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FEEDING FOR EGG QUALITY

A.B.G. LEEK[1]

Summary

Influencing egg quality through feed involves attention to raw material quality, feed manufacture and the nutritional composition of the diet. Food safety ranks high on consumer demands and that starts with what is fed to the hen. Internal quality can be influenced both directly and indirectly by nutrition. Specific egg enrichments add value, fulfil a consumer niche and can represent a significant proportion of some egg markets. External quality is a prerequisite for saleability of the egg into a whole egg market and represents an important quality factor for the producer. Maintaining shell quality requires a "whole life" approach to the nutrition of the laying hen and developing pullet. Nutritional influences on egg weight have been widely studied but complex interactions may occur and further investigation into these may be required in order for the nutritional mechanisms for egg size control to be fully understood.

I. INTRODUCTION

Quality is a primary consideration for every consumer, but what constitutes quality and what defines quality can be market specific. Coutts and Wilson (2007) reported that European studies on consumer perception identified safety and freshness as the primary quality considerations. Nutritional and sensory characteristics were secondary key factors. Whilst these parameters are likely to reflect global consumer preference, regional emphasis may vary. Specific quality factors can be influenced by factors within the egg production process, including specific effects of feed and nutrition. Achieving good quality is not only important for meeting the customers' expectations and thereby encouraging consumption, but as history has shown us, quality is imperative to maintaining consumer confidence and a stable market for eggs and egg products. The aim of this paper is to examine ways in which the laying hen can be fed to produce a high quality product.

II. SAFE EGGS

Perhaps the prime example of what happens with a loss of consumer confidence in egg products is what occurred in the United Kingdom market over 25 years ago. A government report in 1988 highlighted to consumers, through media publicity, the high level of salmonella incidence in eggs. Eggs were identified as a significant factor in the rise in human salmonella infections, driven prominently by *Salmonella* Enteritidis PT4. This report formed a catalyst for a decline in annual egg consumption from just over 200 eggs per person in the late 1980's to a low of around 160 eggs by the late 1990's. Negative publicity on welfare and concerns about cholesterol risks in eggs were also contributory to that decline. However, the UK market saw larger decreases as a direct result of salmonella concerns than any other country in Europe at the time. The response to this was two-fold and serves as an example to other countries, and indeed other industries, on how to respond to a crisis in consumer confidence. The government introduced Salmonella Codes of Practice covering feed production, breeding, hatchery and farming operations. The industry responded by enshrining these codes of practice with industry specific assurance schemes covering feed production (e.g. Universal Feed Assurance Scheme, UFAS) and production assurance schemes.

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Specifically, 85% of UK egg production is audited and approved by the Lion Quality Code of Practice which has required vaccination laying flocks since 1998. Laboratory diagnosed *Salmonella* Enteritidis PT4 cases went from a peak in excess of 18,000 in 1993 to 249 in 2012 (DEFRA 2013). Out of 4,042 laying flocks tested in 2012, one flock was positive for *S.* Enteritidis and 2 flocks for *S.* Typhimurium in the UK (DEFRA, 2013).

Feed is potentially a vector for salmonella into the laying flocks and was linked to the 1980s outbreak in the UK. Feed assurance schemes, which now cover 90% of UK feed manufacture, require upstream raw material assurance schemes that are based around HACCP principals. Whilst this does not assure freedom from salmonella, they do raise the bar in terms of prevention and control of contamination. The feed production process itself introduces a risk of salmonella contamination. A DEFRA commissioned report in 2008 concluded that chemically treating feed was preferable to heat treatment of feed. This was due to the risks of post heat treatment process recontamination, particularly related to cooler hygiene (VLA, 2008). Indeed production of heat treated meals was perceived as a higher risk than heat treatment followed by pelleting.

A further influence on reduced salmonella incidence in feed has been the profile of raw materials used since the removal of meat and bone meal in 1996. Processed animal meals had been shown to carry a high incidence rate of salmonella contamination. Further, over recent years, there has been a trend away from other high risk materials such as fishmeals, full fat soya and vegetable protein blends. Detection rates in extracted soya meals are low but probably still present the greatest risk. Processed by-product meals, e.g. rapeseed meal, sunflower meal and DDGS, may also represent a higher risk of contamination and require careful sourcing and routine monitoring.

The withdrawal of prophylactic antibiotics from feed and increase in salmonella monitoring in many markets has led to more widespread use of non-medicinal alternatives. Broadly speaking, these can be classified into feed sanitizers and gut health promoters, depending on whether their activity is in the feed or in the gut. Formaldehyde based products remain widely used in high risk applications, during material processing, for mill decontamination and as a feed sanitizer for breeding and laying stock. Where formaldehyde use is restricted and in commercial laying flocks there has been an increased use of proprietary organic acid based products for feed hygiene control. A range of products is marketed for the control of pathogens in the gut. To some extent, the choice of product will depend on what and where the desired target activity is. Attention must also be given to water hygiene. Ineffectively sanitised water systems promote poor gut health and consequently, there is potential for a higher pathogenic load. This not only affects the health of the bird and productivity, but may also compromise product safety.

Egg safety linked to feed contamination goes beyond microbial concerns. Feed contamination can cause mycotoxins (e.g. aflatoxin, zearalenone, ochratoxin) (see review by Barnard, 2008), dioxins (de Vries et al., 2006; Schoeters & Hoogenboom, 2006), pesticides (Aulakh et al., 2006) and heavy metals (Abdulaleel & Shuhaimi-Othman, 2011) to accumulate within the egg. Europe has seen several egg recalls as a result of dioxin contamination over recent years, the latest one involving 8 free range farms in Germany in 2012. Although feed was not thought to be the cause of this particular incident, contaminated feed fats were responsible for a larger dioxin associated recall in early 2011. Whilst these instances were identified and contained, there is always the potential for such instances to bring negative media attention to the feed industry and its customers and undermine consumer confidence in egg products.

Results of monitoring for feed contaminates is reported through the European Commission RASSF web portal (https://webgate.ec.europa.eu/rasff-window/portal/). This provides a snapshot of contamination detected in feed materials and feed additives tested in

Europe and is a good reference to where the potential risks lie. Possibly the most significant recent detection reported that had potential to affect the layer industry was the chloramphenicol in an enzyme mixture in July 2013. In this case the system worked, there was a recall and negative publicity was avoided. Despite all the controls in place, the feed industry must remain continually vigilant of potential threats that may arise and affect the consumer safety.

III. INTERNAL QUALITY

The perception of egg freshness and internal quality is principally influenced by storage conditions and time since production. Nutritionally, there are ways to influence the perception of freshness. During storage, several physical changes occur in the egg that is associated with reduced freshness by the consumer. The thick albumen loses viscosity due to changes in its gelatinous structure and an increase in pH and as a result, both the albumen and the yolk appear flatter. Haugh units are a measure of albumen thickness and reflect the albumen viscosity and this measurement provides an index of egg freshness. In Japan, for example, Haugh unit and freshness of the egg is very important due to the increased popularity over recent years of 'tamago-kake-gohan'; a raw egg served over a dish of hot rice accompanied by soy sauce. Oxidative damage during storage can also weaken the vitelline membrane surrounding the yolk causing it to break. This can also be important if the eggs are processed since it affects the separation efficiency of yolk and albumen. Storage oxidation an also affect the taste and smell properties of the egg.

Improving the antioxidant properties of the egg helps to preserve internal egg quality parameters and improve shelf life. Kirunda et al. (2001) reported improved membrane strength when birds were supplemented with 60 IU/kg vitamin E at 34°C against birds supplemented with 20 IU/kg. Dietary antioxidants also help to improve Haugh unit score and maintain freshness for longer. Despite the high protein concentration of albumen, dietary protein appears to have little influence over the Haugh unit score. Some micronutrients; magnesium, zinc and biotin can have a positive effect. Vanadium, a contaminant of feed phosphates and some aquifers, can have a negative effect on albumen quality.

Antioxidants are also important for yolk colour; a weakened vitelline membrane becomes more porous and this leads to a mottling appearance of the yolk. Yolk colour is highly influenced by the diet. Diets rich in xanthophyll pigments, naturally occurring in raw materials or added from concentrated natural or synthetic additives, will yield eggs with higher colour scores. The use of some ingredients can result in yolk discolouration, for example high tannin sorghum and gossypol rich cottonseed meals. Cyclopropene fatty acids also found in cottonseed, can also cause a pink colour to develop in the albumen.

A genetic mutation on the FM03 gene in Rhode Island genetic lines was responsible for reduced activity of triymethylamine oxidase resulting in a fishy taint in brown hens fed on canola (rapeseed) meals. Most major commercial breed lines have now bred out this mutation allowing canola to be fed to laying hens. Fishy taints will still result from feeding of high levels of fishmeal or fish oil in the diet.

The presence of blood and meat spots results in down-grading of eggs due to both appearance and cultural objections. Blood spots on the yolk surface may arise from the rupture of blood vessels at the time the ovum is released from the ovary. Breakdown of this blood may result in it becoming more like a meat spot in appearance. Sloughing of the oviduct epithelium during egg formation gives rise to meat spots as the tissue gets incorporated into the egg white. There are significant genetic influences on blood and meat spots and it is a trait included in selection programs by primary breeders. Disease and stress may also increase the influence. Meat spots are not considered to be influenced by nutrition.

Blood spots can be caused by insufficient vitamin A or vitamin K, the presence of mycotoxins or vitamin K antagonists such as those found in lucerne meals.

IV. HEALTHY EGGS

The egg is often regarded as one of the most natural functional foods; rich in high quality protein, polyunsaturated fats, vitamins and minerals. The message that eggs contain high levels of cholesterol that prevailed in the 60's, 70's and 80's and that egg consumption should be limited, has been rewritten over that last 2 decades. In 2008, the British Heart Foundation removed their advice to limit egg consumption. Health remains a major factor quoted in consumer feedback, as a reason to consume more eggs, but also as a reason to consume fewer eggs (Guyonnet, 2012). In Australia, cholesterol was cited by 40% of respondents as a reason not to consume more eggs (Guyonnet, 2012). The link between eggs and cholesterol is often over simplified, since it makes no distinction between the type, and therefore health risks, of the cholesterol present. Low density lipoprotein (LDL) is the type of cholesterol associated with increased risk of heart disease and stroke and with elevated levels of saturated fat in the diet. Eggs are naturally low in saturated fat and the cholesterol effect to consuming eggs may increase the plasma concentration of high density lipoprotein (HDL) (Mutungi et al., 2008). Unlike LDL, HDL is associated with beneficial effects on vascular disease. In a recent meta-analysis of 17 reports, Rong et al. (2013) concluded that there was no statistical correlation between consumption of up to one egg per day with an increase in coronary heart disease or stroke. It has been well established that the fatty acid profile of the egg reflects the hen's dietary fat source; with unsaturated vegetable oil based diets resulting in higher unsaturated fat content than saturated animal fat based diets. In the UK, a review on egg composition by the British Egg Industry Council concluded that saturated fat content of eggs has reduced from 1.9 g/large egg to 1.5 g/large egg between the 1980s and 2011. This may reflect the change in dietary oils used as a result of a move from animal fats to vegetable fat blends in that period.

Nutritional enhancement of eggs has been the subject of many research trials in recent years, as egg producers seek to differentiate their product offering and add value by manipulating the nutritional characteristics of the egg. In an Australian study reported by Guyonnet (2012), 11% of respondents cited health benefits of nutritionally-enhanced eggs as a purchasing decision factor. The US speciality egg market was reviewed recently by Quant (2013). Between 2011 and 2013, speciality egg production more than doubled in the US to around 8% of production. Omega 3 enriched eggs represent 90% of that speciality market; equating to 15-20 million hens fed on high omega-3 feed. Typically, enrichment is coming from flaxseed meal inclusion. Enrichment using flax seed or canola oil raises the omega-3 concentration in the egg by predominantly increasing the alpha-linolenic acid (ALA, C18:3) concentration and an increase in long chain omega-3 fatty acids; eicosapentaenoic acid (EPA, C20:5), docosapentaenoic acid (DPA; C22:5) and docosahexaenoic acid (DHA; C22:6). Feeding diets containing ingredients rich in long chain omega 3 fatty acids, e.g. algea extracts and deodorised fish oils, can increase the concentration of EPA and DHA.

Zanthophyll carotenoids, found in egg yolks, have been identified as important dietary factors in the prevention of age related macular degeneration and markets are developing for lutein enriched eggs (Lyle et al., 1999). Lutein enrichment of eggs can be achieved with the addition of concentrated marigold extracts or richly pigmented ingredients such as prairie meal (corn gluten meal) or alfalfa. Lutein bioavailability from eggs appears to be higher than other dietary sources making eggs a highly effective means of delivery.

Diet can influence the vitamin concentration found in eggs. Studies in Australia have demonstrated the possibility of enriching eggs with vitamin D3 (Browning & Cowieson,

2103). The concept of vitamin D3 enrichment has been commercialised in Canada where there are concerns about low levels of vitamin D3 in the population due to low sunlight exposure during the winter months. In Europe, supplementation with D3 in the feed is limited by legislative restrictions to a maximum of 3000 IU/kg. This concentration is regarded as close to the birds' basal nutritional requirement and is typical of normal commercial rates of supplementation and so the regulation becomes prohibitive to addition of enrichment levels. A German study has demonstrated that hens kept outdoors, or with indoor/outdoor access, produced egg yolks with 3-4 times as much vitamin D3 (14.3 µg & 11.3 3 µg/100g DM respectively) versus hens kept indoors (3.8 3 µg/100g DM) (Kühn et al., 2014). The same study also reported variable and lower levels in randomly selected free range eggs from supermarkets, inferring a management/behavioural influence. A link between free range flocks and additional vitamin D3 was proposed previously by Ryan (2007), who reported a paling of brown eggs from layers exposed to UV light. A mechanism for this lighter colour has been suggested as hyper-vitamin D3 effect, resulting in over calcification of the egg and an over-layering of calcium carbonate after the pigmented cuticle has been formed. This effect can sometimes be observed in diets containing high calcium concentration or when eggs are retained in the shell gland due to a stressor. Field evidence in free ranging brown layers appears to support a sunlight effect, with pale shells being observed during summer in free range flocks and especially in flocks that use the external area particularly well.

Enrichment of eggs with other vitamins: vitamin A, vitamin E, vitamin B12, thiamine, folic acid, riboflavin, biotin and pantothenic acid, is also possible with variable degrees of biological efficiency. Commercial uptake of these enrichments has been low due to the market development and return on the costs involved. Similarly, enrichment with conjugated linoleic acid (CLA), a health beneficial fatty acid isomer found in ruminant fat and dairy products, is also possible but, as yet, not widely marketed. There have been several media reports on an Italian called Paolo Parisi, producing eggs from hens fed a diet containing goats milk. It is reported that these eggs have very characteristic and pleasing sensory and eating qualities, possibly reflecting the unconventional proteins (casein), short and medium chain fatty acids and carotenoids found in the goats milk. Commanding a price of around € 20/doz (around 29 Australian dollars), this is clearly an extreme example of how feeding a special diet can influence eating quality and add value to the egg!

The possibility of mineral enrichment of eggs is limited to iodine, selenium and manganese. Selenium enriched eggs are the most common form of enrichment, often as part of a vitamin E or omega 3 enrichment. Selenium eggs have been particularly successful in areas of Eastern Europe, where local food products are selenium deficient. Enrichments with other macro minerals (e.g. iron, copper, and zinc) are more challenging, as absorption is a regulated process, so transfer to the egg does not follow in a dose dependant manner.

V. EXTERNAL QUALITY

With the majority of eggs marketed as whole egg, it is unique amongst most animal products in that there is no further processing. Therefore, the production of a unique, consistent product throughout the laying cycle of the bird is highly desirable. External quality is a prerequisite for a saleable product and becomes a higher priority quality parameter for the producer than the final consumer. Shell quality is an important consideration in timing of the cessation of the laying period. Reduced shell quality decreases not only the saleable value of the product from the flock as a result of cracked eggs, but also leads to downgrades of acceptable eggs due to contamination from breakage in the system.

Genetic advancements are developing hens that will lay more consistent egg size profiles from the start of lay throughout; less small eggs at the beginning, less large eggs at

the end. Modern genetic lines are also capable of laying more eggs over the single cycle, as more markets move away from moulting birds. This means that the genetic value of the bird is increasing and it is essential that nutrition programs can capitalise on these enhanced genetic potentials. Feeding for egg quality begins in the rearing house on day one. Growing and developing pullets correctly influences production, not only in the early stages of the laying cycle, but also the persistency of the production and the persistency of shell quality. Correct development of the skeleton, muscle and fat deposits of the bird are critical and highly influenced by nutrition. Whilst early weight gain is important, achieving on or above target weights by 6 weeks and continuing to 13 weeks is critical, as this is when much of the birds muscle and frame development occurs (Kwakkel et al., 1998). Small framed birds carry less bone mass and consequently carry a smaller medullary bone mass and calcium reserve in the laying period and it is more likely shell quality loss will arise earlier in the laying cycle.

The next key stage is to support the development of the medullary bone as the bird reaches sexual maturity and in response to increased oestrogen levels. Medullary bone development continues into the laying period, approaching full development around the point of physical maturity at 30-32 weeks. Dacke et al. (1993) provide an excellent review on the physiology of medullary bone. Some key points to note are that, whilst medullary bone acts as a rapidly available calcium source for shell calcification (it is reported to have the ability supply up to 40% of the shell calcium), there is evidence to suggest that a prolonged restriction on dietary calcium leads to a demineralisation of cortical bone rather than medullary bone. It is for this reason, that we see weakness and deformity in keel bones of calcium deficient birds in later lay even though this bone does not contain the medullary structure. The keel bone is also unique as it calcifies and matures later than other cortical bone, almost at a similar stage as the medullary bone development, and continues to mineralise into the laying period. Since non-invasive assessment can be made on the strength, shape and length of the keel bone, it is a very good indicator of calcium status of the bird. Strong well developed keel bones can be an indicator of potential for good shell quality persistency. Reduced shell quality in later lay cannot simply be addressed by nutritional interventions at that point, since bone development in rearing and positive calcium balance in early lay are critically important. Increasing dietary calcium in later lay can increase shell strength and bone strength but it must also be recognised that higher levels can have significant detrimental effects on feed conversion and production (Nascimento et al., 2014).

Dietary phosphorous often gets mentioned in discussions on shell quality. However, egg shell only contains trace amounts of phosphorous, much less than the egg contains internally. The role of phosphorous in maintaining shell quality is principally through its role in forming and maintaining the bone matrix and its interaction with calcium. Substantially lower levels of dietary phosphorous than are commonly applied in commercial diets have been researched (e.g. Snow et al., 2005) but such low levels are not commonly applied commercially despite the potential for cost and environmental benefits. Indeed, there may be little nutritional benefit from over supplying phosphorous in the diet since elevated phosphorous in the blood will inhibit calcium resorption from the bone and reduce calcium transfer to the egg shell (Dacke et al., 1993). It is possible that the optimum level of phosphorous is dependent on several interacting factors; bone development in rearing and development and maintenance of bone mass in early lay, rate of medullary bone turnover and cortical bone resorption and the proportion of shell calcium supplied directly from absorption to the shell gland. During the laying period the source, dynamics of intestinal solubilisation and availability of calcium as well as the timing of feed (calcium) intake could influence the phosphorous requirement for shell formation and bone homeostasis in lay. These parameters are often not controlled or standardised in experiments into phosphorous requirements. This

may explain the apparent variation in phosphorous response reported in the literature and in the practical situation.

VI. EGG WEIGHT

Control of egg weight is often an important consideration for many producers. Breeding programs are providing flatter egg weight curves with higher weights in early lay and lower weights in later lay compared to previous generations. Egg weight is also influenced by lighting programs applied at both the beginning and end of the rearing period. Ensuring the appropriate rearing programs are used is an important first step, because nutritional intervention to control egg weight often has a secondary, detrimental, effect on production. Dietary linoleic acid concentration is often associated with higher egg weights but Saffaa et al. (2008) concluded that dietary oil concentration had a stronger association with egg weight than linoleic acid. Egg weight responses to linoleic acid concentration are often observed in some situations but not in others. In wheat based diets it appears possible to manipulate egg weight through linoleic acid adjustment, whereas in corn based diets the response appears not to be observed. Complex interactions between fatty acids and hormones on oviduct protein synthesis were described by Whitehead et al. (1993). These interactions could be influencing egg weight and may explain the difference in linoleic acid responses that are observed. Often lower egg weights are reported in diets enriched with omega-3 fatty acids.

Variation in the linoleic acid response may also relate to the energy value being ascribed to the dietary oil. Sources of linoleic acid are often high quality unsaturated vegetable oils and it is possible that the energy values taken on these oils are sometimes underestimated. Higher energy intake can also increase egg weight and it is notable that the current layer energy calculation published by CVB (2009), ascribes a 15% higher energy value of dietary oil than was previously proposed from studies derived from adult cockerels. This indicates that an underestimation of dietary energy value on high oil materials may occur when other energy systems are being applied in the diet formulation. Although the laying hen can adjust her intake in response to dietary energy concentration, the compensation is incomplete (Harms et al., 2004). Metabolism of oil has a lower heat increment than carbohydrate and dietary oil also increases the palatability of feed. In combination these factors may contribute to higher energy, and consequently larger egg weight, in diets containing more dietary oil.

Individually and in combination, dietary amino acid concentration has been demonstrated to impact egg weight as well as productivity. Sulphur amino acid concentration is most associated with egg weight and methionine and cysteine concentrations are often adjusted to obtain the desired egg weight profile. In addition there may be an influence from other nutrients related to sulphur amino acid metabolism. Through involvement in the methylation pathway, choline/betaine and even vitamins B12, B6 and folic acid may be relevant. Field responses have indicated that egg weight can be controlled by reducing methionine + cysteine concentration whilst increasing the ratio of methionine:cysteine in order to minimise the impact on production. Depending on the dietary raw material composition, limiting dietary methionine usually reduces both methionine and methionine + cysteine inclusion. However in diets containing cysteine rich raw materials supplemented with synthetic methionine, a reduction in cysteine concentration may not occur as methionine levels are decreased. In this situation it appears more difficult to reduce egg weight. The possible effect of this methionine:cysteine ratio and egg weight warrants further scientific investigation.

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EGG QUALITY

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Summary

Egg shell quality and egg internal quality are of major importance to the egg industry worldwide and may be measured in a range of ways both commercially and in research facilities. Egg quality is influenced by a range of factors including strain of bird, age of bird, nutrition, moult status, water quality, general stress, heat stress, disease, housing, production system, environmental contaminants and use of proprietary products designed to improve egg quality. Improved understanding of the way in which the egg and egg shell are formed, including knowledge of the proteins comprising the organic matrix of the shell has assisted with diagnosis of the causes of egg shell quality problems and with genetic selection for good quality. Ongoing technological advances have led to improved in-line monitoring of egg shell quality. Egg quality is also important for food safety as eggs are periodically implicated in cases of food-borne illness.

I. WHY EGG QUALITY IS IMPORTANT

Commercial eggs can be broadly divided into table eggs and hatching eggs and egg quality is important for both (Roberts, 2004). For the table egg, eggshell quality affects the visual appearance of the egg and the integrity of the egg until it is used by the consumer. It is also related to the microbiological quality of the egg. For fertile eggs, hatchability depends on the quality of the eggshell which needs to be intact, clean and free from bacteria and other microorganisms, permeable enough to allow gas exchange to occur but not too permeable or the egg will dry out too quickly. The shell of a hatching egg must also allow the chick to hatch out from the egg. Egg internal quality is also important in both table and hatching eggs. For the hatching egg, the contents must contain all the nutrients required by the developing chick (except for oxygen gas which can diffuse through the shell). For a table egg, the consumer prefers yolk of a particular colour (10-12 on the DSM scale in Australia) and has a preference for a particular range of albumen viscosity. There is a known association between albumen viscosity and egg freshness, with fresh eggs having a more firm or viscous albumen (Roberts, 2004). The internal composition of the egg also contributes to the microbiological safety of the egg.

II. THE PROCESS OF EGG FORMATION

Formation of the hen's egg commences with the ovulation of the yolk from the left ovary into the left oviduct. The yolk constitutes part of the oocyte which has developed from a primary oocyte as the result of deposition of proteins and lipids produced in the liver and transported to the ovary in the blood. The ovulated yolk is captured by the infundibulum of the oviduct where the developing egg remains for about 20 mins and acquires the two outer layers of the perivitelline membrane including the chalazae (Nys & Guyot, 2011). In breeder birds, fertilization occurs in the infundibulum. The egg then moves into the magnum where it remains for about 4 hours during which time the egg white or albumen is secreted. The egg spends approximately 1 hour in the isthmus where the inner and outer shell membranes, consisting of a network of fibres made up of proteins and glycoproteins, are deposited onto the egg. The longest period of time, 19 hours is spent in the uterus (shell gland) where the

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eggshell is formed. The terminal region of the isthmus, the red isthmus also known as the tubular shell gland, is where water and electrolytes enter the egg by a process known as "plumping" and the formation of the mammillary cores commences over a period of about 5 hours (Johnson, 2000). The first crystals of calcite form at the sites of organic aggregates present on the surface of the outer shell membrane (Hincke et al., 2011) and grow multidirectionally to form the mammillary bodies which join to form the compact palisade layer (Nys & Guyot, 2011). The eggshell consists of an organic matrix in addition to the inorganic calcium carbonate. There is evidence that the synthesis of the organic matrix, which is composed of proteins, glycoproteins and proteoglycans, controls the deposition of the shell (Hincke et al., 2011). The surface crystal layer is deposited on the palisade layer, followed by the cuticle which appears to play an important role in protection of the egg from bacterial ingress (Samiullah & Roberts, 2014).

III. MEASUREMENT OF EGG QUALITY

Over the years, egg quality has been tested in a variety of ways. Currently, in countries such as Australia, many commercial table eggs are processed using sophisticated equipment which automatically detects cracks, inclusions and dirt on the outside of the egg (Mertens et al., 2011). Such automated equipment frequently includes the step of egg decontamination by washing (Messens et al., 2011). However, some producers use less sophisticated, more manual equipment. In addition, large processing floors also have a quality assurance facility which uses equipment which is more similar to that used in research laboratories. A range of testing equipment is available from companies such as Technical Services and Supplies (U.K.), Nabel Co., Ltd (Japan), Orka Food Technology (China) and Ovobel (Belgium). In the laboratory, measurements of egg shell quality include shell colour (measured by shell reflectivity or spectrophotometry), egg weight, shell breaking strength and deformation to breaking point, shell weight and shell thickness. Egg internal quality is measured as albumen height, Haugh unit and yolk colour. The extent of cuticle coverage of an egg can be determined by staining the cuticle with a histological dye such as the commercially available MST Cuticle Blue dye (MS Technologies) and then measuring the extent of staining with a hand-held spectrophotometer. The quality of construction of an eggshell may be assessed by studying the ultrastructure of the mammillary layer of the eggshell following the removal of the shell membranes (Solomon, 1991).

IV. FACTORS AFFECTING EGG QUALITY

a) Strain of hen

As the result of genetic selection, different strains of laying hen vary in egg shell quality, egg size and production and there are clear differences between modern commercial birds and traditional breeds of laying fowl (Hocking et al., 2003). Selection for increased production may result in reduced egg shell quality (Poppenpoel et al., 1996). Knowledge of the heritabilities of selected egg quality traits and the use of molecular genetic and marker technologies enable targeted selection programs by breeder companies (Dunn, 2011; Dunn et al., 2005).

b) Age of hen

Egg shell quality and albumen quality generally decrease as birds grow older (Nys 1986; Roberts, 2006; Roberts et al., 2013; Travel and Nys, 2011). As hens age, egg size generally increases, eggs become more elongated, shell colour becomes lighter, shell weight generally

increases (but not necessarily in parallel with increases in egg weight leading to decreased percentage shell), shell thickness may stay the same or decrease and egg shell breaking strength decreases. Shell ultrastructural quality also decreases in eggs laid by older hens. Albumen height and Haugh Unit decrease with increasing hen age.

c) Nutrition

Optimal hen nutrition is vital in ensuring good egg quality. The pullet diet has an important effect on hen performance by influencing sexual maturity as well as body weight and composition at the onset of lay (Bouvarel et al., 2011). The protein level of the feed during the growth phase influences body weight (Keshavarz, 1984; Keshavarz & Jackson, 1992; Summers & Leeson, 1994). In mature hens, egg weight is influenced by the quantity of protein consumed. In addition, the amino acid composition is important with methionine being the main limiting amino acid followed by threonine, valine and lysine (Bregendahl et al., 2008). The energy content of the diet affects feed intake (Bouvarel et al., 2011).

Management of the supply of calcium as pullets come into lay is critical because the calcium level of the feed and the form of calcium influences feed intake and egg weight. Feed too low in calcium may result in overconsumption leading to increased levels of fat in the body (Roland, 1986). Typically, a pre-layer diet with approximately 2.5% calcium is introduced 2-3 weeks before the onset of lay (Roland & Brant, 1994). The consensus appears to be that 50-70% of calcium should be in the form of coarse particles (2-5 mm diameter) and the remainder in powder form. Dietary phosphorus levels are important for bone formation and the ratio of calcium to phosphorus can affect the absorption of calcium from the gastrointestinal tract (Boorman & Gunaratne, 2001). The model of Kebreab et al. (2009) illustrates the interaction between these two minerals.

Vitamin D is essential for calcium metabolism and use of the vitamin D metabolite 25-hydroxyvitamin D_3, which is converted into the biologically active form of vitamin D_3 in the bird, can improve shell quality under some circumstances (Al-Zahrani & Roberts, 2014; Keshavarz, 2003). Adequate levels of vitamin C are essential for normal good health and may also help to alleviate the effects of stress (Daghir, 1995b) and it has been suggested that vitamin E assists under conditions of heat stress (Bollengierlee et al., 1998).

Use of non-starch polysaccharide-degrading enzymes has been shown to improve egg quality (Roberts & Choct, 2006) under some circumstances. Phytase supplementation is used in poultry diets to release phytate-bound phorphorus and reduce phosphorus levels in effluent. Phytase supplementation has been shown to improve egg shell quality (Keshavarz, 2003) and there is evidence for a synergistic effect between phytase and xylanase (Ravindran et al., 1999).

Contaminants such as mycotoxins have the potential to reduce production and egg shell quality (Suksupath et al., 1989). There were problems in the past with hens possessing an inherited gene accumulating significant amounts of trimethylamine (TMA) in eggs as the result of an inability to oxidise the TMA found in feed ingredients such as canola meal and fish meal (Pingel & Jeroch, 1997). This is no longer a problem owing to selective breeding.

d) Induced moult

Induced moulting is practised in some countries to extend the laying life of the flock and to improve egg quality (Berry, 2003; Ahmed et al., 2003; Travel et al., 2011). However, the improvements in egg quality may be short-lived which means that moulting tends to be used as a management practice to ensure continuity of supply and at times which costs of replacement flocks are high.

e) Stress

A range of types of general stress can affect egg shell quality. High population densities were shown some time ago to increase the production of body-checked eggs (Dorminey *et al*., 1965). Body-checked eggs are thought to result from contraction of the shell gland while the egg shell is in the early stages of formation. Stress can also induce delays in the timing of oviposition when hens retain their eggs and this can result in an increased incidence of white-banded and slab-sided eggs (Reynard and Savory, 1999). The stressors of relocation (Leary et al., 1997, 1998), or exclusion from nest boxes of birds that normally had access to them, can cause an increase in the incidence of calcium dusted, white-banded, slab-sided and misshapen eggs (Hughes *et al.,* 1986; Reynard and Savory, 1999). Even handling of birds which are not used to handling can increase the incidence of cracked eggs (Hughes and Black, 1976). Many of the deleterious effects of general stress on egg quality can be induced by injections of adrenaline (Hughes *et al.,* 1986; Solomon *et al*., 1987).

The high temperatures experienced in most parts of Australia and also in other countries during the summer can result in smaller eggs and reduced shell quality via a number of physiological processes occurring within the bird (Roberts & Ball, 1998; Usayran *et al.,* 2001). Heat stress reduces feed intake and limits the availability of blood calcium for egg shell formation. It may also reduce the activity of carbonic anhydrase, an enzyme which results in the formation of bicarbonate which contributes the carbonate to the egg shell (Balnave *et al*., 1989). Therefore, sodium bicarbonate supplementation during heat stress may improve egg shell quality (Altan *et al*., 2000). Feeding practices in hot weather should focus on ensuring that birds are receiving adequate levels of essential nutrients (Daghir, 1995a). Diets need to be formulated to match feed consumption and it should be recognized that birds will tend to eat most during the cooler times of the day. The addition of fat to the diet during hot weather has beneficial effects, apparently via a number of mechanisms (Daghir, 1995a). Provision of half the dietary calcium in a coarse particulate form can improve egg shell quality in heat stressed birds. However, there is no evidence to suggest that increasing the calcium level of the diet above that necessary to achieve an adequate intake of calcium has any beneficial effect (Nys, 1999). The phosphorus requirement of laying hens increases slightly at hot environmental temperatures (Garlich *et al.,* 1978, cited in Nys, 1999). Other dietary remedies that have been tried to alleviate the negative effects of heat stress include addition of sodium bicarbonate to the diet (Balnave and Muheereza, 1997) and supplementation of dietary electrolyes and addition of aluminosilicates. However, the results of using these additives have been variable (Nys, 1999). The provision of cool drinking water can alleviate the effects of heat stress (Glatz, 1993).

f) Disease

A number of diseases have been reported to affect egg and egg shell quality and any disease that compromises the health of the bird may result in defective eggs and egg shells by indirect means (Roberts et al., 2011). Infectious bronchitis virus causes loss of shell colour, elongation of egg shells (lower shape index) and reduced albumen quality (Chousalkar & Roberts, 2009). Other viruses that have been shown to affect production and quality around the world include egg drop syndrome, swollen head syndrome, avian encephalomyelitis, avian influenza, Newcastle disease, laryngotracheitis. Bacterial diseases that have been shown to decrease production and egg quality around the world include *Salmonella, Mycoplasma gallisepticum, Escherichia coli, Ornithobacterium rhinotracheale, Gallibacterium anatis, the spirochaetes Brachyspira pilosicoli, B. Intermedia* (see Roberts et al., 2011). Syndromes that can affect egg production and quality include fatty liver syndrome, cage layer osteoporosis.

g) Production system

The type of production system may influence egg shell quality (Guesdon *et al.,* 2006; Rossi & De Reu, 2011). Early problems with cracked eggs in furnished cages have been greatly improved by changes in design of the furnished cages to include egg saver wires and long nest curtains (Wall and Tauson, 2002) as well as increasing nest attractiveness and lowering perch height (Tuyttens et al., 2013). Direct comparisons among the different types of production system (e.g. cage, barn, free range) have been made difficult by the shortage of experiments in which all other variables have been maintained constant. Some of the problems with egg shell quality reported from free range systems may result from an inability to ensure a balanced diet for the hens. Some studies have found effects of cage density on egg shell quality (Mench *et al*., 1986) whereas others report no consistent effects (Lee and Moss, 1995). Some strains of birds appear to be more suitable for particular production systems (Singh et al., 2009; Tumova et al., 2011; Valkonen et al., 2010).

V. EGG QUALITY AND PRODUCT SAFETY

The relationship between egg quality and the safety of the egg for human consumption has received considerable attention in recent years. Table eggs are regularly implicated in outbreaks of food-borne illness (OzFoodNet Working Group 2010, 2012) which makes it essential that the commercial industry conducts regular monitoring to ensure a safe food product. *Salmonella* is the organism of main concern in the egg industry. Australia is fortunate in that the serovar of greatest concern worldwide, *S.* Enteritidis, is not endemic in Australian layer flocks. *Salmonella* can contaminate intact eggs by vertical transmission, where eggs are contaminated via the reproductive tract of the hen while the egg is being formed, and horizontal transmission (Messens et al., 2005; 2011). Egg contamination by horizontal transmission occurs when *Salmonella* penetrates the eggshell during or following oviposition and can lead to contamination of the internal contents (Miyamoto et al., 1998). For salmonellae other than *S.* Enteritidis, horizontal transmission is the most common route for the contamination of egg internal contents (Humphrey, 1994). Bacteria such as *Salmonella* can multiply within the yolk of the egg but in order to reach the yolk, they need to pass through the shell either via defects or pores, cross the two shell membranes, move through the albumen and then across the perivitelline membrane and into the yolk (Musgrove, 2011; Chemaly & Salvat, 2011). The hen and the egg possess many defence mechanisms that protect against bacterial contamination. *Lactobacillus* flora in the cloaca and vagina have an inhibitory effect on bacteria. Eggs with intact cuticle blocking the pores are less likely to be contaminated. The cuticle, egg shell organic matrix and shell membranes have antibacterial properties. The albumen contains antibacterial substances in the form of lysozyme, ovoinhibitor, cystatin and ovotransferrin. The perivitelline membrane has antibacterial properties in addition to acting as a mechanical barrier. Finally, the yolk itself contains antibodies which may afford some protection.

In Australia, the serovar of greatest concern is *S.* Typhimurium, which appears to contaminate eggs via horizontal transmission, although there has been some speculation about types of *S.* Typhimurium being able to enter eggs by vertical transmission. Abnormalities in egg shells can potentiate the entry of food borne pathogens into the eggs (Nascimento *et al.* 1992; DeReu *et al*., 2006, 2008). In many countries, including Australia, eggs are commonly washed to remove external contamination although there is considerable discussion concerning the effectiveness of washing. Washing must be conducted correctly (Messens et al., 2011) or it can damage the cuticle which may, in turn, make eggs more susceptible to bacterial ingress (Gole et al., 2014). Commercial layer flocks are monitored for the presence of *Salmonella* (Davies & Carrique-Mas, 2011) and management and

sanitation programs are used to control *Salmonella* in layer flocks (Ducatelle & Van Immerseel, 2011). Approaches to control of *Salmonella* will vary according to the type of production system (Dewulf et al., 2011). Increasingly high consumer expectations and pressure from public health authorities mean that continued vigilance will be essential for the egg industry.

VI. CONCLUSIONS

Egg quality must be maintained at a high standard in order to ensure both the quality and safety of the product as well as the profitability of the egg industry. Improvements in egg quality and safety have been made possible by advances in understanding of how the egg shell is formed, technological improvements in detection of egg defects and improved ability to select for desirable characteristics in genetic breeding programs.

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INCREASING PERSISTENCY IN LAY AND STABILIZING EGG QUALITY IN LONGER LAYING CYCLES - WHAT ARE THE CHALLENGES?

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Summary

In the past 50 years, selection (initially at the breed level and then using quantitative genetics coupled with a sophisticated breeding pyramid), has resulted in a very productive hybrid for a variety of traits associated with egg production. One major trait currently being developed further is persistency of lay and the concept of the 'long life' layer. Persistency in lay however cannot be achieved without due consideration of how to sustain egg quality and the health and the welfare of the birds in longer laying cycles. These multiple goals require knowledge and consideration of the bird's physiology, nutritional requirements (which vary depending on age and management system), reproductive status and choice of the selection criteria applied. The recent advent of molecular genetics offers considerable hope that these multiple elements can be balanced for the good of all in the industry including the hens. The 'long life' layer, which will be capable of producing 500 eggs in a laying cycle of 100 weeks, is therefore on the horizon, bringing with it the benefits of a more efficient utilisation of diminishing resources including land, water, raw materials for feed as well as a reduction in waste, and an overall reduced carbon footprint.

I. INTRODUCTION

The modern commercial layer is capable of producing over 320 eggs in a single laying cycle if she is kept under optimum conditions. A deterioration in egg numbers combined with a decline in shell quality are the main reasons for replacing a laying flock at or around 72 weeks. Poor shell quality at any time not only results in financial loss but also causes major contamination problems for the highly mechanised egg packing and handling equipment. Poor shell quality at 72 weeks does not mean that all hens in an ageing flock produce eggs of reduced quality, rather the variability in egg quality within the flock increases. The long-term maintenance of the tissues and organs involved in producing eggs is therefore a prerequisite for extending the laying cycle of commercial flocks (Dunn, 2013). However, despite a plethora of research in this area spanning over 50 years we are still ignorant of all the processes and mechanisms controlling the complexity of egg formation nor do we fully understand the functional properties of the individual components of the egg, which are proving to be much more intricate than we ever imagined. Three excellent reviews on these subjects are provided by Nys and Guyot, (2011); Hincke et al., (2012); Rehault-Godbert et al., (2011).

Nevertheless, by 2020, breeding companies claim that they will have developed the 'long life' laying hen capable of producing some 500 eggs in a production cycle lasting 100 weeks. This goal is being achieved using selection programs that base decisions on a triangulation of phenotype evaluation of pure lines extending beyond 55wks, cross breed progeny testing (which is now being carried out in diverse conditions throughout the world) and genotype information derived from DNA markers (microsatellite and SNP) which have been validated to show an association with phenotypic traits (O'Sullivan, 2009). Any

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improvement in persistency in lay must also go hand in hand with sustainable egg quality and the birds must remain healthy throughout the production period. Osteoporosis remains one of the major welfare challenges for the egg industry (Sandilands, 2011) and therefore cannot be ignored in any discussion relating to extending the laying cycle. In this respect the correct nutrition throughout the laying cycle is of paramount importance. The nutritional requirements of the 'long life' layer therefore need to be revised because the results of nutritional trials carried out over 20 years ago (when birds produced fewer eggs) are probably no longer applicable.

This paper begins with an overview of the egg formation and some of the main factors that control and or influence this process. A summary of the progress made by breeding companies in achieving their aim of improving persistency in lay and stabilising egg quality is then provided. The final part of this paper looks at some of the nutritional and welfare considerations which need to be addressed if the concept of extending the laying cycle beyond 72 weeks is to be realised in practice.

II. OVERVIEW OF THE EGG FORMING PROCESS

The almost daily production of an egg by a commercial layer is only feasible due to the simultaneous development of a series of follicles in the left ovary. This follows a defined hierarchy with only one follicle reaching maturation within each 24 hr period. Over 12,000 oocytes are present in the ovary at hatch but only a small percentage of these will ever reach maturity. At ovulation, the yolk mass from the largest follicle is captured by the funnel shaped open end of the proximal oviduct or infundibulum. From here it travels down the oviduct and undergoes successive deposition of the different components of the egg (Romanoff and Romanoff, 1949; Gilbert, 1979; Sauver and de Reviers, 1988). Each component of the egg (the albumen, membranes and the shell) is secreted by different parts of oviduct according to a predetermined sequence of events. During the first four hours, the egg white (albumen) is formed in the magnum, the longest section of the oviduct. The shell membranes are then deposited as the egg mass passes through the isthmus. Five hours after ovulation the egg mass enters the shell gland, where it spends the next 19 hours. It is during this time that the shell forms. The formation of the eggshell occurs in 3 distinct phases (Nys et al., 2004) and is regulated by the precise temporal and spatial secretion of a complex array of proteins (Gautron et al., 1997; Mann et al., 2006), some of which subsequently become incorporated into the calcified structure thereby modifying its biomechanical properties (Hincke et al., 2012) and/or participate in its antimicrobial defences (Rehault-Godbert et al., 2011). The resulting interwoven fabric of organic and inorganic constituents forms the mammillary and palisade layers of the shell. In the last 1.5 hours and just prior to oviposition the shell pigment and then the cuticle (a non-calcified organic layer of variable thickness) are deposited. The egg is then ready for oviposition. The timing and process of oviposition is controlled by neurohyopohyseal hormones and prostaglandins secreted by the ovary and to a lesser extent the shell gland (Nys and Guyot, 2011). Eggs are usually laid during the first few hours after dawn or when the lights come on in the morning. The next ovulation takes place after expulsion of the egg but can also occur just prior to this in some cases.

