease in total nitrogen to inert marker ratio in excreta of DMG supplemented birds. As a consequence, the nitrogen load of broiler manure was substantially diminished through dietary DMG. Besides a decrease in feed cost, the diminished nitrogen waste through faecal and urinary losses provides for an environmental benefit (Nahm, 2007). Yet, improved AFD on account of a low dosage of dietary DMG was not reflected in significantly improved technical performances in the challenge trial. At a higher dosage, in contrast, as demonstrated in the dose-response trial (**Chapter 3**), dietary DMG increased production value by up to +11%$_{rel\,\Delta}$ at 1,000 mg Na-DMG per kg feed. This positive effect on overall technical performance was only present in the ration with vegetal oil as main added fat source, where it showed a linear dose-response relation within the tested range of 0 to 1,000 mg Na-DMG per kg feed. Additionally, abdominal depot fat linearly decreased by up to -38%$_{rel\,\Delta}$ and meat yield tended to linearly increase by up to +6%$_{rel\,\Delta}$ in the entirely vegetal diet.

In order to verify if positive effects of dietary DMG on broiler performance can be repeatedly demonstrated, a series of efficacy trials were performed at three distinct European locations in which broiler strain, rearing conditions and basal diet were each common to the region of the trial site (**Chapter 4**: Kalmar *et al.*, submitted a). The chosen dosage of added DMG to the basal rations was 1,000 mg Na-DMG per kg feed, which was based on the previously revealed linearity of dose-response relations on broiler performance traits, within the range of 0 to 1,000 mg Na-DMG per kg feed. Cumulative feed conversion ratio (FCR) in the control groups showed a wide range of basal flock efficiency, ranging from outstanding (FCR = 1.54), to satisfactory (FCR = 1.69) to rather inefficient (FCR = 1.80). Still, in all three trials, DMG consistently enhanced FCR, in which improvements ranged from -2.0 to -3.9%$_{rel\,\Delta}$. These effects on FCR were generally most pronounced in the earlier growth phases, which is in agreement with the above reasoning. Furthermore, finishing bodyweight and production value were increased in one (+5.5%$_{rel\,\Delta}$) and two (+6.8%$_{rel\,\Delta}$ and +5.9%$_{rel\,\Delta}$) out of three trials, respectively. With regard to slaughter performances, carcass for grilling was not affected by dietary DMG, but one trial revealed a +3.3%$_{rel\,\Delta}$ increase in breast meat yield, which is the most valuable cut yield in broiler carcasses. In one of the other trials, breast meat yield only tended to increase by +2.3%$_{rel\,\Delta}$, but abdominal depot fat vastly lowered by -20.5%$_{rel\,\Delta}$, which resulted in an increase in a meat to fat ratio by +24.7%$_{rel\,\Delta}$.

One of the efficacy trials also included evaluation of organoleptic quality of roasted breast meat, which revealed similar sensory appraisal of roasted breast meat in terms of tenderness, juiciness and taste between control and DMG-supplemented birds.

The consistent presence of positive effects of dietary DMG on several broiler performance traits is likely contributable to a combination of working mechanisms. On the one hand, improved AFD, resultant from an emulsifying action of DMG at the gut level, renders more nutrients and energy to the body, which contributes to an enhanced FCR. On the other hand, leaner growth, as indicated by a higher ratio between meat yield and abdominal depot fat, further improves FCR. This can be explained by a higher energetic cost of fat accretion per mass unit compared to lean accretion (protein plus water).