III. THE ROLE OF THE NEUROENDOCRINE SYSTEM

Reproduction in birds is controlled by GnRH-I neurones in the hypothalamus, the region of the brain that integrates environmental and internal endocrine signals. Dunn (2013) suggested that subtle differences in the neuroendocrine system between individuals may be the reason why some birds are capable of a higher persistency in lay than others. Oestrogen and progesterone are critical to stimulating the growth and maintenance of the left oviduct (Sharp

et al., 1992). These sex steroids are produced by the developing follicles in the ovary at sexual maturity in response to an increase in the circulating levels of gonadotrophins such as pituitary FSH and LH. As hens age, the cells in the hypothalamus that control these processes are thought to become less efficient (Dunn et al., 2009). The net effect is that the oviduct loses weight, and functions less efficiently. The oviduct itself must inevitably suffer damage due to wear and tear, possible low grade infections, and probably it also becomes refractory to the prolonged stimulation. The number of days when no egg is laid subsequently increases as does the number of defective eggs (Solomon, 1991, 2002). However, some individuals are clearly more capable of maintaining a high egg output with good quality shells for longer periods. Thus improving persistency in lay and sustaining egg quality in longer laying cycles should be achievable.

IV. SELECTION AND MAINTAINING LAYING PERSISTENCY

The intensive selection for traits such as age at sexual maturity, peak production and laying persistency to 55 weeks has significantly reduced the genetic and phenotypic variations that previously existed in egg number in commercial lines. Indeed, the biological limit of one egg per day for example has virtually been achieved at peak production. It is now common practice for breeding companies to extend their pure line evaluation beyond 75 weeks. Heritability calculations for egg production at 80-100 weeks are reported to be moderate (h2=0.24) for both white and brown egg layers (source - Institut de Sélection Animale). There is therefore further scope for genetic improvement in laying persistency.

V. SELECTION TO STABILISE EGG QUALITY IN LONGER LAYING CYCLES

For many years breeding companies have focused their efforts on achieving higher egg weights (60 g) by peak production and maintaining egg weight at or around this level for as long as possible (65.5 g by 50 weeks). Beyond this, egg weight creeps up with bird age whilst shell quality tends to deteriorate. Excessively large eggs must be avoided if the laying cycle is increased, as large eggs are notoriously difficult to handle. The selection focus now is on controlling egg weight after peak production and keeping egg weight stable beyond 90 weeks of age (O'Sullivan, 2009). The net effect is that the shape of the egg weight curve has become flatter, and "late egg size" has decreased by 5-7 g.

The Haugh unit is the standard selection measurement for albumen quality. Curtis et al., (2005) reported that Haugh units deteriorate with hen age from an average 89.6 to 68.8 over the laying period. The heritability estimates for Haugh units range from 0.21-0.41 (Dunn, 2011). The heritability estimates for Haugh units calculated over a longer laying cycle at 80-100 weeks are still within this range (source - Institut de Sélection Animale). Thus, through selection it is also possible to maintain acceptable albumen quality in older laying flocks for a longer period in the future.

Egg colour is only included in selection in brown egg laying populations for aesthetic reasons and not because this trait relates to the quality of the egg in any other way. The natural variation in brownness is considered to be important in some markets but this is not universal (Arthur and Sullivan, 2005). Heritability for shell colour in brown lines ranges from 0.3-0.53 depending on the breed (Dunn, 2011).

Eggshell strength on the other hand is vital in ensuring the integrity and safety of the egg contents but the problem here is deciding on which measurement to use. Most companies use a combination of several measurements as they believe that each measures slightly different things. One breeding company for example uses puncture strength as a measure of flexibility and breaking strength as an indirect measure of shell thickness. Heritability estimates for breaking strength measured by quasi-static compression in brown and white

lines have been reported to be 0.28 at 80-100 weeks (source - Institut de Sélection Animale). Thus sustained eggshell strength in older flocks is also a realistic and achievable goal.

Some breeding companies have recently introduced the measurement of dynamic stiffness into their breeding programs. This measurement was developed by researchers at KU Leuven in the late 1990s. The dynamic stiffness measurement was shown by Dunn et al. (2005) to have a high heritability and importantly the measurement was shown to be an accurate predictor of an egg's susceptibility to damage in the field (Bain et al., 2006). This unique feature means that breeding companies (who have been using this measurement for several years now) are reporting beneficial effects at the commercial level in terms of a measurable reduction in the percentage cracks and also in the breeding sector where improved eggshell quality has resulted in improved hatchability and a higher chick output (source - Lohmann Tierzucht).

VI. NUTRITION AND FEEDING FOR PERSISTENCY IN LAY AND GOOD EGG QUALITY

It is important that pullets receive an appropriate diet throughout the rearing phase so that they meet the recommended adult pullet target weight by 14-16 weeks of age and have the correct body composition to sustain egg production beyond 90 weeks. A specific growth curve must therefore be followed. This is particularly important in the case of the 'long life' layer where persistency in lay is expected. Any deviation away from the target pullet weight will influence the mean egg weight during the early laying phase ($r2=0.85$, $p<0.01$) and the total egg output for the entire period of production (Bouvarel et al., 2010). Particular attention must be paid to the energy/ protein ratio between 11-16 weeks, as increased energy content of the diet enhances the fattening score (Cheng et al., 1991). Particle size, if not suitable for beak size, can also result in reduced feed intake and therefore weight gain during the rearing phase (Frikha et al., 2011). Too many dietary changes or rapid changes in diet during the rearing phase should also be avoided. At about 16 weeks of age, the energy and protein content of the ration must be adjusted again ensuring that the hen consumes sufficient feed to cope with growth and the onset of egg production. There are a number of ways of promoting feed intake around this time e.g. use of whole cereals and coarse water-insoluble fibre (Hetland et al., 2005). It is particularly critical that this feed is both appetizing and always available as medullary bone reserves are being formed and the ovary and oviduct are developing at this time.

During the laying period, the first challenge is to adjust the energy and protein requirements to optimize egg output and to carefully control body weight. The growth requirement is only present for the first few weeks at the onset of egg production. Energy required for maintenance thereafter depends on body weight and feather coverage and therefore increases with hen age. Compilation of literature clearly shows a strong and negative correlation between feed intake and dietary energy concentration (Bouvarel et al., 2010, 2011). This adaptation is however only partial and so high energy diets can be used during the first part of the laying period to satisfy the continued requirement for growth and to promote heavier, early egg weight without the risk of overfeeding and producing "fat hens" (Pérez-Bonilla et al. 2012). The hen's energy requirement however decreases as egg production becomes established. To minimise fat deposition a lower energy diet can be used at this time, as the birds will be able to partially compensate by increasing their feed intake. Laying hens also adjust their food intake according to the relative size of the particles in relation to the beak size (Joly, 2004; Safaa et al. 2009). Varying particle size allows further balancing of the energy intake.

The crude protein concentration and amino acids in the layer diet are also important, methionine being the main limiting amino acid (AA). Consumption of an extra 1g of protein per day for example results in an average increase in egg weight of 1.4 g (Bouvarel et al., 2010). However the amount of protein consumed is dependent on the dietary energy concentration and the form of the ration. Ideally the protein and AAs concentration in the diet should be estimated relative to the egg weight (mg/g of egg for AAs) and adjusted to optimise egg production throughout the laying cycle. However an additional difficulty is that the heterogeneity of the flock increases with flock age. The best strategy is therefore to focus on maintaining the production of the higher producing hens and to adjust the supply of proteins and AAs accordingly, providing the cost is not prohibitive.

'Gap' feeding combined with a 'paired feeds' throughout the daylight hours is a useful management tools to improve feed intake efficiency, and flock body weight uniformity during the laying period (personal communication, Lohmann Tierzucht). Sequential feeding methods whereby the energy and protein levels in the morning versus the afternoon feed are varied is also under investigation (Traineau et al., 2013; 2014). Knowledge of the hen's specific needs for energy and protein throughout the day would allow optimisation of the daily intake and improved FCR. At present however there seems to be no clear evidence that the hen's requirement for energy or protein varies throughout the day and that laying hens can adjust their daily intake accordingly. In contrast, the laying hen's specific appetite for calcium in the late afternoon is well established (Mongin and Sauveur, 1979).

VII. DIETARY CALCIUM, EGGSHELL QUALITY AND BONE HEALTH IN LONGER LAYING CYCLES

A laying hen requires 2.2g of calcium on average for every egg she lays. About two-thirds of this calcium is supplied via the hen's diet, and one third by the mobilisation of calcium from the medullary bone that forms under the influence of oestrogen as the bird first comes into lay (Bouvarel et al., 2011). Calcium derived from bone is needed during the final stages of shell formation as this takes place during the night when the bird is not feeding. Medullary bone, unlike structural bone, is capable of undergoing rapid absorption and renewal. Unfortunately resorption of structural bone also occurs causing the symptoms of osteoporosis (Whitehead, 2004). Osteoporosis is caused by a decrease in the amount of fully mineralised structural bone leading to bone fragility and susceptibility to fracture making this one of the major welfare challenges for the egg production industry (Sandilands, 2011). Nevertheless, within a flock it is possible to observe individual birds with high productivity and good bone strength. Therefore it should be possible to select for improved bone strength in the 'long life' layer but since the aetiology of osteoporosis is complex and is not only influenced by genetics but also by the laying environment and by nutrition, genetic selection alone is not the answer (Fleming et al., 2006).

The provision of insufficient dietary calcium during the rearing or laying period has an adverse effect on both eggshell quality (Classen and Scott, 1982; Hartel, 1990) and bone strength (Whitehead, 2004). The laying hens requirement for dietary calcium within the diet for different ages is in the order of 0.9 to 1.2% during the growth period of the pullet, increasing to 2 to 2.5% just prior to the onset of lay and 3.5 to 4.5% once lay is established (Bouvarel et al., 2011). There does not appear to be any benefit of adding more calcium or using a "step up" feeding system (3.5, then 4.5, and finally 5.5%) to limit the age deterioration in shell quality (Keshavarz and Nakajima 1993) although bone reserves of calcium have been shown to increase using this phased approach (Guinotte and Nys, 1991).

Most eggs are laid early in the morning just after the lights come on. Consequently the morning feed probably does not contribute directly to shell formation as this does not

commence for 5 hours after oviposition. Likewise the afternoon feed is not synchronised with shell formation as this continues throughout the night, however a specific appetite for calcium a few hours before the lights go off does ensure that there is some storage of feed including calcium in the crop (Mongin and Sauveur, 1979). Switching on the lights for 2 hours and introducing a midnight feed has been shown to improve the synchronisation of dietary calcium intake with shell formation and to improve eggshell quality (Grizzle et al., 1992). However, the European directive 1999/74/CE requires birds to have 8 hours of uninterrupted darkness in every 24 hour period, and so this type of split lighting program is essentially prohibited in Europe. Studies by Keshavarz, (1998) showed that the daily Ca requirement cannot be reduced by providing the hens with adequate levels of Ca during the afternoon and inadequate Ca level during the morning. Calcium provided in the morning feed is probably important for replenishing medullary bone calcium reserves.

In order to optimise eggshell quality, the calcium particle size should be adjusted according to the density and solubility of the particulate calcium source (quarry or marine). As a general rule a coarse particle of 1-2.4 mm with a low solubility should be introduced to supply two thirds of the calcium along with high soluble marine source as particles of 2-4 mm (Bouvarel and Nys, 2014). Dietary lipid content and the active metabolite of Vitamin D3 have been shown to influence the efficiency of dietary calcium uptake in the gastro-intestinal track but how this relates to the transfer of calcium in the shell gland remains to be determined. High levels of phosphorus or too much or too little salt (NaCl) in the layer diet also has a deleterious effect on eggshell quality and should be avoided (Bouvarel and Nys, 2014). In summary there is still much to learn about the kinetics of intestinal calcium retention throughout the day and such knowledge will clearly be of value in the management of 'long-life' layers.

VIII. NUTRITIONAL CHALLENGES - PREVENTION OF METABOLIC DISEASE

The exportation of massive amounts of protein and lipids into eggs during the laying period challenges hen metabolism and can also cause a range of other metabolic diseases. In birds, all fatty acid synthesis occurs in the liver, mostly from carbohydrate. The egg yolk contains a large amount of lipid. The metabolic activity of the liver therefore has to dramatically increase from sexual maturity onwards to form the lipoprotein precursors of the yolk (Nys and Guyot, 2011). Fatty acid synthesis is coupled with glycolysis so a high dietary consumption of carbohydrate can markedly increase the fat content of the liver resulting in hepatic steatosis (Butler, 1976; Hansen and Waltzem, 1993). Hepatic steatosis in layers tends to occur most often where there is an imbalance in protein:energy ratio and causes a drop in egg production and obese hens. Fatty liver haemorrhagic syndrome (FLHS) has also been observed in hens treated with oestrogens (Butler, 1976; Lee et al., 2010; Choi et al., 2012). This disease, which is not to be confused with hepatic steatosis, is almost invariably fatal but it can also affect egg production (Waltzem et al., 1993; Lee et al., 2010). The provision of dietary 25 (OH) D3, feed restriction with substitution of carbohydrate by dietary fat, supplementation of choline, inositol, vitamin B12, folic acid and vitamin E have all been shown to limit the incidence of hepatic steatosis and FLHS in layers (Bouvarel and Nys, 2014).

IX. DISEASE, ENVIRONMENTAL STRESS AND BIRD BEHAVIOUR

Both disease and environmental stress can induce changes in egg formation at any time during the laying year (Solomon, 1991). Diseases such as infectious bronchitis, egg drop syndrome and Newcastle disease influence egg quality either directly by altering oviduct structure or indirectly by lowering the general health status of the individual. Remedial action

in such cases often involves medicating or vaccinating the entire flock with variable success. Within the laying environment, individual birds also experience a range of 'stress' events of varying magnitude and duration within any 24 hour period. These stress events are likely to be exacerbated in large size, colony management systems where birds are not able to establish a stable dominance hierarchy. When these 'stress' events coincide with a critical point in the egg forming process the result will be the production of an egg of unacceptable quality. Experimentally it has been shown that the oviduct can recover even after experiencing a major stress event, and catastrophic damage to the cells lining the oviduct (Watt, 1989; Solomon, 2002). The recovery process however can take 2-3 weeks. There is no "magic cure" for environmental stress and in many cases it is impossible to identify the stressor and therefore redress the balance especially at the individual bird level.

Feather pecking is a behavioural problem seen in commercial flocks that can result in stress particularly in alternative floor based systems where there are large group sizes (>15 birds). Feather pecking results in feather loss and in extreme cases causes damage to the bird receiving the behaviour and ultimately their death due to cannibalism. Poor feather coverage also means that birds need to eat more to maintain their body temperature (+20 g at 12.8°C if the feather coverage corresponded to 50% normal, +7 g at temperature of 23.9°C) (Peguri and Coon, 1993). Feather pecking is therefore an important welfare and economic issue (Blokhuis et al., 2007) and one that the industry needs to address, particularly if there is an industry wide ban on beak trimming (the main method used to control this behaviour). Indeed, following an EU welfare directive on the issue, beak trimming has already been banned in some European countries and others are working towards this. It is therefore important to be able to identify practical, effective and affordable alternatives to beak trimming. The factors involved in feather pecking outbreaks in commercial flocks, are multifactorial for example nutrition seems to be important. Reports suggest that feather pecking behavior can be reduced by feeding diets high in insoluble nonstarch polysaccharides (Van Krimpen et al., 2007; 2009) or roughage (Steenfeld et al., 2007) and by avoiding protein or amino acid deficiency. Feather pecking behaviour has also been observed in pullets as young as 7 days old. Limiting this behaviour through nutritional means during the early stages of pullet rearing might therefore be important in trying to reduce feather pecking in adult birds (Qaisrani et al, 2013). Feather pecking also has a genetic basis as demonstrated by Kjaer et al., (2001) and others but with variable heritabilities of between 0.06-0.5. There is also an interesting facet in that individuals can affect each other's phenotype – the indirect genetic effect leading to research on selection by family group (Peeters et al., 2012). The first genetic regions (QTL) involved in feather pecking have now been reported (Rodenburg et al., 2004) thus molecular genetics once again offers us potential solutions to this intractable problem.

X. CONCLUSIONS

The benefits of genetic selection for improved persistency in lay and stability in egg quality can only be realised if they are matched by improvements in hen nutrition and careful monitoring, recording and analysis of the effects of this process on the health and welfare of the hens. The opportunities for broader selection indices, thanks to molecular genetics, are increasing and it is evident that poultry geneticists are already harnessing this added tool in an attempt to address some of the major issues that the industry faces for the betterment of all. The economic forces for continuing improvement in productivity are massive but there is also an undercurrent for improved welfare and the industry ignores this at its peril.

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BODY WEIGHT AT POINT OF LAY AND THE ULTRASTRUCTURAL PROPERTIES OF EGGSHELLS

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Ultrastructural studies have demonstrated that the eggshell is comprised of morphological distinct calcified layers with the mammillary layer being the "foundation" of the eggshell. Studies have identified ultrastructural variations in the mammillary layer that can be used as indicators of eggshell quality (Roberts and Brackpool, 1995). Understanding the ultrastructure of eggshells has reinforced the view that the mechanical properties of the eggshell cannot be defined by a simple thickness measurement (Bain, 2005).

Body weight at point of lay is a major factor influencing subsequent egg size, and this applies to both immature and more mature ages (Leeson and Summers 1987). Larger birds consume more feed while producing larger sized eggs with inferior eggshell quality (Leeson and Summers, 1987; Parkinson et al., 2007). The current study was conducted to investigate the relationship between body weight at point of lay and ultrastructural properties of the mammillary layer of the eggshell.

Sixty Hy-Line brown hens were housed individually in cages. Three different groups of body weight (light, medium and heavy - mean body weight at 16 weeks of 1.170 kg, 1.337 kg and 1.507 kg, respectively) were selected. A total of 10 eggshell samples were processed for ultrastructural observation from each group at 10 week intervals during the laying life of the flock: 20, 30, 40, 50, 60, 70 and 80 weeks of age.

A small equatorial piece of eggshell was cut from each eggshell and the inner membrane removed manually. The outer membrane was removed using a Plasma Etcher. Pieces of shell were then mounted, inner surface uppermost, onto an aluminium stub and coated with gold using a sputter coating unit for 5 minutes. Mammillary ultrastructure variables were viewed using a JEOL Neoscope SEM and scored after the method of Solomon (1991).

There was no significant effect of body weight at point of lay on ultrastructural properties except for cap quality which was best in the light birds, and cuffing and changed membrane which were best in the heavy birds. Cap quality estimates the extent of contact between the shell membranes and the shell which is thought to influence shell strength. Cuffing is additional calcite around and between adjacent mammillary cones which also increases shell strength. Changed membrane may reflect abnormal conditions within the oviduct while the eggshell was being laid down (Roberts and Brackpool, 1995). Alignment tended (P=0.09) to have a lower incidence in the heavy group. As hen age increased, the incidence of ultrastructural features known to be associated with poorer shell quality increased: alignment of mammillae, type A bodies, type B bodies, aragonite, late fusion and pitting; cap size became more variable and cap quality poorer. The incidence of ultrastructural features that have been shown to be associated with good shell quality, such as early fusion and confluence decreased as the flock increased in age (Roberts and Brackpool, 1995).

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THE CUTE-EGG PROJECT: A STUDY WHICH AIMS TO QUANTIFY AND IMPROVE THE QUALITY OF THE EGGSHELL CUTICLE

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Commercial poultry production relies on the artificial incubation of eggs. Conditions in the incubators are as ideal for rapid microbial growth as they are for the growth and development of the embryo and keeping micro-organisms out of the egg can be a challenge. Opportunities for horizontal transmission occur during the collection and transportation of eggs and the vertical transmission from breeder hens to production flocks has been identified by EFSA as the most likely route of transfer of antibiotic resistant *E.-coli* and *Salmonella spp*. (Bain *et al*., 2013). Irrespective of the route or site of transfer, the entry of pathogenic or zoonotic organisms to the egg contents is undesirable for food safety, animal and human health.

The cuticle is a glycosylated protein layer which covers the surface of the egg. We have previously presented evidence which confirms that the cuticle forms the first line of defence to the penetration of bacteria and that cuticle deposition can be quantified by staining eggs and measuring the change in % reflectance by spectrophotometry at 640nm. Using this rather cumbersome methodology we were able to establish that cuticle deposition in layer hens is a heritable trait (h2, 0.27) and therefore has the potential for improvement by genetic selection. The cute egg study addresses the physiochemical, physiological and genetic parameters that characterise the cuticle and the development of a new simple one step measurement tool for cuticle assessment. Our preliminary results indicate that the repeatability of our new measurement, which makes use of fluorescence is high (0.76) and better than that previously obtained using the staining method (0.56). The correlation between the original dye method and our new fluorescence measurement is moderate; however this may be because our new spectroscopic method is measuring different parameters than the staining method. We are now at the advanced stages of applying our new one step measurement to collect data from pedigreed birds for the estimation of genetic parameters for cuticle coverage in both meat and egg laying strains of poultry and to determine its genetic relationship with measures of production and bacterial translocation into the eggs. The results generated will be useful for accurate genetic selection to improve cuticle coverage and reduce the risk of pathogens entering the egg. This project will also deliver fundamental knowledge about the biological mechanisms which give rise to the cuticle, its role as a physical and chemical barrier to microbial penetration.

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VITAMIN D, STRONTIUM AND LAYING HENS – WHAT'S THE CONNECTION?

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Summary

Strontium is currently prescribed for patients with osteoporosis to increase bone density and reduce bone fractures but its relevance in animal nutrition is obscure. In order to investigate the effect of supplemental strontium and vitamin D_3 on performance, egg quality and skeletal integrity in poultry a total of 108 laying hens, 99 weeks of age, were fed three levels of strontium (0, 500 mg/kg, 1000 mg/kg) and two levels of vitamin D_3 (2,500 iu/kg, 5,000 iu/kg). There was an improvement ($P < 0.05$) in egg production and feed conversion efficiency with strontium at 500mg/kg and a significant increase in egg weight in those hens fed additional vitamin D_3. Supplemental strontium increased phosphorus, sodium and strontium retention in birds fed 2,500 iu D_3/kg but reduced phosphorus, sodium and strontium retention in birds fed 5,000 iu D3/kg resulting in an interaction ($P < 0.01$) between strontium and vitamin D3. Addition of 5000 iu/kg D_3 increased egg weight ($P < 0.05$); predominantly by increased albumen content ($P < 0.05$) whereas strontium supplementation reduced egg weight ($P < 0.001$). Similarly 5000 iu/kg D_3 increased apparent metabolisable energy ($P < 0.05$); in contrast strontium supplementation reduced ($P < 0.05$) apparent metabolisable energy. The interrelationship between strontium and vitamin D_3 requires further study.

I. INTRODUCTION

The biochemistry and physiology of vitamin D, strontium are uniquely integrated following similar metabolic pathways of intestinal absorption, bone deposition, and renal resorption. Vitamin D_3 is an essential nutrient for bone growth and maintenance and has a critical role in biological pathways such as calcium and phosphorus homeostasis, cellular differentiation, proliferation and immune function (Holick, 2004).

Strontium accumulates in bone with 99% of strontium in a vertebrate animal found in bone (Dahl et al., 2001) . At low dose levels of supplementation (316 – 634 mg/kg per day Sr^{2+}), strontium has been shown to stimulate bone formation by increasing the number of bone osteoblast cells and while at the same time inhibiting bone resorption by inhibiting the action of bone osteoclast cells, leading to an increase in bone volume with no deleterious effect on bone mineralization (Ferraro *et al.*, 1983, Marie *et al.*, 1985, Grynpas and Marie, 1990, Canalis *et al.*, 1996, Dahl *et al.*, 2001). In summary strontium has both an anabolic and an anti-resorptive effect on bone (Marie et al., 2001) . Interestingly among the trace elements found in bone strontium is the only element correlated with bone compression strength (Jensen et al., 1997) .

The commercial laying hen as a large metabolic demand for nutrients particularly those minerals associated with eggshell formation. As such laying hens towards the end of their production cycle (greater than 60 weeks of age), may show similar skeletal abnormalities as older human osteoporotic patients having reduced bone density, increased bone fractures and poorer eggshell quality. The aim of this study was to evaluate the effect of increased low level supplementation of vitamin D_3 and strontium on the performance, egg quality and bone composition of older laying hens.

The older laying hen represents an animal model similar to that of the post-menopausal woman in which strontium and vitamin D_3 supplementation at low levels has shown significant benefit.

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II. MATERIALS AND METHODS

A total of 108 ninety nine week old Isa Brown laying hens were fed for 12 weeks on a layer diet supplemented with two levels of vitamin D_3 (2,500 iu D_3/kg and 5,000 iu D_3 /kg) and three levels of strontium (0, 500 and 1000 mg/kg) in a full factorial arrangement, an outline is shown in Table 1. Each treatment consisted of 18 birds (six replicates of 3 birds per replicate). Each bird was housed separately in cages measuring 25 x 50 x 50 cm^2, with three adjacent cages forming the replicate unit which were placed evenly throughout the experimental laying house. The photoperiod regimen was 16 hours of light and 8 hours of dark. Water and feed was supplied *ad libitum.* On day 92 of this study, a 48 h total collection procedure was undertaken for three out of the six replicate groups whereby the feed input and faecal output were measured to determine energy, nitrogen and mineral retention.

Table 1 - Outline of dietary treatments.

Diet	Diet Description	Added D_3* iu/kg	Added strontium† mg/kg
1	Normal D_3 no added strontium	2500	0
2	Normal D_3 + medium strontium	2500	500
3	Normal D_3 + high strontium	2500	1000
4	High D_3 no added strontium	5000	0
5	High D_3 + medium strontium	5,000	500
6	High D_3 + high strontium	5,000	1000

* DSM Nutritional Products Pty Ltd Rovimix® D_3 500
† Strontium carbonate from Ajax Chemicals

Throughout the 12 week trial all eggs were collected and weighed for each replicate group. In order to determine egg quality a total of thirty eggs per replicate group were randomly collected, weighed and their egg yolks and egg shells weighed. Finally on day 94 of the study, all birds were humanely euthanized and the left tibia excised for analysis.

III. RESULTS

Table 2 - Effect of dietary vitamin D_3 and strontium on production parameters in laying hens.

D_3 (iu/kg)	Sr (mg/kg)	Egg no 94 days	Average egg weight (g)	Egg mass per bird per day (g)	Feed intake per bird per day (g)	Feed per 60g egg (FCR) (g)
2500	0	210.7	65.1 ±1.10	50.7 ±2.87	124.2 ±2.60	147.9 ±11.34
2500	500	239.3	62.5 ±1.10	53.1 ±2.87	133.5 ±2.59	151.4 ±11.34
2500	1000	220.2	62.8 ±1.10	46.8 ±2.87	133.4 ±2.60	176.6 ±11.34
5000	0	196.2	65.6 ±1.10	45.5 ±2.87	126.9 ±2.59	170.4 ±11.34
5000	500	239.0	63.7 ±1.20	53.9 ±3.14	129.9 ±2.84	144.8 ±12.42
5000	1000	194.0	66.9 ±1.10	45.6 ±2.87	128.7 ±2.59	177.7 ±11.34
Model P		*N.S.*	*= 0.0593*	*N.S*	*N.S*	*N.S*
			Main Effects			
2500		223.9	63.47^{b}	50.19	130.39	158.63
5000		209.72	65.38^{a}	48.34	128.47	164.63
P		*N.S*	*<0.05*	*N.S*	*N.S*	*N.S.*
	0	208.17^{b}	65.34	48.11^{ab}	125.54^{b}	159.12^{ab}
	500	239.17^{a}	63.09	53.50^{a}	131.70^{a}	148.12^{b}
	1000	202.33^{b}	64.84	46.20^{b}	131.05^{a}	177.15^{a}
-	*P*	*<0.05*	*N.S.*	*= 0.0518*	*<0.05*	*=0.0551*
		Interaction Terms				
vitaminD_3*strontium		*N.S*	*N.S.*	*N.S.*	*N.S.*	*N.S.*

Means in columns with no common superscript differ significantly.

† Standard Error Mean (SEM)

There was an improvement ($P < 0.05$) in egg production and feed conversion efficiency with strontium at 500 mg/kg however there was deterioration in feed efficiency with strontium at 1000 mg/kg. Egg weight was significantly increased in those hens fed additional vitamin D_3. The results for production parameters are shown in Table 2.

In respect to mineral retention there was a significant ($P < 0.01$) vitamin D_3 and strontium interaction in respect to sodium retention. Also there was a trend for vitamin D_3 and strontium to produce an interaction with phosphorus retention ($P = 0.0587$) whereby higher levels of strontium with the lower level of vitamin D_3 (2,500 iu D_3/kg) improved retention but with the higher level of vitamin D_3 (5,000 iu D_3/kg) the effect of strontium supplementation was to reduce phosphorus retention. Higher strontium (1000 mg/kg) inclusion reduced calcium and sodium retention. The effect on mineral retention is shown in Table 3.

Table 3 - Effect of dietary vitamin D_3 and strontium supplementation on mineral retention in laying hens.

D_3 (iu/kg)	Sr (mg/kg)	Ca retention (%)	P retention (%)	Sr retention (%)	Mg retention (%)	Na retention (%)
2500	0	37.26	26.67^{b}	28.47^{b}	27.13^{b}	28.24^{ab}
2500	500	34.86	28.67^{ab}	18.93^{b}	24.27^{b}	44.75^{a}
2500	1000	51.29	36.14^{ab}	34.63^{b}	25.41^{b}	46.85^{a}
5000	0	46.09	40.35^{a}	73.87^{a}	35.77^{a}	48.53^{a}
5000	500	31.56	35.37^{ab}	23.40^{b}	22.53^{b}	31.20^{ab}
5000	1000	23.22	29.17^{ab}	24.70^{b}	22.21^{b}	14.72^{b}
SEM		*7.717*	*3.923*	*6.444*	*2.655*	*7.078*
Model P		*N.S.*	*N.S.*	*< 0.01*	*< 0.05*	*< 0.05*
			Main Effects			
2500		41.14	30.35	27.34^{b}	25.60	39.94
5000		33.62	34.91	40.66^{a}	26.84	34.48
P		*N.S*	*N.S.*	*< 0.05*	*N.S.*	*N.S.*
	0	41.67	33.51	51.17^{a}	34.45^{a}	38.38
	500	33.21	31.77	21.17^{b}	23.40^{b}	37.97
	1000	37.26	32.63	29.67^{b}	23.81^{b}	30.79
	P	*N.S.*	*N.S.*	*<0.01*	*<0.05*	*N.S.*
		Interaction Terms				
vitaminD_3*strontium		*N.S*	*=0.0587*	*<0.01*	*N.S.*	*<0.01*

Means in columns with no common superscript differ significantly.

† Standard Error Mean (SEM)

IV. DISCUSSION

The significant increase in total egg numbers and the trend towards improvement in FCR in those hens supplemented with strontium at 500 mg/kg was unexpected and to the authors knowledge it is the first time such an outcome has been documented in laying hens. The significant improvement in egg numbers and FCR produced by the laying hens fed strontium at 500 mg/kg may provide an economic advantage for commercial production.

A possible mechanism for the increase in egg production may involve one of the key regulators of muscle and bone development in the chicken and other vertebrates, namely insulin-like growth factor-1 (IGF-1) (Duclos, 2005). Strontium has been shown to significantly increase IGF-1 serum levels in different vertebrate species, namely women (Gulhan et al., 2008), rats Amman (2004), and goats, Li *et al*. (2008). The inter-relationship between strontium and IGF-1 is further exemplified by the studies of Fatayerji *et al*. (2000) who showed that strontium absorption was not related to vitamin D_3 metabolites but was positively correlated with IGF-1.

A second possible mechanism by which strontium may improve laying hen performance is by protecting mitochondrial membranes. Various studies have shown that strontium protects mitochondrial structure by means of a stabilising effect on mitochondrial membranes, and the level of protection is proportional to the strontium content within the extracellular fluid (Tashmukhamedov and Gagel'gans, 1970, Caplan and Carafoli, 1965, Carafoli, 1965).

An increase in vitamin D_3 produced an improvement in phosphorus retention when fed with lower levels of strontium (20 and 500 mg/kg). Furthermore it has been shown that low levels low levels of phosphorus in the diet will directly increase the production of the active metabolite for vitamin D namely 1, 25 dihydroxyvitaminD_3 (1,25-$(OH)_2D_3$) in the kidney (Tanaka and DeLuca, 1973, Han *et al.*, 2009).

V. CONCLUSION

The supplementation of strontium at 500 mg/kg significantly improved egg production and improved feed efficiency. The supplementation of higher vitamin D_3 levels significantly increased egg weight particularly albumin content. This experiment confirmed a unique interrelationship between vitamin D_3 and strontium. Further investigation needs to be undertaken to refine the optimum strontium level required to maximize hen performance. The nature of the relationship between vitamin D_3 and strontium also requires further exploration particularly at the intercellular level. The findings of this experiment may justify the standard inclusion of strontium in commercial layer and breeder diets.

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MICROALGAE IN LAYER DIETS CREATE FUNCTIONAL, DHA ENRICHED EGGS

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Summary

Functional foods have become a hot topic in the food industry due to the push for healthier food products on supermarket shelves. According to the Academy of Nutrition and Dietetics, a functional food is defined as "A food that provides additional health benefits that may reduce disease risk and/or promote good health." Eggs can be marketed well as functional foods as they are increasingly seen to be part of a healthy diet. Enriched eggs offer a way to increase the levels of health promoting micronutrients, without significantly changing eating habits. Consumers buy, cook and eat eggs in the same way, just simply having to choose an enriched brand.

I. INTRODUCTION

Omega-3 PUFA (polyunsaturated fatty acids) are commonly known for their health benefits to the consumer. This family of essential fatty acids provides a host of health benefits. There are three types of fatty acids in the omega-3 family: alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Each of these omega-3 fatty acids is considered essential; however, not all omega-3 fatty acids are created equal. DHA is the most important of these fatty acids and is primarily responsible for the benefits commonly associated with omega-3 foods and supplements.

Alpha-linoleic acid (ALA) is a short-chain omega-3 that serves as a source of energy and as a building block for EPA and DHA. ALA is commonly derived from linseed and flaxseed in livestock diets and thus is often promoted as an omega-3 in foods largely due to low cost and the abundant availability. Whilst this serves as an energy source, its relative inability to be converted in the body to DHA means that its health benefits are minimal.

EPA is a long-chain omega-3 fatty acid important for overall health. Humans do have limited ability to convert dietary ALA to EPA and crucial DHA, however the efficiency of the conversion is very low (less than one percent) and dietary intake of EPA and especially DHA is necessary to maintain sufficient amounts in the body.

DHA is an essential omega-3 fatty acid, most commonly found in wild fish like salmon and mackerel, which feed on marine algae. The active forms of essential omega (n)-3 fatty acids, particularly DHA, in human diets play important roles during pregnancy and early infant development. In adults, high levels of dietary DHA and EPA have been associated with lower rates of coronary heart disease, arrhythmias, atherosclerosis, inflammation, diabetes, and cancers such as breast, prostate, and colon. However, the typical Australian dietary DHA intake falls short of recommendations of 500 mg/day (National Heart Foundation, 2008).

Fish oil is the most commonly known supplement for long chain omega-3 PUFA but it is unsustainable as a result of overfishing which is causing depletion in fish stocks in the sea thus there is an ever increasing need in the animal production sector to find new feed resources. These need to be environmentally friendly and use natural resources efficiently (Rymer et al., 2010). Consumer safety has also been highlighted in the media

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when levels of polychlorinated biphenyls (PCB's), which have been linked to cancer, have been found in some supplements (Warner, 2011).

Algae are gaining attention for their application in the feed and food industries as a highly sustainable source of protein and DHA omega-3. Moreover, the omega-3 content in fish originates from their consuming either the algae directly or other creatures that feed on algae. Algae are a diverse group of simple organisms, ranging from unicellular to multicellular forms such as giant kelp. The use of algae as a source of PUFA is increasingly globally recognized and further research holds great potential for benefits in human pharmacology and nutrition (Robertson et al., 2013).

Microalgae refer to the numerous microscopic algae that grow in marine or freshwater environments, converting water and carbon dioxide to biomass and oxygen in the presence of sunlight. Most commercial production of microalgae is done autotrophically in open outdoor circulating raceways or ponds. Under autotrophic growing conditions, microalgae use light energy to fix carbon dioxide, their carbon source, into hydrocarbons with oxygen discharged as waste product. However, open systems are subject to several disadvantages such as airborne contamination and downstream processing. The growth of zooplankton and other species are also drawbacks of an open system. The other commercial production method in growing algae is the heterotrophic system. Heterotrophic species get their energy from organic carbon compounds. By eliminating light from the production process, any fermenter (such as those used for production of medicines, beverages and food additives) can be used for heterotrophic algal growth (Tsappis, 2013).

The heterotrophic method maintains a closed controlled system that provides a more consistent, traceable and pure algal product. By manipulating the physical and chemical properties of the cultural medium, several species of microalgae can produce and accumulate higher levels of specific fatty acids. Specific strains of heterotrophically grown microalgae thus contain large quantities of high quality eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

II. PRODUCTION OF DHA OMEGA-3 EGGS

A number of global studies conducted by Alltech have examined the improvements to the egg fatty acid composition from feeding layer hens a diet containing *Schizochytrium* microalgae *(All-G-Rich™, Alltech Inc).* These studies were carried out to demonstrate the value of improving the DHA-content in the eggs to the animal, farmer, retailer, consumer and environment. Adding a DHA source into animal diets can naturally increase the DHA levels in food products, providing consumers a way to increase daily intake of DHA to support general health and wellness. Dietary supplementation with All-G Rich™ can affect the DHA omega-3 content of the whole egg, allowing for an egg with almost 2.5 times the DHA content of a conventional egg.

Alltech conducted two research studies at the University of Kentucky. Study one used 288 Hy-Line week 36 layers with 12 replicates and six birds per unit. Eggs were sampled after four weeks and performance was monitored for 16 weeks. The treatment included a corn-soy based commercial diet with 0, 0.5, 1.0, or 2.0% All-G-Rich™ (Table 1). DHA content measured in study one linearly increased with dietary supplementation of All-G-Rich™ (Figure 1). In study two, the trial was performed using 120 Hy-Line week 36 Layers with six replicates and five birds per unit. Eggs were sampled at four weeks. Treatments included a corn-soy commercial diet plus 0, 1.0, 2.0 or 3.0% of ALL-G-Rich™ (Table 1). Performance of the layers as measured in Study two was unaffected by treatment. (Table 2).

Table 1 - Supplementing All-G Rich™ in hen diet increased DHA content in egg yolk and eggs (mg/100g).

Dietary treatment	Study 1		Study 2	
	Egg yolk	Whole egg	Egg yolk	Whole egg
Corn-soy diet (no algae)	283[d]	81[d]	248[d]	70[d]
Diet + 0.5% All-G Rich	419[c]	120[c]	n/a	n/a
Diet + 1% All-G Rich	510[b]	144[b]	509[c]	143[c]
Diet + 2% All-G Rich	656[a]	188[a]	717[b]	198[b]
Diet + 3% All-G Rich	n/a	n/a	776[a]	214[a]
SEM	13.4	4.4	15.6	4.4

Data are means of 12 replicates of three eggs per replicate.
SEM: standard error of the mean
[a,b,c,d] Means differ P < 0.01

Table 2 - Effects of dietary treatments on egg weight, egg and shell quality and egg yolk weight and color.

Trt.		Egg Weight (g)	Haugh Unit	Percent Shell, %	L Color	a* Color	b* Color	Yolk Weight (g)
1	Corn-soy commercial diet (no Algae)	66.3	74.1	8.64	63.9[a]	12.7[b]	59.5	19.2
2	Corn-soy diet + 1% All-G-Rich	66.8	70.7	8.60	64.0[a]	12.7[b]	60.7	19.8
3	Corn-soy diet + 2% All-G-Rich	66.4	72.0	8.35	62.7[b]	13.6[a]	62.6	18.9
4	Corn-soy diet + 3% All-G-Rich	65.8	74.8	8.23	63.0[b]	13.8[a]	62.8	19.0
SEM		1.12	1.83	0.19	0.24	0.35	0.97	0.47
P		0.93	0.40	0.38	0.004	0.006	0.08	0.54

Data are means of 6 eggs per unit sampled at week 20 of feeding treatment diets
SEM: standard error of the mean
[a, b] Means differ (P < 0.01)

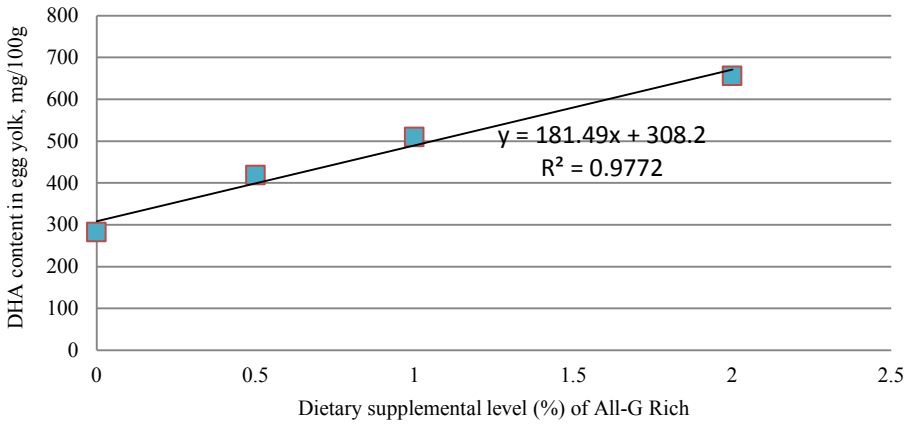

Figure 1 - Linear response of DHA concentration of egg yolk vs. dietary supplementing All-G Rich.

Changes to the fatty acid composition of the DHA enriched eggs did not affect the sensory and storage qualities of the eggs. The eggs looked, cooked and tasted the same as conventional eggs and flavour was significantly preferred in sensory evaluations. There was no reduction in the efficiency of the egg production of DHA enriched eggs when compared to conventional eggs. The DHA enriched eggs can be promoted as a dietary alternative source to fish. The ability to enrich eggs with DHA provides the egg industry with a unique opportunity to produce an innovative, quality and value-added premium product. The Korean concept of YaShikDongWon correlates food and medicine to the same origin. This notion has never been more important and highlights the importance of functional foods for preventative and therapeutic purposes relating to health. Concepts of health and wellbeing across the world are accelerating from global convergence from increased import and export of ingredients (Financial Times, 2013). Functional foods have been a part to this and advancements in modern food backed by scientific evidence have gained public approval.

III. CONCLUSIONS

Consumer demand for superior health quality food products has increased interest in enriching the eggs with DHA omega-3 polyunsaturated fatty acids. This has proven to be a viable method of adding value to eggs for the health-conscious consumers. Algae are gaining attention for their application in the feed and food industries as a highly sustainable source of protein and DHA omega-3. Microalgae-based All-G-Rich™ supplementation in layer diets is a potentially safe, sustainable way to create a more wholesome, naturally enriched DHA eggs to help correct human dietary deficiencies.

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THE EFFECT OF INCORPORATION OF CALCIUM PIDOLATE AND OYSTER SHELL ON THE QUANTITATIVE AND QUALITATIVE PARAMETERS OF EGG PRODUCTION

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Summary

The degradation of egg shell quality from birds over 50 weeks of age leads to loss of collected eggs and an increase in downgraded eggs. This phenomenon is generally attributed to the reduced capacity for absorption and mobilization of body calcium in the aged birds. A second limiting factor is involved: the advanced age of the animals causes a reduction in the capacity to synthesize components of the egg's internal membrane. This experimental study, conducted as part of a thesis, compares the influence of a source of calcium carbonate (oyster shell at 3 grams/layer/day) with calcium pidolate (incorporated at 300 ppm). The treatments are repeated four times on 12 Lohmann White hens (55 weeks old): egg production, egg shell quality and feed conversion ratio (FCR) were studied over 8 weeks of production. In the calcium pidolate group, production was significantly increased by 5% ($p < 0.05$) and the number of downgraded eggs due to shell quality problems was reduced by 25% ($p < 0.05$). The egg weight was also improved. The combination of these improvements led to a decrease in FCR of 10%. The incorporation of calcium pidolate, which is involved in calcium metabolism and synthesis of components of the egg shell internal membrane, allows maintenance of egg shell quality and higher production parameters when compared with the addition of oyster shell- a $CaCO_3$ source considered to be more bioavailable than limestone.

I. INTRODUCTION

From 50 weeks of age, reductions in egg shell quality cause a loss in collected eggs and an increase in downgraded eggs (less valued). This phenomenon is generally attributed to a dysfunction of calcium metabolism (reduced capacity for absorption and mobilization of body calcium). A second limiting factor may be involved: the advanced age of the animals causes a reduction in the capacity of synthesis of components of the egg's internal membrane.

This experimental study, conducted as part of a thesis, at the Research Department on Animal Production of the University of Santiago in Chile, compares the influence of a soluble source of calcium carbonate (oyster shell at 3 grams/layer/day) with calcium pidolate (incorporated at 300 ppm), in the feed of layers between 56 and 63 weeks old. This work focused on quantitative parameters (weekly egg production, egg weight, feed conversion ratio (kg of feed / kg of eggs) and qualitative (% downgraded eggs, % downgraded eggs due to egg shell problems).

II. MATERIALS AND METHODS

56 week old layer birds (Lohmann® white) were used in the trials. There were two dietary treatments and each treatment had 48 layers (12 layers x 4 replicates). The basal diet was formulated according to Lohmann® white specification but without phytase. Calcium carbonate (limestone) was incorporated in the basal diet at 60% grit and 40% powder. Treatment 1 consisted of addition to the basal diet of 300 g/ton of calcium pidolate

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(PIDOLIN® P Ca)-an organic calcium salt patented by Dietaxion. Treatment 2 consisted of birds on control diet and given additional 3 g/hen/day of oyster shells with diameter above 2mm. Both diets had a minimum calcium level of 4%.

The layer birds were housed in a naturally ventilated shed in cages of 4 hens each. The feed was available ad libitum. Daily egg collection was at 1pm. The egg count included total eggs collected, broken eggs, soft eggs, dirty eggs, blood stained eggs and small eggs. All eggs collected on Fridays were weighed.

The study compared the average of production parameters over a period of 8 weeks. The equality of variances of the data was checked with the F-TEST and the averages using a Student T-test.

III. RESULTS AND DISCUSSION

Table 1 below shows that calcium pidolate incorporation provokes a significant increase on egg production of 4 points ($p < 0.05$) during the total period (an increase of more than 5 % production/hen/week) in comparison with the results of the group which received oyster shell. The improvement is partly due to the direct effect of calcium pidolate and could also be attributed to the dilution effect on the diet due to the incorporation of oyster shell at 2.5%.

Table 1 - Effect of the incorporation of calcium pidolate and oyster shell on egg production (% per day).

Week[1]	Calcium pidolate	Oyster shell
56	86.01 ± 2.64	86.90 ± 5.67
57	82.44 ± 6.33	74.70 ± 7.74
58	87.50 ± 3.57	76.79 ± 9.20
59	88.39 ± 4.05	83.11 ± 6.71
60	86.31 ± 6.03	80.93 ± 3.73
61	86.42 ± 8.46	81.60 ± 5.10
62	84.60 ± 6.60	82.12 ± 2.54
63	78.02 ± 5.78	78.62 ± 5.51

Test of variances equality (F-Test)

	Calcium pidolate	Oyster shell
Average	84.8117	79.6939
Variance	12.7852	9.5333
F	1.3411	

Student-Test: $p = 0.029$ (significant difference)

The percentage of total downgraded eggs of calcium pidolate group was different from the oyster shell group ($p=0.05$). It appears that this parameter only differs in the second phase of treatment (from week 60).

The incidence of downgraded eggs represents the sum of broken and soft-shelled eggs divided by the total number of eggs collected. The type of defect is not identified. The calcium pidolate group had a lower proportion of eggs downgraded due to shell quality. There is a difference of 4 points compared with oyster shell, which relates to 25% lower ($p < 0.05$)

Table 2 - Effect of the incorporation of calcium pidolate and oyster shell on downgraded eggs for shell quality problems (broken and dirty) (% of total collected eggs).

Week[1]	Calcium Pidolate	Oyster shell
56	10.39 ± 3.29	10.28 ± 1.21
57	7.22 ± 3.15	9.46 ± 5.43
58	8.12 ± 3.15	12.45 ± 9.32
59	11.20 ± 4.09	11.87 ± 4.14
60	9.16 ± 7.29	13.55 ± 4.29
61	6.28 ± 1.41	12.98 ± 5.29
62	8.90 ± 3.57	16.11 ± 9.37
63	10.34 ± 6.43	16.91 ± 5.05

Test of variances equality (F-Test)

	Calcium Pidolate	Oyster shell
Average	8.7443	13.33
Variance	2.9241	6.4331
F	0.4545	

Student-Test: p = 0.003 (significant difference)

Average eggs weight shows a positive effect of the use of calcium pidolate: higher effect on average of 4% compared to oyster shell (p = 0.08). FCR of calcium Pidolate group is significantly improved by 10% compared to oyster shell group (p = 0.05).

Table 3 - Effect of the incorporation of calcium pidolate and oyster shell on feed conversion ratio.

Week[1]	Calcium Pidolate	Oyster shell
56	2.73 ± 0.15	2.58 ± 0.26
57	2.74 ± 0.38	2.77 ± 0.26
58	2.72 ± 0.09	3.13 ± 0.21
59	2.62 ± 0.29	2.89 ± 0.33
60	2.86 ± 0.17	3.31 ± 0.28
61	2.79 ± 0.39	3.32 ± 0.57
62	2.84 ± 0.26	3.51 ± 0.79
63	2.59 ± 0.21	2.69 ± 0.17

Test of variances equality (F-Test)

	Calcium Pidolate	Oyster shell
Average	2.7366	3.0233
Variance	0.0089	0.1161
F	0.0769	

Student-Test: p = 0.05(significant difference)

IV. CONCLUSIONS

The production is enhanced significantly but egg weight was not significantly higher for the calcium pidolate group, as compared with the oyster shell group. Lower incidence of broken and soft eggs was found in the calcium pidolate group and feed conversion ratio was also significantly improved.

This study shows that calcium pidolate use is clearly preferable to an addition of oyster shell for improving production parameters and shell quality.

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EFFECT OF SUPPLEMENTATION WITH TWO LEVELS OF 25-HYDROXYCHOLECALCIFEROL ON EGG INTERNAL AND EXTERNAL QUALITY IN COMMERCIAL LAYING HENS

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Summary

Hy-Line Brown layer hens were housed in individual cages from 19 to 80 weeks of age to study the effects of dietary supplementation with two levels of 25-hydroxycholecalciferol ($25(OH)D_3$) on egg internal and external quality, and the amount of cuticle present on the eggshells. Ninety birds were divided into group A (control group) fed with normal commercial layer mash feed, and groups B and C (treated groups) fed with normal commercial layer mash feed plus 0.5 g of $25(OH)D_3$ [premix (68.9 µg $25(OH)D_3$)] per kg of feed and 1 g of $25(OH)D_3$ [premix (137.8 µg $25(OH)D_3$)] per kg of feed, respectively.

There was a significant main effect ($P<0.05$) of hen age and treatment group on albumen height, Haugh unit, and yolk colour score. There was a significant main effect of both hen age and treatment group on most of the eggshell characteristics such as shell reflectivity before and after staining, difference in shell reflectivity, egg weight, shell weight, percentage shell, shell deformation, shell thickness, and cuticle single score value, whereas a significant interaction between these two factors was seen for yolk colour score and percentage shell. There was a significant effect only of hen age on shell breaking strength and translucency score. The results indicated that the highest albumen height, Haugh unit, yolk colour score, difference in shell reflectivity, and cuticle single score value and lowest shell reflectivity before and after staining were found in group A. In addition, the highest shell weight, percentage shell, and shell thickness and lowest shell deformation were for group C (which is the higher supplemental Hy-D group). Group B was lower than group C for shell weight and percentage shell and higher for shell deformation with group A intermediate, whereas group B was lower than the other groups for egg weight. Shell breaking strength and translucency score were affected only by hen age and there was no significant difference between the three treatment groups for these variables.

I. INTRODUCTION

In recent years, there has been a focus on egg quality to minimize yolk content and eggshell quality deterioration (Rossi et al., 2013). The big concern in the poultry industry in Australia and around the world is eggshell quality problems which cause around 10% of eggs to be downgraded (Roberts, 2005). Over the laying life of the hen, egg quality may represent the major factor that affects the entire poultry industry (Dunn, 2011). Much research has been conducted on the metabolism of calcium and vitamin D_3 in attempts to improve egg quality (Kaur et al., 2013). In addition, scientists have investigated ways to decrease the fall in egg production that happens with increasing hen age (Chennaiah et al., 2004). A number of quality characteristics of the eggshell such as shell breaking strength, deformation, and percentage shell deteriorate with increasing hen age (Roberts, 2005).

Age and nutrients are two of many factors that may affect eggshell quality (Dhawale & Nagpur, 2008). Vitamin D_3 is converted to $25(OH)D_3$ in the liver and in the kidney to $1,25(OH)D_3$ which is the active metabolite (Bar, 2008; Cohen et al., 1978; de Matos, 2008; Frost et al., 1990; Keshavarz, 2003; Morris et al., 2014). The production of $1,25(OH)D_3$ is

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related to the demands for Ca for formation of eggshell and bone, and growth (Bar, 2008). Other factors that affect eggshell quality include the amount of Ca^{2+} and/or phosphorus (P) in the diet and the Ca:P ratio (Bar, 2008). Studies have shown no effect on shell quality of adding vitamin D_3 to the feed of older hens (Jones & Scott, 2006). The objective of this experiment was to examine the effects of dietary supplementation with Hy-D®, at the recommended dose of 25(OH)D_3 (69 µg/kg of diet, Keshavarz, 2003) and at twice the recommended dose (137.8 µg/kg of diet), on egg internal and external quality from the point of lay to 80 weeks of age.

II. MATERIALS AND METHODS

Ninety Hy-Line Brown laying hens were divided into three groups (A, B, and C), 30 replicates per group with one laying hen per replicate. Hens were housed individually in commercial laying cages. Group A was the control fed with normal commercial layer mash feed, group B was fed with normal layer mash plus 0.5 g of 25(OH)D_3 premix (68.9 µg 25(OH)D_3) per kg of feed, and group C was fed with normal layer mash plus 1 g of 25(OH)D_3 premix (137.8 µg 25(OH)D_3) per kg of feed. Layer mash feed was formulated by a nutritional consultant and mixed by a specialist feed company, then Hy-D® premix was added according to the above doses for B and C groups in the mixing room at the University of New England. Treatments were applied from 19 weeks of age (when birds reached 5% production) to 80 weeks of age. Birds were given *ad libitum* access to the feed.

Eggs were analysed weekly for egg internal quality (Haugh unit, albumen height, and yolk colour score), eggshell quality (shell colour, eggshell translucency score, shell breaking strength, shell deformation, egg weight, shell weight, percentage shell, shell thickness), and the amount of cuticle present on the eggshells. Specialised equipment (Technical Services and Supply U.K. egg quality equipment, Konica Minolta Spectrophotometer, shell thickness gauge based on a Mitutoyo Dial Comparator gauge), available in the Egg Quality Laboratory at UNE, was used for these analyses. Cuticle cover was estimated using cuticle blue dye, a Konica CM-2600d hand-held spectrophotometer and a single score value was calculated after the method of Leleu et al. (2011).

Data were analysed by ANOVA using StatView software. Differences between means were established using Fisher's Least Protected Difference test.

III. RESULTS

There was a significant main effect ($P<0.05$) of hen age and treatment group on some egg quality measurements. Albumen height, Haugh unit, and yolk colour score were lower when Hy-D was added to the hen diets, (table 1). There was a significant interaction between hen age and treatment group only for yolk colour score. Some external egg quality parameters were influenced by both hen age and dietary treatment and some only by hen age. Percentage shell was influenced by the two variables and the interaction between the two. Shell reflectivity before and after staining was higher for groups B and C than for the control group A. Shell weight and percentage shell were highest for group (C) and lowest for group (B) with group A intermediate. Shell thickness was higher for group C than for groups A and B. Difference in shell reflectivity and the cuticle single score value were higher for the control group than for the other groups. Shell deformation was highest for group B and lowest for group C with group A intermediate. There was no significant main effect ($P>0.05$) of hen age or treatment group on shell breaking strength and translucency score (Table 1).

Table 1: The effects of additional Hy-D in the diets on egg internal and external quality parameters[1].

Response variable	Treatment Group + Hy-D level		
	A[2] (0 µg)	B[3] (68.9 µg)	C[4] (137.8 µg)
Haugh unit	86.89 ± 0.228 [a]	86.05 ± 0.232 [b]	86 ± 0.227 [b]
Albumen height	7.8 ± 0.034 [a]	7.6 ± 0.034 [b]	7.61 ± 0.033 [b]
Yolk colour score	9.61 ± 0.033 [a]	9.51 ± 0.031 [b]	9.55 ± 0.032 [b]
Shell reflectivity before staining (%)	26.11 ± 0.090 [b]	26.7 ± 0.112 [a]	26.6 ± 0.094 [a]
Shell reflectivity after staining (%)	21.15 ± 0.098 [b]	22.18 ± 0.126 [a]	22.3 ± 0.111 [a]
Difference shell reflectivity (%)	4.96 ± 0.054 [a]	4.48 ± 0.062 [b]	4.34 ± 0.058 [b]
Egg weight (g)	62.18 ± 0.129 [a]	61.61 ± 0.137 [b]	62.27 ± 0.141 [a]
Shell weight (g)	5.91 ± 0.015 [b]	5.82 ± 0.015 [c]	5.98 ± 0.014 [a]
Percent shell (%)	9.52 ± 0.019 [b]	9.46 ± 0.018 [c]	9.62 ± 0.018 [a]
Shell thickness (µm)	410.96 ± 0.756 [b]	412.3 ± 0.675 [b]	416.46 ± 0.683 [a]
Shell deformation (µm)	259.7 ± 0.884 [b]	263.41 ± 1.03 [a]	255.8 ± 0.970 [c]
Translucency score	1.53 ± 0.019	1.53 ± 0.019	1.5 ± 0.018
Shell breaking strength	40.68 ± 0.174	40.89 ± 0.175	40.55 ± 0.184
Cuticle single score value	18.53 ± 0.130 [a]	17.38 ± 0.166 [b]	17.06 ± 0.140 [b]

[a-c] Within a row, means with no common superscript letters are significantly different (P<0.05).
[1]Means ± Standard Error.
[2]A is control diet.
[3]B is control diet supplemented with 68.9 µg 25(OH)D_3/kg feed.
[4]C is control diet supplemented with 137.8 µg 25(OH)D_3/kg feed.

IV. DISCUSSION

Addition of 25-hydroxycholecalciferol to the diet of the hens resulted in reductions in egg internal quality. The results for Haugh unit differ from those of Zang et al. (2011) who reported no significant effects of inclusion of Hy-D in the diet of Lohman pink-shell commercial laying hens at 25 weeks of age (Zang et al., 2011). The small but statistically significant effects of Hy-D on egg internal quality have not previously been reported and the underlying mechanism is not understood. In the present study, supplementation of the diet with 25-OH-D_3 improved some shell quality characteristics, such as shell weight, percentage shell and shell thickness, particularly at the higher inclusion of Hy-D. Although the differences between groups were small numerically, they indicate that the addition of Hy-D to poultry diets has the potential to improve shell quality and this may be particularly important in older flocks. Shell deformation was highest for group B. In contrast, Keshavarz (2003) reported that there was no improvement in shell quality from inclusion of 25-OH-D_3 at 69 µg of 25-OH-D_3/kg diet. However, McLoughlin and Soares (1976) results agree with ours in that they found an improvement in shell quality of eggs laid by older hens (74 weeks of age) and eggs laid from hens reared for the second year of production with supplementation of 25-OH-D_3 with oyster shell (McLoughlin and Soares, 1976). In the present study, there was no significant effect of Hy-D supplementation on shell breaking strength which differs from the findings of Zang (2011) who reported an improvement in eggshell strength with supplementation of Hy-D to the diet of Lohman pink-shell commercial laying hens (Zang et al., 2011). Difference in shell reflectivity and cuticle single score value (ΔE*ab value) results were lower for eggs laid by group B and C hens than for the group A (control group), indicating that the cuticle cover was better for the control group than for the vitamin D supplemented groups. How the addition of Hy-D to the diet affects cuticle deposition is not known.

In conclusion, supplementation of 25-hydroxycholecalciferol to the diet of laying hens decreased internal egg quality but improved some traits of shell quality, particularly at the higher level of inclusion.

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QUANTIFICATION OF PROTOPORPHYRIN IX FROM EGGSHELL OF BROWN EGG LAYING HENS CHALLENGED WITH VACCINE AND WILD STRAINS OF INFECTIOUS BRONCHITIS VIRUS

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Summary

Infectious bronchitis virus (IBV) strains primarily infect the epithelial tissues of the respiratory tract and kidneys but they also multiply in the egg forming region of the oviduct, causing paleness of shell colour in brown egg laying hens. Protoporphyrin IX (PP IX) is the main eggshell pigment in the eggs of laying hens, in addition to other pigments such as biliverdin. The brown pigment has a number of functions including specific gram positive antibacterial action and positive influence on consumer perceptions. The aim of the current study was to assess any significant effect of different IBV strains on the shell colour in brown shelled eggs. Eggs were collected from day 2 to day 22 post infection (p.i) from unvaccinated and vaccinated laying hens challenged with IBV wild strains (T and N1/88) and vaccine strains (A3 and Vic S) in addition to a control group hens. Eggshells were processed for measurement of shell reflectivity (%), spectrophotometry (SCI L* component), and protoporphyrin IX (PP IX) quantification from shells with and without cuticle. There was a significant effect ($P<0.05$) of day p.i and viral strain on shell reflectivity, SCI L* and PP IX in eggshells with and without cuticle. The values for shell reflectivity and SCI L* increased and those for PP IX decreased with increased day p.i until day 12, suggesting an increasing viral load in the shell gland. The shell reflectivity and L* values decreased insignificantly after day 12 and slightly increased again towards day 22. The amount of PP IX tended to increase after day 12 p.i. but this was not statistically significant, suggesting that after day 12 p.i., the viral load started declining and thus shell colour was restoring in the challenged hens. The higher shell reflectivity and SCI L* values, and lower PP IX values, of eggshells from T and N1/88 followed by Vic S strain infected birds suggests that the T strain was most severe in its effect followed by N1/88 and Vic S with A3 being the more mild one. The values of shell reflectivity, SCI L* and PP IX were not significantly different for eggshells from unvaccinated and vaccinated laying hens in the whole eggshell, but were significant in shells from which cuticle had been removed.

I. INTRODUCTION

Infectious bronchitis virus (IBV) can infect chickens of all ages and has the capability to multiply in various epithelial tissues including trachea, lungs, kidneys, ovaries and oviduct (Ignjatovic *et al.,* 2002). IBV is one of the factors responsible for eggshell deterioration and lighter shell colour (Chousalkar and Roberts, 2009). The Australian strains of IBV have the ability to multiply in the shell gland of vaccinated and unvaccinated Isa Brown hens (Chousalkar and Roberts, 2007). The T strain of IBV is mainly nephropathogenic but its infection also results in the production of lighter colour shells (Chousalkar and Roberts, 2009). The N1/88 and Vic S strains infect the oviduct in laying hens causing lighter shell production (Chousalkar and Roberts, 2009). The eggshell colour of brown eggs is a quality aspect for consumers (Curtis *et al.,* 1985). Shell colour has been linked to egg quality parameters in brown eggs (Jones *et al.,* 2010) and with some known antimicrobial properties

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(Ishikawa *et al.,* 2010). Protoporphyrin IX, the main eggshell pigment (Kennedy and Vevers, 1973), is also used as a tool to assess the stress level and disease conditions of laying hens (Martinez-de la Puente, *et al.,* 2007). The objective of the present study was to measure shell colour and quantify the amount of PP IX from the cuticle and true shell in brown shelled eggs of Isa Brown laying hens challenged with two wild (N1/88 and T) and two vaccine (Vic S and A3) strains of IBV, as compared with a control group of hens.

II. MATERIALS AND METHODS

The amount of protoporphyrin was quantified from eggshells (day 2 to 22, p.i) of unvaccinated and vaccinated Isa Brown laying hens challenged with T, N1/88, Vic S or A3 strains of IBV and from a negative control group.

Shell reflectivity (%) and shell colour (L* component of L*a*b*) were measured on the shells using a reflectivity meter and a Konica Minolta spectrophotometer (CM-2600d), respectively. Shell reflectivity, expressed as a percentage, is an indicator of shell colour lightness – the higher the value, the lighter the colour of the eggshell and vice versa. In the L*a*b* colour space system, L* represents the grading between white (100) and black (0). The higher the value for L*, the lighter the shell colour and vice versa. Briefly, each eggshell, individually, was soaked in an EDTA solution (0.34 M, pH 7.5) for 5 minutes and the cuticle was scrubbed off in running tap water using a soft brush. Shell reflectivity and shell colour L* were measured as described earlier on the eggshells with cuticle removed.

Eggshells from the one control and four challenge groups on days 2 to 22 post infection were analysed for PP IX in whole eggshell and shell without cuticle layer. The dried shells were soaked for 2 hr in water, the shell membrane was removed manually and the shells were allowed to dry thoroughly. A 0.250 g sample from each shell was weighed into a 10 mL plastic centrifuge tube into which 4 mL of methanol- concentrated (36%) HCl (2:1) solvent was added. All tubes were wrapped in aluminum foil and placed in a refrigerator for 3 hr, avoiding exposure to light. The samples were centrifuged at 3000 rpm for 30 minutes. After centrifugation, the supernatant solution was decanted into spectrophotometer cuvettes (4mL) and the absorbance of the supernatant was read at 412 nm (Shimadzu, UV-1201).

A standard curve was constructed from solutions of protoporphyrin (Sigma Aldrich Australia) ranging from 0 mM to 6.87 x 10^{-6} mM and used to calculate the amount of protoporphyrin in 1g of eggshell (with and without cuticle present). For determination of the amount of PP IX in the cuticle, the values of the eggshell samples without cuticle were subtracted from the values of the eggshell samples with the cuticle. Data were analysed using Statview Software (SAS Institute Inc., Version 5.0.1.0). A two way analysis of variance (ANOVA) was conducted taking day (p.i.) and group as independent variables and reflectivity, shell colour L* and amount of PP IX in shell with and without cuticle as dependent variables. Level of significance was indicated by probability of less than 5%. The Fishers LSD test was used to differentiate levels of significance between mean values.

III. RESULTS

There was a significant main effect ($P < 0.0001$) of day (p.i.) and challenge strains (i.e. control, N1/88, T, Vic S and A strains) but no significant interaction between the two ($P > 0.05$) for shell reflectivity (%) and shell colour L* measured on eggshells with and without cuticle. The shell reflectivity and L* values increased with the day p.i until day 12 and then slightly decreased up to day 22, indicating that the amount of pigment first decreased and then increased slightly with day p.i (Table 1). The eggshells of hens infected with wild strains of IBV were lighter in colour compared with the vaccine strains. The values were not significantly different between unvaccinated and vaccinated hens for the whole eggshells but

the difference was significant when values were measured on shell without cuticle and cuticle itself (Table 3). Eggshells from control group and A strain infected hens were significantly different from the other groups with lower shell reflectivity, L* values and higher PP IX values which means that these hens deposited a higher amount of PP IX into shells. The reflectivity and L* values were very similar throughout the experiment.

For a 1g piece of eggshell, there was more PP IX in the shell with cuticle intact, as compared with a piece of shell from the same eggshell with cuticle removed. The total amount of PP IX in 1g of shell both with and without cuticle was significantly higher in day 2 eggshells compared to the other days up until day 22 (Table 1). Among the treatment groups, for shells with and without cuticle, control group eggshells had the most protoporphyrin, T strain the lowest, with the N1/88, Vic S and A groups intermediate (Table 2).

Table 1 - Effect of day p.i. on shell colour variables.

Day p.i.	Reflectivity % with cuticle	Reflectivity % without cuticle	PP IX (mM) with cuticle	PP IX (mM) without cuticle	PP IX (mM) in cuticle
2	^{c}31.18	^{ab}37.48	$^{a}1.1x10^{-7}$	$^{a}8.5x10^{-8}$	$^{a}2.4x10^{-8}$
4	^{abc}32.41	^{ac}38.18	$^{cde}8.7x10^{-8}$	$^{cde}6.8x10^{-8}$	$^{bcd}1.9x10^{-8}$
6	^{a}32.79	^{a}38.76	$^{de}8.4x10^{-8}$	$^{d}6.7x10^{-8}$	$^{d}1.6x10^{-8}$
8	^{ab}32.61	^{ab}38.46	$^{e}8.2x10^{-8}$	$^{cd}6.5x10^{-8}$	$^{cd}1.7x10^{-8}$
10	^{c}31.30	^{bcde}37.45	$^{cde}8.6x10^{-8}$	$^{bcd}6.7x10^{-8}$	$^{bcd}1.9x10^{-8}$
12	^{abc}31.74	^{de}36.85	$^{cd}8.8x10^{-8}$	$^{bcd}6.8x10^{-8}$	$^{bcd}1.8x10^{-8}$
14	^{c}30.78	^{de}36.20	$^{c}9.0x10^{-8}$	$^{bc}7.1x10^{-8}$	$^{bc}1.9x10^{-8}$
16	^{c}31.15	^{cd}36.89	$^{cde}8.7x10^{-8}$	$^{cde}6.9x10^{-8}$	$^{bcd}1.8x10^{-8}$
18	^{c}30.86	^{e}36.09	$^{c}9.1x10^{-8}$	$^{c}7.2x10^{-8}$	$^{bcd}1.9x10^{-8}$
20	^{bc}31.50	^{e}37.27	$^{b}9.9x10^{-8}$	$^{b}7.8x10^{-8}$	$^{bc}2.0x10^{-8}$
22	^{c}30.85	^{cde}36.89	$^{b}9.9x10^{-8}$	$^{b}7.8x10^{-8}$	$^{b}2.1x10^{-8}$
P value	0.0017	0.0002	<0.0001	<0.0001	0.0007

PP IX is protoporphyrin IX; Values are Mean
[a,b,c,d,e] Within a column, values with different superscripts are significantly different from each other

Table 2 - Effect of infectious bronchitis virus challenge strains on brown eggshell colour.

Variable	Control	T strain	N1/88 strain	Vic S strain	A strain	P value
Reflectivity % with cuticle	^{b}30.66	^{a}32.40	^{a}32.40	^{a}31.73	^{b}30.44	0.0006
Reflectivity % without cuticle	^{c}35.85	^{a}38.39	^{ab}37.51	^{ab}37.73	^{b}37.14	0.0017
PP IX (mM) with cuticle	$^{ab}9.3x10^{-8}$	$^{c}8.7x10^{-8}$	$^{bc}8.9x10^{-8}$	$^{bc}9.0x10^{-8}$	$^{a}9.4x10^{-8}$	<0.0001
PP IX (mM) without cuticle	$^{a}7.4x10^{-8}$	$^{c}6.8x10^{-8}$	$^{ab}7.3x10^{-8}$	$^{bc}7.1x10^{-8}$	$^{ab}7.2x10^{-8}$	0.0012
PP IX (mM) in cuticle	$^{bc}1.9x10^{-8}$	$^{ac}1.9x10^{-8}$	$^{c}1.7x10^{-8}$	$^{b}1.9x10^{-8}$	$^{a}2.2x10^{-8}$	0.0006

PP IX is protoporphyrin IX; Values are Mean
[a,b,c] Across a row, values with different superscripts are significantly different from each other

Table 3 - Effect of vaccination status on eggshell colour of hens challenged with infectious bronchitis virus.

Variable	Reflectivity % with cuticle	Reflectivity % without cuticle	PP IX (mM) with cuticle	PP IX (mM) without cuticle	PP IX (mM) in cuticle
Unvaccinated	31.63	[a]37.79	9.0 x10^{-8}	[b]7.0 x10^{-8}	[a]2.0x10^{-8}
Vaccinated	31.45	[b]36.86	9.2 x10^{-8}	[a]7.3 x10^{-8}	[b]1.8x10^{-8}
P value	0.2781	0.0198	0.0932	0.0001	0.0124

PP IX is protoporphyrin IX; Values are Mean
[a, b] Within a column, values with different superscripts are significantly different from each other

IV. DISCUSSION

There was more protoporphyrin IX in the calcareous part of eggshells of vaccinated hens compared with unvaccinated hens. The reduction in shell colour resulting from challenge of unvaccinated hens with N1/88 strain and T strain IBV has been reported previously (Chousalkar and Roberts, 2009) and the current study utilized eggshells collected during that experimental trial. The results of protoporphyrin confirmed that the loss of shell colour resulting from IBV challenge correlates with a smaller amount of the pigment protoporphyrin being deposited into the eggshell during the eggshell formation in the shell gland of the brown laying hen. The non-significant difference between unvaccinated and vaccinated hen eggshell values for shell reflectivity, L* and PP IX could explain that the vaccinations had little or no protective effect against IBV infection of shell gland on the secretion and/or deposition pattern of protoporphyrin IX in the shell gland and that the viral strains could infect the shell gland of unvaccinated and vaccinated hens at the same level of intensity. The current study is on-going and will investigate protoporphyrin levels in the shell gland tissues of IBV infected hens.

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EFFICACY OF *BUTTIAUXELLA* PHYTASE IN CORN SOYA BASED LAYER DIET ON EGG PRODUCTION PERFORMANCE AND NUTRIENT DIGESTIBILITY

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Summary

This study was aimed at determining the efficacy of phytase on egg production performance and nutrient digestibility in laying hens. Three dietary treatments were tested including a positive control (PC), negative control (NC) and NC + 300 FTU/kg of a phytase derived from *Buttiauxella spp*. expressed in *Trichoderma reesei*. PC diet was based on corn/soy and formulated to provide 2820 kcal ME and 163 g protein per kg feed in phase-1 and 2811 kcal ME and 163 g protein per kg feed in phase-2 diets. NC diet had 0.20% and 0.18% lower available P in phase 1 and 2 diets respectively vs PC, while Ca was 0.15% lower. Performance parameters were measured using 240 laying hens (ISA brown, 16 reps per treatment X 5 hens/rep) over a period of 34 weeks during their first laying cycle. At the end of the study faecal and tibia samples were collected for total tract nutrient digestibility and bone mineralization measurements, respectively. Hen housed egg production, egg weight and egg mass were significantly lower in NC diet fed group than those fed the PC group. *Buttiauxella* phytase supplemented NC diet significantly improved hen housed egg production, egg weight, egg mass, FCR and feed efficiency compared to the unsupplemented NC diet. Feed intake and egg quality parameters were not significantly influenced by dietary treatments. Liveability was significantly higher in the phytase supplemented group compared to both PC and NC groups. Gross energy and nitrogen digestibility was not affected either by dietary calcium and phosphorus levels or phytase supplementation. Dry matter digestibility was higher ($P<0.05$) in NC and phytase treatment compared to PC. Phytate phosphorus digestibility was significantly higher in phytase supplemented group than the unsupplemented group.

I. INTRODUCTION

More phosphorus is present in the form of indigestible phytate phosphorus than free phosphorus in plant based feed ingredients. It has been shown that only 30-40 per cent of phosphorus from plant sources is freely available for digestion and absorption by poultry. The rest is in the form of phytate phosphorus, which is not efficiently utilised by poultry (Perney *et al*., 1993). The phytate not only binds phosphorus but also exhibits other anti-nutritional properties in the feed (Camovale et al., 1998). Due to a lack of endogenous phytase enzyme in poultry to hydrolyse the phytate bound phosphorus (Nelson, 1976), it is necessary to add inorganic phosphorus to meet the phosphorus needs of poultry. Undigested and unutilised inorganic and phytate phosphorus in faeces and urine cause a major environmental problem when poultry excreta is used as a manure for growing crops (Ravindran et al., 1998). It has been shown that phytase supplementation of the feed improves the egg production performance and nutrient digestibility in laying hens (Hughes et al.,2008; Zyal et al., 2012). This study was designed to investigate the efficacy of *Buttiauxella* phytase on egg production performance and nutrient digestibility in layers.

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II. MATERIALS AND METHODS

A total of 240, 26 week old, ISA brown hens were randomly allocated five to a cage in a randomised block design. Prior to feeding the experimental diets, egg production was recorded for three weeks to allocate the treatments to achieve uniform egg production among all the treatment groups. There were three experimental diets and each diet was offered to 80 hens. Each diet had 16 replications (five hens per replicate) for egg production, egg weight, egg mass, egg quality measurements, feed consumption, feed conversion ratio (FCR), feed efficiency (FE), mortality, tibia and nutrient digestibility measurements. All hens in the study were weighed and checked for their health status before offering experimental diets. Maize based positive control (PC) and Negative control (NC) experimental diets were formulated to provide 2820 kcal ME and 163.5 g protein per kg feed in phase-1 and 2811kcal ME and 163.5 g protein per kg feed in phase-2 diets. The major differences between the PC and NC diets were in their available phosphorus and calcium content. PC diet had available phosphorus content of 0.33 and 0.29 % and calcium content of 3.63 and 3.80 % in phase-1 and phase-2 diets, respectively. NC diet had available phosphorus content of 0.13 and 0.11 in phase-1 and phase-2 diets, respectively. Phase-1 diets were offered for the first 17 weeks and phase-2 diets were offered for the last 17 weeks of the study. NC diet without or with 300 FTU of *Buttiauxella* phytase (Axtra® PHY) was offered *ad libitum* for a period of 34 weeks during their first laying cycle. Ingredient and nutrient composition of the experimental diets are presented in Table 1.

Table 1 - Ingredient and nutrient composition of the experimental diets (%)

	Phase-1		Phase-2	
Ingredient	Positive control	Negative Control	Positive control	Negative Control
Maize	57.67	59.31	57.14	58.64
SBM 48	22.09	21.87	22.17	21.93
Soya oil	2.5	1.95	2.56	2.09
Rice Bran	5.0	5.0	5.0	5.0
Salt	0.33	0.33	0.30	0.3
Lysine HCl	0.032	0.007	0.032	0.027
DL Met	0.167	0.165	0.169	0.166
L-Tryptophan	0.005	0.005	0.005	0.005
Limestone	8.54	8.86	9.14	9.40
Dicalcium Phosphate	1.26	0.10	1.03	0
Sodium bicarbonate	0	0	0.048	0.046
Vit / Min	0.4	0.4	0.4	0.4
Marker	2.0	2.0	2.0	2.0
Nutrients				
CP	16.35	16.35	16.35	16.35
MEP kcal/kg	2820	2818	2811	2811
Ca%	3.63	3.47	3.8	3.65
Total P%	0.62	0.41	0.58	0.39
Av P%	0.33	0.13	0.29	0.11

Means with the same superscripts are not significantly different (P<0.05)

Towards the end of the study, experimental diets with indigestible marker were offered to hens for a period of one week and faecal samples were collected over three days to measure nutrient digestibility. At the end of study, one bird from each cage was euthanized to collect a tibia sample to study bone mineralization. Data were subjected to analysis of variance (ANOVA) and General Linear Model Procedure (GLM) to test for the probability of significant differences between the means. Least significant differences (LSD) were used to test for significant differences between means using the Statistical Analysis System (SAS 2005).

III. RESULTS AND DISCUSSION

Egg production performances, nutrient digestibility and bone mineralization of hens fed either the non-supplemented or phytase enzyme supplemented diets are presented in Table 2 and 3. Hen housed egg production was 2.48 per cent lower in the negative control (NC) diet fed group than those hens fed on the positive control diet (PC). However, the difference in hen housed egg production between positive control and negative control groups were not statistically significant. Hen housed egg production of the NC diet supplemented with phytase showed this group laid significantly more ($p<0.05$) eggs than those groups fed the unsupplemented diet. Average egg weight and egg mass per day was significantly higher in PC diet fed group than those groups fed the NC diet. Hens fed the NC diet supplemented with phytase laid significantly heavier eggs and produced higher egg mass than those fed the unsupplemented NC diet. A similar response for phytase supplementation in layers was also reported by Zyla et al. (2012) and Hughes et al. (2008). Daily feed intake was numerically lower in the negative control and phytase supplemented NC groups than the PC group. However, the difference in feed intake between the treatment groups was not statistically different. Feed conversion ratio and feed efficiency was not significantly different between PC and NC fed groups. Hens fed the NC diet supplemented with phytase had a significantly improved feed conversion ratio and feed efficiency compared to those fed the unsupplemented diets.

Table 2 - Efficacy of phytase on the performance of laying hens fed corn/soya based diet.

Measurements	**PC**	**NC**	**NC+300 FTU**	**LSD**	**P**
Production performance					
Egg production (HH) (%)	89.96^{ab}	87.48^{b}	92.36^{a}	3.28	0.01
Egg production (HD) (%)	91.06^{ab}	89.03^{b}	92.36^{a}	3.09	0.10
Egg weight (g/egg)	66.69^{a}	64.72^{b}	67.21^{a}	1.53	0.005
Egg mass (g/hen/day)	60.00^{a}	56.65^{b}	62.18^{a}	2.66	0.0006
Feed intake(g/hen/day)	119.37	115.81	117.23	3.80	0.17
FCR (g feed/g egg)	1.971^{a}	2.012^{a}	1.886^{b}	0.06	0.0015
Feed Efficiency (%)	50.88^{b}	49.78^{b}	53.06^{a}	1.68	0.001
Mortality (%)	5.00^{ab}	6.25^{a}	0^{b}	5.38	0.05
Weight gain (g/hen)	8.69^{a}	-173.69^{b}	-47.5^{a}	63.67	0.0001
Egg quality Parameters					
Haugh Unit	82.75	87.25	83.37	5.69	0.23
Yolk Colour	10.75^{b}	10.68^{b}	11.37^{a}	0.58	0.04

Means with the same superscripts are not significantly different ($P<0.05$)

Liveability was significantly higher in the phytase enzyme supplemented group than those fed the unsupplemented diets. Hens fed the unsupplemented NC diet lost significantly more weight than those groups fed the PC diet or NC diet supplemented with phytase enzyme. Yolk colour was significantly improved by phytase supplementation in NC diet.

Table 2 - Efficacy of phytase on nutrient digestibility and bone mineralization of laying hens fed corn/soya based diet.

Measurements	PC	NC	NC+300	LSD	P
Dry matter (%)	66.5[b]	69.62[a]	70.00[a]	2.54	0.01
Gross Energy (%)	77.42	78.45	78.53	1.78	0.38
Nitrogen (%)	48.40	48.77	48.77	4.66	0.98
Phytate Phosphorus (%)	28.24[c]	48.49[b]	62.80[a]	6.92	0.001
Fecal Phosphorus (%)	26.66[b]	33.51[ab]	38.24[a]	7.01	0.06
Fecal Calcium (%)	55.02	51.88	57.17	0.56	0.17
Tibia Ash (%)	55.69[a]	53.68[b]	54.71[ab]	1.61	0.05
Tibia Calcium (%)	44.62	44.18	43.56	2.80	0.74
Tibia Phosphorus (%)	18.93	18.18	18.62	1.30	0.51

Means with the same superscripts are not significantly different (P<0.05)

Dry matter digestibility was significantly lower in the PC diet fed group than those fed the unsupplemented or phytase supplemented NC group. Gross energy, nitrogen, fecal phosphorus and calcium digestibility were not significantly improved by phytase supplementation of NC diet. Liebert et al. (2005) and Hughes et al. (2009) also reported a non significant effect of phytase supplementation on nutrient digestibility in layers. Phytate phosphorus digestibility was significantly improved by phytase supplementation. Improved phytate phosphorus digestibility in phytase supplemented layer diets were also reported by Lim et al. (2003) and Keshavarz. (2013). Tibia ash content was significantly lower in NC diet than those fed the PC diet. Phytase supplementation in NC diet improved tibia ash content equal to that of hens fed the PC diet. Tibia calcium or phosphorus content was not significantly influenced either by dietary phosphorus level or phytase supplementation of NC diet.

IV. CONCLUSION

Egg production performance was significantly better in the positive control diet fed group than those fed the negative control diet. Phytase supplementation of negative control diet significantly improved production performance, phytate P digestibility and tibia ash content of laying hens. This study demonstrates that *Buttiauxella* phytase can replace 0.18 to 0.2 % available P and 0.15 % Ca in corn/soy based layer diets without affecting egg production performance.

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THE EUCLIDIAN PATHWAY TO MORE INSTRUCTIVE BROILER BIOASSAYS: NUTRITIONAL GEOMETRY

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Summary

Factorial designs involving dietary treatments are widely applied in poultry nutrition research to identify the significance of multiple influential factors. Factorial experimental designs require that dependent variables are held constant so that their effects on independent variables can be investigated. However, the number of treatments in full factorial experiments can become cumbersomely large, involving several levels of a number of different factors. Poultry diets are mixtures of various feed ingredients including grains, protein meals, fats, oils sources of phosphorus and calcium and other minor ingredients. In investigations into the impact of different feedstuffs or feed additives in diets, the protein level and/or the energy density of the diet is usually held constant. Any alternation of the proportion of one component in the blend may of necessity change the proportions of other components. Conventional factorial experimental designs cannot distinguish between the effects of inclusion levels of a certain ingredient *per se* and the effects of proportions of multiple ingredients in the blend. Mixture experimental designs are extremely helpful in studies involved isoenergetic or isonitrogenous diets. Over the past two decades, a unifying framework for nutrition, described as the geometric framework for nutrition, has been developed to identify how a diversity of animal species can regulate their intake of a range of nutrients when experimentally challenged with perturbations to their nutritional environment or status. The present review compares approaches based on nutritional geometry with conventional full factorial experimental designs and other response surface methodologies. Examples of recent studies completed by the Poultry Research Foundation, where nutritional geometry was applied to broiler feeding studies are briefly included in this review.

I. FACTORIALLY DESIGNED EXPERIMENTS VS RESPONSE SURFACE METHODOLOGY

A conventional experimental approach investigates the effects of one independent variable while keeping all other variables constant. This approach faces two challenges (i) it is only valid when the underlying principles linking cause and effect are known with some certainty; (ii) variables may interact with each other and the magnitude of the effect caused by altering one factor will depend on the magnitude of one or more of the other variables (Armstrong, 2006). Factorial designs were developed to meet these challenges. They evaluate the impact of multiple variables simultaneously, assesses their relative importance, and determine the interactions between these factors, although careful consideration must be given to the factors to be investigated and the manipulated levels of each factor (Fisher, 1926). Full factorial designs are probably the most widely applied experimental approach in poultry nutrition research. They are particularly useful for identifying the significance of multiple influential factors, a useful step in preliminary stages of research to filter out unimportant variables. Once the importance of independent variables has been established, quantification

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of the magnitude of the response is usually required in further research for the purposes of optimisation and prediction. Response surface methodology can visualise the effects of variables by using contour plots or three-dimensional diagrams. The common approaches of response surface experimental design include Box-Behnken Design (BBD) and Central Composite Design (CCD) and to a lesser extent, mixture design.

Compared with response surface methodology, one major disadvantage of factorial experiments is that the number of treatments and/or replicates can become excessive very quickly. Table 1 compares the number of treatments and efficiency in factorial designs with two response surface designs. The efficiency was calculated by dividing the number of coefficients in the second order model with the number of treatment combination needed per number of factors being tested. As shown in Table 1, the central composite design and Box-Behnken design requires smaller number of treatments than factorial design; therefore, they have been used in poultry nutrition research occasionally.