There are several reasons for the observed leaner growth. First, increased bioavailability of amino acids, through higher protein digestibility, not only increases the supply of building blocks for protein accretion, but also lowers accretion of depot fat. The latter has been demonstrated by Namroud *et al*. (2008) who revealed an inverse relation between dietary protein content and abdominal depot fat in broiler chickens, which coincided with a more efficient FCR. Second, a choline sparing effect of dietary DMG, as described above, might have indirectly facilitated mitochondrial long chain fatty acid oxidation through an increase in choline-mediated carnitine tissue uptake (Daily and Sachan, 1995; Dodson and Sachan, 1996). Facilitated fatty acid oxidation, in turn, lowers its deposition in depot fat. In addition, it lowers the metabolic demand of amino acids catabolism for energy supply, which renders amino acids more available for protein accretion. As demonstrated in the challenge trial, lower amino acid catabolism through dietary supplementation with DMG is further substantiated by diminished uric acid excretion whilst increased nitrogen bioavailability, which indicates enhanced anabolic nitrogen utilisation. Third, altered DNA-methylation might as well have diminished accretion of depot fat through a reduction in the expression of lipogenic enzymes. This could be either directly or indirectly in consequence of a betaine sparing effect (Huang *et al.,* 2008). Finally, in relation to the methylation capacity of DMG, enhanced remethylation of homocysteine to methionine might have spared methionine, which is often the first limiting amino acid in broiler diets (Klasing, 2000; Slow *et al.*, 2004).

3. TOLERANCE, SAFETY AND BIOACCUMULATION OF DMG IN BROILERS

Previous clinical trials on human subjects and New Zealand white rabbits did not reveal adverse health effects of orally administered DMG in these species (Gascon *et al*., 1989; Reap and Lawson, 1990). Unfavourable effects of dietary DMG on broiler health are thus not expected. Still, the recommended dosage in standard broiler rations, which is based on the results of the dose-response trial and substantiated by the efficacy trials, is much higher compared to the experimental setups of these literature data. Moreover, although detrimental effects were never observed in the previous broiler trials, toxicity of DMG had never been explicitly evaluated in avian species before. Hence, a tolerance and safety trial was performed (**Chapter 5**, Kalmar *et al*., submitted b). Herein, 1-day-old broiler chicks were fed one of three test diets added with 0, 1,000 or 10,000 mg Na-DMG per kg feed until slaughter age, representing the negative control, supplementation at the recommended dosage and at the tenfold of the recommended dosage, respectively. Besides evaluation of tolerance through assessment of technical performance traits, indicators of adverse health effects were investigated in order to evaluate safety of DMG in the target species. Production performance was significantly improved at the recommended dosage and was at the tenfold dosage in between this of the negative control and the recommended dosage. This demonstrates a wide tolerance range for dietary DMG in broiler chickens. Blood cell counts, plasma enzymology and histological examination of liver, kidney and heart samples did not reveal indications of pathological changes due to dietary DMG. These data substantiate a wide safety range of dietary DMG in broilers, and are in agreement with earlier reports in mammals, in which oral DMG neither evoked adverse health effects (Gascon *et al*., 1989; Reap and Lawson, 1990).

In response to a general concern of unintentional consumption by humans of residues of animal feed additives (Bird, 1961), bioaccumulation of DMG was assessed in broiler tissues from the tolerance and safety trial. To facilitate interpretation of the results, DMG content was also determined in several unrelated food items for human consumption. Although DMG content was below the detection limit in dairy products - yolk, egg white and full-cream milk - and corn, it was detected as naturally present in most analysed food items. Herein, salmon steak, beef steak and wheat contained only low levels: 20, 50 and 50 µg DMG per kg, respectively; whereas wheat bran and spinach contained much more DMG: 300 and 380 µg DMG per kg, respectively. These data represent values of uncooked (raw) samples.

DMG content in raw breast meat of chickens was much higher compared to beef or salmon steak and averaged 156 and 155 µg per kg meat of control birds and birds fed 1,000 mg Na-DMG per kg feed for the whole rearing period, respectively. Within these groups, DMG content in abdominal depot fat was nearly absent and was one third the content of meat in liver and kidney samples. Therefore, DMG did not appear to accumulate in chicken meat, fat, liver or kidney when supplemented to diets of poultry at the recommended dosage. Excreta samples as well contained similar DMG levels in control and recommended DMG dosage groups, and averaged 30 and 37 µg DMG per kg in the respective groups. In whole blood, on the other hand, DMG was, although not significantly, much higher in the 1,000 mg Na-DMG per kg feed group compared to the control: 408 compared to 278 µg DMG per kg. This confirms a high absorption rate of intact DMG through the gut wall, which was as expected. Moreover, DMG is a small, water-soluble molecule with sufficient lipophilic properties to cross cellular membranes (Cupp and Tracy, 2003). Also, these data indicate that in broilers, dietary DMG at a dosage of 1,000 mg Na-DMG per kg feed is metabolised after absorption, instead of being excreted or accumulated in body tissues.

DMG was either less well absorbed across the gut wall or to a higher extent excreted at tenfold of the recommended dosage, as excreta contained excessively high DMG levels (620 µg DMG per kg) compared to the other test groups (30 to 37 µg DMG per kg). Nevertheless, at this dosage, DMG accumulated in meat, liver, kidney and whole blood. DMG level in adipose tissue, in contrast, remained very low. Bioaccumulation was, except for whole blood, the highest in meat, in which DMG content was almost twofold compared to the control or the 1,000 mg Na-DMG per kg feed group. Still, the resulting DMG content of 302 µg DMG per kg raw meat reached only a similar value as in wheat bran and remained far below the natural content of, for instance, uncooked spinach.

At the recommended dosage, DMG content remains unaltered in the consumer parts of broiler chickens. Hence, supplementation of broiler diets with 1,000 µg Na-DMG per kg feed does not appear to pose a risk of increased DMG intake through consumption of chicken meat or liver. Moreover, raw meat or chicken liver reared on a ration in which DMG is included at tenfold the recommended dosage does not result in higher DMG concentrations compared to uncooked, regular spinach. In addition, cooking might decrease DMG content of these ingredients, but this was not investigated in detail.

CONCLUSION

The current work showed that DMG can to a large extent attenuate broiler ascites syndrome, and improve technical and slaughter performance in broilers. In addition, it was demonstrated that dietary DMG is safe and well-tolerated in the target species, does not alter organoleptic characteristics of meat, and does not accumulate in consumer parts at the recommended dosage of 1,000 mg Na-DMG per kg feed. Also, when added at a tenfold dosage, the resulting DMG content in raw chicken meat was much lower then found in, for instance, uncooked spinach. In conclusion, DMG revealed to be a valuable feed additive to broiler diets.

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SUMMARY

Important objectives in the meat-type broiler industry remain to improve feed conversion ratio (FCR), mortality rate and carcass yield. Moreover, alleviating welfare problems is currently considered essential in enhancing animal production systems. Metabolic disorders may give rise to a compromised animal welfare next to financial losses due to deterioration of production efficiency as a result of increased mortality rate, less efficient utilisation of nutrients, or to condemnation of carcasses. Potential means to achieve the above mentioned goals comprise genetic improvement and refinements in rearing conditions, next to optimising nutrition, including development of new feed additives. The main objective of the current research was to evaluate the potential of *N*,*N*-dimethylglycine (DMG), a naturally occurring metabolite in living organisms, as a new feed additive in broiler diets. A first aim was to examine if DMG alleviates pulmonary hypertension syndrome or broiler ascites syndrome, whereas a second aim was to investigate if DMG enhances nutrient digestibility. A third aim included determination of the dose-response relationship of DMG on technical performance traits and health in broilers and the effect of dietary fatty acid profile. A fourth aim was to evaluate persistency of DMG effects in different rearing conditions, basal diets and broiler strains. The final objective was to evaluate tolerance, safety and bioaccumulation of dietary DMG in the target species.