Another potential challenge of factorial designs is that the interactions between independent variables often become difficult to interpret when several factors are involved. Response surface methodology may visualise the interaction clearly. For example, Liu *et al.* (2014a) investigated the effects of phytase supplementation on growth performance, nutrient utilisation and the importance of starch and protein digestive dynamics in relation to feed conversion efficiency. Figure 1 illustrates how response surface methodology detects the relative importance between the digestion rates of starch and protein in broiler chickens. The relationship indicates that feed conversion efficiency may be enhanced by manipulating starch and protein digestion rates. The synchrony of protein and carbohydrate inputs can influence protein synthesis and of feed efficiency so that starch and protein digestive dynamics should not be treated separately but in combination. A balanced provision of glucose and amino acids at sites of protein synthesis is a fundamental prerequisite for efficient growth (Geiger, 1950). In order to achieve the optimal feed conversion ratio, which is shown as the bright white area in Figure 1, the protein digestion rates are more sensitive and demanding than starch. This suggests that the digestion rate of protein is more integral to optimal feed conversion ratios in broiler chickens. Thus strategies to accelerate protein relative to starch digestion should be advantageous. Such strategies could include the incorporation of 'fast' proteins, free amino acids, and exogenous phytase or protease into broiler diets. Alternatively, whole grain feeding may retard starch digestion rates (Liu *et al.*, 2014b) to generate a better balance between protein and starch digestion rates.

Table 1 - Comparison of treatment numbers and efficiency in factorial design and response surface methodology [adapted from Ferreira *et al.* (2007); De Leon *et al.* (2010)].

Factors (k)	Number of Coefficients (p)	Number of Treatment Combinations (N)			Efficiency (p/N)		
		CCD	BBD	Factorial	CCD	BBD	Factorial
2	6	9	-	9	0.67	0.86	0.67
3	10	15	13	27	0.67	0.77	0.37
4	15	25	25	81	0.60	0.71	0.19
5	21	43	41	243	0.49	0.68	0.09
6	28	77	61	729	0.36	0.65	0.04
7	36	143	85	2187	0.25	0.63	0.02
8	45	273	113	6561	0.16	0.62	0.007

CCD: $N = 2^k + 2k + N_c$ (where k is the number of factors and N_c is the number of centre runs).
BBD: $N = 2k\ (k\text{-}1) + N_c$ (where k is the number of factors and N_c is the number of centre runs).
Full Factorial in CRD: $N = 2^k$ (where k is the number of factors).

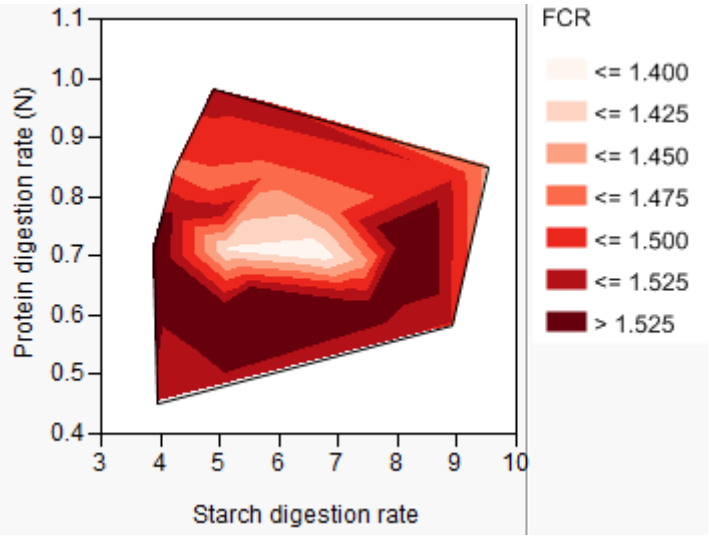

Figure 1 - Thin-plate spline response surfaces for the relationship of feed conversion ratio (FCR) with starch and protein (nitrogen) digestion rates ($\times 10^{-2}$ min^{-1}) (Liu et al., 2014a).

Response surface methodology was adopted in two recent studies to predict requirements of methionine, lysine, and threonine in broiler chickens (Mehri *et al.*, 2012, Mehri, 2014). As shown in Figure 2, Mehri *et al.* (2012) reported that the maximum weight gain at the stationary point may be obtained with 0.54, 1.12, and 0.78% of digestible methionine, lysine and threonine in the diet, respectively, and the optimal feed conversion may be obtained when the diet contains 0.53, 1.13, and 0.75% of digestible methionine, lysine and threonine, respectively. Estimated ideal ratios of digestible methionine and threonine to lysine were 48 and 70% for weight gain and 47 and 66% for FCR. Basically, response surface method is suitable to examine the effects of numerical variables; alternative applications in poultry nutrition include the investigations of exogenous enzyme efficacy (Zheng *et al.*, 2014), interactions between minerals (Bradbury *et al.*, 2014, Wilkinson *et al.*, 2014), interactions between anti-nutritional factors in feed and optimal feed processing strategies.

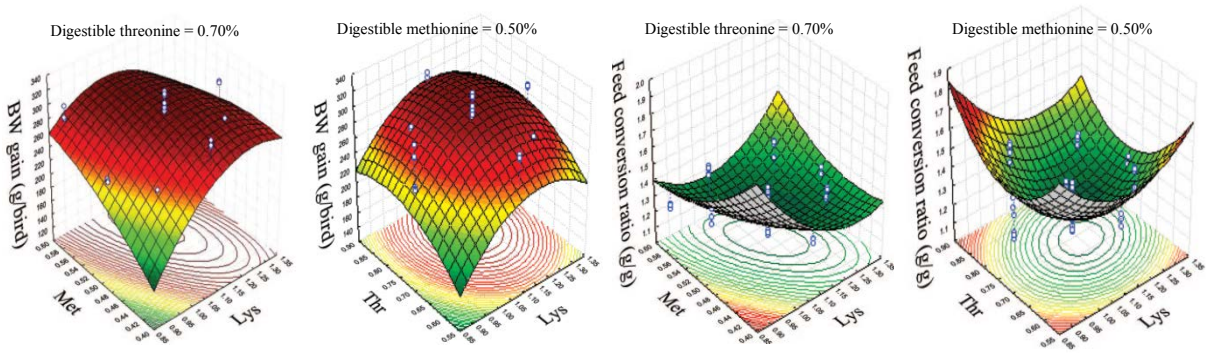

Figure 2 - Response surface plots of weight gain and feed conversion ratio from broilers offered diets with different levels (%) of lysine, methionine and threonine from 3 to 16 days post-hatch (Mehri et al., 2012).

II. RIGHT-ANGLED MIXTURE TRIANGLE

Response surface methodology can be applied to explore the effects of numerical variables, for example, environmental temperature, humidity and lighting regimen on feed intake, or the steam-pelleting conditioning temperatures, retention times and feed additive inclusions on growth performance. However, poultry diets are mixtures of various feedstuffs including

grains, protein meals, and a range of feed ingredients. Any alteration of the proportion of one component in the mixture must of necessity change the inclusion rate of at least one other component. Moreover, in practice, nutritionists formulate poultry diets based on apparent metabolisable energy. In poultry nutrition research, total dietary metabolisable energy (ME) is often maintained constant when investigating the effects of different nutritional components in the diet. For example, the change of dietary protein concentration will alter the concentrations of fat and starch in the diet to maintain total ME constant. Conventional factorial experimental designs and response surface methods introduced in the previous section cannot distinguish between the effects of protein concentration *per se* and the effects of starch and fat in the mixture. This demonstrated that mixture design is extremely suitable for poultry nutrition research, especially in studies involving isoenergetic and/or isonitrogenous diets.

Gous and Swatson (2000) used a mixture experiment to determine weight gain and food conversion efficiency of broiler chickens offered 1 of 13 combinations of three protein sources (fishmeal, sunflower oilcake meal and soybean oilcake meal) from 7 to 21 days post-hatch. Figure 3 shows the contour plots of the effects of the three protein sources on growth performance of broiler chickens. This is one of the very few studies embracing a mixture design in poultry nutrition. They compared the selections made by the birds when given a choice and the prediction of optimal weight gain and feed conversion based upon response surfaces. The study showed that the choices made by the broiler chickens coincided with those mixtures that maximised body weight gain and feed conversion efficiencies.

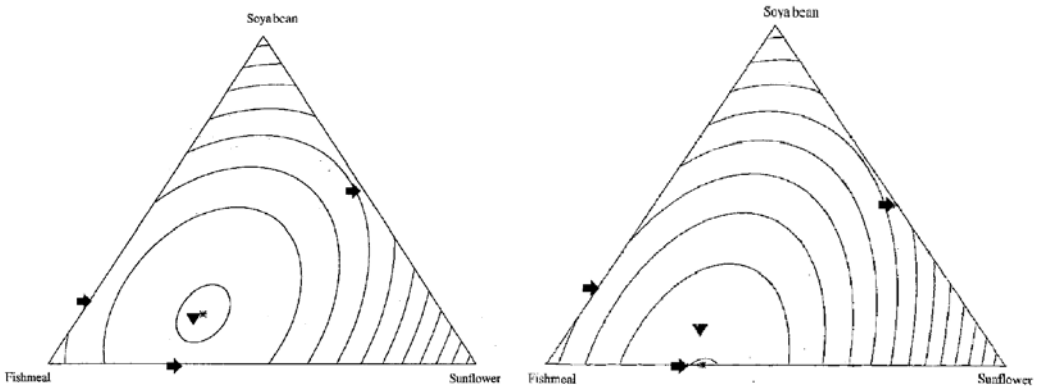

Figure 3 - Contours in a 3-component mixture representing different combinations of soya bean oilcake meal, fishmeal and sunflower oilcake meal that result in similar weight gain (left) and food conversion efficiencies (right) (maximum*) (Gous and Swatson, 2000).

In 1993, an integrated approach to animal nutrition and feeding behaviour was introduced. It is termed the geometric framework for nutrition, and it satisfies the multiple-food-components requirement using a *nutrient space* which is a geometric space built of two or more axes, where each axis represents a food component that is suspected to play a role in influencing the responses of animals to the environment (Raubenheimer and Simpson, 1993, Simpson and Raubenheimer, 1993) (for a recent comprehensive review see Simpson and Raubenheimer (2012)). The geometric framework generates a spatial metaphor whereby relationships among biological components can be visualised and interpreted within the same multi-nutrient context. Such components might include the target intake towards which it would regulate if unconstrained ("intake target", represented by a point or small region), its current nutritional state (a point), foods (lines called "nutritional rails" radiating from the origin into the nutrient space at an angle determined by the ratio of the nutrients in the food), and the constrained responses when the animal has available only foods that do not enable it to reach the intake target ("rules of compromise"). Together, these concepts provide a

powerful means to study the appetite systems for different nutrients, and how these interact in the regulation of food intake and its consequences.

The right-angled mixture triangle (RMT) was introduced into the geometric framework system, which differs from the conventional equilateral mixture triangle (EMT) design. Figure 4 illustrates the geometric difference between EMT and RMT designs (Raubenheimer, 2011). Although the information content of RMT and EMT is equivalent, in EMT the angle subtending any two axes is 60°; whereas, RMT provides rectangular spaces which are more straightforward and readily interpreted. The rectangular space has the advantage that it more intuitively parses the relationships among components in the model into two- and three-way interactions, thus aiding the user to interpreting complex patterns (Raubenheimer 2011). To illustrate, figure 5 provides a comparison between EMT and RMT where the same data, fructose–glucose–sucrose composition of floral nectar in 26 plant species from temperate forests in South America, are plotted in both formats. The pattern of data scatter is equivalent in the two plots, but the point of difference between the two plots is that for many the patterns can more readily be related to the axes in the RMT than in the EMT. Fig. 5A clearly shows that the balance of fructose and glucose was comparatively constant across samples (the positive relationship between these variables), while the sucrose concentration varied widely (the spread of data along the glucose: fructose balance vector).

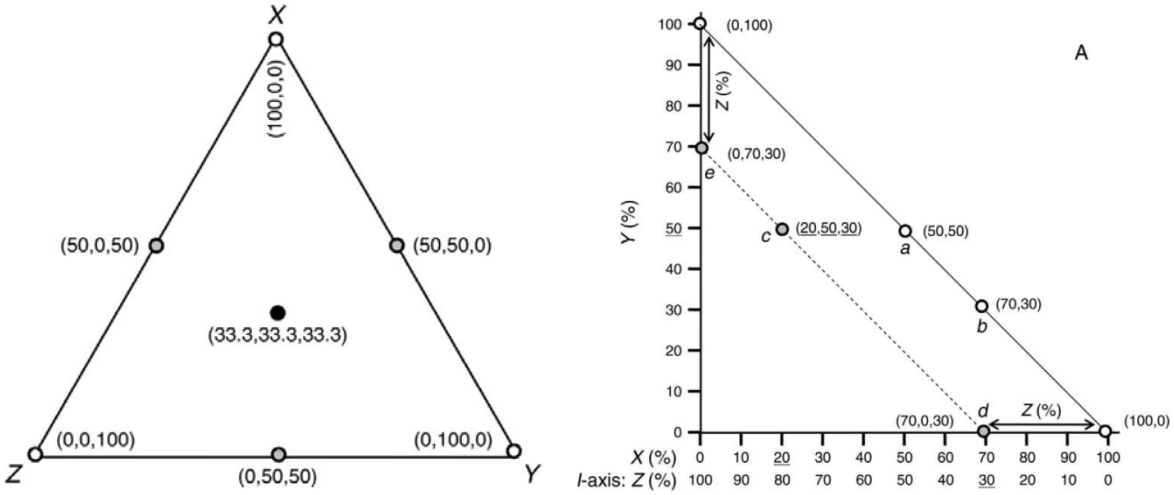

Figure 4 - *Left:* Equilateral mixture triangle defining the mixture space of all possible blends of three components *X, Y*, and *Z*. Shown in parentheses are the *X*, *Y*, *Z* coordinates for seven mixtures: pure ingredients of *X*, *Y*, and *Z* (open circles), 50%:50% mixtures of two ingredients (grey circles), and an equal mixture of all three components (black circle). *Right:* A three-component right-angled mixture triangle with focal axes *X* and *Y*, and implicit axis *Z* (labelled using the generic notation *I*). Any two-component mixture of *X*+*Y* (i.e., where *Z*=0%) will fall on the concentration isoline described by equation *Y*=100-(*X*+0) (i.e., *X*+*Y*=100%), shown as a solid diagonal in the figure (e.g., mixtures *a* and *b*). If *Z* is added at 30% then, by the same logic, the possible mixtures fall on the dashed isoline *X*+*Y*=70% (e.g., mixtures e, c, and d). In general, the *X*+*Y* isoline for any value of *Z* can be plotted as a line joining the point 100%-*Z* on the *X*-axis with the same value on the Y-axis (an exception is where *Z* = 100%, in which case the isoline reduces to a point with (*X, Y*) coordinates (0, 0)). Because these *X*+*Y* isolines are given by the equation *Z*=100-(*X*+*Y*), in the special case where the isoline contacts the *X*-axis (i.e., *Y*=0), this reduces to *Z*=100-*X*. The value of *Z* can thus be read off an axis that transforms *X* by this function (axis I in the figure). As an example, the (*X*, *Y*, *Z*) coordinates for point *c* are underlined on the three axes (20, 50, 30).

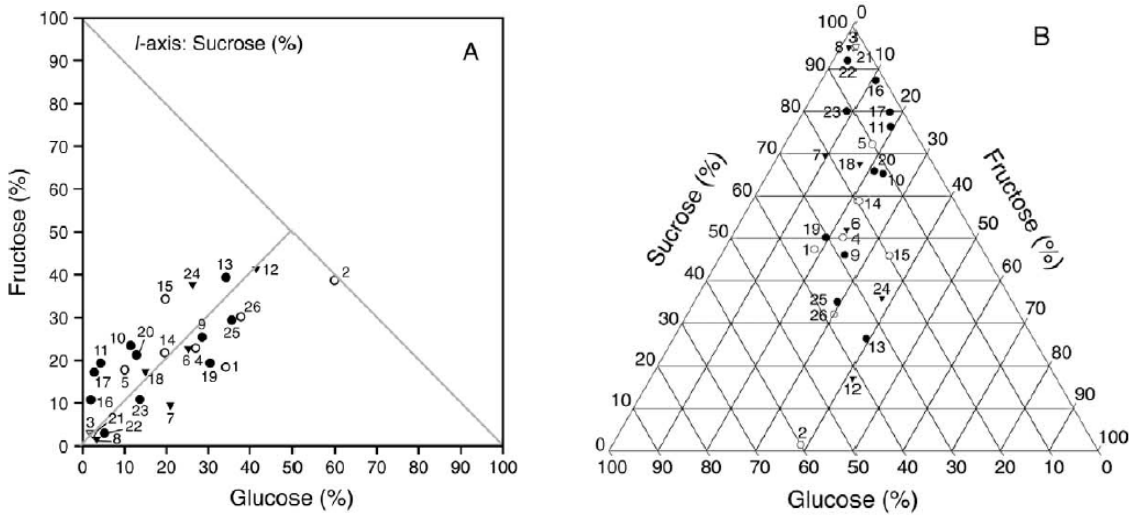

Figure 5 - (A) RMT and *(B)* EMT showing the fructose–glucose–sucrose composition of floral nectar in 26 plant species from temperate forests in South America, which are associated with one of four pollinator groups (solid circles, hummingbirds; open circles, diurnal longtongued insects; solid triangles, diurnal short-tongued insects; open triangles, nocturnal insects) (Raubenheimer, 2011).

III. APPLICATION IN POULTRY NUTRITION

Only recently, the geometric framework has been applied in poultry nutrition (Bao et al., 2012, Bradbury et al., 2014, Wilkinson et al., 2014). The interaction between calcium (Ca) and phosphorus (P) represents a complex relationship in broiler chickens, which is influenced by vitamin D and phytase (Selle *et al.*, 2000). Numerous studies have been reported in the literature to determine Ca and P requirements for optimal growth performance and skeletal integrity. However, the interactions between Ca, P and possibly, other minerals have resulted in widely inconsistent recommendations for dietary levels of Ca and P in the literature. By using geometric framework approach, Bradbury et al. (2014) examined the interaction between calcium (Ca) and non-phytate phosphorus (nPP) in growth performance and skeletal integrity. Fifteen dietary treatments with five different ratios of Ca : nPP (4, 2.75, 2.1, 1.5 and 1.14 : 1) and three total densities of Ca and nPP (12, 13.5 and 15 g/kg) were offered to broiler chickens from 7-28 days post-hatch. It was found that broilers are willing to over consume nPP to defend a Ca intake target more so than they are willing to over consume Ca to defend an nPP target; overall dietary nPP was more influential on performance metrics. However, from the data, it appears that birds assign higher priority to Ca than nPP intakes – i.e. when confined to one of a range of diets varying in the Ca:nPP ratio, they will let nPP intake vary with diet composition to meet the Ca target. Figure 6 illustrates the interactive effect of dietary Ca and nPP on broiler body weight gain and feed conversion ratio from 7 to 28 days post-hatch in the Bradbury *et al.* (2014) study. At low Ca and high nPP or high Ca and low nPP diets, birds had reduced feed intake, body weight gain, poorer feed efficiency and lower tibia ash. Similarly, from the same feeding study, Wilkinson *et al.* (2014) reported that the ratio of Ca : nPP is more influential to mineral digestibility than the absolute dietary concentration of each macro-mineral and nitrogen digestibility showed discrete optima around 10.0 and 5.0 g/kg nPP and Na digestibility was maximised around 8 to 9.0 g/kg Ca and 4.5 to 5.4 g/kg nPP (Figure 7).

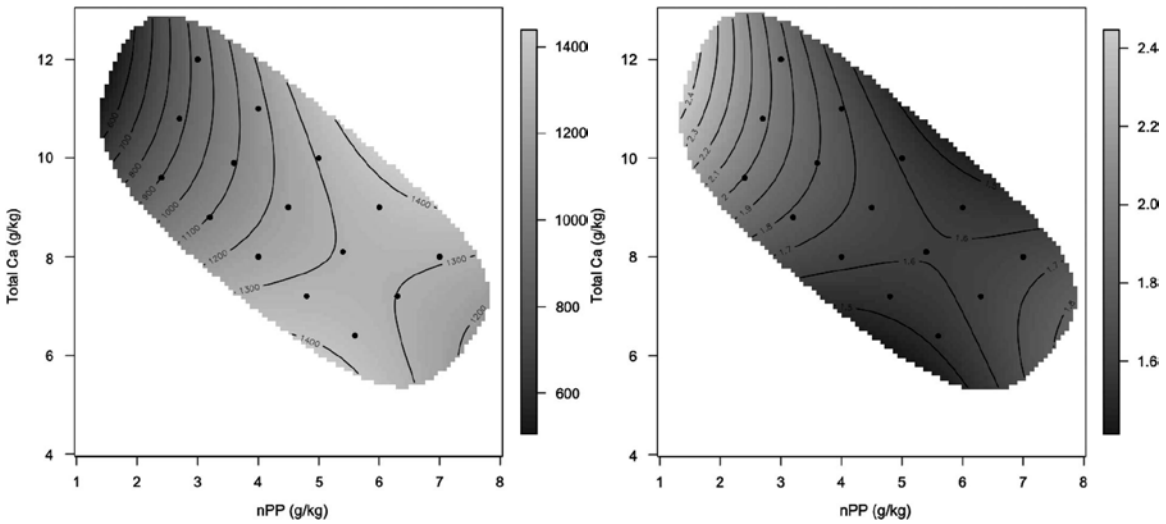

Figure 6 - Graphical representation of the interactive effect of dietary Ca and nPP on broiler body weight gain (g/kg) (left) and feed conversion ratio (g/g) (right) from 7 to 28 days post-hatch (Bradbury *et al.*, 2014).

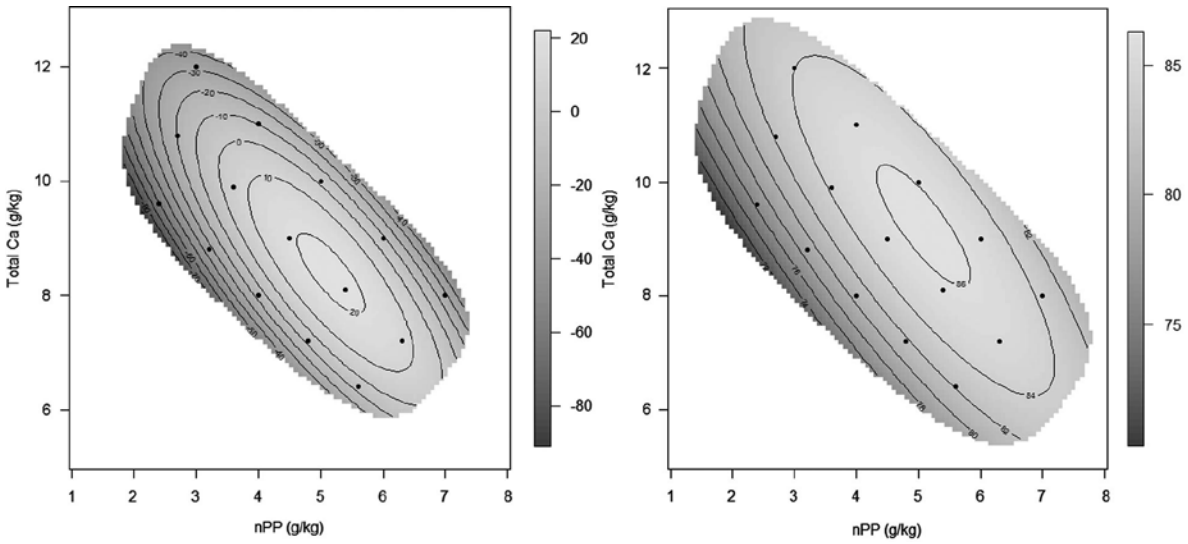

Figure 7 - Graphical representation of the interactive effect of dietary Ca and nPP on digestibility of sodium (left) and nitrogen (right) at 28 days post-hatch (Wilkinson *et al.*, 2014)

A recently completed study by the Poultry Research Foundation assessed interactions between starch, protein and fat on growth performance in broiler chickens offered synthetic diets (unpublished data). The feeding study comprised 21 dietary treatments with various concentrations of starch and protein by using right-angled mixture triangle design. The experimental diets were based on corn-starch, casein, isolated soy protein, synthetic amino acids, sunflower oil and other minor ingredients. These isoenergetic diets were offered to broiler chickens from 10-23 days post-hatch. Interestingly, energy derived from protein has a more pronounced impact on weight gain than 'non-protein energy'. Fat had nearly no impact on feed conversion ratios in comparison with protein energy intake; however, fat may influence energy utilisation and carcass compositions, which will be reported in subsequent publications (Figure 8). More importantly, as shown in Figure 9, the optimal weight gain (529g/bird) was achieved when dietary energy contributions of starch, protein and fat equalled to 39.98%, 47.21% and 12.81%, respectively. These equate to 326, 414 and 51 g/kg dietary starch, protein and fat, respectively, in a diet containing 3250 kcal/kg (13.60 MJ/kg) of metabolisable energy. The predicted optimal FCR (1.398) was achieved when energy

contributions of starch, protein and fat equalled to 48.19%, 41.00% and 10.81%, respectively, which equates to 393 g/kg starch, 360 g/kg protein and 43 g/kg fat in the diet.

IV. CONCLUSIONS

Relationships between food compositions, diet composition, diet utilisation and performance are complex and multi-dimensional. The geometric framework offers an approach for conceptualising these relationships, and constructing models which identify key relationships needed to understand how the nutritional regulatory systems of animals interact with the compositions of foods to determine outcomes such as growth, body composition, feed conversion efficiency, fecundity and so forth. Understanding these relationships is an important step towards identifying the key managing the key control points to manage these for optimal production outcomes. As yet, the use of this approach in poultry production is in its infancy, but a detailed example illustrating these points can be found for finfish production in aquaculture (Ruohonen *et al.*, 2007).

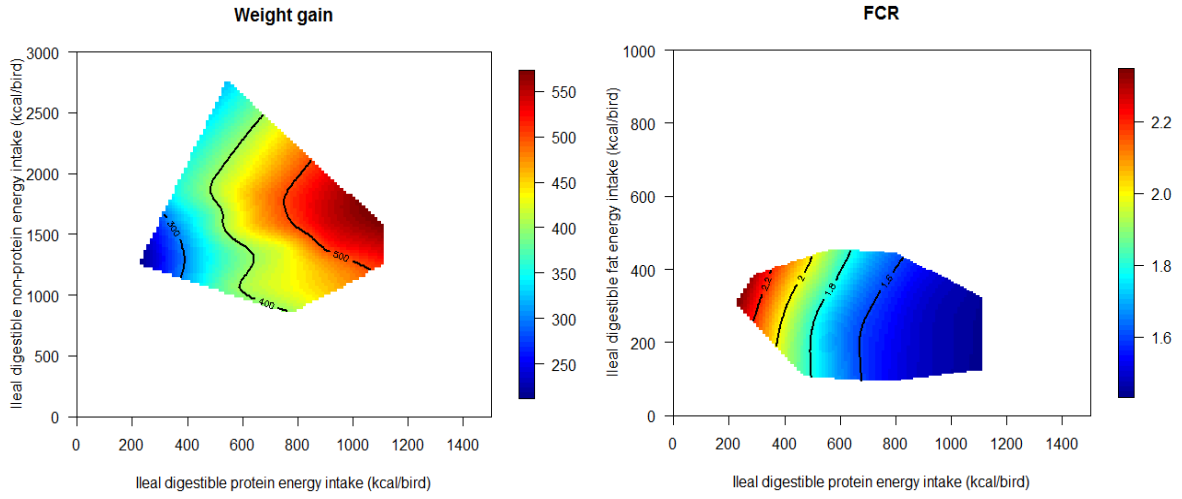

Figure 8 - Thin-plate spline response surfaces of weight gain and FCR for protein, starch, or fat (unpublished data).

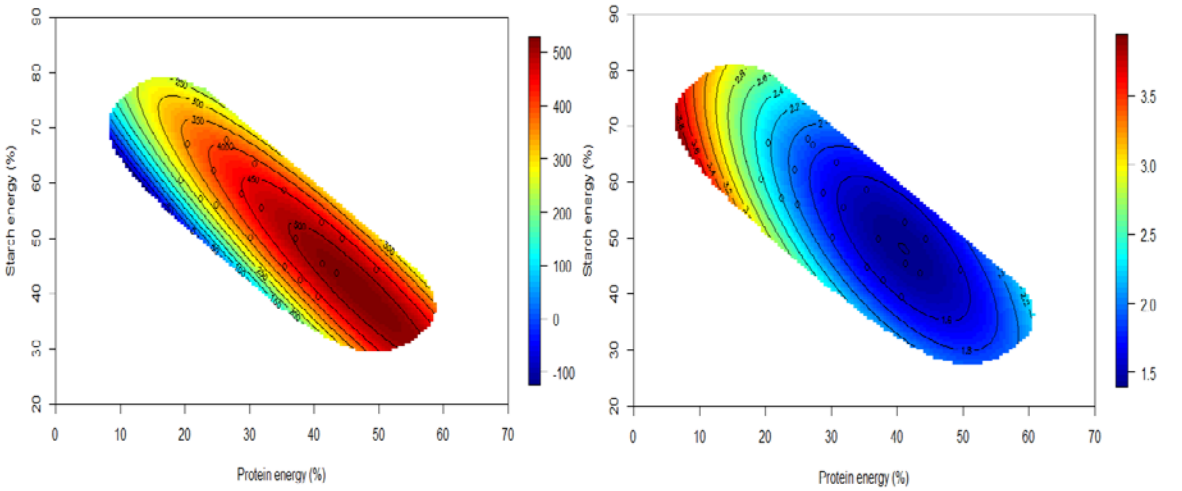

Figure 9 - The contour of starch and protein percentage energy contributions on weight gain (left) and FCR (right) (unpublished data).

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PRACTICAL APPLICATIONS OF NUTRIGENOMICS IN POULTRY NUTRITION

K.M. BRENNAN[1]

Summary

Novel molecular techniques such as microarray technologies have spurred the development of the field of nutrigenomics. Using microarrays to evaluate gene responses allows us to assess the activity of thousands of genes at the same time, thus allowing a rapid measure of physiological changes. Nutrigenomics can provide a powerful tool for understanding how nutrition impacts performance, health and disease in poultry. This paper will serve as a review of current applications of Nutrigenomics in poultry nutrition. The wealth of information obtained from nutrigenomics studies can help nutritionists and producers better understand how to feed poultry to ensure optimal health and performance.

I. INTRODUCTION

Nutrigenomics is the study of how nutrients, forms of nutrients, and nutritional strategies impact the genome. The use of DNA microarray technology allows us to begin to understand how nutrition modulates gene expression and how this modulation relates to animal health and performance. These molecular technologies also allow for the rapid evaluation of nutritional strategies. Advancements in the area of bioinformatics have enabled investigators to decipher the functional and biological relevance of nutrigenomic data sets. Together these technological advances are creating new opportunities in poultry research that could lead to improved animal health and production.

II. GASTROINTESTINAL HEALTH

The avian gastrointestinal tract (GIT) plays a central role in the digestion of feed and absorption of nutrients. Its proper function is essential for optimal health and growth. The GIT not only includes the tissue and cells of the intestine but also the complex community of microbes it harbors. Nutrigenomics can provide a systems biology approach to understanding how nutrients influence intestinal health through interaction with gut cells and the microbiota in the intestine (which in turn can change gut cell dynamics).

Both prebiotics and probiotics can improve gut health resulting in improved feed efficiency and growth in broilers. The cellular mechanisms behind these improvements have been elucidated utilizing nutrigenomics. For example, adding mannan oligosaccharides (MOS) to broiler diets not only increases the expression of mucin, an important component of the protective intestinal mucosal barrier, but also surprisingly down-regulates selected genes involved in cell turnover and proliferation, potentially conferring an energy-sparing effect (Brennan et al., 2013b). Similarly, probiotic bacteria introduced to poultry diets after bacterial challenge have been show to down-regulate selected genes associated with intestinal pathogens (Yang et al., 2014).

An even newer area of research that is increasing our knowledge about the interaction of nutrition and GIT health is microbiomics. Microbiomics uses molecular biology techniques, such as advanced DNA sequencing, to study the ecology of the GIT microbial communities (Yeoman et al., 2012). Currently this field is in its infancy and research is limited to general characterization of the unique communities present in each segment of the

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intestine. Eventually molecular profiling should provide the data needed to equip us to optimize the microbiome through nutrition for improved bird health and production.

III. NUTRIENT FORM VERSUS FUNCTION

Nutrigenomics can be used to explain why we sometimes see differences between nutrient forms. For example, vitamin E supplementation of broilers is known to confer antioxidant benefits and to improve meat quality and shelf life. Nutrigenomics studies have also shown that vitamin E acts a transcriptional regulator in lipid metabolism and oxidation, helping to further explain its beneficial effects (Li et al., 2009). In other studies, nutrigenomics has been used to determine why different nutrients can sometimes have the same effects on physiological markers. For example, Xiao et al. (2011) showed that a commercial algae-based antioxidant had effects similar to those of vitamin E on total antioxidant status of broilers and meat shelf life. Nutrigenomics data suggest that the antioxidant mimics vitamin E at a transcriptional level by inducing the expression of genes involved in lipid metabolism, cell morphology and cellular oxidation (Xiao et al., 2011).

Nutrigenomics has also been used to determine the underlying reasons why we see a production response with one nutrient form but not with another (e.g., organic versus inorganic minerals). Hall et al. (2012) found that different forms of zinc can differently regulate the expression of transport proteins in the intestine. For example, Brennan et al. (2011 & 2012) showed that, in both male and female birds, different forms of selenium activate transcription of different functional groups of genes, explaining why organic selenium improves reproduction over inorganic selenium.

IV. NUTRITIONAL PROGRAMMING

Over the past several years, researchers have begun to investigate how restricted, "programmed" nutrition during the neonatal and early-life periods can affect animals during adulthood. In chickens, for example, more than 24 h of fasting post-hatch has been shown to have unfavorable effects on weight gain, gut health and meat quality in adult broilers (Gonzales et al., 2003, Halevy et al., 2000). Conversely, feeding chicks post-hatch diets with reduced protein levels has been shown to benefit bird growth and development throughout adulthood, even after chicks are switched to a traditional diet (Everaert et al., 2010). Delayed access to feed post-hatch has been shown to alter hepatic gene expression (Richards et al., 2010).

Nutrigenomics can help us understand how nutritional programming works by revealing the gene expression patterns associated with early-life nutrition. For example, feeding 100% of the NRC recommendations (normal) for trace minerals for the first 96-h post-hatch can have positive effects on gene expression even after birds are switched to low (20% of the NRC recommendations) mineral diets (Brennan et al., 2013a). In this study, higher levels of minerals fed during the post-hatch period were shown to increase the expression of genes such as Cyclin D1, which play a key role in cell cycle regulation, a biological function essential for gut mucosal growth and repair. The expression of solute carrier proteins, a family of transporters essential for the active and passive transport of nutrients in the small intestine, were upregulated in adult birds that received the normal post-hatch diet, as was the expression of genes involved in the uptake of minerals and other nutrients. In the study by Brennan et al., there was no effect on performance. However, considering the importance of proper intestinal function and health, these changes are considered beneficial. Overall this study demonstrated that post-hatch nutrition can alter gene expression patterns long-term, which can have lifelong implications for bird health.

V. COMMERCIAL APPLICATIONS

We anticipate that the application of nutrigenomics research will lead to the implementation of improved precision feeding strategies by the poultry industry. The gene-level findings of nutrigenomics combined with the performance data (e.g., weight gain, egg production) from whole-animal studies help us to better understand how nutrients affect animal health and production. Nutrigenomics provides a way to identify precisely which nutrients or nutrient combinations are optimal and when they should be delivered to elicit maximum benefits. Through the application of nutrigenomics, we anticipate improved poultry production economics stemming from streamlined feeding strategies, leading to improved feed efficiency and bird health.

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PROGENY PERFORMANCE: CURRENT RESEARCH AND U.S. FIELD PERSPECTIVES

M.T. KIDD[1]

Summary

Optimizing meat yields in broilers begins with good chick quality. Hen nutrition is a key component for good chick viability, welfare, growth, and meat yields. This paper covers some historical and current research on hen feeding to impact progeny, and how the U.S. breeder nutritionist is poised to implement some of these opportunities in the current market conditions. Namely, hen dietary fortification of the tocopherols, cholecalciferol, selenium, manganese and zinc are discussed with regard to needed levels and source effects.

I. INTRODUCTION

Dietary hen needs that impact hatchability have been reviewed (Beer, 1969; Wilson, 1997). A subsequent review assessed the impact of dietary hen needs on progeny live performance, health, and processing traits (Kidd, 2003). Despite the broad knowledge of hen needs for the developing embryo and chick post hatch in published literature, industrial implementation of hen feeding regimes for progeny productivity by nutritionists is still being assessed. This paper reviews studies on hen nutrition for progeny performance, primarily published after 2003, and the perspective of the U.S. nutritionist in implementing effective hen nutrition strategies.

II. HEN PROTEIN AND ENERGY

Meat-type hen protein and energy nutritional status for progeny improvements has been assessed for some time (Aitken et al., 1969) and most recently to improve chick weight and viability from young breeder hens (Moraes et al., 2014). Crude protein (19 versus 25 g) and energy (325, 385, and 450 kcal ME/kg) were fed to broiler breeders from 19 weeks onward and in all hatches male chicks fed 450 kcal energy, but not female chicks, had improved early growth compared to chicks from hens fed 325 kcal energy (Spratt and Leeson, 1987). Independent of dietary protein, high energy (450 kcal) in hen diets increased male progeny (day 41) carcass protein and decreased carcass fat (Spratt and Leeson, 1987). It was postulated that male, but not female, chicks responded to increased dietary maternal energy due to heightened early growth rate.

Moraes et al. (2014) evaluated progeny from hens fed diets differing in protein (13.7 and 15.3%) and energy (2,528 and 2,736 kcal) during rearing, and energy (2,800 and 2,900 kcal) during lay. Improvements in broiler breast meat yield were noted as the energy to protein ratio was increased from the rearing phase to the laying phase, suggesting pullet nutrition can also impact progeny growth and yields (Moraes et al., 2014).

Two recent studies in broiler breeders evaluated dietary lysine on progeny (Mejia et al., 2013; Ciacciariello and Tyler, 2013). Mejia et al. (2013) used corn based distillers grains with solubles to reduce hen dietary lysine and found that broilers from young breeders (26 weeks) had low body weight and breast yield, but higher dark meat yields when hens were fed the lowest lysine (600 mg lysine/hen/day). The former response mimics broiler responses when fed suboptimal lysine diets. Ciacciariello and Tyler (2013) found a strong correlation

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to hen digestible lysine and progeny live performance (day 21) and concluded that changes in hen feed allowance over time to improve egg production could reduce progeny live performance.

Hen feed restriction and feeder space affects hen reproduction, but effects on progeny as mediated by protein and energy intake are sparse. Eusebio-Balcazar et al. (2014) found that hens given more feed space during photostimulation fed on corn, but not wheat, based diets had progeny with heavier femurs and longer shanks. Chick length is a key measurement for chick quality, the former beneficial bone attributes may be a function of overall nutrient intake and digestion.

Commercial U.S. nutritionists have embraced feeding distillers grains with solubles to breeder hens and have implemented the dietary use of low fat- distillers grains with solubles as well. Based on available ingredients and hen growth rate, U.S. nutritionists lean on the side of lower energy and lower digestible lysine than in past. Trials assessing hen feeding programs should monitor progeny, when possible, as amino acid and energy needs for hens to optimize, or at least maintain, progeny performance are still subject to debate.

III. HEN MICRONUTRIENTS AND CHICK HEALTH

Key micronutrients impacting progeny viability discussed herein are the fat soluble vitamins D and E and trace metals manganese, selenium, and zinc.

Maternal vitamin D_3 is critical for optimal progeny development (Sunde et al., 1978). Atencio et al. (2005a) fed broiler breeder hens dose responses of cholecalciferol (vitamin D_3) ranging from 0 to 2,000 or 4,000 IU/kg of diet. Progeny were evaluated in six hatches from hens ranging from 27 to 52 weeks of age. Viability of progeny was improved when hens were fed vitamin D_3 between 2,000 and 4,000 IU/kg of diet, but early growth needs of progeny chicks may be higher than 4,000 IU/kg of diet (Atencio et al. 2005a). Subsequent work by Atencio et al. (2005b) demonstrated improved biological value of 25-OHD_3 in hen diets over vitamin D_3 for embryo mortality and bone ash of progeny.

The tocopherols (vitamin E) have long been known for their role in disease prevention and antioxidant status. Surai et al. (1997) correlated the α-tocopherol levels in yolk to tissues in the chick post hatch. The heightened immunity from vitamin E has been shown to improve adaptive antibody transfer from hen to chick (Boa-Amponsem et al., 2001). Zhao et al. (2011) fed White Leghorn hens 0, 40, and 100 IU vitamin E/kg of diet and found that antibody titers to both Newcastle disease virus and avian influenza were highest with 100 IU of vitamin E.

Regarding fat soluble vitamins, although the level of hen cholecalciferol for optimal progeny is a range, clearly feeding 25-OHD_3 to hens is more efficacious. Vitamin E at levels reaching 100 IU/kg in hen diets will improve chick health, but more research is needed to quantify cost benefit responsiveness in progeny.

Similar to the tocopherols, selenium acts as a potent antioxidant. Pappas et al. (2006) found that chicks from hens fed less than 0.1 mg/kg selenium versus chicks from hens fed 0.5 mg/kg selenium from Sel-Plex (Alltech Inc., Nicholasville, KY) has higher tissue selenium concentrations 14 days post hatch. Wang et al. (2011) evaluated Lingnan yellow broiler breeders fed supplemental selenium from either sodium selenite or selenomethionine (0.3 mg/kg) versus unsupplemented 0.04 mg/kg hens. They found that selenomethionine supplementation improved hatchability, selenium tissue status, and antioxidant capability 8 weeks post hatch compared to an equal level of sodium selenite.

Both manganese and zinc are heavily involved in metalloenzyme functionality. Virden et al. (2003) fed hens diets with inorganic manganese and zinc or supplemental levels of (Availa® zinc and manganese). Progeny were reared in a floor pen facility and overall

livability was improved (P=0.06) in broilers from hens fed supplemental organic minerals. Moreover, progeny from hens fed organic manganese and zinc tended to have improved breast meat yield over that of the progeny from hens fed inorganic levels. Although past research has shown the carryover effects of zinc on progeny immunity and bone quality (Kidd et al., 1992), future research should assess manganese and zinc ability to mediate overall health and protein accretion in progeny (e.g., 75 to 150 mg Zn and Mn/kg varying by source).

IV. HEN FEEDING PROGRAMS AND CHICK HEALTH

Other than the micronutrients cited above, commercial nutritionists are assessing prebiotic/probiotics in hen diets on progeny performance. Kidd et al. (2013) fed broiler breeder hens diets with and without supplemental 0.68 kg Saccharomyces cerevisiae and evaluated progeny. Progeny from the 39 week, but not the 32 week hatch, had better feed conversion and breast meat yield than broiler control fed hens. Also, hatching eggs from the 32 week hatch, but not the 39 week hatch, has less bacterial contamination. This work points to improved hen nutrient utilization and possibly reduced pathogens as a function of dietary Saccharomyces cerevisiae (Kidd et al., 2013).

Hatchery sanitation is paramount for good chick viability and subsequent 7 day mortality. U.S. breeder nutritionists can help to promote sanitary incubation conditions with feeding programs that reduce internal and external hatching egg contamination. In addition to the work cited above using a dried fermentation product for commercial poultry, feeding programs that minimize external egg contamination (e.g., corn particle size, betaine, sodium chloride, and inorganic copper) can boost hatch and progeny viability.