The first two aims were addressed in a challenge trial in which a total of 64, 14-day-old broiler hens (Ross-308) were randomly assigned to two groups of eight replicate pens of four birds each. Test diets contained 0 or 167 mg Na-DMG per kg feed. The broilers were raised under cold environmental temperature conditions and fed a high energy diet in order to incite pulmonary hypertension syndrome. The main objectives of this study were to evaluate the effect of dietary supplementation with a low dosage of DMG on incidence of pulmonary hypertension syndrome and on nutrient digestibility in broilers. The results showed an important protective effect of DMG on progression towards broiler ascites syndrome as demonstrated by a reduction in incidence of pulmonary hypertension from 44.8% in the control group to 14.6% in the DMG group. This effect is likely related to an effect of DMG on the fat metabolism through which plasma non-esterified fatty acids (NEFA) increased significantly less with age in the DMG groups. This hypothesis is based on the noxious effect of NEFA on the pulmonary vasculature, which eventually leads to endothelial dysfunction and progression towards pulmonary hypertension. As DMG strongly diminished plasma NEFA level in the finisher phase of broilers, this likely coincided with an equivalent reduction in NEFA-mediated damage to the pulmonary vasculature through which pulmonary

hypertension was abated. Next, dietary DMG supplementation resulted in a significant increase in apparent faecal digestibility of crude protein from 81.4 to 86.5% and nitrogen-free extract from 62.5 to 70.6%. The most likely working mechanism to explain these findings is the surfactant potential of DMG, through which nutrients become less insulated by fat droplets, rendering them more available for digestive enzymes and the absorptive brush border of the small intestine. Concomitantly to improved protein digestibility, nitrogen emission was significantly reduced by dietary supplementation with DMG.

A second trial addressed the third aim and entailed 10 dietary treatments, which were tested in a complete block design with 5 replicate pens of 30 1-day old broilers (Cobb-500) per treatment. Herein, 5 dosages of DMG, ranging from 0 to 1,000 mg Na-DMG were tested in broiler diets with one of two fat sources: animal or vegetal. The main conclusions were that dietary supplementation with DMG can improve technical and slaughter performance, and may reduce oxidative stress and pulmonary hypertension, but the degree of the effect is modulated by fatty acid profile of the diet. In this respect, effects are more pronounced in a diet rich in polyunsaturated fatty acids (PUFA) compared to a diet rich in saturated and monounsaturated fatty acids (SFA and MUFA). A recommended dosage of 1,000 mg Na-DMG per kg feed was suggested based on this trial.

The next trials intended to assess persistence of efficacy of DMG at the recommended dosage to improve broiler performance over a range of broiler strains, rearing conditions and basal flock efficiency. Three trials were conducted at 3 European locations: trial 1 in Settimo Torinese, Italy, trial 2 in Kraig, Austria and trial 3 in Berlin, Germany. In all trials, 1-day-old chicks were randomly divided over several pens with birds having *ad libitum* access to one of two diets during the entire rearing period. The test diets were either a control diet or the same diet supplemented with the recommended dosage of DMG. Control diets, broiler strain, and rearing conditions as well as period were according to standard practices of the respective region. Technical performance (feed conversion ratio [FCR], finishing bodyweight [BW_{fin}] and production value [PV]) and carcass traits were determined. In each trial, two of three tested technical performance traits were significantly improved by DMG. Moreover, meta-analysis showed an overall, significant improvement in FCR, BW_{fin} and PV by -2.8%, +2.3% and +4.3%, respectively. Organoleptic quality was similar between treatment groups and, irrespective to trial site, DMG supplementation resulted in an overall increase in breast meat yield and meat to fat ratio by +2.2 and +12.6%, respectively.

The final study included a tolerance and safety trial to investigate eventual deleterious effects of DMG on broiler health and performance traits at the recommended or ten times the recommended dosage. Furthermore, bioaccumulation of DMG was examined. The trial consisted of 3 treatment groups with each 8 replicate pens of 20, 1-day old broiler chicks (Cobb, Avimex GmbH) that were fed 1 of 3 test diets until slaughter age at 39 days. The treatment groups were: control (0 mg Na-DMG per kg), the recommended dosage of 1,000 mg Na-DMG per kg and a tolerance dosage of 10,000 mg Na-DMG per kg. Technical performance parameters were recorded at pen level and 12 replicate blood samples were obtained at slaughter age for determination of cell counts, hemoglobin, electrolytes, enzymes and metabolites in plasma. Liver, heart and kidney samples of the 12 replicate chicks were histopathologically examined. The recommended DMG level showed a significant improvement in BW_{fin}, FCR and PV compared to the control, similar to the results found in the previous trials. Technical performance of the chicks fed the tolerance dosage was not further improved but was in between those of the control and recommended DMG groups. Neither of the assessed blood parameters showed a significant difference between treatment groups. Pathological changes in target organs were absent in either treatment group. Bioaccumulation occurred in blood at both doses, and in meat, liver and kidney at the tenfold dose. Nevertheless, DMG content in raw meat at the tenfold dose was comparable with wheat bran and much lower than uncooked spinach. In conclusion, the experimental results showed dietary DMG to be well-tolerated and safe in broilers.