V. HOW CAN WE USE THE CURRENT KNOWLEDGE TO MOVE FORWARD?

Hen nutrition and hatchery management can aid to generate a robust chick. For many micronutrients, progeny needs are higher than for hen productive performance. In this paper, energy, a dried fermentation product from Saccharomyces cerevisiae, tocopherols, cholecalciferol, selenium, manganese and zinc have been shown to impact progeny from hen research studies. With fat soluble vitamins, cost benefit studies should be conducted based on 7 day mortality. More research is warranted with amino acids and energy on hen productivity and progeny impact. Commercial test evaluating levels and sources of antioxidant vitamins and minerals (including zinc and manganese) should be fed to hens to assess hatchery contamination and subsequent 7 day mortality. Finally, hen levels of micronutrients and additives thought to improve progeny viability should be further assessed in their ability to impact yields. The interaction of in ovo nutrition with hen nutrition remains unclear.

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'CONTOUR PLOT' BIOMETRICS ENHANCE INTERPRETATION OF BROILER BIOASSAYS

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Summary

Using an equilateral triangular design, 'contour plot' biometrics were utilised to generate response surfaces to interpret variations in performance of broilers offered nutritionally equivalent diets based on three different grain sorghums, Block I, Liberty and HP. These steam-pelleted, basal diets were blended to constitute ten dietary treatments and were offered to male broiler chicks from 7 to 28 days post-hatch. Parameters of growth performance and nutrition utilisation were determined. There were no significant effects of dietary treatments on performance with the exception of ME:GE ratios ($P < 0.03$) or, effectively, efficiency of energy utilisation. However, response surfaces predicted that increasing inclusions of diets based on Block I sorghum compromised FCR, AME, ME:GE ratios and AMEn. This may possibly be due to higher concentrations of kafirin, phytate and 'non-tannin' phenolic compounds in Block I as opposed to HP and Liberty sorghums. Optimal growth performance and nutrient utilisation in broiler chickens can be predicted by contour plot biometrics and the influence of listed anti-nutritional factors, which have been quantified, are being investigated in relation to growth performance and additional parameters of nutrient utilisation.

I. INTRODUCTION

Sorghum is used, either partially or entirely, as the cereal grain base in Australian pig and poultry diets but it has been associated with sub-optimal broiler growth performance (Selle *et al.*, 2010, Liu *et al.*, 2013). Cereal grains of different varieties and from different locations may be blended into poultry diets but varying the inclusion rate of one feed ingredient will spontaneously change inclusion rates of other feedstuffs. Conventional full factorial designs cannot detect these interactions but this obstacle can be circumvented by alternative experimental designs involving multivariate optimisation. Such designs generate response surfaces which graphically depict interactions between ingredients or nutrients. It requires the total amount of the mixture to be held constant and the response is entirely dependent on the relative proportions of the ingredients or nutrients in the blend. The intention of the present study is to examine how inclusion rates of three different sorghums, with divergent inherent properties, influence broiler performance and nutrient utilisation by using an equilateral triangle mixture design.

II. MATERIALS AND METHODS

Three characterised sorghum with different protein, kafirin and phytate contents were used to formulate three nutritionally equivalent basal diets (Table 1). Following steam-pelleting, the three basal diets were proportionally mixed to form ten dietary treatments (Table 2) which filled the geometric space in an equilateral triangle as shown in Figure 1. Each of the ten dietary treatments was offered to six cages (6 birds per cage) or a total of 360 male Ross 308 chicks from 7 to 28 days post-hatch. Initial and final body weights were determined, feed intakes were recorded from which feed conversion ratios (FCR) were calculated. The incidence of dead or culled birds was recorded daily and their body-weights used to adjust

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FCR calculations. Total excreta collected from day 25-27 to generate data for parameters of nutrient utilisation [apparent metabolisable energy (AME); ME:GE ratios, N retention, N-corrected AME (AMEn)] on a dry matter basis. Excreta were air-forced oven dried for 24 hours at 80°C. The gross energy (GE) of diets and excreta were determined by bomb calorimetry using an adiabatic calorimeter (Parr 1281 bomb calorimeter, Parr Instruments Co., Moline, IL). Nitrogen contents of diets and excreta were determined using a N determinator (Leco Corporation, St Joseph, MI, USA). Differences were considered significant at $P < 0.05$ by using the JMP® 9.0.0 (SAS Institute Inc. JMP Software, Cary, NC). Response surface and contour plots are generated by R 3.0.3.

Table 1 - Composition and nutrient specifications of sorghum-based experimental diets

Item (g/kg)	Block I	Liberty	HP	Item (g/kg)	Block I	Liberty	HP
Diet composition				Calculated dietary			
Sorghum	620.0	620.0	620.0	nutrients			
Soybean meal	224.6	225.4	230.0	AME(MJ/kg)	12.95	12.93	12.95
Canola meal	75.0	75.0	75.0	Protein	217	186	187
Sunflower oil	28.0	24.0	20.0	Starch	378	388	388
Limestone	7.4	6.9	6.9	Calcium	7.5	7.5	7.5
Dicalcium phosphate	16.5	16.1	16.1	Phosphorus	7.5	6.9	6.9
Sodium chloride	1.0	0.6	0.7	Phytate-phosphorus	2.8	2.7	2.7
Sodium bicarbonate	4.5	4.9	4.9	Nonphytate phosphorus	4.7	4.2	4.2
Arginine	0.5	1.3	1.1	Sodium	1.8	1.8	1.8
Isoleucine	-	0.4	0.3	DEB	208.0	207.7	211.0
Lysine	2.5	3.5	3.3				
Methionine	2.3	2.9	2.9	Nutrients in sorghum			
Threonine	0.7	1.3	1.2	Crude protein	137.1	109.1	80.9
Valine	-	0.7	0.6	Kafirin	67.1	41.4	50.5
Vitamin-mineral premix	2.0	2.0	2.0	Phytate	9.8	7.8	4.9
Celite	15.0	15.0	15.0				

Table 2 - The proportions (%) of each basal diets in ten dietary treatments.

Treatment	Block I	HP	Liberty
1A	100	0	0
2B	0	100	0
3C	0	0	100
4D	50	50	0
5E	50	0	50
6F	0	50	50
7G	66.6	16.7	16.7
8H	16.7	66.6	16.7
9I	16.7	16.7	66.6
10J	33.3	33.3	33.3

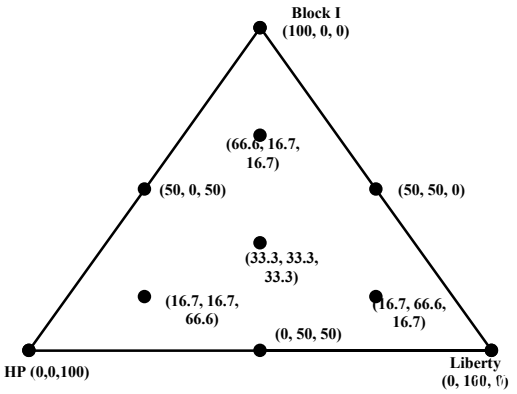

Figure 1 - Dietary treatments in an equilateral triangle design.

III. RESULTS

The overall mortality from 7 to 27 days post-hatch was 2.32% which was not influenced by dietary treatments ($P > 0.70$). Table 3 showed the results of growth performance and nutrient utilisation. There were no significant treatment effects on AME, AMEn, N retention and growth performance. However, diets based on Liberty sorghum had significantly higher ME:GE ratios than Block I and HP sorghums ($P < 0.05$).

Table 3 - Effects of dietary treatment on growth performance [WG, weight gain (g/bird); FI, feed intake (g/bird); FCR, feed conversion ratio (g/g)] and nutrient utilisation [AME, apparent metabolisable energy (MJ/kg); AMEn, nitrogen-corrected AME (MJ/kg); ME:GE, the ratio of metabolisable energy and gross energy (MJ/MJ); N retention, nitrogen retention (%)].

Treatment	WG	FI	FCR	AME	AMEn	ME:GE	N retention
1	1387	2301	1.660	13.38	11.87	0.800[a]	68.22
2	1431	2334	1.631	13.26	11.85	0.800[a]	68.36
3	1409	2342	1.663	13.47	12.14	0.827[c]	72.39
4	1421	2334	1.664	13.39	11.81	0.802[a]	72.01
5	1420	2343	1.650	13.46	12.00	0.814[abc]	71.58
6	1441	2316	1.608	13.63	12.20	0.832[c]	73.41
7	1359	2238	1.647	13.48	12.05	0.807[ab]	70.11
8	1432	2313	1.615	13.52	12.03	0.816[abc]	71.81
9	1432	2299	1.606	13.54	12.14	0.823[abc]	71.40
10	1392	2261	1.626	13.37	11.90	0.811[abc]	72.12
SEM	20.424	32.215	0.0183	0.1238	0.1103	0.0075	1.3775
P-value	0.139	0.318	0.142	0.673	0.158	0.029	0.156

IV. DISCUSSION

Compared to conventional statistical designs, response surfaces generated by mixture designs are more sensitive in detecting significance of treatment effects. The relationship between weight gain and the proportions of three basal diets as shown in Figure 2a was predicted by the following equation ($r^2 = 0.99$, $P < 0.001$):

$$WG = 13.792P_{BlockI} + 14.377P_{HP} + 14.173P_{Liberty} - 0.003158P_{BlockI}P_{HP} + 0.001309P_{BlockI}P_{Liberty} + 0.003499P_{HP}P_{Liberty}$$

Where P_{BlockI}, P_{HP}, $P_{Liberty}$ represents the percentage of diets based on sorghum Block I, HP and Liberty. Increasing the inclusion level of diet based on Block I compromised weight gain and the predicted optimal weight gain 1439 g/bird was achieved when the proportions of diets based on Block I, HP and Liberty were equal to 0.0%, 79.2% and 20.8%, respectively.

As shown in Figure 2b, the relationship between FCR and the three basal diet proportions was predicted by the following equation ($r^2 = 0.99$, $P < 0.001$):

$$FCR = 0.01655P_{BlockI} + 0.01631P_{HP} + 0.01651P_{Liberty} - 1.783 \times 10^{-5}P_{HP}P_{Liberty}$$

Similarly, higher inclusion rates of Block I compromised feed efficiency and the predicted optimal FCR 1.486 g/g was achieved when the proportions of diets based on Block I, HP and Liberty equal to 0.0%, 55.6% and 44.4%, respectively.

As shown in Figure 2c,the relationship between AMEn and the proportions of three basal diets was predicted by the following equation ($r^2 = 0.99$, $P < 0.001$):

$$AMEn = 0.01191P_{BlockI} + 0.1186P_{HP} + 0.1214P_{Liberty} - 1.927 \times 10^{-5}P_{BlockI}P_{HP} - 4.226 \times 10^{-6}P_{BlockI}P_{Liberty} + 7.799 \times 10^{-5}P_{HP}P_{Liberty}$$

Increasing the inclusion rates of diet based on Block I and HP compromised AMEn and the predicted optimal AMEn 12.38 MJ/kg was achieved when the proportions of diets based on Block I, HP and Liberty equal to 0.0%, 32.0% and 68.0%, respectively.

As shown in Figure 2d, the relationship between ME:GE and the proportions (%) of three basal diets was predicted by the following equation ($r^2 = 0.99$, $P < 0.001$):

$$ME{:}GE = 0.008P_{BlockI} + 0.008016P_{HP} + 0.008262P_{Liberty} + 6.520 \times 10^{-6}P_{HP}P_{Liberty}$$

Increasing the inclusion rates of diet based on Block I and HP would also compromise the ME:GE ratio. The predicted optimal ratio 0.845 MJ/MJ was achieved when the proportions of diets based on Block I, HP and Liberty equal to 0.0%, 31.1% and 68.9%, respectively.

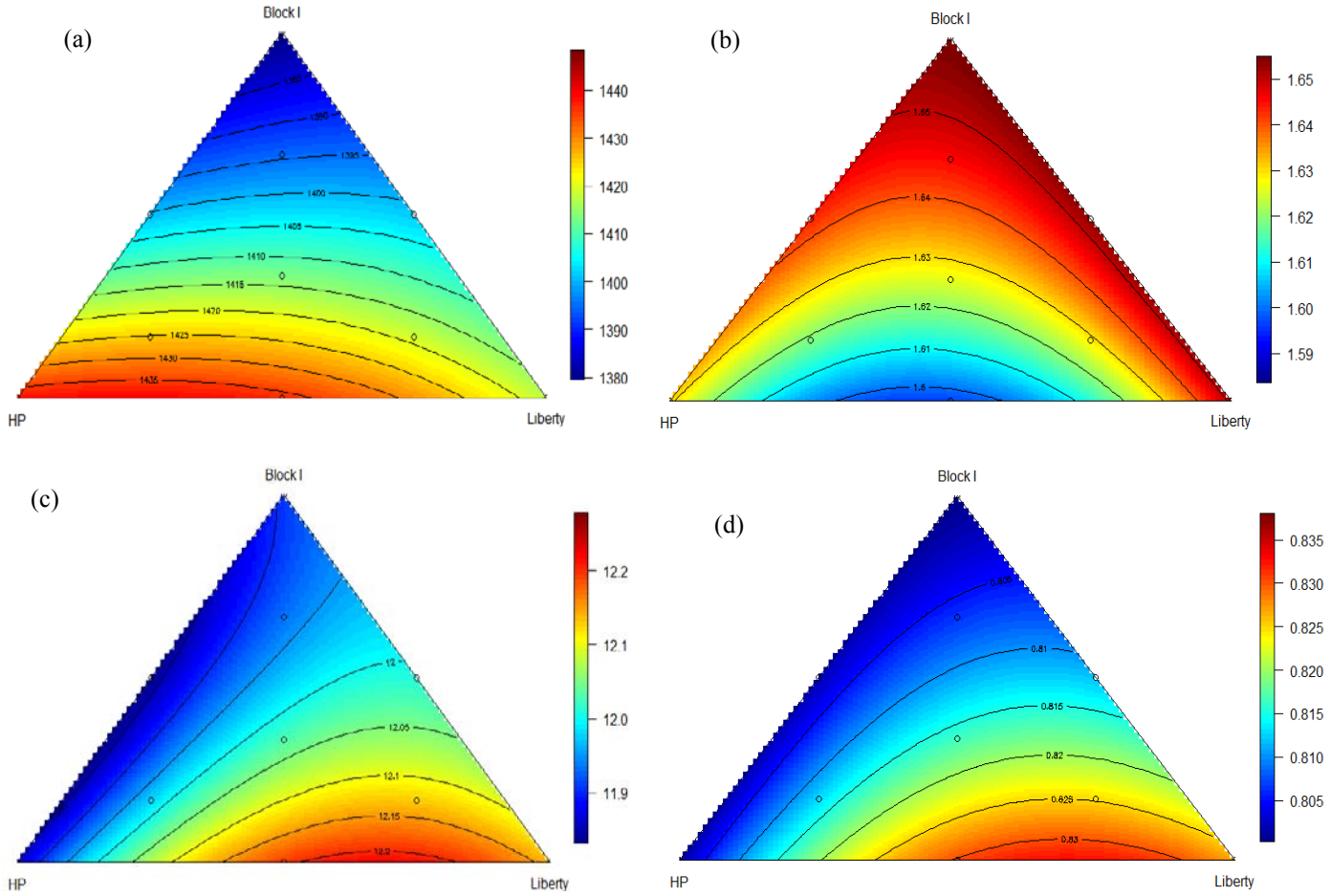

Figure 2 - Response surfaces of the relationships between the proportions of three basal diets and weight gain (a), feed conversion ratio (b), nitrogen-corrected apparent metabolisable energy (c) and the ratio of metabolisable energy and gross energy (d).

All three sorghums did not contain condensed tannin on the basis of the Clorox bleach test; however, non-tannin phenolic compounds may possess anti-nutritive effects. Sorghum Block I had the highest kafirin and phytate concentrations and both factors in sorghum may contribute to inconsistent or sub-optimal growth performance in poultry. In the present study, increasing inclusions of Block I in experimental diets compromised weight gain, feed efficiency and energy utilisation. Future investigations will use contour plot biometrics to generate response surfaces to understand the relationship of growth performance, nutrient digestion and utilisation with concentrations of phytate, kafirin and also non-tannin phenolic compounds in sorghum-based broiler diets. As Liberty is a white sorghum it is expected to contain lower concentrations of phenolic compounds than the other two red sorghums.

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EFFECT OF GUANIDINOACETIC ACID SUPPLEMENTATION ON PERFORMANCE OF BROILER BREEDERS AND THEIR PROGENIES

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Summary

A study was conducted to investigate the effects of supplementation of creatine precursor, guanidinoacetic acid (GAA, CreAMINO®) on the reproductive traits of broiler breeders. The carryover effects of GAA on performances of their progenies were also evaluated. A total of 150, Cobb-500 broiler breeders at 50 weeks of age were randomly allocated to 5 dietary treatments supplemented with various levels of GAA (0.00%, 0.04%, 0.08%, 0.12% and 0.16%) and the diets were fed for a period of 10 weeks. In progeny trial, 360 one-day old male broilers originating from the broiler breeders fed with different levels of GAA were randomly allocated to 5 dietary treatments with no added GAA and were fed for 42 days. Feeding increasing levels of GAA to broiler breeders improved the fertility (P=0.063) and hatchability (P=0.014). Egg production and embryo mortality however were not affected with GAA supplementation. Supplementation of GAA in the breeder diets significantly improved FCR (P<0.05) of the broiler chickens. In conclusion, GAA supplementation optimized reproductive performance of broiler breeders as well as FCR of their progenies.

I. INTRODUCTION

Creatine (Cr) and its phosphorylated form phosphocreatine (PCr) play a vital role in cellular energy metabolism of animals. The Cr/PCr system functions as a backup to the adenosine tri-phosphate (ATP)/adenosine di-phosphate (ADP) system in order to store and mobilize energy when required on short notice. The animal's demand for Cr can partly be fulfilled directly from Cr, which is present in animal by-products. In addition, Cr is formed by *de-novo* synthesis through methylation of guanidinoacetic acid (GAA) which itself is formed from the amino acids glycine and arginine. Part of this Cr pool (1.5-2.0% per day) is irreversibly lost and excreted as creatinine through urine (Wyss and Kaddurah-Daouk, 2000; Lemme et al., 2007). This indicates the need of a steady refilling of the Cr pool either by *de-novo* synthesis or by dietary intake. Breeder diets are commonly vegetable-based which lack Cr as plants do not contain Cr. The developing embryo is totally dependent on the nutrients which are available in the egg. Eggs contain low levels of Cr (Ramirez et al., 1970; Murakami et al., 2014) but during incubation, the synthesis of Cr steadily increases as indicated by an increasing activity of the key enzyme L-arginine-glycine amidinotransferase. This leads to increasing Cr content in the whole embryo and Cr can be detected in the embryo after 6 days of incubation (Ramirez et al., 1970). The *de-novo* synthesis of Cr requires arginine, glycine and energy. It is well-established that GAA (CreAMINO®) significantly improves broiler performance. Thus, the question arises if the supplementation of a Cr source to breeder diets might have an effect on the performance of the parents, embryos and progenies as it might relieve the metabolism and enhance the cell's energy transport and buffer capacity. However, there is a lack of information about the use of GAA on the performance of broiler breeders and its effect on their progeny. Therefore, the aim of this trial was to investigate the effect of GAA (CreAMINO®) supplementation on the reproductive traits of broiler breeders and performance of their progeny.

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II. MATERIALS AND METHODS

Broiler breeder trial: A total of 120 Cobb-500 broiler breeders were used in a 10 week trial period between 50 and 60 weeks of age and were randomly distributed to 5 dietary treatments supplemented with GAA (CreAMINO®, minimum GAA content of 96%) at 0.00% (T1), 0.04% (T2), 0.08% (T3), 0.12% (T4) and 0.16% (T5) with six replicates per treatment and four broiler breeders per pen. A corn-soybean meal breeder mash diet was formulated to meet the nutrient recommendations of Rostagno et al. (2011). The amount of feed per day was provided based on the recommendation of the Cobb Breeder Management Guide. The breeding hens were artificially inseminated twice beginning in week 60 with the second insemination done 4 days after the first. Semen was collected from roosters (not fed with GAA) using the abdominal massage method. Semen was pooled and sperm cell concentration was determined to ensure all breeding hens were inseminated with the same number and volume of sperm cells. Egg collection was done for a 10-d period. Number of eggs placed in the incubator according to the different treatments was as follows: 125 (T1), 121 (T2), 128 (T3), 128 (T4) and 121 (T5). Egg production, fertility, hatchability of eggs and embryo mortality were evaluated.

Progeny trial: 360 one-day old male broiler chickens originating from the broiler breeders fed with different levels of GAA were used in the progeny trial. They were allocated in a completely randomized design using 5 treatments with 6 replicate pens of 12 birds per pen. Dietary treatments of progeny trial were corresponded to the treatments of broiler breeder trial in order to correlate performance of broiler chickens to the respective treatment groups of breeders. The diets were based on corn and soybean meal, with no added GAA and formulated to meet the nutrient recommendations of Rostagno et al. (2011). Feed in mash form and drinking water were provided *ad libitum*. Environmental conditions during the trial were appropriated to the birds following the breeder's recommendations. Weight gain, feed intake and feed conversion were calculated for the trial period (1 to 42 days). At the end of experimental period, three birds per pen with average pen weight were selected to measure carcass yield, breast meat yield and leg meat yield.

Data were analyzed using the GLM procedure of SAS (2009) and the significant differences among treatments were compared by the Tukey's test.

III. RESULTS AND DISCUSSION

Broiler breeder trial: There were no effects of the treatments on egg production and embryo mortality. However, fertility ($P<0.07$) and hatchability ($P<0.05$) improved with increasing supplementation of GAA (Table 1). Supplementation of 0.08% of GAA (CreAMINO®) optimized fertility and hatchability. Increasing the dietary level of GAA in quail feed resulted in a significant linear increase ($P<0.05$) of the GAA, Cr and creatinine content of eggs (Murakami et al., 2014). This clearly shows that GAA is successfully absorbed by quails and that there is a carry-over into the egg. GAA is used to synthesis Cr which is indicated by the increasing Cr levels in the eggs. Murakami et al. (2014) further reported that GAA supplementation significantly ($P<0.05$) improved hatchability and fertility which is in accordance with the current study. Even though Cr concentration in eggs was not determined in this study, it could be assumed that GAA supplementation increased the Cr content in the eggs and the utilization of this nutrient by embryos improved the hatchability. Furthermore, the embryo is totally dependent on ATP synthesis to fulfill the energy demand during the growth and hatching process (Molennar, 2010). It is proposed that as precursor of Cr, supplementary GAA increased the egg Cr content, contributing to ATP availability to meet the needs of embryonic metabolism, and thereby improve hatchability. Ramirez et al. (1970) showed that the development of chicken embryos during the incubation period required Cr.

The authors found a low concentration of Cr in the egg, indicating that the content of arginine and glycine in the egg and activity of the enzyme L-arginine-glycine amidinotransferase can be important for the synthesis of Cr in the chicken embryo in order to support its development during the hatching process. This result suggests that the embryo might have a high enzymatic demand for Cr synthesis in the presence of arginine and glycine in the egg.

Table 1 - Effect of GAA on production, mortality, fertility and hatchability of broiler breeders.

Parameters	GAA , %						
	0.00 (T1)	0.04 (T2)	0.08 (T3)	0.12 (T4)	0.16 (T5)	SEM	P
Egg production, %	52.12	50.42	53.18	53.44	50.50	5.99	0.604
Embryo mortality							
Initial %	4.29	6.11	4.40	2.38	3.17	8.16	0.514
Intermediate %	3.20	3.43	2.99	1.12	2.08	3.15	0.399
Final %	3.00	3.27	3.03	3.17	5.66	5.89	0.591
Pecked %	1.39	4.98	1.21	3.03	3.17	3.74	0.121
Contaminated %	2.73	1.67	1.12	2.78	2.64	7.26	0.222
Fertility %	80.27b	85.15ab	96.51a	96.43a	81.76b	12.09	0.063
Hatchability %	65.66b	65.59b	83.76a	83.95a	65.04b	17.88	0.014

[a,b] Values within a row with different superscripts are significantly different (P<0.05, hatchability) and (P<0.07, fertility).

The improvement in fertility with increasing supplementation might be related to the fact that Cr is used by the spermatozoa. Cr improves the flagella motility and therewith improves the process of gaining access to the ovum (Lee et al., 1988).

Progeny trial: Progeny originating from broiler breeders which had been fed diets with different levels of GAA, were fed the common diet without GAA during the whole trial. Nevertheless, GAA supplementation of the parent's feed significantly (P<0.05) improved the feed conversion of the progeny up to 0.08% GAA (Table 2). However, there were no significant effects of GAA supplementation in breeder diets on feed intake or body weight gain of their progeny. The positive impact on FCR might be explained by findings of Murakami et al. (2014), where GAA supplementation increased the Cr content in eggs of meat-type quail breeders as well as the Cr content in breast muscle of newborn progeny. Furthermore, myoblast fusion is essential for muscle development, postnatal growth and muscle repair after injury. O'Connor et al. (2008) showed that Cr treatment enhanced cell fusion in a creatine-kinase (CK)-dependent manner suggesting that ATP consuming reactions are replenished through the PCr/CK system. The findings implicate roles for PCr as a high-energy phosphate buffer in the fusion of multiple cell types including muscle and sperm/oocyte. This showed that muscle development is enhanced in the presence of increased PCr. Thus, one could hypothesize that in the current trial the presumed increased levels of Cr in the egg might have increased myogenesis of the embryo resulting in more muscle cells at day of hatch. This might partly explain the improved FCR of the progeny as they may have had an increased capacity for muscle growth. There were no significant effects of GAA supplementation in breeder diet on progeny carcass weight, breast meat yield (BMY) or leg meat yield (LMY). However, when expressing BMY and LMY relative to carcass weight there was a numerical increase with increasing GAA supplementation in breeder diets (59.4, 62.4, 61.2, 60.5 and 61.4%), which underpins the aforementioned assumptions.

Table 2 - Performance of progeny from 1 to 42 days of age.

GAA, %	BWG, g	FI, g	FCR g/g	Carcass, g	BMY, g	LMY, g
0.00 (T1)	2.866	4.615	1.61b	2.128	642	621
0.04 (T2)	2.790	4.194	1.51ab	2.074	672	621
0.08 (T3)	2.816	4.052	1.44a	2.270	721	668
0.12 (T4)	2.780	4.102	1.48a	2.105	640	634
0.16 (T5)	2.949	4.724	1.61b	2.245	694	684
SEM	247	856	0.12	206	65	77
P	0.351	0.533	0.038	0.279	0.182	0.440

[a,b] Values within column with different superscripts are significantly different (P<0.05); BMY=breast meat yield; LMY=leg meat yield.

In conclusion, the results from this study showed that supplementation of GAA (CreAMINO®) improve the reproductive performance of breeders as well as the growth performance of their progeny.

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THE EFFECT OF DIRECT FEED ACCESS AFTER HATCH AND DIET TYPE ON BROILER CHICKEN GROWTH PERFORMANCE

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Summary

Newly designed housing systems enable feed and water access directly after hatch. In addition to feed availability it remains unclear to what extent diet type (determining the nutritional value) has an additive effect on growth performance. Therefore, the effects of diet type and moment of feed and water access after hatch were studied using a 2 × 2 factorial design. In the current study, provision of 100g of a prestarter diet resulted in a lower FCR during the first week after hatch when compared to a starter diet, although this effect did depend on the moment of feed and water access. Furthermore, breast meat percentage at d 35 improved due to prestarter supply, but only when fed without any delay in time after hatch. Irrespective of moment of feed and water access, a prestarter diet resulted in significantly improved body weight gain and feed intake during the entire grow-out period. Delayed feed access resulted in lower BW gain and feed intake during the first 14 d post hatch. In the current study it took chickens 14 days to compensate for a delayed access period of 53 hours after hatch. Feeding a prestarter diet improves growth performance of broiler chicken on short and long-term, especially when fed directly after hatch.

I. INTRODUCTION

In commercial hatcheries, hatch windows up to 36 hours can be found (Decuypere et al., 2001). Combined with chicken handling in the hatchery and transportation to broiler farms, the time between hatch and first feed intake may be up to 48-72 hours (Noy and Uni, 2010; Willemsen et al., 2010). During this period, chickens are kept in a warm environment without water, potentially causing dehydration. In addition, they are not able to consume feed, solely relying on their residual yolk as an energy supply for growth and development. It is estimated that the residual yolk contributes for only 10-11% of the total energy and protein consumption during the first three days after hatch (Wijtten, 2011), suggesting that the residual yolk may not entirely fulfill the nutritional requirement of just hatched broiler chickens. Delayed feed and water access likely impairs the development of the thermoregulatory system as well as maturation of the immune system and gastrointestinal tract (GIT) (Christensen, 2009).

Newly designed housing systems enable chickens to have feed and water access directly after hatch, eliminating the initial delay in first nutrient and water intake and improving growth performance (van de Ven et al., 2009). Feeding programs for broilers often start with a starter diet provided during the first week after hatch, although nowadays more often a so called prestarter is fed. A prestarter diet is intended to be more aligned with the physiological needs and digestive capacity of the chicken at that age. To date, research focused on the interaction between direct feed access and diet type is limited and these two effects are often confounded by each other. Therefore in the current study the effect of diet type (prestarter versus starter diet) was evaluated for broiler chickens receiving direct or delayed feed and water access after hatch.

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II. MATERIALS AND METHODS

In the current study the effects of diet type and moment of feed and water access after hatch were studied using a 2 × 2 factorial design. Treatments were diet type (a commercially available starter vs. prestarter diet) and moment of feed and water access after hatch (direct or delayed). The average biological age of broiler chickens with direct feed and water access was 491 h after onset of incubation. The average biological age of broiler chickens with 24 h delayed feed and water access was on average 544 h after onset of incubation, having defined d 0 as 520 h after onset of incubation. Hatching eggs of Ross 308 broiler breeders (38 wk of age) were transferred at E18 to the Cargill Animal Nutrition Innovation Center Velddriel (the Netherlands) to hatch in a grow-out facility. Eggs (n = 360) were evenly distributed over 24 cages and placed on elevated egg-trays. Half of the cages were provided feed and water ad libitum from the moment of hatch onwards. In the remaining cages feed and water were provided from d 1 onwards (544 h after onset of incubation). Chickens were fed either a commercially available prestarter diet (2.0 mm pellet; 3,000 kcal AME and 1.45% lysine; 100 g per chicken) followed by a commercial starter diet (2.5 mm pellet; 2,982 kcal AME and 1.18% lysine) or only received a commercial starter diet during the first 14 days after hatch. All chickens were fed a commercially available grower diet (3.0 mm pellet; 3,098 kcal AME and 1.12 lysine) from d 14 to 34. Feed intake (FI) and individual body weights were recorded at d 0, 1, 3, 7, 14, 21, and 34. Based on body weight gain (BW gain) and feed intake, the feed to gain ratio (F:G ratio; kg of feed consumed / kg of weight gain) was calculated. At d 35, 5 chickens per cage were selected to collect the weights of the breast fillet and abdominal fat pad. Data were subjected to mixed model analysis using the PROC MIXED procedure in SAS (version 9.3, 2011, SAS Institute Inc., Cary, NC) using cage as the experimental unit. Contrasts were used to determine relationships for 1) the effect of diet type (starter versus prestarter diet), 2) the effect of moment of feed and water access (direct versus delayed), and 3) the interactive effect of diet type and moment of feed and water access. Data are expressed as least square means. Effects were considered to be significant when $P \leq 0.05$.

III. RESULTS

Feeding a prestarter diet resulted in a lower FCR from d 0 to 7 ($P < 0.001$; Table 1), although this effect depended on the moment of feed and water access ($P = 0.023$): when chickens were delayed feed, the difference in FCR during d 0 to 7 was much more profound. Furthermore, feeding a prestarter diet without delay resulted in improved breast meat percentage at d 35 compared to feeding a starter diet (+0.9%; $P = 0.040$; Table 1). BW gain, FI and FCR d 0 to 14 significantly improved in case of a prestarter diet compared to a starter diet (Table 1). The improved BW gain and FI persisted for the entire grow out period, resulting in a 3.6% higher BW at d 34 (2285 versus 2206 g; $P = 0.041$). Also carcass size increased when feeding a prestarter diet compared to a starter diet when expressed as percentage of body weight ($P = 0.05$; Table 1). Delayed feed access resulted in lower body weight gain and feed intake during the first 14 days post hatch ($P < 0.05$; Table 1), however, this effect did not persist during the grower period. No effect of feed access was found on FCR or slaughter characteristics.

Table 1 - The effect of diet type and moment of feed access after hatch on BW, BW gain, feed intake, feed to gain ratio (F:G ratio) and slaughter characteristics of broiler chickens, expressed as least square means.

Feed and water access		Direct		Delayed				Contrasts[3]		
	Diet type	Starter	Prestarter	Starter	Prestarter	Pooled SEM	Model *P* value	DT	FWA	DT × FWA
n[1]		6	6	6	6					
BW, g	0 d	44.1	45.5	44.5	44.3	0.6	<.001	0.203	0.416	0.110
	34 d	2206	2285	2183	2252	49	<.001	0.041	0.433	0.876
BW gain, g/d	0-7 d	19.5	22.3	17.1	19.9	0.9	<.001	<.001	0.001	0.961
	0-14 d	32.6	35.9	30.4	33.4	0.9	<.001	<.001	0.002	0.876
	14-34 d	85.2	86.9	85.7	87.0	1.9	<.001	0.287	0.837	0.907
	0-34 d	63.6	65.9	62.9	64.9	1.3	<.001	0.029	0.404	0.886
Feed intake, g/d	0-7 d	19.7	21.1	17.7	18.6	0.9	<.001	0.095	0.002	0.720
	0-14 d	37.9	41.4	35.7	38.6	1.1	<.001	<.001	0.004	0.723
	14-34 d	130.2	134.9	130.7	133.4	3.3	<.001	0.144	0.824	0.692
	0-34 d	92.2	96.4	91.5	94.3	2.1	<.001	0.034	0.390	0.662
F:G ratio	0-7 d	1.010	0.946	1.036	0.932	0.010	<.001	<.001	0.504	0.023
	0-14 d	1.163	1.154	1.175	1.154	0.007	<.001	0.007	0.298	0.233
	14-34 d	1.528	1.554	1.526	1.533	0.015	<.001	0.152	0.317	0.431
	0-34 d	1.451	1.464	1.456	1.453	0.011	<.001	0.571	0.726	0.355
BW at 35 d, g[2]		2213	2281	2261	2309	71	0.055	0.288	0.483	0.851
Carcass, % of BW[2]		76.5	77.6	76.9	77.4	0.5	0.014	0.050	0.734	0.480
Breast, % of carcass[2]		34.2	35.1	34.8	34.6	0.3	<.001	0.171	0.851	0.040
Fatpad, % of carcass[2]		1.4	1.4	1.6	1.5	0.1	0.002	0.232	0.087	0.130

[1]Individual cages with 15 broiler chickens.
[2]Average of 5 birds per cage, 6 cages per treatment.
[3]Contrast statements: DT – Effect of diet type, FWA - Effect moment of feed and water access, DT × FWA - Interactive effect diet type and moment of feed and water access.

IV. DISCUSSION

The aim of the current study was to evaluate the effect of diet type (prestarter versus starter diet) with and without delayed feed and water access after hatch on broiler chicken growth performance. Interactions found were only limited to the period during in which the treatments were provided and to slaughter characteristics. The improved FCR during d 0 to 7, but also improvements in BW gain and FI throughout the grow-out period when feeding a prestarter diet, may be explained by differences in dietary composition and the physiological status of the chicken during the first days after hatch. Digestibility rates for nitrogen, starch and fat are highly dependent on chicken age (Batal and Parsons, 2002; Batal and Parsons, 2004). This relates to development of the intestine itself, but also other components required for digestion like enzyme secretion (Noy and Sklan, 1995). It can be hypothesized that an improved FCR limited to the first week after hatch supports these findings as this is largely determined by the digestive capacity of the chicken. Prestarter diets include ingredients that align with the digestive capacity of the chicken directly after hatch. Protein sources like soy protein concentrate and soy protein isolate are known to be more digestible compared to other protein sources such as soybean meal and may therefore be preferable (Batal and Parsons, 2003). Differences in carcass development and breast meat percentage are commonly linked

in literature with the moment of feed access, as delayed feed access was found to result in delayed satellite cell proliferation, involved in muscle development (Moore et al., 2005). However, hatch moment also appears to affect breast meat yield (Lamot et al., 2014). In addition to earlier studies, current results suggest that diet type may affect breast meat yield too. The general effect of impaired growth performance of chickens due to delayed feed access, as also observed in the current study, has been well documented in literature. This effect is most likely to be explained by deterioration of the GIT during the time the residual yolk functions as a limited nutrient supply while feed and water are still unavailable, resulting in a delayed maturation of the GIT. The latter has been well quantified and is reflected by a reduced absolute weight, shortened intestinal length, impaired enzymatic activity, changed villi density, crypt cell density and reduced crypt depth and villi height (Maiorka et al., 2003). Although the chicken may recover from delayed feed and water access via compensatory growth, this depends on the duration of delayed access. In the current study, broiler chickens required 14 days to compensate for a delayed feed and water access period of 53 hours compared to broiler chickens with direct feed and water access.

V. CONCLUSIONS

Delayed feed access resulted in lower body weight gain and feed intake during the first 14 days post hatch. Compensatory growth may help the chicken to recover from initial delayed feed and water access, although this depends on the duration of delayed feed and water access: in the current study the recovery time was about 14 days to compensate for a delayed feed and water access period of 53 hours. Feeding a prestarter diet improves growth performance and slaughter characteristics (carcass percentage and breast meat percentage) of broiler chickens, especially when supplied directly after hatch.

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DIETARY SELENIUM SUPPLEMENTATION OF BREEDERS INFLUENCES GROWTH AND SELENIUM STATUS OF PROGENY

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Summary

This study aimed evaluating the impact of selenium sources fed to breeders on muscle Se status and growth performances of their progeny. The breeders were divided into 3 groups differing by the dietary Se source as followed: sodium selenite (SS), selenized-yeast (SY) and seleno-hydroxy-methionine or HMSeBA (SO). Each breeders group provide 290 day-old chick. On Day 0, 30 chicks per groups were selected to measure muscle Se content and the 260 remaining chicks were distributed into 13 replicates pens of 20 chicks for 21 days. Broilers from the 3 groups received the same standard broilers diet to observe only the effect of breeders Se nutrition. On Day 12 and 21, growth performances were measured and one chick per pen was selected to measure breast muscle Se content. The breeders Se source had no effect on the body weight at hatch, body weight gain or feed intake during the entire experimental period ($P > 0.05$). However, FCR of broilers issued from breeders fed with SO showed significantly improvement between Day 0 to 12 and Day 0 to 21 comparatively to SS ($P < 0.05$). Muscle Se content of day-old broiler chicks was higher for SO group compared to other treatments and higher for SY group compared to SS ($P < 0.05$). At 12 and 21 days of experiment, the muscle Se content was similar between each group and was decreased compared to hatch level ($P < 0.05$). In addition, muscle Se content of broiler at one-day-old appeared to be significantly related to FCR for the periods: Day 0 to 12 and Day 0 to 21 ($P < 0.001$). These results showed the higher ability of HMSeBA compared to other Se source in breeders' diet to improve chicks' muscle Se content at hatch which is related to FCR improvement during 0-21 day period of growth.

I. INTRODUCTION

Several studies showed the benefits of dietary organic selenium (Se) supplementation in breeders on egg quality and fertility compared to inorganic Se (Pappas et al. 2005a). However, few studies observed the effect of breeders' nutrition on the performance of the offspring. Indeed, the young broilers chicks are facing strong oxidative stress during their first days of life due to important physiological changes, such as, adaptation to aerobic life, active development of physiological systems such as digestive tract, hormonal and neuronal systems. As a consequence, chicks' antioxidant reserves such as vitamin E and carotenoids in liver is decreased by 20-fold during the 10 first days of life (Suraï, 2002). Fortunately, in the same time, liver glutathione peroxydase activity (GPx) is increased, showing the switch between antioxidant systems during this period.

Suraï (2000) had shown an increase of the Se content of day-old-chick at hatch and higher glutathione level and GPx activity in liver of their chicks at least 5 days after hatch when breeders were supplemented with selenized yeasts. Moreover, Pappas et al. (2005b) had observed a positive effect of the breeders Se intake on the GPx activity in tissues of their offspring throughout the 4 weeks following hatch. Recently, Jlali et al. (2013) demonstrated that eggs' selenium content can be tremendously increased with different organic selenium sources when compared to inorganic selenium sources, resulting in selenium deposition higher with seleno-hydroxy-methionine (HMSeBA) compared to selenized yeast. According

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to these results, we could suggest a higher Se status of the offspring of breeders fed with HMSeBA compared to selenized-yeast.

The objective of this study was to compare the effect of HMSeBA in breeders nutrition compared to other organic and inorganic Se source on the evolution of the Se status of their offspring and if this higher Se status had an effect on their growth performance.

II. MATERIALS AND METHODS

A total of 870 day-old chicks from 3 different breeders' broilers groups (3 x 290) were used in this study. The 3 breeders groups differing with the dietary Se sources and level as followed: sodium selenite at 0.3 mg Se/kg (SS); selenized yeast at 0.2 mg Se/kg (SY); seleno-hydroxy-methionine or HMSeBA at 0.2 mg Se/kg (SO). On the 290 day-old-chicks per group, 30 were randomly selected and euthanazied at the arrival of birds to measure their birth Se status. The pectoralis major were collected and pooled by 5 chicks in order obtaining 6 pools of muscle per breeders groups. The 260 other day-old-chick were allocated in 13 replicates pens of 20 chicks for 21 days. Whatever the chicks' origin, they received the same starter and grower commercial broilers diet containing only sodium selenite as Se supplementation at 0.2 mg Se/kg of feed. The body weight, feed intake and FCR of birds were measured on day 12 and 21 and muscles samples have been collected on 1 bird per pens at D12 and D21 to follow Se status of birds.

The total selenium concentration in diets and muscles was determined by inductively coupled mass spectrometry (ICP-MS) according the method of Bierla et al. (2008). Brielfy, one gram of feed sample and 250 mg of muscle samples were mineralized in a mixture (2:1, vol/vol) of HNO_3 (69-70%) and H_2O_2 (35%) at 85°C for 4 h within a closed vessel heating block system. The solution was further diluted with water and total selenium content was subsequently measured by inductively coupled plasma mass spectrometry (Agilent 7500cx, Tokyo, Japan) analysis. Isotopes 76, 77 and 78 were used for quantification.

All data were analyzed using GLM procedure of SAS 9.1.3 (SAS Inst. Inc., Cary, NC). The pen was used as experimental unit for growth performance and muscle Se content criteria analysis. Comparisons of least square means for each significant effect were performed by Tukey-Kramer test. The linear regression of FCR in relation with muscle Se content at hacth was performed with the REG procedure. Statistical significance was set at $P < 0.05$.

III. RESULTS AND DISCUSSION

The feed analyses of the starter and grower broiler diets gave 0.26 and 0.35 mg Se/kg respectively. The Se level of the breeders' diet was close to the expected level with an average background Se level of 0.25 mg Se/kg during the 3 months before egg collection for this study (data not shown).

The growth performances of day-old-chicks are presented in Table 1. The breeders Se sources had no effect on hatch weight of the offspring. Feed intake (FI) and body weight gain (BWG) from Day 0 to 21 wasn't affected significantly by breeders' dietary treatments. However, broilers from breeders feed with SO had a better FCR between Day 0 to 12 and Day 0 to 21 ($P < 0.05$) compared to the broilers from breeders feed with SS, while chicks issued from SY treatment presented intermediary FCR.

Table 1 - Broilers growth performance in relation to selenium sources distributed to breeders.