As a general conclusion, the trials revealed DMG to be a valuable feed additive in broiler diets that is safe and well-tolerated in the target species and does not accumulate in consumer parts at a recommended dosage of 1,000 mg Na-DMG per kg feed. This new feed additive effectively abates progression towards broiler ascites syndrome, which is a metabolic disease that causes large financial losses as well as impaired animal welfare. In addition, DMG improves nutrient digestibility and lowers nitrogen load of poultry manure. Finally, DMG enhances feed utilisation, even in high performing birds, and may improve slaughter performance.

SAMENVATTING

Optimalisatie van voederefficiëntie en karkaskarakteristieken, alsook het verlagen van het sterftecijfer, vormen nog steeds belangrijke objectieven in de opfok van vleeskuikens. Bovendien vormt het verminderen van welzijnsproblemen een uitdaging binnen deze industrie. Het veelvuldig voorkomen van metabole stoornissen, eigen aan een intensieve opfok, heeft namelijk niet enkel een weerslag op de productiviteit door een minder efficiënte voederbenutting, een verhoogde mortaliteit of afkeuring van karkassen, maar leidt ook tot welzijnsproblemen. De voornaamste strategieën om aan deze doelstellingen tegemoet te komen, zijn enerzijds gerichte genetische selectie en verfijning van de opfokcondities, en anderzijds optimalisatie van het rantsoen. Dit laatste houdt onder andere de ontwikkeling van nieuwe voederadditieven in. De doelstelling van de dierproeven in dit proefschrift was de evaluatie van *N*,*N*-dimethylglycine (DMG), een lichaamseigen metaboliet, voor de toepassing als nieuw voederadditief voor de opfok van vleeskuikens. In eerste en tweede instantie werd nagegaan of DMG enerzijds de incidentie aan broiler ascites syndroom of pulmonaire hypertensie kan verlagen en anderzijds de nutriëntverteerbaarheid gunstig beïnvloedt. Een volgende onderzoeksvraag richtte zich naar dosis-respons effecten van DMG op productiviteit en diergezondheid in vleeskuikens, en op het effect van het vetzuurprofiel in het voeder op deze relaties. Een vierde luik peilde naar de efficaciteit van DMG in verschillende broilerlijnen opgekweekt in standaard opfokcondities. Een laatste objectief was de evaluatie van DMG als voederadditief op vlak van veiligheid, tolerantie en bioaccumulatie in vleeskuikens.

In een eerste voederproef werden 64 vleeskuikens (Ross-308, hennen, 14 dagen oud) ingedeeld in twee voedergroepen van elk acht hokken met vier kuikens per hok. De testvoeders waren enerzijds een controlevoeder, en anderzijds hetzelfde voeder gesupplementeerd met 167 mg Na-DMG per kg voeder. In deze challengeproef werd ascites uitgelokt door de kuikens op te fokken met een ernergierijk voeder en een koude omgevingstemperatuur te handhaven. De voornaamste objectieven waren het nagaan van de effecten van een lage voederdosage met DMG op de incidentie van pulmonaire hypertensie en op de nutriënt verteerbaarheid. De incidentie van pulmonaire hypertensie was 44.8% in de controle groep en slechts 14.6% in de DMG groep. Hiernaarst, was het vrije vetzuurgehalte in plasma meer dan dubbel zo hoog in de kuikens uit de controlegroep in vergelijking met de DMG groep. Vrije vetzuren oefenen een schadelijk effect uit op de pulmonaire vasculatuur, welke leidt tot endotheliale dysfunctie en progressie naar pulmonaire hypertensie. Het werkingsmechanisme van het protectief effect van DMG op de ontwikkeling van ascites, is aldus vermoedelijk gelinkt aan een DMG-effect op het vetmetabolisme, welke op haar beurt