Breeders diet	SS	SY	SO	SEM	P-value
D 0-12					
BW D 0 (g)	40.31	40.23	40.31	0.19	0.876
BW D 12 (g)	327.4	328.3	331.0	43.7	0.366
BWG (g)	287.1	288.1	290.7	45.0	0.381
FI (g)	357.4	354.0	355.9	60.6	0.544
FCR (g/g)	1.245[a]	1.229[ab]	1.225[b]	0.0003	0.025
D 12-21					
BW D 21 (g)	710.6	711.6	717.5	180.7	0.389
BWG (g)	383.2	383.3	386.5	75.9	0.566
FI (g)	613.6	610.4	612.8	124.7	0.743
FCR (g/g)	1.602	1.593	1.586	0.0003	0.096
D 0-21					
BWG (g)	670.3	671.4	677.2	182.3	0.395
FI (g)	971.0	964.4	968.7	277.0	0.591
FCR (g)	1.449[a]	1.436[ab]	1.431[b]	0.0002	0.007

a,b: Means with different superscripts are statically different ($P < 0.05$).

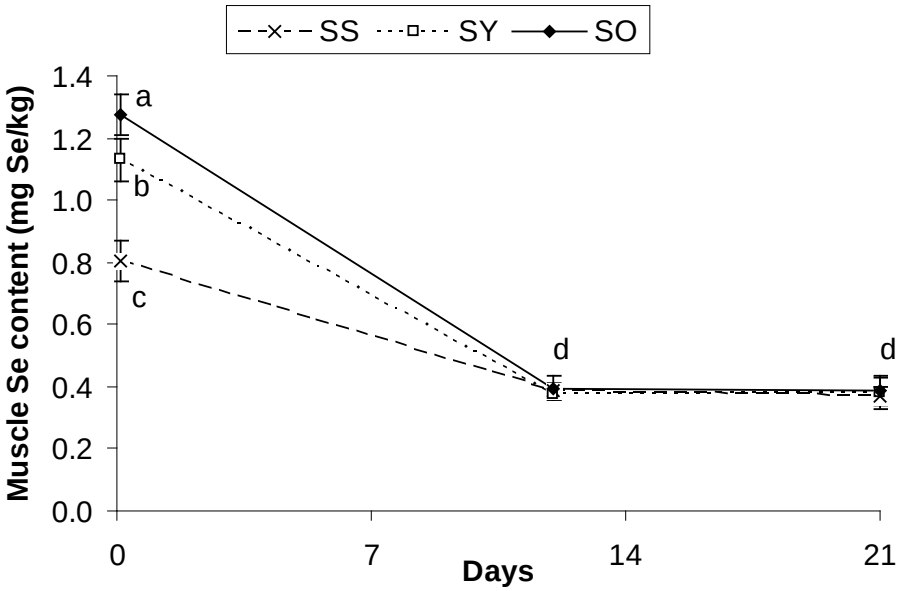

Figure 1 - Changes in broilers muscle Se content in relation with dietary selenium source of breeders.

Chicks muscles' Se content at the different sampling days are presented in Figure 1. At hatch, broilers from breeders fed with organic Se sources (SO and SY) had a higher muscle Se content ($P < 0.001$) compared to broilers issued from breeders feed with SS. In addition, the broilers from SO treatment had a higher muscle Se content compared to broilers from SY treatment ($P < 0.001$). These results are consistent with those of Jlali et al. (2013) who reported egg Se content in the same order when comparing these sources. The link between egg selenium content and day old chicks' selenium status had been also reported by Suraï (2000). However, at 12 days old, breast muscle selenium content in the different groups appeared similar irrespective of the breeders' treatment. The muscle selenium concentration at Day 12 appeared also significantly lower compared to Day 0 ($P < 0.05$). The decreased muscle selenium concentration between hatch and Day 12 could be explained on one hand by the low efficiency of inorganic Se source supplied during growth to support muscle Se content and on the other hand by the selenium turnover to build selenoprotein, such as GPx,

to improve the antioxidant defense of young broilers during the challenging period following hatch as reported by Suraï (2002). At Day 21 muscle selenium content appeared similar in the three treatments and equivalent to Day 12. Wang et al. (2011) report the comparison of supplementing breeder hence with sodium selenite or selenomethionine on the offspring performances. Like in our study the authors reported FCR improvement in broilers issued from breeders hence fed with selenomethionine comparatively to sodium selenite. In their study the authors reports, like in our work, a higher tissues selenium deposition in chicks issued form the organic selenium group. They also reported significantly higher antioxidant status of chicks from the organic selenium group with higher GPx and SOD activities in breast muscle compared to inorganic group.

The improvement of the FCR during the 0-12 and 0-21 day growing period appeared significant between SO group compared to SS group whereas SY group appeared intermediary. Interestingly, we observed a significant linear relation between hatch (Day 0) muscle Se content and the improvement of FCR for the periods 0-12 and 0-21 but with a low correlation coefficient (R^2= 0.22; $P < 0.01$ and R^2= 0.17; $P<0.01$) respectively. The higher muscle Se content at hatch suggest better Se reserve for the animal of SO groups compared to other treatments. This better Se reserve could facilitate the transition of antioxidant system from vitamin E and carotenoids to the GPx mentioned by Surai (2002) during the first days of life of chicks. In addition, Pappas et al. (2005b) and Wang et al. (2011) had shown a higher GPx activity in tissues at hatch for the chicks with the higher hatch muscle Se content. According to literature data, we could hypothesis that higher tissues selenium content led to a better GPx activity in chicks that allowed FCR improvement. The present results point-out the importance of day old chicks' selenium status on their subsequent performances. In this case, the use of HMSeBA in place of SS in breeders' diet permits an improvement of 1.25% of the FCR of their offspring during the 21 first days of life.

This study shows the higher ability of HMSeBA to supply selenium in eggs and chicks comparatively to selenized yeasts and sodium selenite. This better Se status of chicks at hatch with HMSeBA had a positive effect on the improvement of the FCR between Day 0 to 21.

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SEVEN DAYS GROWTH TRIAL ALLOWS ACCURATE SELENIUM SOURCES BIOAVAILABILITY EVALUATION IN CHICKENS

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Summary

The aim of this study was to develop a rapid assessment method to determine the selenium bioavailability of different selenium sources in birds. The first step was to measure the kinetic of selenium deposition in muscle and feathers of broilers fed different selenium sources. A total of 576 chicks were divided into 4 treatments with 8 replicates of 18 birds per pen for 21 days. The diets used in the experiment were supplemented with different Se sources as followed: negative control (NC) not supplemented in Se; sodium selenite (SS); selenized yeast (SY) and seleno-hydroxy-methionine or HMSeBA (SO) at 0.2 mg Se/kg of feed. Total selenium content in breast muscle and feathers was assessed on days 0, 7, 14 and 21. Only SO increased muscle Se concentration at day 7 compared to Day 0 ($P < 0.05$), while all other treatments exhibited decrease of Se muscle concentration ($P < 0.05$) from day 0 to day 7. On day 21, chicks fed SO had a higher ($P < 0.05$) muscle Se concentration compared to the level observed at hatch (day 0), birds fed SY had a similar muscle Se concentration than at day 0, while in SS and NC treatments, muscle Se concentration at day 7 was significantly lower than at day 0 ($P < 0.05$). At Day 21, muscle and feather Se content were highly correlated ($R^2 = 0.927$; $P < 0.0001$). This study shows that breast muscle selenium measured as early as at Day 7 allows discriminating selenium sources as well as at day 21. Moreover, the feather Se content measured at day 21 appeared a good and convenient indicator of selenium status in birds that can be use at farm level. These results also underline the benefit of a pure organic selenium source such as seleno-hydroxy-methionine to rapidly increase selenium status during the early growth phase.

I. INTRODUCTION

The tissues of chick embryos and newly hatched chicks contain a high proportion of polyunsaturated fatty acids. During their first days of life, birds face a rather very stressful period due to adaptation to aerobic life and important metabolic changes, and are thus highly susceptible to oxidative stress (Surai and Fisinin, 2014). In addition, their hepatic concentration of vitamin E and carotenoids decrease by 20-fold during the 10 first days of life (Surai 2002). However, the glutathione peroxidase (GPx) liver activity increased in the same time and this selenoprotein had a major place in the antioxidant defense (Surai 2002). Thereby the early selenium (Se) nutrition of chicks is very important. Indeed, the signs of Se deficiency are generally observed around 7 days post-hatch, when vitamin E level is low and GPx activity not yet sufficient (Surai, 2002). Traditionally, to ensure adequate antioxidant status of young broilers, Se supplementation is provided in the diets, usually using sodium selenite (SS) or organic forms mainly seleno-yeasts (SY). However, organic Se forms in the yeast depend on the strain and growing conditions (Surai and Fisinin, 2014). Recently, a new pure organic Se source, the seleno-hydroxy-methionine or HMSeBA had show a better muscle Se enrichment of broilers compared to seleno-yeast after 21 and 42 days of supplementation (Briens et al. 2013, 2014), but the effect of this source on Se status was not evaluated in early chick nutrition. The inconvenient of the measurement of muscle Se content is the death of animal to collect muscle. Alternative methods based on the measurement of

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feather Se content were used on wild birds to follow their Se status (Burger, 1996). However, feather Se content varies with the geographic area and the age of birds (young vs. adult). In addition some studies have shown an increase of feather Se content with the Se supplementation of birds (Sevcikova et al., 2006).

The purpose of this study was to evaluate the possibility to use muscle and feather Se contents to compare the bioavailability of selenium sources. Measuring the kinetic of Se deposition in young broilers, it will also be possible to determine the shortest time allowing discrimination between selenium sources and if feathers could be used as an indicator of the birds Se status.

II. MATERIALS AND METHODS

A total of 576 day-old chicks from a commercial hatchery were allocated to 4 treatments with 8 replicates of 18 birds per pen. The birds received the starter diet from Day 0 to Day 11 and the grower diet from Day 12 to Day 21 and each treatment was supplemented with different Se sources as follow: NC, basal diet not supplemented with Se; SS, SY and SO, basal diet supplemented with 0.2 mg Se/kg from sodium selenite, selenized yeast and HMSeBA respectively. At the end of each phase: body weight (BW) and pen feed intake (FI) were recorded and used for calculation of feed conversion ratio (FCR). Moreover, on Days 0, 7, 14 and 21, one bird per pen (8 per treatment) was randomly selected and euthanized. The wings feathers (1g per bird) and the 2 *Pectoralis major* muscle were collected and pooled for two birds before being frozen at -20°C for subsequent analysis. The total selenium concentration of diets, feathers and muscles were determined by inductively coupled mass spectrometry (ICP-MS) according to Bierla et al. (2008). Briefly, one gram of feed sample or 250 mg of tissue samples (muscle, feathers) were mineralized in a mixture (2:1, vol/vol) of HNO_3 (69-70%) and H_2O_2 (35%) at 85°C for 4 h within a closed vessel heating block system. The solution was further diluted with water and total selenium content was subsequently measured by inductively coupled plasma mass spectrometry (Agilent 7500cx, Tokyo, Japan) analysis. Isotopes 76, 77 and 78 were used for quantification.

All data were analyzed using GLM procedure of SAS 9.1.3 (SAS Inst. Inc., Cary, NC). The pen was used as experimental unit for growth performance, whereas the pooled samples were used as experimental unit for Se concentrations in feathers and muscle. Comparisons of least square means for each significant effect were performed by Tukey-Kramer test. To assess the relationship between selenium concentration in feathers and in *Pectoralis major* muscle, Pearson correlation coefficients were analyzed with the CORR procedure. Statistical significance was set at $P < 0.05$.

III. RESULTS AND DISCUSSION

The feed analyses confirmed that the expected Se levels in each diet were obtained with a background Se level of 0.13 and 0.10 mg Se/kg in starter and grower diet respectively (data not shown).

The different treatments had no effect on growth performance during the 21 days of experiment (average BW = 918 g and average FCR = 1.39 g/g). These results are consistent with other works that did not show effects on performance in relation with dietary selenium supplementation in broilers under standard conditions (Briens et al. 2013; Yoon et al., 2007).
Changes in muscle Se content through the experiment depending on the Se source is presented in Figure 1. Selenium sources significantly affect the Se deposition in pectoralis muscle ($P < 0.001$) at Day 7 and the same effects remain similar until 21 days old. At Day 7, chicks fed with the diet supplemented with SO treatment showed a significant increased of muscle Se content compared to hatch level (Day 0) (+ 41%) whereas other treatments showed

significant decrease in muscle selenium content (-15, -37 and -43 % for SY, SS and NC respectively). SY supplementation restored muscle selenium content after 14 days at a similar level than Day 0. SS treatment appeared to keep muscle selenium content stable from Day 7 to Day 21, whereas for NC treatment muscle selenium content decreased until Day 14. These results are consistent with previous works on the difference of muscle Se concentration with different Se sources supplemented for 21 days (Briens et al. 2013, 2014). In addition, these results show that selenium measurement in breast muscle as early as at 7 days allow to compare dietary selenium sources as efficiently as at 21 days of age. The decrease of muscle Se content for SS and NC during the first 7 days was also observed by Payne and Southern (2005) and could be explain by the mobilization of Se muscle storage to build selenoproteins and the lowest transport and absorption efficacy of SS compared to organic Se source (Suzuki 2005). Between Day 7 to D21, the muscle Se content of broiler fed SS diet remains stable, whereas the muscle Se content of broiler fed NC continues to decrease. This finding shows that even a high feed Se background like in this study (0.13 to 0.10 mg Se/kg in NC) cannot support a sufficient Se status of broilers and a good production of selenoproteins compared to supplemented diets.

The slight decrease of muscle Se content of chicks fed with SY during the first days of life and the subsequent recovery between Day 7 and Day 14 could be related to selenium form in yeasts. Indeed, the main part of selenium from yeast is SeMet incorporated into yeast protein; considering that gastrointestinal function of chick in the first week of life is not optimal (Sklan & Noy; 2000), it could be speculated that incomplete digestibility of these proteins led to decrease the Se uptake during the first week of life. Inversely, the Se present in a pure organic selenium form such as SO, namely HMSeBA or seleno-hydroxy-methionine, could be considered as free seleno amino acid in the gut, that does not need preliminary digestion process leading to a more efficiently absorption at early stage.

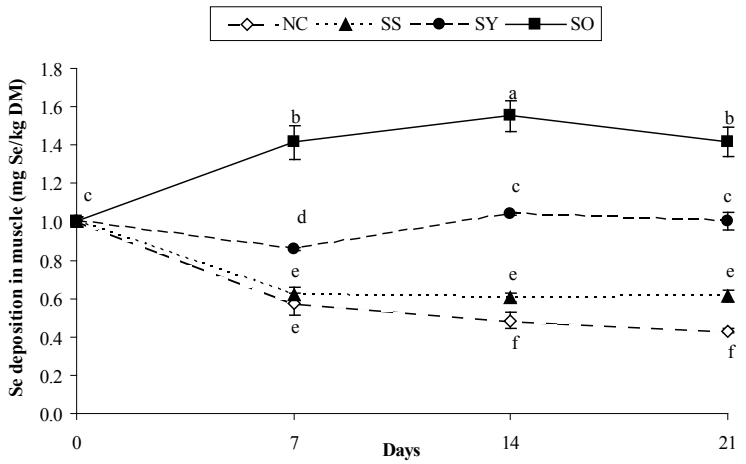

Figure 1 - Kinetic of muscle Se content in Pectoralis major muscle in function of the dietary Se source. Results are expressed as mean ± SD and different letters indicate statistical differences at $P < 0.05$.

Se concentration in feathers collected on day 7 and 21 on birds fed NC, SY and SO diet was summarized in Figure 2A. Whatever the dietary treatment, the Se concentration in feathers decreased ($P < 0.001$) with age of the birds. At both sampling time (7 or 21 days) feather selenium content appeared significantly affected ($p<0.05$) by dietary selenium content and source as follow: NC < SY < SO. The present results are in agreement with the study of Sevcikova et al. (2006) who observed higher Se content in feather of Se supplemented groups compared to groups without Se supplementation. The larger difference between Se sources in feather Se content at Day 21 compared to Day 7 could be explained by the development of feather during growth. Indeed, at hatch, the second generation of feathers starts to grow and

they push out the down feather (Leeson and Walsh, 2004). However, although at 7 days of age, the chicks have just start their first moulting and feather growth, feather selenium analysis at Day 7 allows to discriminate selenium treatments.
The correlations between muscle and feather Se content at Day 7 and Day 21 were highly significant ($P < 0.001$) with a better accuracy at Day 21 ($R^2 = 0.927$) than at Day 7 ($R^2 = 0.542$) (Figure 2B). Our results suggest that the measurement of feather Se content at 21 days of age is a simple and accurate indicator to monitor muscle Se status of birds and to compare bioavailability of selenium sources.

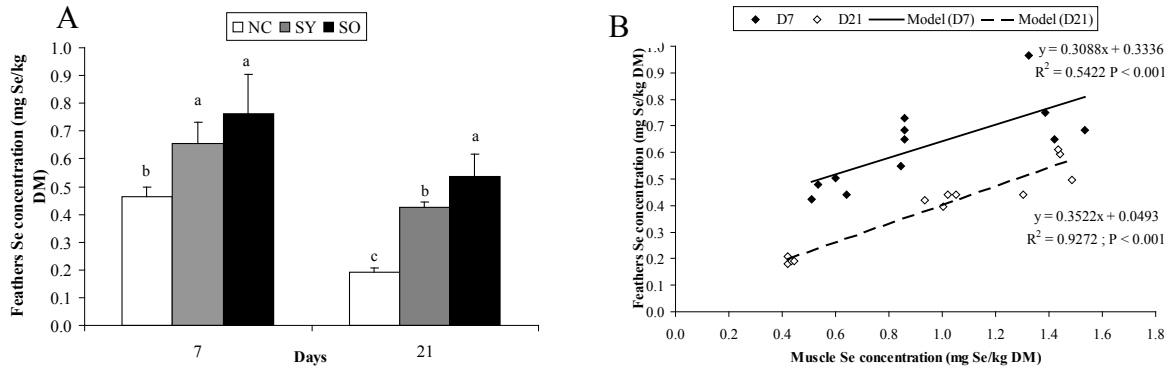

Figure 2 - A: Se deposition in feathers in function of dietary selenium source at day 7 and 21. Results are expressed as mean ± SD and different letters indicate statistical differences at $P < 0.05$; B: Correlation between Se deposited in feathers and in Pectoralis major muscle.

This study shows the higher ability of HMSeBA to increase Se status during the first days of life compared to selenized yeast and inorganic Se. This study also allows to develop a method to evaluate simply the bioavailability of different Se sources using the muscle Se content at Day 7. Moreover, feather Se content appeared highly correlated with muscle Se content in broilers at 21 days of age and could be used as an easier and non-destructive method compared to muscle sampling to follow Se status of broilers in the field.

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EFFECTS OF VITAMIN E AND SELENIUM DEFICIENT DIETS ON THE PATHOLOGY OF GEESE AND MUSCOVY DUCKS

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Summary

This study 100 geese and 100 Muscovy ducks were fed diets of banana stalk and rice bran. At two months of age, 50 geese showed ataxia, myoclonus and finally died while 30 Muscovy ducks showed anorexia and had difficulty walking. Three geese and three Muscovy affected carcasses were necropsied. Blood samples from affected and healthy birds, feed and water samples were collected. Microscopically, encephalomalacia and diffuse satellitosis were found in brains of affected geese while diffuse satellitosis without encephalomalacia was found in the brains of Muscovy ducks. In addition, degeneration of the gizzard smooth-muscle cells and cardiac-muscle cells, necrosis of pancreatic acinar cells were found in both affected geese and Muscovy ducks. Importantly, the concentration of vitamin E in geese and Muscovy ducks was 0.15 µg/mL and 1.56 µg/mL, respectively which were significantly ($P < 0.05$) lower than concentrations in unaffected birds. Furthermore, the concentration of Selenium (Se) in geese and Muscovy ducks were 2.01 µg/dL and 1.93 µg/dL, respectively, which were also significantly ($P < 0.05$) lower than concentrations in unaffected birds. In the follow-up study 2 months after supplementation with Vitamin E and Se, the conditions had improved and the serum concentrations of Vitamin E and Se were significantly higher than when birds were on the banana stalk and rice bran diet ($P < 0.05$) and at 7 months all birds were healthy and the serum concentrations of vitamin E and Se were as the same as control birds. It was concluded that the feeding of banana stalk and rice bran which is considered to be vitamin E and Se deficient could affect brain lesions and muscular dystrophy in geese and Muscovy ducks. The data also suggested that geese were more sensitive to vitamin E and Se deficiency than Muscovy ducks.

I. INTRODUCTION

The deficiency of vitamin E is one of nutritional diseases that affect poultry including chickens, ducks and turkeys. Diets deficient in vitamin E can produce encephalomalacia, exudative diathesis, and muscular dystrophy in chicks; enlarged hocks and dystrophy of the gizzard smooth muscle in turkeys; and muscular dystrophy in ducks (Klasing and Austic, 2003*)*. Encephalomalacia is a nervous syndrome characterized by ataxia, myoclonus and prostration. The signs usually begin between 15-56 days of age. In exudative diathesis, the lesions include vasculitis and in muscular dystrophy, the fibers of skeletal, smooth and cardiac muscles can degenerate (Klasing, 2008).Importantly, vitamin E deficiency mostly occurs in poultry that are fed rations high in polyunsaturated fats which support vitamin E becoming rancid and the vitamin is no longer bio-available (Mezes et al., 1997).

Selenim (Se) is an essential mineral in poultry, The deficiency of Se in chickens,especially in combination with low vitamin E supply is responsible for the development of a range of diseases including exudative diathesis (Nouguchi et al., 1973), encephalomalacia (Combs and Hady, 1991) and pancreatic atrophy (Cantor et al., 1975). The relationship between vitamin E and Se is not fully understood, however, Se appears to

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effectively prevent myopathies of gizzard and heart in young poults (Surai, 2002).The objective of this study was to investigate the pathology of geese and Muscovy ducks fed vitamin E and Se deficient diets.

II. MATERIALS AND METHODS

100 geese and 100 Muscovy ducks were reared from about one week after hatching. At about two months of age, 50 geese showed ataxia, myoclonus and prostration while 30 Muscovy ducks showed anorexia and difficulty walking. Mostly affected geese died but there was no mortality in Muscovy ducks. All were fed with diets of rice bran and banana stalk. Three geese and three Muscovy ducks carcasses, and 10 blood samples from affected geese and Muscovy ducks, along with feed and water samples were submitted for analysis. Blood samples from five healthy geese and Muscovy ducks were collected as control samples. After supplementation with vitamin E 100 IU and Se 0.2 mg/kg of diet for two months, the pathological conditions improved. At this time, blood collection from 10 geese and Muscovy ducks were taken. Seven months after treatment in a second follow-up study, blood samples from 10 geese and Muscovy ducks were collected. Statistical analysis was calculated on data using parametric (ANNOVA). The difference in means was considered statistically significant when $P< 0.05$.

III. RESULTS AND DISCUSSION

Banana stalk is high fiber but has no fat, and vitamin E or Se (Poyyamozhi and Kadirvel, 1986; Viswanathan et al., 1989). Rice bran has been use as a feed ingredient in poultry but it contains a large amount of oil and lipase which are susceptible to oxidation (Linfield, 1985). It was likely that the diets used in this study would be considered, as being deficient in vitamin E especially when it was found that rice bran had a long storage time and became rancid. At first, geese showed severe nervous signs and microscopic examination revealed encephalomalacia in brain (Table 1). The concentrations of vitamin E in affected geese was 0.15 µg/mL which was significantly ($P < 0.05$) lower than in the control samples taken from unaffected birds (Table2). Moreover, the lesion was typically consistent with that of vitamin E deficiency in chicks described by Klasing and Austic (2003), Van Vleet and Ferrans (1976) and Ikumo (1980). To our knowledge, this is the first report which confirms that encephalomalacia can occur in geese. Microscopic examination of affected Muscovy ducks revealed diffuse satellitosis in the brain(Table1).The average concentrations of vitamin E in Muscovy ducks was 1.56 µg/mL which was significantly ($P < 0.05$) lower than in the control samples (Table2). It is possible that the lesions were caused by vitamin E deficiency which allows accumulation of excessive lipid hydroperoxides, that result in brain tissue damage (Garland and Pritchard, 2008). In this study, geese showed obvious brain lesions at death while Muscovy ducks showed a mild degree of brain lesions and were able to recover from the disease after treatment. Both geese and Muscovy ducks revealed degeneration of cardiac myocytes and gizzard smooth muscle cells. In this study, the concentrations of Se in affected geese and Muscovy ducks were 2.01 µg/dL and 1.93 µg/dL, respectively, and were significantly ($P < 0.05$) lower than concentrations seen in the control samples (Table 2). These findings are supported by those of Klasing and Austic, (2003); Dhillon and Winterfield (1983) and Van Vleet and Ferrans (1976) who have reported on the characteristics of vitamin E and Se deficiency in ducklings. In addition, it has been confirmed that vitamin E and Se deficiency in chickens is responsible for nutritional pancreatic degeneration (Thompson and Scott, 1969; Cantor et al., 1975). In this study, the low concentrations of Se was associated with necrosis of acinar cells in the pancreases. Additionally, from Table 2 In the follow – up study in 2 months and 7 months after

treatment, the condition had improved and the concentration of vitamin E and Se were significantly ($P < 0.05$) increased.

Table 1 - Histopathologic changes in Geese and Muscovy ducks fed diets formulated predominantly from rice bran and banana stalk.

Tissues	Lesions	Geese No.			Muscovy No.		
		1	2	3	1	2	3
Brain	- Encephalomalacia	+	+	+	-	-	-
	- Diffuresatellitosis	+	+	+	+	+	+
Heart	Degeneration of cardiac myocytes	+	+	+	+	+	+
Gizzard	Degeneration of gizzard smooth muscle cells	+	+	+	+	+	+
Pancreas	Necrosis of acinar cells	+	+	+	+	+	+

- = lesion absent; + = lesion present

Table 2 - The average concentrations of vitamins E and Selenium in serum of Geese and Muscovy ducks.

	Geese(Mean ±SE)		Muscovy (Mean ±SE)	
Investigation time:	Vitamin E	Selenium µg/dL	Vitamin E	Selenium µg/dL
Before treatment	0.15^{a}±0.02(3.65^{b})	2.01^{a}±0.10(3.95^{b})	1.56^{a}±0.13(3.86^{b})	1.93^{a}±0.12(3.87^{b})
2 months after treatment	2.60^{a}±0.11	4.37^{a}±0.13	3.72^{b}±0.17	4.03^{b}±0.16
7 months after treatments	4.90^{b}±0.19	3.88^{b}±0.1	4.82^{b}±0.22	4.35^{b}±0.0

Values in brackets are the mean concentration for the control birds.
Within columns values with uncommon superscripts are significantly different ($P < 0.05$).

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EFFECT OF *IN OVO* ADMINISTRATION OF INSULIN-LIKE GROWTH FACTOR-I ON HATCHING CHARACTERISTICS AND PERFORMANCE OF BROILER CHICKENS

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Insulin-like Growth Factor-1 (IGF-I) is a key regulator of muscle development and metabolism in birds and other vertebrate species (Duclos, 2005). The early expression of IGF-I during the embryonic chicken development suggests that this peptide plays a significant role in chicken embryonic growth (Kocamis et al., 1998). The current study was conducted to investigate the effect of *in ovo* injection of IGF-I on hatchability and productive performance of broiler chickens. Accordingly, 600 fertile eggs were randomly assigned to five groups with five replicates of 24 eggs; including non-injected control, vehicle injected control (10 mM acetic acid and 0.1% BSA), and rhIGF-I injected groups (100, 200 and 300 ng per egg with vehicle injected). For injection application, on the fifth day of incubation (120-124 hrs of incubation) following the eggs being placed in the incubator, all the set eggs were candled and the air sacks located. Injections were made into the albumen right under the air sack using 23-gauge needles cut to 13 mm, then the holes were sealed with liquid paraffin and the eggs were replaced in the incubator in order to be hatched. Eggs which did not hatch until day 21 of incubation were cracked and the dead embryos were assayed for mortality. The hatched chicks were reared for 6 weeks to evaluate their post-hatch performance.

Hatchability, embryonic mortality and birds' liveability post-hatch were not affected by administration of 100 and 200 ng of IGF-I ($P \geq 0.05$). However, eggs injected with 300 ng IGF-I did not hatch, with the majority of mortality occurring between 17 to 19 days of incubation. It could be speculated that the accelerated growth rate and basal metabolism of embryos in 300 ng IGF-I group would have resulted in embryonic asphyxia and consequently embryos death. Birds in 100 and 200 ng IGF-I groups had a higher feed intake, gained significantly higher weight and exhibited improved FCR ($P \leq 0.05$) at the end of production period (42 d). The body weight coefficient of variation at day 42 was statistically ($P \leq 0.05$) higher for IGF-I treated birds, as a result of a marked difference in body weight between male and female birds in these groups. Individual body weight of male and female birds indicated a greater impact of IGF-I on weight gain of male birds compared to their female counterparts. The current results and our previous studies (Mohammadrezaei et al., 2014) indicate that estrogen synthesis could be a stumbling block for the IGF-I action mechanism in female embryos and hatchings.

Table 1 - Performance parameters of *in ovo* treated birds over the 6 weeks of production (0-42 d).

	Treatments			
Performance Parameters	Control	Sham Control	IGF-I 100	IGF-I 200
Body weight (g/bird)	2495.2 ± 25[b]	2489.8 ± 36[b]	2641.4 ± 34[a]	2670.8 ± 29[a]
Body weight gain (g/bird/day)	59.41 ± 0.29[b]	59.28 ± 0.21[b]	62.89 ± 0.91[a]	63.59 ± 0.44[a]
Feed intake (g/bird/day)	111.12 ± 0.74[b]	110.94 ± 0.61[b]	113.42 ± 0.91[a]	114.44 ± 0.79[a]
Feed conversion ratio (g/g)	1.870 ± 0.014[a]	1.871 ± 0.015[a]	1.802 ± 0.013[b]	1.798 ± 0.016[b]
CV% of body weight	3.451 ± 0.839[b]	3.519 ± 0.773[b]	5.892 ± 0.215[a]	5.971 ± 0.912[a]

[a-b] Means in the same row followed by different letters differ significantly (Tukey test; $P \leq 0.05$)

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AMINO ACIDS VERSUS ENERGY: TRENDS IN FEEDING MODERN COMMERCIAL BROILERS

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Summary

Over the past twenty five years dietary amino acid density has been increasing, and dietary energy density has been decreasing. A historical perspective and references from the year 2005 onward have been included to discuss this trend. Indeed, as broilers are being selected for more efficient meat yields, bird weight targets to age are decreasing and further processed-high valued meat products are increasing. Optimal amino acid and energy feeding programs are presented in these proceedings. However, company decisions regarding diet density should be tied to profit index and overall efficiency, both in the field and processing plant.

I. INTRODUCTION

As commercial broilers are improved yearly by primary breeder companies, it is important to continuously monitor amino acid and energy needs for optimal feeding. A common and straightforward experimental approach to measure nutrient need changes with genetic advancements is to test dietary lysine needs per therm. However, much of the recent experimentation with advancing modern commercial strains has focused on dietary amino acid density, typically without consideration to energy. Hence, as modern broiler strains are improved for whole body protein accretion with less feed intake and whole body fat, optimal live and processing performance dictate aggressive amino acid nutrient inputs in feed formulation. The following paper assesses some recent findings that can serve as a foundation for future research.

II. RECENT DIETARY AMINO ACID DENSITY RESEARCH

Prior to the year 2000, U.S. nutritionists routinely fed low amino acid density diets to broilers. With the consistent improvement in meat yielding genetic broiler strains and introduction of value added poultry products, dietary amino acid density among U.S. broiler complexes began to increase in the early 2000's. Significant research that has led to this formulation change has been reviewed (Dozier et al., 2008). Conclusions from this review were: 1) both body weight gain and breast meat yield were more sensitive to early rather than late dietary amino acid density; 2) economic predictions for feeding high dietary amino acid density are more sensitive to meat prices than feed ingredient prices; and 3) regression equations based on past literature for dietary amino acid nutrient predictions were most accurate for lysine ($R^2 = 0.93$) yielding an equation of $y = 0.0009x^2 – 0.014x + 1.44$ were y = dietary total lysine and x = day of age (Dozier et al., 2008).

Some of the key references in the review by Dozier et al. (2008) were amino acid density by genotype studies (Corzo et al., 2005a) or amino acid density studies in modern commercial broiler strains (Corzo et al., 2005b; Dozier et al., 2006a and 2006b; Dozier et al., 2007; Kidd et al., 2005). The former studies were conducted in similar management styles and all studies provided analyzed amino acid values, of which regression equations were

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estimated (Dozier et al., 2008). All of the broiler strains used for the regression equation were slow feather strains with one Ross x Cobb cross and seven Ross crosses (Corzo et al., 2005a; Corzo et al., 2005b; Dozier et al., 2006a and 2006b; Dozier et al., 2007; Kidd et al., 2005). Hence, amino acid density work in Cobb slow and fast feather crosses is warranted.

Corzo et al. (2010) was the first to source Cobb x Cobb 500 fast-feathering broilers and utilize the vent sexing procedure to test responses to amino acid density in males. This study utilized 17 treatments and reared birds to 42 days of age with a processing weight of 2.6 kg/bird (Corzo et al., 2010). It was concluded that Cobb birds responded well to amino acid density feeds, but feeding amino acid density feeds in the starter period was not warranted, a regime that differs greatly from Ross counterparts. Coneglian et al. (2010) conducted similar work in both slow- and fast-feathering Cobb x Cobb 500 broilers rearing them to 40 days of age (2.8 kg at final processing). Similar results to that of Corzo et al. (2010) were obtained except that alternating amino acid density (Low and High versus High and Low) produced equal results (Coneglian et al., 2010).

Recent work (Taschetto et al., 2012; Vieira et al., 2012) has also assessed amino acid density of Cobb 500 broilers. Taschetto et al. (2012) utilized both male and female slow-feathering Cobb 500 broilers and noted no sex by amino acid density interactions except for feed intake and abdominal fat as male broilers responsed to higher amino acid levels. Results of Taschetto et al. (2012) mirror that of Corzo et al. (2010). Additional research by Vieira et al. (2012) utilized slow-feathering Cobb x Cobb 500 male broilers fed diets varying in amino acid density from 1 to 7, 8 to 21, 22 to 35, and 36 to 42 days of age. Optimal analyzed total lysine for the former periods were 1.59, 1.46, 1.14, and 0.98%, respectively (Vieira et al., 2012). Using the regression equation from Dozier et al. (2008) to compare results to Vieira et al. (2010) in the former periods resulted in total lysine levels of 1.36, 1.26, 1.12, and 1.04% of diet.

The former data compiled from research reports evaluated amino acid density across test diets surfeit in energy. Hence, amino acid responses per therm, which were consistently evaluated in the 1980's and 1990's, lack recent attention.

III. RECENT DIETARY ENERGY RESEARCH

Starting birds benefit with high amino acid density, but less is known concerning energy density. Dozier et al. (2008) fed birds either 3,040 or 3,140 kcal ME/kg from 1 to 15 days of age. Birds were exposed to two ambient temperatures to 15 days of age (32 versus 27 C averaged) and reared to 40 days of age. Differences in 40 live performance or yields did not occur (Dozier et al., 2008).

Regarding growing broilers (14 to 28 days of age), recent research evaluated energy graduations (3,000 to 3,150 in 30 kcal/kg increments) in both Hubbard x Cobb 500 and Ross 708 male broilers (Dozier and Gehring, 2014). An energy estimate for Hubbard x Cobb broilers, which weighed on average 68 g/bird more than Ross 708 at 28 days, for feed conversion was 3,062 kcal ME (Dozier and Gehring, 2014). Ross 708 male broilers did not respond to energy.

In the finishing period, a number of studies have assessed ME needs of broilers (Dozier et al., 2006a; 30-59 days); (Dozier et al., 2007a; 36-60 days); (Dozier et al., 2007b; 42-56 days). This work demonstrated consistent decreases in feed intake and feed conversion as energy was increased, but no effect on breast meat yields. However, increasing amino acids density in birds fed the high energy treatments restored breast meat yields to control levels, pointing towards an amino acid to energy need in modern heavy broilers.

Vieira et al. (2014) evaluated both amino and energy density (3 x 3 factorial setting) from 1 to 29 days of age. The moderate amino acid program was set at 1.28, 1.22, and 1.07%

digestible lysine and AMEn of 3,050, 3,100, and 3,200 with the low and high treatments being derived by reducing or increasing levels by 10 and 1.5%, respectively. Amino acid and energy trends were discussed at the Arkansas Nutrition Conference (Vieira et al., 2014) for both bird performance and profit margins. Consistent improvements in feed conversion were noted with increasing amino acid and energy density. It was concluded that commercial settings in warm areas with poor management should feed higher than normal energy for optimal bird performance (Vieira et al., 2014). A similar finding by Zhai et al. (2014) who fed both Cobb 500 and 700 broilers diets differing in energy and amino acids from placement to 55 days of age.

IV. FEEDING THE BIRD OF THE FUTURE

The compilation of experimental results with modern high yield broilers, with both rapid and slow early growth, indicate commercial broilers have become increasingly sensitive to dietary amino acid density, and to a lesser extent dietary energy density. Indeed, as indicated by Vieira et al. (2014), modern broilers reared in tropical climates with suboptimal housing respond to increasing dietary energy. But this is not the case for most the world's production in environmentally controlled housing. Carré et al. (2014) evaluated diet, feed intake, age, and body weight from 42 publications to predict future reductions in slaughter time for 2.5 kg broilers. Although genetic improvements in slaughter age and feed conversion were noted, dietary digestible lysine was modeled for past, current and future slaughter times. Hence, broilers slaughtered at 2.5 kg in 34 days require dietary digestible lysine of 1.26, 1.23, and 1.00% whereas future genetic improvements resulting in 2.5 kg slaughter weights at 20 days of age will require 1.60, 1.59, and 1.38% digestible lysine across starter, grower, and withdrawal periods, respectively (Carré et al., 2014). What will be the energy needs in birds grown to 20 days in diets based primarily on soybean meal and feed grade amino acids to yield the lysine levels predicted in the former work? In future, a balanced approach in feed formulation (i.e. ideal protein) will be critical for nutrient need extrapolations because genetic advances continue to be steadfast and global poultry research facilities are on the decline.

A recent review (Kidd et al., 2013) presented a historical perspective on the use of linear programming in feed formulation and subsequent acceptance and use of feed grade amino acids. The poultry industry has a fifty year history with using feed grade amino acids, and currently amino acid fermentation companies are contemplating commercial strategies for L-valine and L-isoleucine. Although amino acid density research has provided lysine-based formulation strategies, more research is warranted for minimum branched-chain amino acids digestible minimums and minimum ratios to lysine. More requirement research with valine and isoleucine, coupled with a better understanding of arginine, glycine, leucine, and non-essential amino acid nitrogen for commercial broilers, will pave the way to optimize or minimize dietary amino acid needs for broilers based on the financial outlook, especially as modern broilers are marked at increasingly earlier ages. Clearly, we have not mastered semi-purified diets (high crystalline amino acid inclusion and low levels of protein) for commercial broilers (Corzo et al., 2005). Current research at the University of Arkansas, Division of Agriculture Poultry Research Farm is underway to assess the branched-chain amino acid needs and crude protein-feed grade amino acid balance in Cobb male and female broilers.

Bolden (2014) presented an overview of dietary amino acid and energy density in Cobb 500 broilers at the 2014 Arkansas Nutrition Conference indicating the flexibility of feeding modern broiler strains. Hence, broiler nutrition should be tied to economic priorities, and in doing so, the highest amino acid density known to improve breast meat, feed conversion, and days to market shouldn't always be fed (i.e., companies focused on feed costs/kg of bird weight or in companies with inefficient processing plants). Further, it was

noted that industry average calories (kcal ME/kg of diet) fed to broilers have been reduced by over 60 kcal the last 25 years (Bolden, 2014).

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ON FREE AMINO ACIDS: THEIR ROLE IN STARCH AND PROTEIN DIGESTIVE DYNAMICS

P.H. SELLE[1], H.H. TRUONG[1] and S.Y. LIU[1]

Summary

That increase in body-weight of broiler chickens from 42 g to 3,000 g at 42 days post-hatch is an ultra-dynamic process that is not properly reflected in static apparent digestibility coefficients of starch, protein and amino acids determined at the terminal ileum. Determinations of digestibility coefficients at the terminal ileum eliminate the confounding influence of hind-gut fermentation but the large majority of glucose and amino acids are absorbed in the jejunum. The quantity of glucose and amino acids absorbed from the small intestine is a function of dietary concentrations, feed intakes and digestibility coefficients. Moreover, the rates and sites of absorption of glucose and amino acids need to be taken into considerations of digestive dynamics. A tangible proportion of these absorbed nutrients are not transferred into the systemic circulation but are utilised by the gut mucosa for energy to drive digestive processes and for structural and secretional protein synthesis. Glucose, glutamic acid and glutamine are important energy substrates but, to varying extents, the balance of amino acids undergoes catabolism to meet the copious energy requirements of the gut. The provision of balanced quantities of glucose and amino acids at protein deposition sites is critical for efficient growth. Therefore, the inherently different digestive dynamics of free versus protein-bound amino acids, and their post-enteral availability, should be taken into account. This is necessitated by the likelihood that free amino acids will be incorporated into broiler diets to increasing extents in the future. Thus this review paper is a tenuous forecast of the likely impacts free amino acids on digestive dynamics of starch and protein in the framework of chicken-meat production.

I. INTRODUCTION

That a mounting array of synthetic amino acids will be incorporated into broiler diets to increasing extents would appear to be an entirely reasonable prediction. The caveat is that predictions are fraught with difficulty; particularly when they concern the future. However, nearly 50 years ago, Beames *et al.* (1968) made the following prognostication: “It is probable that the price of synthetic amino acids will continue to fall and that they will be used increasingly as replacements for conventional protein supplements”.

The inclusion of free or synthetic amino acids in broiler diets, methionine, lysine and threonine, has been routinely practiced for decades. Now additional essential amino acids including arginine, isoleucine, tryptophan and valine are commercially available. The likelihood is that the increasing global protein demand for both human food and animal feed will continue to drive protein prices higher, which has been the case for soybean meal. Against this outlook, the prices for synthetic amino acids are relatively static or even declining. In one feeding study (Selle *et al.,* 2007), typical wheat-based broiler diets were supplemented with 2.06 g/kg methionine, 2.95 g/kg lysine hydrochloride and 0.87 g/kg threonine. This represented 40, 20 and 11%, respectively, of the specific amino acid concentrations in the diet and 2.5% of the dietary protein content. While the proportions for specific amino acids are substantial; the likelihood is that these proportions will increase and embrace a broader range of amino acids in response to escalating prices for protein meals.

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This review is a speculative consideration of the implications of the probable increase in free amino acid inclusions in broiler diets in the interactive context of starch and protein digestive dynamics.

II. BACKGROUND

Golberg and Guggenheim (1962) compared the digestion of amino acids and their appearance in portal circulation in rats offered casein or soy flour as protein sources. Both the small intestinal digestion and the entry into the portal circulation of lysine from casein were more rapid than from soy flour. Moreover, the data suggests that a greater proportion of lysine entered the portal circulation from casein, the more rapidly digested protein source. Thus differences in digestive dynamics of protein-bound amino acids exist between feedstuffs but differences between free and protein-bound amino acids will be of an even greater magnitude. Free amino acids do not undergo digestion and are directly available for absorption in the upper small intestine and appear in the portal circulation more rapidly than protein-bound amino acids (Wu, 2009). Thus the digestive dynamics of free amino acids, that is the rate, quantity and site of absorption is very different to that of protein-bound amino acids. This was demonstrated by Ted Batterham (Batterham, 1974; Batterham and O'Neill, 1978) in respect of lysine monohydrochloride in grower pigs. In a review of these studies, Batterham and Murison (1981) concluded that the utilisation of free lysine with once daily feeding was 0.53 for weight gain and 0.56 for feed conversion efficiency on the basis of carcass weight, relative to frequent feeding. Thus the differential absorption rates and the asynchronous availability of free and protein-bound lysine at sites of protein accretion was pivotal. Indeed, Kondos and Adriaans (1982) claimed that a dry-extruded, 'slow release' lysine outperformed lysine HCl in grower pigs fed on a twice-daily basis by 9.33% in average daily gain (820 versus 750 g/day), 8.12% in feed conversion efficiency (2.49 versus 2.71) in association with a 16.5% reduction in N urinary losses. While similar outcomes had been previously reported in rats (Rolls *et al.,* 1972), Batterham's proposition that the digestive dynamics of free and protein-bound amino acids were asynchronous did not meet with unanimous acceptance at their time of publication. Nevertheless, it was subsequently demonstrated that the utilisation of free amino acids by broiler breeders fed once- or twice-daily was inferior on the same basis (Nonis and Gous, 2006).

An open question is how efficiently can broiler chicks utilise free amino acids? Broilers are offered diets on an *ad libitum* basis and their feed intake under a '24-hours-ons' lighting regime is ostensibly continuous. Nevertheless, Pinchasov *et al.* (1990) reported that 7-21 day weight gains and feed conversions were compromised by offering chicks maize-based diets with increasing inclusions of free amino acids on an unrestricted basis but the lighting regime was not specified.

The definition of digestive dynamics in this review includes the digestion of nutrients in the gut lumen, absorption of end-products into the gut mucosa, and their post-enteral availability. This process involves both static and kinetic components and static ileal digestibility coefficients are used to calculate the kinetic parameters. That is the rate, quantity and site of absorption of glucose and amino acids along the small intestine. The rate of digestion may be deduced by fitting apparent digestibility coefficients and mean retention times in intestinal segments into an exponential mathematical model. The quantity of a nutrient absorbed at the terminal ileum (g/bird/day) or other intestinal segments is a simple function of feed intakes, dietary concentrations and digestibility coefficients. The site of absorption may be defined as one of four small intestinal segments (proximal and distal jejunum, proximal and distal ileum) and the amounts of starch or protein that apparently disappear or are absorbed as glucose and amino acids may be deduced.

More than sixty years ago, Geiger (1950) opined that the relative timing of amino acid and carbohydrate uptakes can markedly affect protein synthesis and the efficiency of feed utilisation. Nevertheless, the complex impacts of starch and protein digestive dynamics in broilers still require further elucidation and the importance of digestive dynamics in relation to chicken-meat production merits greater recognition (Liu and Selle, 2014; Liu *et al.,* 2014a). Instructively, Rerat (1993) made the following (condensed) statements:

> *"The food intake of single-stomached animals is discontinuous and varies in amount and composition. These quantitative and qualitative variations in food intake pose a number of questions. Do they affect the digestive processes which are related to food transit and enzyme hydrolysis, especially the extent of nutrient absorption and its kinetics? These questions cannot be answered merely by measuring digestibility. Digestibility is used to evaluate the disappearance of ingested nutrients either in the whole digestive tract or in its proximal part (ileal digestibility). Whichever technique is used, these digestibility measures are global and static and do not account for the different absorption rates which vary for different nutrients. Study of kinetics associated with differences in the relative absorption rate of the various nutrients is necessary for a fuller understanding of variations in the nutritive value of feeds."*

The validity of the Rerat (1993) summation of kinetics or dynamics is evidenced in one study (Selle *et al.,* 2012; Liu *et al.,* 2013a) completed by the Poultry Research Foundation. This involved broiler diets based on red, white and yellow sorghums and mash, reground pellet and intact pellet feed forms in a 3x3 factorial array of dietary treatments. As shown in Table 1, ileal digestibility coefficients of starch and nitrogen were not significantly correlated to key performance parameters. However, there were predominantly significant correlations between starch and nitrogen digestion rates and predicted glycaemic indices (or glucose absorption) with weight gain, FCR and N retention.

Table 1 - Pearson correlation coefficients (*probability values in parentheses*) between static and dynamic digestibility parameters and performance of broiler chicks from 7 to 27 days post-hatch (adapted from Selle *et al.*, 2012; Liu *et al.*, 2013a).

Parameter	Weight gain	FCR	N retention
Apparent ileal digestibility of nitrogen	0.088	-0.181	0.206
	(0.528)	*(0.191)*	*(0.134)*
Apparent ileal digestibility of starch	-0.170	0.040	0.064
	(0.218)	*(0.776)*	*(0.648)*
Nitrogen digestion rate ($K_{nitrogen}$)	0.353	-0.341	-0.146
	(0.009)	*(0.012)*	*(0.291)*
Starch digestion rate (K_{starch})	0.311	-0.238	-0.387
	(0.022)	*(0.083)*	*(0.004)*
Predicted glycaemic response	0.400	-0.512	-0.380
	(0.003)	*(< 0.001)*	*(0.005)*

Further confirmation of the importance of digestive dynamics is illustrated in Figure 2. In a recent study (Truong et al., 2015) there was a negative linear relationship between starch:protein disappearance rate ratios and 40-day weight gains. The negatively correlation has an R^2 value of 0.587; thus 59% of the variation in weight gain may be attributed to the disappearance rate ratio and, importantly, weight gains will be enhanced following an increase in the disappearance rate of protein (g/bird/day) relative to starch from the proximal ileum.

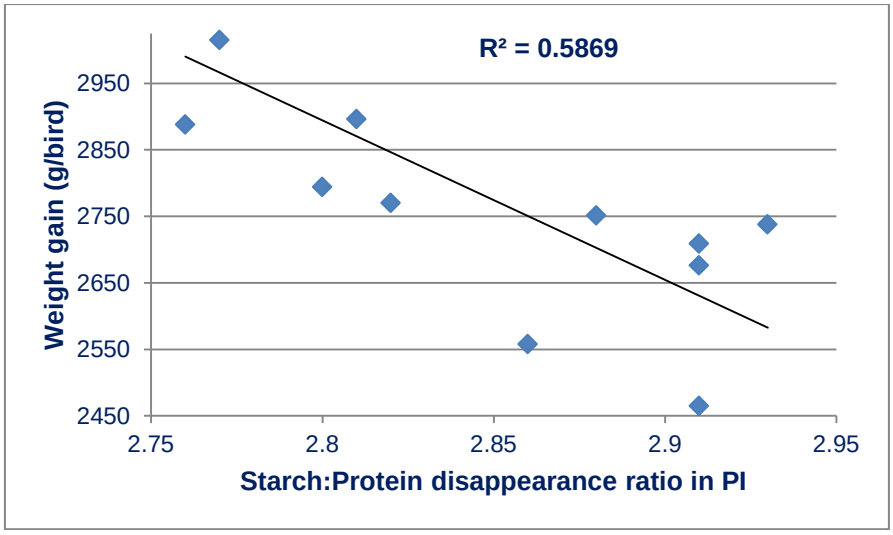

Figure 1 - Linear relationship (r = -0.766; P = 0.003) between starch:protein disappearance rate ratios in proximal ileum and 40-day weight gains in broilers offered maize-based diets without and with 500 FTU/kg phytase (Truong *et al.,* 2015).

III. INTERMITTENT FEED INTAKES

Broiler chickens are fed *ad libitum* but this unrestricted access to feed does not equate to continuous feeding and feed intakes of broilers may be best described as intermittent and are also subject to lighting regimen. Broilers exhibit diurnal feeding patterns and consume most of their ration either at the start or end of the day (Savory, 1980). Moreover, Jensen *et al.* (1962) found that chicks offered pelleted diets under a '12-hours-on' lighting regime spent only 4.7% of illuminated time in eating and Fujita (1974) reported that chicks spent 10.8% of time eating under a '14-hours-on' lighting regime. Based on five of our studies, the average retention time in the jejunum and ileum was 2.98 hours. Coupled with the data of Denbow (2000) for retention times in the crop, proventriculus, gizzard, and duodenum, digesta reaches the terminal ileum in broilers in approximately 4.25 hours. Thus, despite essentially unrestricted access to feed in commercial production systems, intermittent feed consumption patterns still provide scope for asynchronies in digestion and absorption of nutrients in the small intestine to influence broiler performance. This logical interpretation is supported by both the data in Table 1 and a review of starch and protein digestive dynamics (Liu and Selle, 2014) in which the impacts of digestive dynamics in broilers are evident under standard lighting regimen. Starch and protein digestion occurs mainly in the jejunum through which digesta will pass in approximately 3 hours; therefore, as lighting regimen influence feed consumption patterns and extended periods without illumination should intensify imbalances in digestive dynamics.

IV. AMINO ACID CATABOLISM

The efficient anabolic incorporation of amino acids into skeletal protein is the desired outcome for chicken-meat production. However, a substantial proportion of amino acids undergo inevitable and preferential catabolism which represents a shortfall of amino acids for protein deposition. Estimates of lysine catabolism range from 15 to 30% in pigs and 20 to 24% in broiler chickens (Moughan, 2014). From a growth simulation model for 50 kg pigs, Moughan (2014) predicted that the inevitable catabolism of lysine accounted for 24-44% of losses which exceeded losses from undigested (25-32%) and endogenous flows (12-18%) of lysine. Sites of amino acid catabolism are not limited to intestinal mucosal cells but surprising quantities of amino acids are catabolised in enterocytes for the provision of energy

to meet the copious requirements of the gut (Stoll *et al.,* 1998; Wu, 1998). Initially Windmueller and Spaeth (1975) reported that the rat intestine utilised nearly all absorbed dietary glutamic acid and circulating glutamine but 60% of glutamate/glutamine was metabolised to CO_2 and this catabolism made 'an important contribution to the energy requirement of the gut'. Subsequently, Stoll *et al.* (1999) reported that glucose, glutamate and glutamine met 75% of the energy requirement of portal drained viscera in pigs and that dietary glutamic acid was fundamental in this respect. Poultry studies are limited but Watford *et al.* (1979) found that both glutamate/glutamine and glucose were oxidised in enterocytes but glucose generated 27% more oxygen in chicken enterocytes than glutamate/glutamine. Wu *et al*. (2005) even suggested that antimicrobial agents depress catabolism of dietary amino acids in enterocytes by reducing the intestinal mass and that this may be one mechanism for their growth promoting effects. The implication is that catabolism of amino acids in the gut mucosa is manipulable and its reduction would trigger the entry of more amino acids into the systemic circulation to become available for protein accretion with a corresponding increase in the more efficient utilisation of glucose for energy provision to the gut.

V. SLOWLY DIGESTIBLE STARCH

Data has been generated (Weurding, 2002; Weurding *et al.,* 2003a,b) to indictae that the provision of some slowly digestible starch, or starch digested in the lower small intestine, in broiler diets enhances feed conversion efficiency. More recently it was demonstrated that slowly or gradually digestible starch enhanced energy utilisation (AME, AMEn) in addition to FCR in sorghum-based broiler diets (Liu *et al.,* 2014b; Selle *et al.* 2013b, 2014). Gradually digestible starch was defined as starch digested in the distal jejunum, proximal and distal ileum as opposed to the proximal jejunum. Graded inclusions of the reducing agent sodium metabisulphite generated more gradually digestible starch which appeared responsible for the improvements in energy utilisation and FCR. A more continuous absorption of glucose from the gut pursuant to the provision of slowly digestible starch could beneficially modify insulin-induced responses. However; any interpretation of possible glucose/insulin responses is complicated by the differences between avian and mammalian species in glucose and insulin metabolism, although these differences may be overstated (Simon, 1989; Simon *et al.,* 2011). An alternative hypothesis is that slowly or gradually digestible starch may enhance FCR in broilers via a protein-sparing effect (Weurding *et al.,* 2003b). Enting *et al.* (2005) included amino acids as glutamine and casein in broiler diets based on unprocessed or hydrothermally-treated maize and peas as hydrothermal processing increases starch digestion rates and there were significant interactions between amino acid inclusions and starch digestion rates. In slowly digestible starch diets amino acids marginally improved FCR (0.60%) but, in rapidly digestible starch diets, amino acids improved FCR by 4.66% (1.638 versus 1.718). These researchers suggested that slowly digestible starch might "prevent the use of amino acids as an energy source for the gut wall", thereby increasing their availability for protein accretion.

The gastrointestinal tract consumes in the order of 20% of incoming dietary energy for digestive and absorptive processes (Cant *et al.,* 1996) and much of this energy is derived from catabolism of amino acids rather than glucose (Fuller and Reeds, 1998). Nevertheless, Fleming *et al.* (1997) found starch/glucose and glutamic acid/glutamine were approximately equally important energy substrates for small intestinal mucosal cells in the rat. Moreover, net ATP production from glucose was greater than glutamine by a two-fold factor. Similarly Posho *et al.* (1994) reported that, in comparison to glutamine alone, the provision of both glucose and glutamine to porcine enterocytes reduced ATP production from glutamine. This

suggests that enterocytes can derive energy from glucose provided it is available. It follows that glucose from rapidly digested starch sources would be depleted in the upper small intestine, effectively forcing mucosal cells in the lower small intestine to catabolise amino acids for energy provision. Thus slowly digested starch sources would provide more glucose to the lower small intestine thereby sparing protein from oxidation and energy generation from glucose would be substantially more efficient than from amino acids.

A pivotal study was completed by van der Meulen *et al.* (2007) in pigs. Less rapidly digested native pea starch significantly increased the net portal flux of essential amino acids by 22.9% in comparison to more rapidly digestible maize starch. More specifically, slowly digestible starch increased portal fluxes of tryptophan and lysine by 42.3 and 28.6% in absolute terms. Also, slowly digestible starch increased proportions of tryptophan and lysine in portal circulation by 49.3 and 32.5%, respectively Relatively, as a proportion of ileally digested amino acids. Some of the van der Meulen *et al.* (2007) findings are shown in Figure 2 and indicate that amino acid catabolism for energy provision to the gut is an important source of amino acid losses that can be averted by slowly digestible starch.

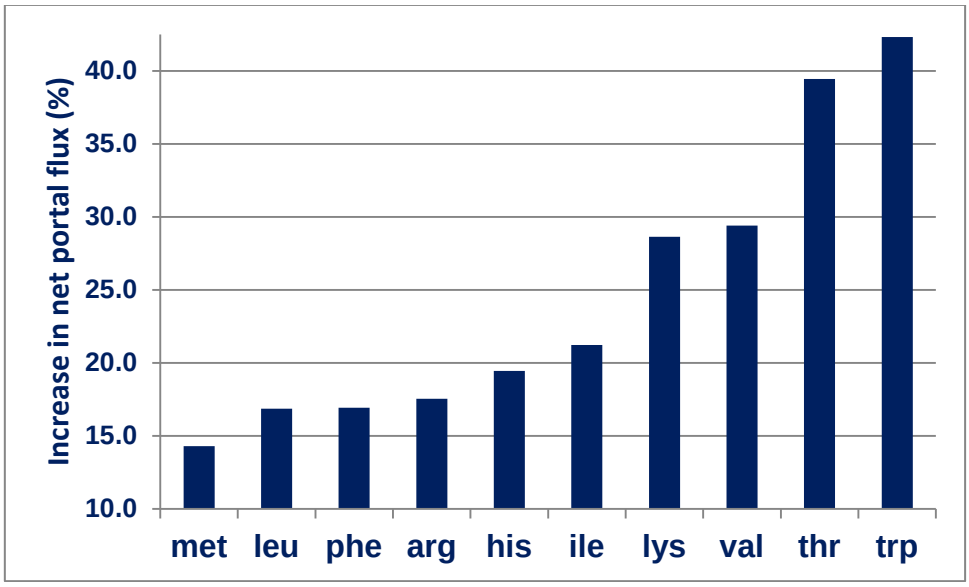

Figure 2 - Percentage increase in net portal flux (mmol) of essential amino acids following the provision of a more slowly digestible starch source (pea versus maize starch) to pigs (adapted from van der Meulen *et al.,* 1997).

VI. FREE AMINO ACIDS

Free amino acids do not require digestion and it follows that they will be rapidly and almost completely and rapidly absorbed in the upper small intestine to far greater extents than protein-bound amino acids. Schwartz *et al.* (1959) compared free and protein-bound lysine in chicks and at the highest level of supplementation, free lysine HCl supported nearly 20% greater weight gains than bound lysine in dehulled soybean meal. The more rapid digestion rates of free amino acids is illustrated in Table 2 where sorghum-based broiler diet were supplemented with methionine, lysine and threonine as free amino acids (Liu *et al*., 2013b). The average digestion rate for nine essential amino acids was 2.96 $k_{amino\ acid}x10^{-2}min^{-1}$ with the branched chain amino acids being noticeably slower. In contrast, methionine and lysine had a remarkably higher mean digestion rate of 4.83 $k_{amino\ acid}x10^{-2}min^{-1}$ because of their dietary presence in both free and bound forms. Thus free amino acids accelerate 'protein' digestion rates relative to starch, which could provide a better post-enteral balance of glucose and amino acids for protein deposition.

While speculative, but perhaps importantly, free amino acids may be less susceptible to catabolism in the gut mucosa because of their proximal absorption in segments of the small intestine where more glucose is available as an alternative energy substrate. On the basis of the van der Meulen *et al.* (2007) data, this would apply especially to tryptophan and lysine, less so to threonine, valine and isoleucine and least to methionine and arginine. If, in fact, free tryptophan and lysine are less prone to catabolism in the gut mucosa than their protein-bound counterparts, then the implications are quite profound.

Table 2 - Digestion rate ($k_{amino\ acid}$ x $10^{-2}min^{-1}$) in descending order of amino acids in a sorghum-based broiler diet supplemented with 3.37 g/kg methionine, 3.74 g/kg lysine and 1.29 g/kg threonine as free amino acids (adapted from Liu *et al.*, 2013b).

Amino acid	Digestion rate	Amino acid	Digestion rate
Methionine	5.51^{a}	Proline	2.28^{efgh}
Lysine	4.14^{b}	Serine	2.27^{efgh}
Arginine	3.25^{c}	Alanine	2.18^{efgh}
Glutamic acid	2.95^{cd}	Glycine	2.11^{fgh}
Threonine	2.58^{de}	Leucine	2.07^{gh}
Histidine	2.57^{de}	Isoleucine	2.05^{gh}
Aspartic acid	2.52^{ef}	Valine	2.04^{gh}
Phenylalanine	2.39^{efg}	Tyrosine	1.85^{h}
	SEM	0.1525	
	Significance (P =)	< 0.0001	
	LSD (P < 0.05)	0.50996	

^{abcdefgh}Mean values not sharing a common superscript are significantly different (P < 0.05)

Fang *et al.* (2010) compared the first-pass intestinal metabolism and post-enteral availability of DL-methionine (DL-MET) with DL-2-hydroxy-4-methylthiobutyrate (DL-HMTB) in pigs; however, a diet with only protein-bound methionine was not assessed. The fractional net portal balance of methionine in diets containing DL-HMTB (~80%) was significantly higher than DL-MET (~60%) by approximately one-third over a six hour post-prandial period. These results suggest that free amino acids can be chemically modified in order to decrease their metabolism in the gut mucosa and increase their entry into the systemic circulation. Also, it appears that the first-pass intestinal usage of free DL-HMTB methionine was modest which lends some support to the suggestion that the propensity of free amino acids to undergo cartabolism in the gut mucosa is less than their protein-bound counterparts.

Alternatively, dietary inclusions of a given free amino acid may disrupt the digestive dynamics of that particular amino acid and/or the balance of amino acids to the detriment of protein deposition. Free lysine has been shown to impact negatively on the utilisation of [14^{C}]phenylalanine in pigs offered diets on a restricted basis (Batterham and Bayley, 1989).

Daenzer *et al.* (2001) offered diets to rats containing protein as either casein (150 g/kg) or an equivalent mix of free amino acids. Predictably, the absoprtion of free amino acids was generally more rapid than from casein. However, weight gains were lower and urinary N losses higher in rats offered free amino acids, promting the researchers to conclude that whole-body net protein synthesis was better supported by protein-bound amino acids. Importantly, the meals were offered twice-daily to rats trained to consume their ration in less than 30 minutes. Wheat starch was the primary dietary energy source and it appears that a better dynamic balance was struck between starch and casein than with free amino acids but this was observed under restricted feeding regimen.

In conclusion, in general terms, increasing usage of free amino acids will benefit chicken-meat production because they will attenuate the impact of escalating protein meal prices which is pivotal. Free amino acids will facilitate the fine-tuning of least-cost ration formulations in respect of key digestible amino acid ratios, including lysine, arginine and branched-chain amino acids. In addition, free amino acids should increase the overall rate of "protein" digestion relative to that of starch which may be of benefit. More specifically, a potential advantage is that the propensity of free amino acids to undergo catabolism in the gut mucosa may be less than protein-bound amino acids because of the more proximal absorption of free amino acids in the small intestine. This would result in the inherently more efficient derivation of energy from glucose to drive digestive processes. Alternatively, a potential disadvantage is that free amino acids may lead to an unbalanced provision of free and bound amino acids at sites of protein deposition which would compromise the utilisation of both the specific and the balance of amino acids. However, this impact would appear to be largely dependent on consumption patterns under ostensibly *ad lib* feeding regimes and whether or not broiler chickens are continuous or discontinuous feeders. Feeding patterns may be modified by more extended patterns of illumination and the practice of whole grain feeding may encourage more continuous feed intakes than intact pellets. Certainly, investigations into these possible positive and negative outcomes are justified as the likelihood is greater advantage can be taken from the use of free amino acids following the development of a better appreciation of their role in starch and protein digestive dynamics. Nevertheless, in overall terms, the roll-out of more free amino acids should be a positive development for the chicken-meat industry.

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EFFECT OF NET ENERGY FORMULATION ON BROILER PERFORMANCE AND CARCASS COMPOSITION

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Energy has become the major cost of broiler production yet heat increment is not taken into account when formulating poultry feed. Heat increment can be measured by indirect calorimetry and is related to the relative composition of protein, fat and other constituents in the diet (Swick et al., 2013; Carré et al., 2014). This relationship can be used to predict the heat increment and net energy of individual ingredients and then can be used to formulate feed. The current experiment used 576 Ross 308 male broilers fed a common diet to 10 days. On day 10 the birds were place into floor pens (12 per pen) and fed grower (10 to 24 days) and finisher (24 to 35 days) diets formulated on an ME basis or an NE basis using the NE value calculated using a prediction equation developed earlier (Swick et al., 2013). The summit diets were then blended to produce 2 intermediate diets containing 60% of the NE diet or 60% of the ME diet (3NE:2ME or 2NE:3ME). Formulations were based on current commercial ingredient costs at the time. Bird performance was determined on day 34 and carcass evaluations performed on 5 birds per pen on day 35. Performance exceeded Ross308 breeder guidelines between day 10 and 34 (Table 1). Formulation method had no effect on feed intake (FI), weight gain (WG), feed conversion (FCR), livability (LIV), breast or thigh as a percent of body weight ($P > 0.05$). Abdominal fat (percent of body weight) however, was greater in the intermediate blended diets than the ME formulated diet ($P < 0.05$) but the NE diet was not different from the ME formulated diet ($P > 0.05$). Because of lower diet costs with NE formulation, the cost per kg liveweight gain was 4.1% lower in the NE formulated diets as compared to ME formulated diet. These results show that NE formulation offers potential cost savings for the broiler industry with no adverse impact on carcass quality.

Table 1 - Performance of broilers fed diets formulated on and NE or ME basis from 10 to 34 days and carcass composition at 35 days.

Treatment	FI, g	WG, g	FCR	LIV, %	AUD/kg live	[1]Breast, %	[1]Thigh, %	[1]Abd. fat, %
ME	3272	2201	1.487	94.4	0.6180[a]	20.3	11.1	1.09[b]
3ME:2ME	3287	2202	1.492	96.5	0.6090[a]	20.4	11.1	1.16[a]
2ME:3NE	3301	2188	1.509	95.8	0.6094[a]	20.5	11.2	1.21[a]
NE	3229	2158	1.496	95.8	0.5924[b]	20.7	10.9	1.12[ab]
P	0.34	0.45	0.87	0.80	0.001	0.51	0.19	0.03
CV, %	3.07	2.49	2.26	5.39	2.66	3.17	3.06	8.31

[a,b] $P < 0.05$.
[1] Percent of live body weight on day 35.

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IMMEDIATE POSTHATCH NUTRIENT RESTRICTION: THE EFFECT ON BROILER MUSCLE DEVELOPMENT AND OCCURENCE OF INTRAMUSCULAR FAT

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Neonatal nutrition is an important facet of broiler production which can effect muscle growth and development through to harvest. A critical component of muscle development during this period are the myogenic stem cells, called satellite cells (SC). Satellite cells are maximally active in the first week posthatch during which time they fuse with muscle fibres to facilitate increased muscle protein synthesis. During this critical period SC activity can be impaired by feed restriction which has been shown to affect muscle growth (Halevy et al., 2000). Additionally, as SC are stem cells they can, when stimulated by nutritional manipulation, transdifferentiate to an adipogenic lineage to form fat cells (Asakura et al., 2001). In vitro studies have shown that SC are responsive to an increasing severity of nutritional restriction, observed as reduced myogenic activity and elevated levels of adipogenic gene expression and lipid accumulation (Powell et al 2013, 2014). To investigate if these in vitro observations are representative of SC activity in growing broiler chickens, four diets with increasing restriction of digestible methionine (dMet) were fed to broilers in the first week posthatch, coinciding with the period of maximal SC activity. This study sought to assess the sensitivity of posthatch SC activity to nutritional restriction and its impact on muscle development.

Four hundred male Ross-308 broiler chicks were obtained on the day of hatch. They were divided equally among four treatments with five replicate pens and 20 birds per pen. Four corn-soy based starter diets with titrated levels of dMet were formulated. The control diet contained 0.53% dMet, and three treatments had 120%, 80%, and 60% of the dMet adequate control diet. The treatment diets were fed for the first 7 d, after which all birds were fed the adequate dMet starter until 14 d. Birds then changed to a grower (15-28 d), and finisher (29-42 d) diet. Ten birds from each treatment were sacrificed at 0, 1, 4, and 7 d and weekly thereafter. Samples of breast muscle were collected and fixed in 10% neutral buffered formalin for microscopic evaluation of muscle fibre and fat cell width, perimysial spacing, muscle morphology and presence of intramuscular fat.

The four inclusion rates of dMet in the starter diet from 0-7 d had no effect on body weight, breast weight or feed conversion ratio throughout the study. No significant difference in muscle fibre width, fat cell width, perimysial fibre bundle spacing, objective scoring of the muscle morphology nor the presence of intramuscular fat were observed between treatments. This indicates that there was no significant effect of the dMet treatments on SC activity during the posthatch period, although gene expression analysis is ongoing to confirm this conclusion.

Although Powell et al. (2013) observed a significant effect of the restriction of Met and Cys on SC activity in vitro, this was only observed when the inclusion level of Met and Cys was reduced to 25% or less of the control. These results indicate that SC can function satisfactorily under a degree of Met restriction, with no significant effect on muscle development or intramuscular fat accumulation.

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A DEFICIENT SUPPLY OF METHIONINE CHANGES THE COMPOSITION AND METABOLISM OF THE *PECTORALIS MAJOR* MUSCLE IN GROWING BROILERS

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Summary

Although dietary sulphur amino acids (AA) have been extensively studied, little is known about how differential supplies and sources can affect muscle composition. The aim of this study was to investigate the growth performance, meat quality, composition and protein metabolism of the *pectoralis major* muscle (PM) in broilers receiving a diet either deficient or sufficient in sulphur AA and either supplemented with DL-Met or DL-2-hydroxy-4-methylthiobutyric acid (HMTBA) during a 3-wk period. For both Met sources, the supply of Met and sulphur AA in the deficient diets was respectively 43% and 30% below those of the sufficient diets. The deficient Met supply reduced the bird final BW, ADG and G:F ($P < 0.05$). In Met-deficient birds, the relative weight and protein content of PM were lower, and the meat colour was more red and green, whereas the water content was higher ($P < 0.01$). The absolute protein synthesis and protein mass of PM were lower in Met-deficient birds ($P < 0.05$) whereas proteasome activity of PM was conversely greater ($P < 0.01$). In these birds, the His and OH-Pro contents of retained protein in PM were higher ($P < 0.01$), whereas the Lys, Ile, and Leu contents tended to be lower ($P < 0.10$), with no change in the Met and Cys contents. Free contents of β-Ala, anserine and balenine of PM were lower in Met-deficient birds, but free contents of His, carnosine, and taurine were higher ($P < 0.01$). In conclusion, the AA and protein contents of the muscle together with bird growth are changed by a Met deficiency with a potential impact on protein degradation and meat quality. There are no differences between dietary Met (DL-Met) and hydroxy-Met (HMTBA) sources on muscle growth and protein metabolism.

I. INTRODUCTION

The sulphur amino acids (AA) Met and Cys are usually the first-limiting AA for protein deposition in poultry diets. As an indispensable AA, Met is also an important methyl donor and precursor for the biosynthesis of compounds such as creatine, carnitine, and polyamines (Baker et al., 1996; Kim, 2005). Cysteine is considered a semi-essential AA because it can be synthesized from Met and Ser by transsulfuration. Cysteine is also a precursor of molecules such as glutathione (Cys-Glu-Gly), co-enzyme A and taurine (Shoveller et al., 2005, Brosnan and Brosnan, 2006). Although dietary sulphur AA have been extensively studied, little is known about how differential supplies and sources can affect tissue composition. A deficient Met supply has been shown to affect the chemical composition of different tissues and certain aspects of breast meat quality (Conde-Aguilera et al., 2013). Also, there are indications that the use of DL-2-hydroxy-4-methylthiobutyric acid (HMTBA) results in better relative bioefficacy for bird performances compared with DL-Met when provided to meet the recommended requirement, but that DL-Met may be more bioefficient in birds receiving a deficient sulphur AA supply (Kratzer and Littell, 2006; Vazquez-Anon et al., 2006). Therefore, the objective of this study was to investigate the growth performance, meat

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quality, composition and protein metabolism of the *pectoralis major* muscle of broilers to a deficient sulphur AA supply with two different Met sources.

II. MATERIALS AND METHODS

Eighty birds (181 ± 7 g) were divided into 4 homogeneous groups of twenty 7-d-old birds each to receive a diet either deficient or sufficient in sulphur AA and either supplemented with DL-Met or HMTBA: DL-Met+ and DL-Met– (sufficient and deficient in sulphur AA, respectively with DL-Met) and HMTBA+ and HMTBA– (sufficient and deficient in sulphur AA, respectively with HMTBA) during a 3-wk period. To account for changes in nutrient requirements during the experiment, different diets were formulated to cover crude protein (23-20%) and AA requirements (other than Met) at the beginning and end of the experiment, and birds received a different diet each week. The anticipated true digestible (TD) Lys content decreased from 1.16 to 1.02% from wk 1 to 3, which is in accordance with the recommendations of the breeder (Aviagen, 2009). The supply of Met and sulphur AA in DL-Met– and HMTBA– diets was respectively 43% and 30% below those of DL-Met+ and HMTBA+. The birds were pair-fed to ensure the same feed intake, housed individually in wire-floored cages (length 30 cm × width 30 cm × height 36 cm) and room temperature ranged from 29-22°C from 7-23 d of age. Artificial lighting was 23 h/d during the 3 first d of age and 18 h/d thereafter. Forty birds (10 birds per treatment) were used to determine the composition of breast meat (composition group) and the other 40 birds (10 birds per treatment) were used to determine breast meat quality, protein synthesis and proteolytic enzyme activities (sampling group). In composition group birds, after electrical stunning and exsanguination, *pectoralis major* muscles (PM) were separated from the carcass, weighed, cut into small pieces, frozen and freeze-dried later. All PM were independently homogenized in a cutter mill (Grindomix GM200, Restsch, Newton, PA) and subsamples were taken and ground with a ball mill (Vangoumil 300, Darmstadt, Germany) for protein, fat, ash, dry matter, and free and total AA analyses using methods of the International Organization for Standardization (ISO methods, http://www.iso.org). In sampling group birds, subsamples from PM were frozen in liquid N and stored at –20°C for measuring protein synthesis using the flooding dose procedure (Sève et al., 1993, Tesseraud et al., 1996), at –80°C to determine proteolytic enzyme activities (Le Naou et al., 2012), and at 4C° for meat colour and the pH 24 h after slaughter (Petracci and Baeza, 2011). Statistical analyses were performed using the GLM procedure of SAS by two-way ANOVA including Met supply, source and their interaction effects.

III. RESULTS AND DISCUSSION

The sulphur AA supply affected performance with lower final BW, ADG and G:F values in chickens receiving the Met-deficient diets ($P < 0.05$; Table 1). These results are consistent with other studies that investigated the effect of inadequate supply of AA regardless of Met source; Vazquez-Anon et al., 2006). In these deficient birds, the colour of PM was more red and green (a* and H°) compared with birds receiving the Met-sufficient diets ($P < 0.01$), which could be attributed to a changed myoglobin content (Lindahl et al., 2001). A significant Met supply x source interaction was observed for pH 24 h post mortem, which was higher in HMTBA+ birds compared with DL-Met+ birds ($P < 0.05$). Accordingly, a change in ultimate pH was also observed in the muscle of birds depending on HMTBA and/or Met supply that may suggest differential glycogen storage resulting in different drip losses (Mercier et al., 2009).

Table 1 - Performance and meat quality traits of broilers offered a diet deficient (DL-Met– and HMTBA–) or sufficient (DL-Met+ and HMTBA+) in sulphur AA by supplementation of Met (DL-Met) or hydroxy-Met (DL-2-hydroxy-4-methylthiobutanoic acid; HMTBA), respectively from 7 to 24 d of age[1].

	Treatment				*P*-value		
Item	DL-Met–	DL-Met+	HMTBA–	HMTBA+	RSD	Met level	Met source
Initial BW (kg)	0.15	0.16	0.15	0.15	0.01	NA	NA
Final BW (kg)	1.02	1.10	1.00	1.11	0.05	<0.01	0.67
Empty BW (kg)	1.02	1.10	1.00	1.11	0.05	<0.01	0.72
ADFI (g/d)	72.7	74.6	74.0	74.2	3.2	NA	NA
ADG (g/d)	47.9	49.9	47.9	49.4	3.0	0.01	0.73
FCR (g/g)	1.52	1.50	1.54	1.50	0.06	0.03	0.29
Meat quality trait							
Lightness (L*)	49.7	50.5	50.2	48.6	2.9	0.68	0.44
Redness (a*)	0.24	-0.99	0.72	-1.28	0.72	<0.01	0.67
Yellowness (b*)	10.5	10.3	10.9	10.0	1.0	0.08	0.83
Hue (H°)	87.4	84.1	85.1	82.6	3.2	0.01	0.07
Chroma (C°)	10.5	10.4	11.0	10.1	1.0	0.10	0.73
pH (24 h)	5.94^{ab}	5.84^{a}	5.90^{ab}	5.97^{b}	0.11	0.70	0.21

[1]Least-squares means, n = 20 for performance and n = 10 for meat quality traits. No statistical analysis was performed for daily feed intake because feed was not offered *ad libitum*. There was no Met supply and source interaction, except for pH (24h) where $P = 0.02$ and [a,b] values within a row without a common letter differ by pairwise comparisons, $P < 0.05$. NA, not applicable; RSD; residual SD.

Relative weight and protein content of PM were lower in Met-deficient birds compared with Met-sufficient birds, whereas the water content was higher ($P < 0.01$; Table 2). In Met-deficient birds, the His and OH-Pro contents of retained protein in PM were higher compared with the Met-sufficient birds ($P < 0.01$), whereas the Lys, Ile, and Leu contents tended to be lower ($P < 0.10$); there was no change in Met and Cys contents. Free contents of β-Ala, anserine and balenine of PM in Met-deficient birds were lower compared with the Met-sufficient groups, but free contents of His, carnosine, and taurine were higher ($P < 0.01$). The absolute protein synthesis and protein mass of PM were lower in the Met-deficient birds than in the Met-sufficient groups, but the efficiency of protein synthesis of PM was lower ($P < 0.05$). The proteasome activity of PM was greater in Met-deficient birds compared with the Met-sufficient groups ($P < 0.01$; Table 3). There was no effect of Met source on performance, meat quality traits, nutrient composition, protein synthesis and proteolytic enzymes of PM.

The lower PM weight in Met-deficient bird was associated with a higher proteolytic enzyme activity. Similarly, it has been observed that a Lys restriction increased protein degradation rate in PM muscles (Tesseraud et al., 1996, 2001 and 2009). Our results also indicate that animal can cope with a limiting AA supply by changing the AA composition, possibly by changes in the proportions of different types of tissue proteins (Conde-Aguilera et al., 2013). For instance, an increased collagen content of the muscle could explain the change in the AA composition in the deficient groups because collagen is rich in OH-Pro, poor in Lys, and also needs water to stabilize its conformation (Kar et al., 2006), as suggested with the higher water content observed in the Met-deficient groups. This 25% higher His content in retained protein of the Met-deficient birds reported in the present study was also previously observed (Conde-Aguilera et al., 2013), and may be partly explained by the increase in the free carnosine content, a His-containing dipeptide abundant in muscles. Besides its pH buffering capacity, carnosine has antioxidant properties providing an important defense mechanism against oxidative stress in fast-twitch glycolytic muscles (Boldyrev et al., 2013), which is in accordance with the increased content of other antioxidant molecules (e.g. taurine) observed in PM of Met-deficient animals. However, Morand et al.

(1997) reported lower glutathione synthesis when rats received a diet deficient in sulphur AA. If the antioxidant status may have been compromised by the limiting Cys supply for glutathione synthesis, other antioxidant mechanism could have been initiated against oxidative stress, such as the carnosine system.

Table 2 - Composition of *pectoralis major* muscles in broilers offered a diet deficient (DL -Met– and HMTBA–) or sufficient (DL-Met+ and HMTBA+) in sulphur AA by supplementation of Met (DL-Met) or hydroxy-Met (DL-2-hydroxy-4-methylthiobutanoic acid; HMTBA), respectively from 7 to 24 d of age[1].

Item	Treatment				RSD	*P*-value	
	DL-Met-	DL-Met+	HMTBA –	HMTBA +		Met level	Met source
% of empty BW	14.0	16.0	13.4	15.7	1.4	<0.01	0.35
Composition (g/kg)							
N x 6.25	208	220	199	218	12	<0.01	0.15
Lipid	9.7	10.1	11.2	10.6	2.2	0.84	0.14
Ash	12.6	12.6	11.5	13.1	1.3	0.06	0.42
Water	770	758	779	758	13	<0.01	0.25
Composition (g/kg protein)							
Retained AA							
Lys	84.3	87.6	86.6	88.9	3.3	0.06	0.20
Met	23.1	23.7	21.0	19.6	11.8	0.94	0.53
Cys	11.2	11.5	11.3	11.5	0.5	0.39	0.88
Ile	45.9	46.8	46.6	48.0	1.6	0.10	0.17
Leu	74.4	76.3	75.8	78.1	2.7	0.07	0.17
His	42.1	32.2	42.1	34.9	2.3	<0.01	0.17
OH-Pro	2.73	2.15	2.84	2.26	0.33	<0.01	0.41
Free AA & peptides							
β-Ala	0.64	1.13	0.53	1.07	0.42	0.01	0.63
His	0.12	0.03	0.11	0.03	0.02	<0.01	0.56
Anserine	14.5	24.8	15.9	23.4	2.8	<0.01	0.99
Balenine	0.12	0.30	0.09	0.27	0.04	<0.01	0.02
Carnosine	20.9	7.3	20.1	9.7	2.0	<0.01	0.32
Ornithine	0.12	0.09	0.11	0.13	0.07	0.87	0.60
Taurine	1.04	0.69	1.05	0.76	0.39	0.05	0.82

[1]Least-squares means, n = 10. There was no Met supply and source interaction. AA, amino acid; RSD; residual SD.

In conclusion, the contents of protein and especially AA of the muscle together with bird growth are changed by a Met deficiency with a potential impact on protein degradation and quality. There are no differences between dietary Met (DL-Met) and hydroxy-Met (HMTBA) sources on muscle growth and protein metabolism.

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Table 3 - Protein synthesis and activities of proteolytic enzymes of *pectoralis major* muscles in broilers offered a diet deficient (DL-Met– and HMTBA–) or sufficient (DL-Met+ and HMTBA+) in sulphur AA by supplementation of Met (DL-Met) or hydroxy-Met (DL-2-hydroxy-4-methylthiobutanoic acid; HMTBA), respectively from 7 to 24 d of age[1].

Item	Treatment				RSD	*P*-value	
	DL-Met–	DL-Met+	HMTBA–	HMTBA+		Met level	Met source
Protein synthesis							
Fractional rate (%/d)	21.9	19.9	22.0	20.4	3.7	0.14	0.80
Absolute (g/d)	2.66	3.08	2.75	3.07	0.51	0.03	0.82
Efficiency	17.2	14.1	17.0	15.6	3.5	0.05	0.54
RNA:protein ratio (mg/g)	13.0	14.3	13.4	13.3	2.0	0.34	0.64
Protein mass (g)	12.2	15.5	12.6	15.2	1.6	<0.01	0.95
Proteolytic enzyme activities							
Proteasome	13.2	10.2	13.2	10.8	1.4	<0.01	0.47
Calpain	3.68	3.66	3.64	3.68	0.87	0.97	0.99

[1]Least-squares means, n = 10. There was no Met supply and source interaction. RSD; residual SD. Efficiency was expressed as (g protein) · d^{-1} · (mg RNA)$^{-1}$. Activity enzymes are expressed as (relative fluorescence units) · min^{-1} · (g protein)$^{-1}$.

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IMPACT OF INCREASING DIETARY AMINO ACID DENSITY IN BROILERS FED LOW METABOLIZABLE ENERGY

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Summary

The objective of this study was to evaluate the effects of reducing dietary metabolizable energy (ME) and increasing dietary amino acid (AA) densities via supplemental AA in reduced ME diet on growth and carcass indices of broiler chickens. A total of 450 newly-hatched male Ross 308 broilers were randomly distributed to five dietary treatments consisting of a basal corn-soybean meal diet formulated to contain dietary ME levels of 11.51, 11.93 and 12.35 MJ/kg during starter (1-14 d), grower (15-28 d), and finisher (29-42 d) phases, respectively. The ME concentrations were then reduced by 0.42 MJ/kg to create the low ME (LoME-100AA) diet. The AA density of LoME diet was increased by 5, 10 and 15% corresponding to 105, 110 and 115% of Evonik's AMINOCHICK® 2.0 recommendations. At day 42, there was no significant effect of dietary treatments on growth and carcass traits of broiler chickens. Reducing dietary ME, however numerically improved body weight gain, feed intake and increased breast meat yield. Increasing the dietary AA density of low ME diet by 5 to 15% did not significantly influence the growth performance or carcass parameters. In conclusion, dietary ME can be reduced without negatively affecting the growth or carcass performance, however increasing the AA density of such a low ME diets might not be beneficial as no additional energy is available for birds to utilize higher AA levels. Further improvements in the broiler performance could be possible by increasing AA density if the ME level of such diet is increased.