leidt tot een daling in vrije vetzuur gemediëerde endotheliale schade. Naast een gunstig gezondheidseffect, leidde supplementatie ook tot sterke verhoging in schijnbare verteerbaarheid van ruw eiwit (+5.1%) en van de overige koolhydraten (+8.1%). Dit kan verklaard worden door een emulsifiërende werking van DMG ter hoogte van het spijsverteringskanaal waardoor de niet-vet nutriënten beter beschikbaar worden voor vertering en absorptie. Een betere schijnbare eiwitverteerbaarheid in de DMG groep ging bovendien gepaard met een lagere stikstofuitscheiding.

De volgende voederproef was een dosis-respons studie met als proefopzet een gerandomiseerd block design met tien behandelingsgroepen van telkens 5 hokken van 30 ééndagskuikens (Cobb500) per behandelingsgroep. Hierbij werden 5 dosages van DMG uitgetest in een range van 0 tot 1,000 mg Na-DMG per kg in vleeskuikenvoeders met ofwel voornamelijk dierlijk vet ofwel plantaardig vet als vetbron. De voornaamste conclusies uit deze studie zijn dat DMG een gunstige invloed kan hebben op zoötechnische productiviteit en karkaskarakteristieken, alsook oxidatieve stress en pulmonaire hypertensie kan verminderen, maar de mate van effect hangt af van, enerzijds de dosis en anderzijds het vetzuurprofiel in het voeder. Hierbij zijn de effecten sterker uitgesproken bij een voeder rijk aan meervoudig onverzadigde vetzuren dan een voeder rijk aan verzadigde en enkelvoudig onverzadigde vetzuren. Op basis van de resultaten uit deze proef werd een dosage van 1,000 mg Na-DMG per kg voeder aangeraden.

Vervolgens werden een aantal proeven uitgevoerd om de efficiëntie van de aangeraadde dosage aan DMG na te gaan in praktijkrelevante situaties. Hiertoe werd in drie Europese teststations een efficaciteitsproef uitgevoerd, waarbij telkens zowel de vleeskuikenlijn, de opfokcondities en het basisrantsoen representatief was voor de regio. De testlocaties waren: Settimo Torinese in Italië (studie 1), Kraig in Oostenrijk (studie 2) en Berlijn in Duitsland (studie 3). In elke proef werden ééndagskuikens verdeeld over een aantal hokken en *ad libitum* voer verstrekt gedurende een volledige opfokronde met één van twee testvoeders: controle versus 1,000 mg Na-DMG per kg. De onderzochte parameters waren enerzijds zoötechnische productiviteit (voederefficiëntie, eindgewicht en productiegetal) en anderzijds karkasrendement. In elk van de drie efficaciteitsproeven werd minstens twee van de drie geteste zoötechnische parameters gunstig beïnvloedt door supplementatie met DMG aan de aangeraade dosis. Bovendien toonde meta-analyse van de overkoepelende data een significante, algemene verbetering in voederefficiëntie, eindgewicht en productiegetal van respectievelijk -2.8%, +2.3% en +4.3%. De organoleptische vleeskwaliteit was niet

verschillend tussen de behandelingsgroepen en de overkoepelende data toonden een toename in borstvlees van +2.2% en in vlees:vet verhouding van +12.6%

Tot slot werd een tolerantie en veiligheidsproef uitgevoerd om uit te sluiten of de aangerade alsook tienvoudige dosage van DMG schadelijke gevolgen heeft op de diergezondheid en productieprestaties van vleeskuikens. Verder werd nagegaan of DMG aan deze dosages accumuleert in lichaamsweefsels van deze doelgroep. Deze proef bestond uit acht herhaalde hokken van telkens 20 eendagskuikens (Cobb, Avimex GmbH) per behandelingsgroep: controle, 1,000 mg Na-DMG per kg en 10,000 mg Na-DMG per kg. De kuikens werden *ad libitum* gevoederd tot de slachtleeftijd van 39 dagen, en de zoötechnische prestaties werden geregistreerd op hokniveau. Op het einde van de proef werden uit elke behandelingsgroep 12 kuikens geëuthanaseerd voor bloedname en staalname van lever, hart en nier. Op de bloedstalen werd het aantal rode bloedcellen, hemoglobine gehalte, witte bloedcel formule, electrolyten, en plasma enzymen -en metabolieten bepaald. Op de weefselstalen werd histopathologie verricht. De prestaties van de DMG-gesupplementeerde kuikens aan de aangerade dosage weren significant hoger in vergelijking tot de controle groep, terwijl de tienvoudige dosage leidde tot prestaties tussenin deze van de kuikens gevoederd met het controlevoeder en het voeder met 1,000 mg DMG per kg. De bloedparameters toonden gelijkaardige resultaten tussen de verschillende behandelingsgroepen en histopathologisch onderzoek duidde niet op pathologische afwijkingen ten gevolge van DMG. Bij beide DMG-dosages werd accumulatie van DMG vastgesteld in vol bloed, en in de tolerantie-dosis alsook in spier, lever en nierweefsel. Desalniettemin was het DMG-gehalte in rauw vlees van kuikens uit de tolerantiegroep vergelijkbaar met deze in bijvoorbeeld tarwezemelen of ongekookte spinazie. Deze resultaten tonen aldus aan dat DMG goed wordt getolereerd en veilig is voor supplementatie in rantsoenen voor vleeskuikens.

Het algemeen besluit van dit proefschrift is dat DMG een waardevol en veilig voederadditief is in de opfok van vleeskuikens. Bovendien accumuleert DMG niet in consumeerbare delen van vleeskuikens waneer hun voeder voor de volledige opfokperiode werd gesupplementeerd met DMG aan de aangerade dosage van 1,000 mg Na-DMG per kg voeder. Ten eerste beschermt dit nieuwe voederadditief vleeskuikens tegen broiler ascites syndroom, een metabole ziekte dat enerzijds een grote financiële kost en anderzijds belangrijke welzijnsproblemen veroorzaakt. Ten tweede wordt de nutriëntverteerbaarheid verhoogd en de stikstof uitscheiding verlaagd bij supplementatie met DMG. Tot slot wordt, over een ruime range van basale efficientie, de voederefficiëntie en mogelijks het karkasrendement gevoelig verbeterd door DMG.

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& **Aagje**,
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SUBMITTED BOOK CHAPTERS

Kalmar ID. Calcium metabolism in rabbits. In *Small Mammal Nutrition*. Versele-Laga Ltd., Deinze, BE. (submitted)

Kalmar ID. Nutrition of the ferret. In *Small Mammal Nutrition*. Versele-Laga Ltd., Deinze, BE. (submitted)

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Moons CPH and **ID Kalmar**. Nutrition of the Mongolian gerbil. In *Small Mammal Nutrition*. Versele-Laga Ltd., Deinze, BE. (submitted)

CURRICULUM VITAE

Isabelle Dominique Kalmar werd geboren op 9 december 1977 te Gent, België. In 1995 behaalde ze het diploma van het hoger secundair onderwijs (Wiskunde-Wetenschappen) aan het Sint-Bavo Humaniora te Gent. Datzelfde jaar begon zij haar studies aan de Universiteit Gent en behaalde in 1998 het diploma Bachelor in de Geneeskunde. Vervolgens vatte ze de studie Diergeneeskunde aan en behaalde in 2004 met grote onderscheiding de academische graad van Dierenarts. Haar afstudeerrichting was Onderzoek & Industrie, met de klinische optie: vogels, bijzondere diersoorten en laboratoriumdieren. Tijdens haar studies liep ze gedurende twee maanden een klinische stage in de dierenkliniek van Mangai Zoo en Jurong Bird Park in Singapore. Voor haar afstudeerthesis verrichtte ze in samenwerking met de vakgroep Voeding, Genetica en Ethologie van de Faculteit Diergeneeskunde en Versele-Laga Ltd. gedurende drie maanden voedingsonderzoek op papegaaien in het onderzoekscentrum van de Loro Parque Fundación te Tenerife. Voor deze scriptie behaalde ze een award voor 'Excellent Research in Parrot Nutrition'.

Aansluitend op haar afstuderen trad ze in dienst aan bovenvermelde vakgroep. Haar onderzoek betrof enerzijds voedingsonderzoek op papegaaien, waarop ze op 18 februari 2011 doctoreerde aan de Faculteit Diergeneeskunde van de Universiteit Gent. Anderzijds, deed ze in samenwerking met Taminco NV onderzoek naar nieuwe veevoederadditieven, welke deels beschreven is in dit proefschrift. Verder was zij aangesteld als studiemonitor voor de registratieproeven van Taminco NV. Andere activiteiten waren onder andere het geven van practica en gastlessen, het begeleiden van masterproeven van studenten Diergeneeskunde, dienstverlening en projectcoördinator van Project Pathway. Dit laatste bestond enerzijds uit de ontwikkeling van een flowcytometrie model voor het *in vitro* testen van de antimicrobiële activiteit van nieuwe voederadditieven en anderzijds uit het onderzoek van effecten van gemethyleerde voederadditieven op het niveau van genexpressie aan de hand van microarray en PCR methoden. In 2006 behaalde ze het certificaat van proefleider (FELASA categorie C). Vervolgens behaalde ze in 2007 met grootste onderscheiding het diploma Master in Laboratory Animal Science (FELASA categorie D). In 2008 behaalde ze het post academisch certificaat Praktijkgerichte Statistiek aan het Instituut voor Permanente Vorming.

CURRICULUM VITAE

Isabelle Dominique Kalmar was born on December 9th, 1977 in Ghent, Belgium. In 1995 she finished secondary grammar school (Mathematics and Science) at Sint-Bavo Humaniora in Ghent. That year, she started her education at Ghent University and graduated as Bachelor in Medicine in 1998. Subsequently, she took up Veterinary Medicine studies and graduated magna cum laude in 2004. Her graduation option was Research & Industry, with the clinical option: birds, special animals and laboratory animals. During her studies she did a two-month clinical internship at the Animal Clinic of Mangai Zoo and Jurong Bird Park, Singapore; and a three-month research internship at the Research Centre of the Loro Parque Fundación in Tenerife. Her master thesis, which was performed in cooperation with the Department of Nutrition, Genetics and Ethology and Versele-Laga Ltd., is entitled "Nutrient Requirements and Digestibility in Parrots". For this research, she was granted an award for "Excellent Research in Parrot Nutrition".

Immediately after graduation, she got employed at the above mentioned department where she conducted research activities on parrot nutrition, on which she defended a PhD in Veterinary Medicine at Ghent University on February 18th 2011. Next, she investigated new feed additives in production animals in cooperation with Taminco NV, which is partly described in this PhD thesis. Other activities included teaching, supervision of master's projects in Veterinary Medicine, consultancy, and project coordinator of 'Project Pathway'. The latter included on the one hand development of a flowcytometry model for *in vitro*-testing of antimicrobial potential of new feed additives, and on the other hand investigation of effects of methylated feed additives at the level of gene expression using microarray and PCR methods. She was also appointed as study monitor of the registration trials of Taminco NV. In 2006, she earned the FELASA category C certificate and in 2007 she acquired the degree of Master in Laboratory Animal Science (FELASA category D) summa cum laude. In 2008, she earned the post academic certificate in Practice-Based Statistics.

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