I. INTRODUCTION

Optimizing growth and minimizing diet cost depends on precision of diet formulation to meet bird's requirement while minimizing nutrient excesses. However, considerable variation exists in terms of level of dietary metabolizable energy (ME) maintained under commercial broiler production (for e.g., 11.72 to 12.56 MJ/kg for broilers aged 1-14 d). Redefining the optimal dietary ME levels for today's high performing birds is of utmost importance to poultry producers for higher economic returns. Hidalgo et al. (2004) reported that reducing the dietary ME formulated with constant ME to protein ratio adversely affects the broiler performance. Modern broilers grow rapidly and have higher nutrient requirements but proportions of these nutrients may vary (Vieira and Angel, 2012). Furthermore, modern broilers have higher AA requirements in proportion to their energy requirements (Gous, 2010). Dozier et al. (2008) concluded that modern broilers consume less feed per unit gain with higher potential for more protein deposition and that feeding diets containing higher AA density will result in higher breast meat yield (BMY). Other studies have suggested that modern broiler chickens no longer adjust their feed intake according their energy requirement, rather feed intake is regulated by the level and balance of circulating AA in the blood (Ferket and Gernet, 2006; Plumstead et al., 2007). Our previous research has shown that reducing the dietary ME from 12.34 to 11.51, 12.76 to 11.92 and 13.18 to 12.34 MJ/kg in starter, grower and finisher diets respectively did not negatively affect growth or carcass performance, moreover reduced abdominal fat and improved overall carcass quality (Girish and Payne, 2013). Similarly, Oliveira Neto et al. (1999) showed that increasing the dietary ME from 12.56 to 13.82 MJ/kg linearly increased fat deposition, while protein deposition was optimized at 13.01 MJ/kg in 42 day-old broilers. Furthermore, Leeson et al. (1996) has also shown that reducing the dietary ME from 13.82 to 11.30 MJ/kg linearly decreased the carcass fatness of broilers at 49 d. These studies suggest that the dietary energy above what is required

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for maximum protein accretion would lead to fat deposition and clearly there is an opportunity to reduce the dietary ME content in order to minimize cost of production and improve overall carcass quality. However, there is limited information in defining the effects of increasing the AA density in broilers fed reduced energy diets where in supplemental AA are contributing to the larger proportion of dietary AA rather than intact protein sources. Therefore, objective of this study was to evaluate the effects of feeding low ME diet and increasing dietary AA density via supplemental AA on growth and carcass characteristics of broiler chickens from 0 to 42 days.

II. MATERIALS AND METHODS

A total of 450 one-day-old male Ross 308 broiler chicks were randomly distributed to five treatments with 9 replicate pens per treatment and 10 birds per replicate. This experiment was conducted in a closed house with tunnel ventilation and evaporative cooling system. Birds were raised on the solid-concrete-floor pens using rice hull as bedding material. Basal corn-soybean meal control diet was formulated to contain minimum AA concentrations based on ideal protein for starter (0-14 days), grower (15-28 days) and finisher (29-42 days) phases according to AMINOCHICK® 2.0 and dietary ME in respective phases were 11.51, 11.93 and 12.35 MJ/kg (BaME-100AA). ME levels of the basal diets in the respective growth phases were chosen based on the lowest ME levels tested by Girish and Payne (2013) as there was no negative impact on growth and carcass parameters of broilers fed the lowest ME diets. To further explore if there is any effect of reducing the dietary energy, ME concentration was reduced by 0.42 MJ/kg to create the low ME (LoME-100AA) diet. In such LoME diet, AA density was increased by 5, 10 and 15% to formulate LoME-105AA, LoME-110AA, LoME-115AA corresponding to 105, 110 and 115% of AMINOCHICK® 2.0 recommendations. Dietary nutrient concentrations are provided in Table 1. Feed in pellet form and water were provided *ad libitum* for 42 days.

Body weight and feed intake were recorded at 14, 28 and 42 days of age to calculate body weight gain (BWG) and FCR. At the end of experiment (day 42), two birds from each pen with body weight close to the average body weight of each pen were slaughtered for carcass measurements. Data were subjected to analysis of variance as a randomized complete block design using SAS, 2007. Treatment differences were considered significant when $P < 0.05$.

III. RESULTS AND DISCUSSIONS

There were no significant differences in growth performance or carcass parameters measured across the dietary treatments (Table 2) possibly because lack of dietary energy to utilize the higher AA concentrations to support higher protein accretion (Classen, 2013) or AA requirement of broilers was optimally met at level of LoME-100AA. Numerically, birds fed LoME-100AA diet had higher BWG and BMY by 77 g and 3%, respectively compared with BaME-100AA diet.

FCR of broilers fed diets BaME-100AA and LoME-100AA was similar while there was marginal increase in the BWG and BMY in birds fed LoME-100AA diet, which could be attributable to efficient utilization of dietary AA as dietary ME was still sufficient to meet bird's requirement. On contrary, Hidalgo et al. (2004) reported that decreasing the ME content of diets keeping the ME to CP ratio constant significantly affects the growth performance of broiler chickens. Based on several experiments (Evonik, 2007) it was concluded that reducing the AA and concomitant decrease in the dietary energy will reduce the broiler performance. Moreover balanced dietary protein should not be adjusted to the same extent as that of energy and possibly should not be reduced at all in order to maintain optimum performance and profitability. It is noticeable from results of the present study that reducing the dietary ME by 0.42 MJ and keeping the same AA density (LoME-100AA) did not significantly influence broiler performance.

Table 1 - Nutrient content (as-is basis) of dietary treatments.

Phases	Diets	ME, MJ/kg	CP, %	Minimum standardized ileal digestible AA contents, %							
				LYS	MET	M+C	THR	TRP	ARG	ILE	VAL
Starter (0-14 d)	BaME-100AA	11.51	22.55	1.23	0.59	0.89	0.78	0.24	1.37	0.84	0.97
	LoME-100AA	11.09	22.69	1.23	0.59	0.89	0.78	0.24	1.37	0.84	0.97
	LoME-105AA	11.09	21.93	1.29	0.64	0.93	0.82	0.23	1.31	0.88	1.02
	LoME-110AA	11.09	21.69	1.35	0.70	0.98	0.86	0.22	1.27	0.92	1.07
	LoME-115AA	11.09	21.16	1.41	0.75	1.02	0.90	0.23	1.21	0.97	1.12
Grower (15-28 d)	BaME-100AA	11.92	19.83	1.05	0.50	0.78	0.68	0.20	1.18	0.73	0.83
	LoME-100AA	11.51	19.96	1.05	0.51	0.78	0.68	0.21	1.20	0.73	0.83
	LoME-105AA	11.51	19.52	1.10	0.55	0.82	0.71	0.20	1.15	0.77	0.87
	LoME-110AA	11.51	19.35	1.16	0.60	0.86	0.75	0.19	1.12	0.80	0.91
	LoME-115AA	11.51	18.87	1.21	0.65	0.90	0.78	0.20	1.06	0.84	0.95
Finisher (29-42 d)	BaME-100AA	12.34	19.02	0.98	0.48	0.75	0.64	0.19	1.13	0.70	0.79
	LoME-100AA	11.92	18.99	0.98	0.49	0.75	0.64	0.19	1.13	0.70	0.79
	LoME-105AA	11.92	18.78	1.03	0.53	0.79	0.67	0.19	1.10	0.74	0.83
	LoME-110AA	11.92	18.59	1.08	0.58	0.83	0.70	0.18	1.07	0.77	0.87
	LoME-115AA	11.92	18.11	1.13	0.61	0.86	0.74	0.18	1.01	0.81	0.91

Table 2 - Effect of increasing AA concentrations in low ME diets on growth performance and carcass parameters of broilers at d 42.

Treatments	BWG, g	FI, g	FCR	DY, % LW	BMY, % LW	AF, % LW
BaME-	3008	5041	1.68	78.09	28.39	2.13
LoME-	3085	5168	1.68	78.60	29.26	2.34
LoME-	3068	5128	1.68	78.73	29.26	2.15
LoME-	3065	5128	1.67	78.65	28.71	2.35
LoME-	2994	5032	1.68	78.45	28.81	2.40
SEM	40.3	43.1	0.02	0.23	0.48	0.04
P – value	0.42	0.13	0.99	0.32	0.66	0.68

BWG=body weight gain; FI=feed intake; FCR=feed conversion ratio; DY=dressing yield; LW=live weight BMY=Breast meat yield; AF=Abdominal fat

In a previous study (Girish and Payne, 2013) reducing the dietary energy, linearly decreased the abdominal fat associated with decrease in the carcass yield while there were no differences in BMY indicating dietary energy in excess of optimal protein deposition will simply be deposited as fat. Hence, supplementary hypothesis for the present study was that whether providing additional AA might positively influence the protein synthesis and deposition in LoME-100AA diet. Previous studies (Corzo et al., 2005; Dozier et al., 2008; Vieira and Angel,

2012) have shown an increase in BWG, feed efficiency and BMY as a result of increasing dietary AA density. Nonetheless, in the present study birds receiving diets containing 5 to 15% AA density above the basal diet did not significantly influence the carcass or BMY possibly because energy was limiting for further protein accretion. On contrary, feeding very high density AA (LoME-115AA) resulted in numerical decrease in feed intake (by 136 g) resulting in corresponding decrease in the BWG (by 90 g) which might be partially related to the metabolic heat stress caused due to catabolism of excess of AAs. Thyroid hormones mainly 5-triiodothyronine (T3) and thyroxine (T4) affects protein and energy metabolism, particularly T4 may stimulate protein degradation to meet the energy demands in birds fed inadequate amount of energy (Guyton, 1991). Similar results were also observed (Cho, 2011) where in feeding high levels of lysine associated with low dietary ME resulted in higher FCR and lower BWG.

Based on the results of present study, clearly there is an opportunity to reduce dietary *energy* from the current industry practice without affecting the broiler performance. Furthermore, energy and AA needs of broilers also might vary depending on the environmental conditions and therefore, future research needs to be directed towards establishing the ME to digestible lysine and/or balanced protein ratio under different feeding conditions.

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PERFORMANCE OF ROSS 308 BROILERS FED DIFFERENT LEVELS OF ENERGY AND BALANCED PROTEIN UNDER MODERATE HEAT STRESS

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Summary

A study was conducted to examine the response of live and carcass performance in Ross 308 broilers to diets contained 20% above and below balanced protein (BP), and 15% or 7.5% less dietary metabolisable energy (ME) than Aviagen nutrient specification. Male chickens were offered nine different dietary treatments and reared to 42 days.

The maximum body weight was achieved using 85% of ME and 120% of BP at 35d, and 92.5% of ME and 100% of BP at 42d as compared to the 2007 Aviagen recommended nutrient specification for Ross 308. The best FCR corrected for mortality and body weight were achieved by birds at 35d given the 20% BP above of, and the same ME (100%) to that recommended by Aviagen; and at 42d, 120% of BP and 92.5% or 100% of ME. The best carcass yield was attained using 85% or 92.5% of ME and 100% or 120% of BP at 35 and 42d. These results suggest that diets for Ross 308 under moderate heat stress conditions may be formulated at the same or higher BP, and lower or the same ME as that recommended by Aviagen (2007) to give better bird performance.

I. INTRODUCTION

The concept of ideal balanced protein (BP) has been widely adopted in broiler feed formulation and feeding (Kemp and Kenny, 2003, Lemma et al., 2006, Sklan and Noy, 2003, Vieira et al., 2004) and used by primary breeders such as Aviagen (Aviagen, 2007). Under this concept, the emphasis is no longer on minimum crude protein but rather on having all the essential amino acids correctly balanced in appropriate ratios relative to lysine.

However there is much interest in finding out the performance response to different levels of dietary BP and energy as compared to Aviagen recommendation. This study was conducted to investigate the response of Ross 308 broilers to various dietary combinations in relation to BP and metabolisable energy (ME) under tropical conditions. The data gathered would provide valuable information for the breeding company to update its advice on the appropriate levels of ME and BP in its next publication of nutrient specification for newer generations of genetically improved broiler chickens.

II. MATERIALS AND METHODS

A total of 864 male Ross 308 birds were allocated as a 3x3 factorial in a randomized complete block design – three BP (80/100/120%) and three ME levels (85/92.5/100%) of Ross 308 Broiler Nutrition Specification (Aviagen 2007) for each growth phase. The digestible lysine and ME for Starter, Grower and Finisher diets were 1.27, 1.10, 0.97% and 3025, 3150, 3200 kcal/kg* respectively. There were nine experimental treatments, six replicates each with 16 birds per replicate pen, giving a sub-total of 96 birds per treatment.

Experimental diets were of a corn/soybean meal based composition, together with meat meal and canola meal. Starter diets were presented in crumble form for the first 10 days, with grower (11-24d) and finisher feeds (25-42d) in pelleted form. Suitable coccidiostats were included. No feed enzymes were incorporated. Birds were kept in a closed environmentally-controlled housing with tunnel air ventilation and evaporative cooling

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system, with temperature at 25-28^{o}C and humidity around 60-80%[#]. Birds were raised to 42 days of age. All live bird performance data were collected. Four birds were randomly sacrificed at 35 days and five birds at 42 days of age for carcass measurements. Data were statistically analyzed using the LSM method of JMP (SAS, 2012).

III. RESULTS AND DISCUSSION

The overall mortality is 3.5% and there was no dietary difference in mortality. Table 1 showed the results of feed intake (FI), live body weights (BW) and adjusted FCR at 10, 24, 35 and 42 days post-hatch. Carcass characteristics at 42d are presented in Table 2.

As expected, in diets contained constant level of BP, increasing the dietary ME level from 85 to 92.5 to 100% of Aviagen recommendation lowered feed intake at each growth phase. When ME was constant, feed intake was improved when BP increased from 80-100% and reduced when BP further increased to 120% (Table 1). BW of broilers at 35 and 42 days old fed 20% less BP (80%) of current Aviagen advice did not increase as the dietary ME increased from 80 to 100%. However, at 100 or 120% of BP, BW increased as dietary ME increased from 80 to 92.5% or 100% of ME. These BW achieved are well above the published performance objectives for Ross 308 (Aviagen, 2012).

The best FCR and BW of Ross 308 were achieved using the 100% of ME and 120% of BP at 24d and 35d, and at 42d, both 92.5% and 100% of ME and 120% of BP. As with BW results, these FCR results achieved are well above the published performance objectives for Ross 308 (Aviagen, 2012). There were interactions between ME and BP on feed conversion. FCR of Ross 308 birds fed 80% of BP at 35 or 42d did not improve as the dietary ME was increased, whereas these birds fed 100% or 120% of BP had better FCR as the dietary ME was increased from 80 to 92.5%. This interaction may be accounted for the drastic feed intake reduction from birds fed 80% of BP and 92.5% of ME as compared to that from birds fed 80% of BP and 80% of ME. It is possible that highest ME/CP ratio when the balance protein is deficient may reduce the ME intake below the potential ME intake.

The highest carcass yield (expressed as percentage of live weight) was attained using 85% of ME and 100% of BP at 35 or 42d and highest breast yield with 85% of ME and 120% of BP at 35d and 85% of ME and 100% or 120% of BP at 42d (Table 2). There were interactions between dietary ME and BP on carcass and breast yield at both 35 and 42d. Carcass and breast yield of birds fed 80% of BP at 35 or 42d drastically reduced as the dietary ME was increased, whereas these birds fed 100% or 120% of BP had similar or slightly smaller carcass or breast yield as the dietary ME was increased from 80 to 92.5% or 100%. This effect was due mainly to the fact that the birds fed 85% of ME had the highest ratio of BP/ME intake as compared to those at 92.5 or 100% of ME. Further, the reduced feed intake from birds fed 80% of BP and 92.5% or 100% of ME also reduced the amino acid intake below the potential body protein deposition. Processing results for Ross 308 showed that lowest abdominal fat was found in chickens fed 85% of ME and 120% of BP. As the BP was increased the abdominal fat was reduced and as the dietary ME was increased the abdominal fats were increased. However, this increment of abdominal fat was more pronounced in chickens fed 80% of BP as ME was increased.

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[*] For ease of comparison, ME is expressed as kcal/kg rather as MJ as this is the unit used in Aviagen's publications.

Table 1 - Performance (FI, BW and FCR) of broilers at 10, 24, 35 and 42days fed different levels of ME and BP.**

Treatment	ME (%)	BP (%)	Feed intake (g)				Bodyweight (g)				Adjusted FCR+			
			10d	24d	35d	42d	10d	24d	35d	42d	10d	24d	35d	42d
Energy	85		277[a]	1754	3840	5108	287	1307	2521	3156	0.966	1.342	1.509	2.052
	92.5		254[b]	1598	3456	4689	282	1272	2394	3115	0.903	1.273	1.461	1.914
	100		241[c]	1519	3314	4557	278	1240	2346	3085	0.870	1.250	1.447	1.880
BP		80	256[b]	1541	3270	4433	260	1057	1976	2583	0.986	1.492	1.732	2.253
		100	264[a]	1693	3724	5014	292	1354	2597	3329	0.902	1.235	1.393	1.854
		120	252[b]	1637	3616	4907	295	1408	2689	3443	0.851	1.137	1.292	1.739
Energy x BP	85	80	272	1745[ab]	3783[b]	4987[bc]	272[c]	1181[d]	2271[d]	2838[e]	1.018[a]	1.493[a]	1.690[b]	2.285[a]
		100	295	1798[a]	3947[a]	5218[a]	295[ab]	1363[b]	2629[b]	3260[d]	0.956[c]	1.301[b]	1.461[c]	2.006[b]
		120	294	1720[b]	3789[b]	5119[ab]	294[ab]	1376[b]	2662[ab]	3369[c]	0.924[d]	1.231[c]	1.375[d]	1.866[c]
	92.5	80	256	1465[e]	3003[e]	4074[f]	256[d]	1008[e]	1834[e]	2423[f]	0.989[b]	1.499[a]	1.742[a]	2.246[a]
		100	293	1694[b]	3741[b]	5064[abc]	293[ab]	1376[b]	2627[b]	3411[ab]	0.889[e]	1.212[d]	1.373[d]	1.802[d]
		120	298	1633[c]	3624[c]	4927[c]	298[a]	1432[a]	2722[a]	3511[a]	0.832[g]	1.109[f]	1.268[e]	1.696[e]
	100	80	253	1413[e]	3023[e]	4239[e]	253[d]	983[e]	1822[e]	2488[f]	0.952[c]	1.485[a]	1.765[a]	2.230[a]
		100	289	1588[cd]	3485[d]	4759[d]	289[b]	1322[c]	2535[c]	3316[bc]	0.861[f]	1.192[e]	1.345[d]	1.755[d]
		120	293	1557[d]	3434[d]	4674[d]	293[ab]	1416[a]	2682[ab]	3450[a]	0.798[h]	1.072[g]	1.232[f]	1.654[e]
	SEM		3.14	19.67	40.85	55.10	3.14	14.02	28.66	24.64	0.008	0.007	0.012	0.02
Source			---------- *P value* ----------											
Energy			<0.0001	<0.0001	<0.0001	<0.0001	0.0058	<0.0001	<0.0001	0.0454	<0.0001	<0.0001	<0.0001	<0.0001
BP			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Energy x BP			0.9621	<0.0001	<0.0001	<0.0001	0.0295	<0.0001	<0.0001	<0.0001	0.0011	<0.0001	<0.0001	<0.0001

+Adjusted for bodyweight and mortality (3.5% overall).

**Treatment with 100% ME and 100% BP refers to the 2007 Aviagen recommendation for Ross 308.

a, b, c, d, etc = Values within column with no common superscript differ significantly ($P < 0.05$).

Table 2 - Carcass composition (%BW) of 42d broilers fed different levels of ME and BP.**

Treatment	ME, %	BP, %	Carcass Yield	Breast	Thigh	Drum	Wing	Abdominal Fat
Energy	85		76.2	23.5	13.7	10.0	7.5	1.4
	92.5		75.3	21.6	13.7	10.1	7.5	1.6
	100		75.0	20.8	13.9	10.1	7.5	1.9
BP		80	74.4	19.3	13.6^{b}	10.0	7.5	2.4
		100	76.3	23.0	13.8^{a}	10.0	7.5	1.6
		120	75.9	23.6	13.9^{a}	10.0	7.4	0.9
Energy x BP								
	85	80	75.6^{b}	22.4^{de}	13.4	9.8^{d}	7.3^{c}	2.1^{c}
		100	76.4^{a}	23.9^{ab}	13.8	10.1^{ab}	7.6^{a}	1.3^{f}
		120	76.5^{a}	24.3^{a}	13.9	10.1^{ab}	7.5^{abc}	0.8^{h}
	92.5	80	73.5^{c}	17.9^{f}	13.5	10.3^{a}	7.6^{a}	2.3^{b}
		100	76.5^{a}	23.3^{bc}	13.7	9.8^{cd}	7.4^{bc}	1.6^{e}
		120	75.7^{b}	23.6^{b}	14.0	10.0^{bc}	7.4^{bc}	0.9^{gh}
	100	80	73.9^{c}	17.8^{f}	13.8	10.0^{bc}	7.5^{abc}	2.7^{a}
		100	75.9^{ab}	21.8^{e}	13.9	10.1^{ab}	7.5^{ab}	1.8^{d}
		120	75.4^{b}	22.8^{cd}	13.9	10.0^{bcd}	7.4^{bc}	1.1^{g}
	SEM		0.24	0.23	0.12	0.09	0.06	0.06
Source			----------*P value*----------					
Energy			<0.0001	<0.0001	0.0545	0.6101	0.9372	<0.0001
BP			<0.0001	<0.0001	0.0007	0.9123	0.1723	<0.0001
Energy x BP			<0.0001	<0.0001	0.2547	<0.0001	0.0033	0.0043

**Treatment with 100% ME and 100% BP refers to the 2007 Aviagen recommendation for Ross 308.
a, b, c, d = Values within column with no common superscript differ significantly ($P < 0.05$).

In conclusion, Ross 308 broilers in this study were more responsive to increasing levels of BP than ME in terms of live bird performance. It is apparent that under moderate heat stress conditions the dietary ME should be reduced whereas the dietary amino acids could be increased to attain maximum bird performance.

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THE SID OF AMINO ACIDS OF WHEAT-DDGS MEASURED BY TWO DIFFERENT METHODS

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Summary

The SID of AA for wheat-DDGS was determined using two methods; the direct method using a semisynthetic diet background and by difference method using both corn and wheat based diet backgrounds. Digestibility of methionine, cysteine, methionine plus cystine and arginine in DDGS were significantly affected by method but not diet background used ($P < 0.05$). It appeared that the semi-synthetic diet (direct method) exhibited significantly lower SID values than those derived using a corn or wheat-based diet (difference method), which both gave similar results ($P > 0.05$). This has implications for ingredient evaluations studies and for interpretation of values in the literature. This is possibly also true for the digestibility of other nutrients, such as phosphorous and calcium.

I. INTRODUCTION

As novel ingredients, such as DDGS, become more commonplace in animal feed formulation, it is important that their nutritional value is accurately established. This is especially true given the variability of such ingredients is often high, thus a robust and repeatable method is necessary. There are several methods commonly used to determine the digestibility of nutrients and it is known that the values obtained are influenced by the methods themselves, and that these differences are dependent upon the ingredient of interest. The diet type employed is one such example of a factor which may affect the outcome and the use of semi-synthetic diets has been questioned due to the effects of the basal diet components on the digestive tract (Becker et al., 1955). Given the significant differences between the basal diets and methodologies described, an experiment was designed to determine and compare the direct and regression methodologies for the determination of the SID values of AA of wheat-DDGS.

II. EXPERIMENTAL DETAIL

Eighty day-old male Ross 308 broilers were obtained and on d 21, two birds of similar weight were re-housed to one of 8 replicate cages per treatment. From day 1 to 21, prior to the trial period, chicks were fed a corn:soybean meal mash diet formulated to be sufficient in energy, AA, vitamins and minerals. At d 21 the birds were assigned to trial diets. From d 24 to 27, (a total of 72 hours) feed intake was measured and excreta collected. At all times, feed and water were provided on an ad libitum basis. All birds were culled on d 28 of the experiment by asphyxiation with carbon dioxide and cervical dislocation to confirm death. The weight of each bird was recorded and the ileal region of the gut was dissected out from the Meckel's diverticulum to the ileal-caecal junction. Ileal digesta was collected to determine the AID and the SID of crude protein (CP) and AA. Digesta were pooled per cage (two birds). All protocols were approved by the relevant Ethical Review Committee and all experimental conditions followed official guidelines for the care and management of birds.

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There were 5 dietary treatments (not shown in the interest of space), which were designed to allow determination of AA digestibility of wheat DDGS in different diet backgrounds. Diet S-DDGS was a semi-synthetic diet including 500 g wheat DDGS/kg and 205 g starch and 200 g glucose/kg. Diets C and W were based on 660 g corn or wheat/kg and 245 g soybean meal (SBM)/kg, respectively. Diets C-DDGS and W-DDGS contained corn or wheat (respectively) at 295g/kg, together with 110 g SBM/kg and500g wheat DDGS/kg. With the exception of the semi-synthetic diet, the proportions of cereal to SBM in the diets were maintained at a constant ratio.

For samples of diets, dry matter (DM) was determined in triplicate in a forced air convection oven. Digesta samples were frozen and then freeze-dried to a constant weight when determining dry matter. The concentration of titanium dioxide (employed as an inert marker) was determined in diet and digesta samples using the spectrophotometric method described by Short et al. (1996). Amino acids analysis was conducted as described by Llames and Fontaine (1994).

Firstly, the AID of AA in the assay diets were calculated according to equation: AIDD = 1- [(ID x AI)/(AD x II)], where AIDD = AID of AA in the diet, ID = marker concentration in the assay diet (g/kg DM), AI = AA concentration in ileal digesta (g/kg DM), AD = AA concentration in the assay diet (g/kg DM) and II = marker concentration in ileal digesta (g/kg DM).

The final SID values attributed to each diet background (not shown in the interest of space) were calculated in two stages as follows: *Part 1: SIDD= AIDD + [EELaa (g/kg DMI)/AD]*, where SIDD = the SID of AA in the individual 5 diets, AIDD=AID of AA in those diets, EELaa = the mean assumed endogenous loss of AA/kg DM intake (Lemme et al., 2004) and AD = AA concentration g/kg in the assay diet.

Part 2: The content of SID of each AA in each diet (Table 1) was then calculated by multiplying the content of that AA in the diet by its SIDD value. The coefficient of SID of AA attributed to the wheat DDGS was assumed directly in the S-DDGS diet (SID multiplied by 2 as it was included at 500g/kg of diet and was the only AA source present). It was calculated by difference between the 2 corn and 2 wheat diets according to the difference method (Fan and Sauer, 2005).

All data were exported to JMP v9.0 (SAS Institute, Cary, NC, USA) and subjected to analysis of variance. Means were separated by Tukeys LSD and were considered significant at $P<0.05$.

III. RESULTS

The SID values of DDGS for lysine, threonine, isoleucine, leucine, valine, histidine and phenylalanine were not significantly different ($P > 0.05$), as determined in the different diet backgrounds (Table 1). However the SID values of methionine, cysteine, methionine plus cystine and arginine of DDGS were significantly affected by the diet ($P < 0.05$), with birds fed the semi-synthetic diet (direct method) exhibiting significantly lower SID values than those fed a corn or wheat-based diet (difference method), which did not differ ($P > 0.05$) between each other.

Table 1 - The coefficient of standardised ileal digestibility (SID) of amino acid in wheat DDGS measured broilers affected by diet type.

	Diet types							
	Semi-synthetic[1]		Corn[2]		Wheat[2]			
	Mean	SD	Mean	SD	Mean	SD	P	RMSE
Lysine	0.26	0.102	0.27	0.036	0.32	0.039	0.056	0.064
Methionine	0.64b	0.046	0.70a	0.012	0.71a	0.039	0.004	0.035
Cystine	0.52b	0.058	0.65a	0.049	0.68a	0.049	<0.001	0.057
Methionine + Cystine	0.58b	0.048	0.68a	0.029	0.69a	0.049	<0.001	0.043
Threonine	0.56	0.058	0.56	0.022	0.58	0.024	0.463	0.037
Isoleucine	0.62	0.061	0.62	0.023	0.63	0.031	0.871	0.040
Leucine	0.66	0.038	0.68	0.021	0.68	0.023	0.357	0.028
Valine	0.52	0.073	0.56	0.027	0.54	0.037	0.250	0.048
Histidine	0.6	0.057	0.59	0.022	0.61	0.029	0.548	0.038
Phenylalanine	0.73	0.049	0.74	0.014	0.74	0.014	0.491	0.029
Arginine	0.58b	0.042	0.68a	0.018	0.69a	0.022	<0.001	0.043

[a,b] Within a row, means without common superscripts are significantly different as indicated by the P value.
[1]Using direct method; [2]Using the difference method.

IV. DISCUSSION

The values of SID of AA determined in this study were similar to, although slightly lower, than those expected, comparing to those reported elsewhere. The lower levels may be attributable to heat treatment (Cozannet et al., 2010) or poor quality of the starting wheat stock.

The SID values for some AA in the current study were significantly reduced when derived in a semi-synthetic background that contained glucose and starch at 200 and 205 g/kg, respectively compared to a corn or wheat-based diet. Presumably this is attributable to the semi-synthetic portion of the diet and is supportive of previous anecdotal findings and the work of Becker et al. (1955). The potentially negative effect of dextrose has recently been reported for example (Kong and Adeola, 2013). In other literature, monosaccharides have also been shown to be detrimental when included in animal feed (Douglas et al., 2003). In contrast, Fan and Sauer (1995) did not find any differences in the SID of AA in canola meal fed to growing pigs based on either the direct method (using 517g corn starch/kg) or derived by the difference method. However, recent work by the authors (Perryman, 2014 personal communication) has suggested from similar experiments that semisynthetic dietary ingredients may also affect phosphorous digestibility values. When phosphorous digestibility is assessed in a high corn starch/dextrose diet relative to a more complete diet, digestibility is reduced. This is possibly due to irritation of the gut mucosae, inducing increased epithelial loss. As such, dextrose and to a lesser extent starch, may be acting as anti-nutritional factors in purified or semi-synthetic diets once a certain inclusion rate threshold is breached.

A clear conclusion from this experiment is that the method of determination of the SID of certain AA of wheat-DDGS affects the value obtained; the use of a semi-synthetic diet and the direct method may result in lower values. This has clear implication, both for ingredient evaluation studies but also for studies using digestibility as a metric of interest when determining the affect of various dietary treatments. When comparing data on digestibility values, caution is advised and it is suggested that only values obtained using like for like methodologies are compared.

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EFFECT OF CANOLA MEAL SOURCE ON BROILER PERFORMANCE

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Canola meal is a potentially competitive alternation to soybean meal in poultry feed formulations. However, variation in metabolisable energy, digestible amino acids and the presence of glucosinolate (GS) limits its inclusion level in broiler diets. A feeding study was conducted to compare performance of broilers fed canola meals produced by solvent extraction (SE), expeller extraction (EE) and cold expeller extraction (CE) methods. Seven canola meals were formulated into wheat-soybean meal based diets at 100 g/kg to 10 days and 200 g/kg from 10 to 24 days taking into account assumed oil and protein content as per their method of production. A non-canola meal treatment served as a control. Canola meal samples were analysed for GS, reactive lysine (RL) by 1-fluoro-2,4-dinitro-benzene (FDNB, Carpenter, 1960) or guanidination with ortho-methyl isourea (OMIU, Rutherford and Moughin, 1997) using NIRS prediction (DPI, Wagga Wagga, NSW) and heat damage index (HDI) using NIRS (Amino Red®, Evonik Singapore).

The CE meal had the highest RL (as a percentage of total lysine), lowest heat damage index and intermediate GS levels as compared to other meals. Birds fed SE meal B, EE meals D and F and CE meal G had both BW and FCR not different than controls ($P > 0.05$). Birds fed EE meal E had lower BW and poorer FCR compared to control fed birds ($P < 0.05$). Bird FCR was negatively correlated to RL (FDNB method) expressed as a percent of total lysine ($r = 0.89$). Bird FCR was not correlated to GS levels ($r = 0.14$) however FI was negatively correlated to GS ($r = 0.78$). The study indicates that bird performance can be more affected by RL than GS levels. While GS did appear to have a negative impact on FI, comparable performance relative to control was achieved using the higher GS meals. The results suggest that higher quality canola meals can be formulated into diets at levels up to and perhaps exceeding 100 and 200 g/kg from 0 to 10 days and 10 to 24 days respectively.

Table 1 - Performance of broilers and chemical analysis of various canola meals (0-24 d).

Trmt	[p]BW	[q]FI	[r]FCR	[s]EE	[t]GS	[u]RL	[v]RL	[w]HDI
Control	1191^{ab}	1629^{b}	1.418^{c}	ND	ND	ND	ND	ND
SE A	1225^{a}	1816^{a}	1.537^{ab}	38	1.1	94	86	97
SE B	1179^{abc}	1666^{b}	1.466^{bc}	19	3.7	95	88	95
EE C	1122^{bc}	1654^{b}	1.535^{ab}	79	5.3	92	84	99
EE D	1151^{abc}	1610^{b}	1.453^{bc}	81	5.3	93	91	96
EE E	1083^{c}	1683^{b}	1.625^{a}	86	4.2	88	87	99
EE F	1168^{abc}	1682^{b}	1.497^{bc}	111	5.2	94	87	97
CE G	1234^{a}	1690^{b}	1.419^{c}	99	4.0	98	95	90

[abcd] $P < 0.05$; [p] body weight of birds; [q]feed intake; [r]FCR corrected for mortality; [s]ether extract of canola meals; [t]GS µmol/g of canola meals; [u]free lysine/total lysine of canola meals, FDNB; [v]free lysine/total lysine of canola meals, NIRS; [w]heat damage index (AminoRed®) of canola meals; ND = not determined.

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PHYTASE INFLUENCES THE INHERENTLY DIFFERENT STARCH DIGESTIVE DYNAMICS OF WHEAT- AND MAIZE- BASED BROILER DIETS

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Summary

Phytase at 500 FTU/kg was included in wheat- and maize-based diets which were offered to broiler chickens in order to determine apparent digestibility coefficients and disappearance rates of starch from four small intestinal segments. Starch from wheat-based diets was 34.8% more digestible in the proximal jejunum (0.822 versus 0.610; $P < 0.001$) but, in the distal ileum, starch digestibility coefficients were 4.53% higher for maize-based diets (0.947 versus 0.906; $P < 0.02$). Thus, maize starch was more slowly and extensively digested than starch from wheat-based diets. Phytase increased proximal jejunal starch digestibility coefficients by 17.6% (0.774 versus 0.658; $P < 0.005$) and significantly increased starch disappearance rates by from 7.89% (distal ileum) to 23.7% (proximal jejunum). Phytate may impede starch digestion and consideration is given to the mechanisms whereby phytase influences starch digestive dynamics.

I. INTRODUCTION

Phytase supplementation of broiler diets is a routine practice and is used primarily to liberate the P component (282 g/kg) of the phytate molecule (*myo*-inositol hexaphosphate; IP_6). Phytase also generates 'extra-phosphoric' responses because phytate interacts with protein, starch, fat, calcium and trace minerals but these responses are less well understood and accepted (Selle and Ravindran, 2007). In particular, interactions between phytate and starch have not been clarified and the effects of phytase on starch digestion are elusive. Rickard and Thompson (1997) nominated several mechanisms whereby phytate may influence starch digestion, which included inhibition of amylase activity either directly or via chelation of Ca, which is a requisite co-factor for amylase and it was also suggested that phytate may complex starch directly or indirectly by binding proteins that are closely associated with starch. The present study was designed to evaluate the inclusion of exogenous phytase in broiler diets based on wheat and maize and to identify whether phytase has an effect on starch digestive dynamics.

II. METHOD

Data from two separate but similar 40-day feeding studies were compiled and analysed as a 2 x 2 factorial array of treatments consisting of wheat- and maize-based diets, without or with bacterial phytases at 500 FTU/kg. Protein contents of wheat and maize were 110 and 81 g/kg, respectively, and the wheat-based diet was formulated to be 12.97 MJ and 217 g/kg protein while the maize-based diet was formulated to be 12.92 MJ and 178g/kg protein. The two cereals were mediumly-ground (3.2 mm hammer-mill screen) prior to incorporation into the diets that were steam-pelleted at 84^0C. Celite^{TM} (World Minerals, Lompoc, CA, USA) was included in diets at 20 g/kg as an inert marker to determine apparent digestibility coefficients of starch in four small intestinal sites. The small intestines were removed from birds euthanized by intravenous injection of sodium pentobarbital and digesta in their entirety were gently expressed from the proximal jejunum (PJ), distal jejunum (DJ), proximal ileum (PI) and distal ileum (DI) and pooled for each cage. Proximal jejunal samples were taken from the end of the duodenal loop to the mid-point with Meckel's diverticulum and distal jejunal samples from the mid-point to the diverticulum. Proximal ileal samples were taken from Meckel's diverticulum to the mid-point with the ileo-

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caecal junction and distal ileal samples were taken from below this mid-point. The digesta samples were freeze-dried and acid insoluble ash (AIA) concentrations were determined by the method of Siriwan *et al.* (1993).

Starch concentrations in diets and digesta were determined by a procedure based on dimethyl sulfoxide, α-amylase and amyloglucosidase, as described by Mahasukhonthachat *et al.* (2010). The apparent digestibility coefficients for starch at four small intestinal segments were calculated from the following equation:

$$\text{Apparent digestibility coefficient} = \frac{(\%\text{nutrient}/\%\text{AIA})_{\text{diet}} - (\%\text{nutrient}/\%\text{AIA})_{\text{digesta}}}{(\%\text{nutrient}/\%\text{AIA})_{\text{diet}}}$$

Starch disappearance rates (g/bird/day) were calculated from the following equation from the defined regions of the small intestine:

$$\text{Disappearance rate} = \text{feed intake}_{\text{(g/bird/day)}} \times \text{dietary starch}_{\text{(g/kg)}} \times \text{starch}_{\text{(digestibility coefficient)}}$$

Experimental data was analysed as a 2x2 factorial array of dietary treatments using the IBM® SPSS® Statistics 20 program (IBM Corporation. Somers, NY USA). A probability level of less than 5% was considered to be statistically significant.

III. RESULTS

The effects of dietary treatments on apparent starch digestibility coefficients and starch disappearance rates are shown in Table 1. Interactions between grain-type and phytase were not observed for any of the parameters determined. There were significantly different starch digestibility coefficients between wheat and maize in the PJ and DI. Starch from wheat-based diets was 34.8% more digestible in the proximal jejunum (0.822 versus 0.610; $P < 0.001$) but, in the distal ileum, starch digestibility coefficients were 4.53% higher for maize-based diets (0.947 versus 0.906; $P < 0.02$). Maize had significantly slower starch disappearance rates compared to wheat in the PJ (50.7 vs 67.5 g/bird/day; $P < 0.001$), however in the DI, this was reversed where wheat had lower starch disappearance rates than maize (74.2 vs. 78.6 g/bird/day; $P< 0.04$). Phytase increased proximal jejunal starch digestibility coefficients by 17.6% (0.774 versus 0.658; $P < 0.005$). Phytase significantly ($P < 0.01$) increased starch disappearance rates in all segments of the small intestine. Starch digestibility rates with phytase supplementation were increased by 23.7% (65.3 vs. 52.8 g/bird/day) in the PJ, 10.1% (73.8 vs. 67.0 g/bird/day) in the DJ, 9.91% (78.7 vs. 71.6 g/bird/day) in the PI and 7.89% (79.3 vs. 73.5 g/bird/day) in the DI.

IV. DISCUSSION

This study presents evidence that suggests that phytate interacts with starch and this is declared by the accelerated starch disappearance rates generated by phytase. One possibility is that phytate impedes starch digestion and, as mentioned, several mechanisms have been proposed. However, there is little evidence to support the existence of direct starch-phytate complexes and it seems more likely that phytate may indirectly bind starch via starch granule-associated proteins (SGAP) as either binary or ternary protein–phytate complexes involving starch and cereal proteins. In theory, phytate has the capacity to complex proteins that are located in and on starch granules (Baldwin, 2001). Interactions between starch granules with soy and wheat proteins have been investigated (Mohamed and Rayas-Duarte, 2003) where SGAP removal from the surface of starch granules decreased protein-starch interactions.

Table 1 - The effects of 500 FTU/kg phytase on apparent digestibility coefficients and disappearance rates (g/bird/day) of starch in the proximal jejunum (PJ), distal jejunum (DJ), proximal ileum (PI) and distal ileum (DI) in broilers offered wheat- or maize-based diets.

Treatment		Starch digestibility coefficients				Starch disappearance rates			
Grain-base	Phytase	PJ	DJ	PI	DI	PJ	DJ	PI	DI
Wheat	0	0.777	0.833	0.896	0.894	62.2	66.7	71.7	71.5
	500	0.868	0.882	0.915	0.918	72.7	73.9	76.7	76.9
Maize	0	0.538	0.833	0.884	0.936	43.4	67.2	71.4	75.5
	500	0.681	0.865	0.948	0.959	58.0	73.6	80.8	81.7
SEM		0.0342	0.0224	0.0224	0.0129	2.956	2.332	2.401	1.965
Main effects: Grain-base									
Wheat		0.822	0.858	0.905	0.906	67.5	70.3	74.2	74.2
Maize		0.610	0.849	0.916	0.947	50.7	70.4	76.1	78.6
Main effects: Phytase		0.658	0.833	0.890	0.915	52.8	67.0	71.6	73.5
0		0.774	0.873	0932	0.938	65.3	73.8	78.7	79.3
500 FTU/kg									
Significance		< 0.001	0.664	0.609	0.015	< 0.001	0.963	0.434	0.038
Grain-base (GB)		0.002	0.062	0.062	0.155	< 0.001	0.008	0.007	0.008
Phytase (Phy)		0.432	0.682	0.299	0.954	0.505	0.868	0.360	0.851
GB x Phy interaction									

The first study to investigate the effect of phytate on sodium was by Cowieson *et al.* (2004), where broilers were fed atypical diets of glucose and phytate displayed increased total tract sodium excretion but with phytase addition total tract sodium excretion was reduced by 44%. These findings were subsequently confirmed where phytase increased Na digestibility at the ileal level (Ravindran *et al*., 2006; 2008; Selle *et al*., 2009). There is evidence that phytate depresses sodium pump activity in rats which was associated with reduced blood glucose levels (Dilworth *et al*., 2005). In addition, Liu *et al*. (2008) found that phytase increased sodium pump and glucose concentrations in duodenal and jejunal enterocytes in chickens. For example in the jejunum, 1000 FTU/kg phytase significantly increased concentrations of the sodium pump by 18.4% (13.59 versus 11.48 mmol/mg) and of glucose by 46.8% (13.73 versus 9.35 mmol/mg) in birds offered maize-based diets containing 2.2 g/kg phytate-P. The implication is that phytate was depressing sodium pump activity and, in turn, intestinal uptakes of glucose.

It has been proposed that phytate may impede absorption of glucose by depressing the functionality of the so-called 'sodium pump' (Na^+-K^+-ATPase) (Truong *et al*., 2014). In essence, glucose absorption from the gut lumen into the systemic circulation is driven by the sodium pump, which is located in the baso-lateral membrane of enterocytes in extreme numbers (Glynn, 1993). The sodium pump maintains an electrochemical gradient across the enterocyte by the active exchange of three Na^+ ions exiting for two K^+ ions entering the enterocyte. The Na-dependent absorption of glucose from the gut lumen into enterocytes via SGLT-1 co-transporters is driven by the activity of the sodium pump. Concentrations of Na within enterocytes are pivotal for sodium pump function (Therein and Blostein, 2003). Phytate may deplete Na concentrations in enterocytes, thereby depressing sodium pump activity. By limiting P bioavailability phytate could interfere with the rephosphorylation of the sodium pump but the diets in the present study were P-adequate. However, the depletion of Na may be a consequence of sodium bicarbonate hypersecretion into the duodenum especially from the pancreas. It has been proposed that this is a

compensatory mechanism because phytate increases secretions of HCl and pepsin as phytate-bound protein is refractory to pepsin digestion in the stomach (Selle *et al.*, 2012).

The differences in starch digestive dynamics of wheat and maize are noteworthy as the disappearance rate of wheat starch from the proximal jejunum was 33% more rapid than maize. As a consequence, 91% of wheat starch digestion took place in the PJ as opposed to 64% in maize-based diets. Slowly digestible starch is that which is digested in the three posterior segments of the small intestine and therefore 36% of maize starch was slowly digestible as opposed to only 9% for wheat. Giuberti *et al*. (2012) reported that the digestion rate of wheat starch was double that of maize under *in vitro* conditions. Differences in rates and sites of starch digestion along the small intestine can influence broiler performance, where several studies (Weurding *et al*. 2001, 2003) have indicated that the provision of slowly digestible starch improves feed conversion ratios in broilers. This suggests that the rate at which wheat starch is digested may be excessively rapid.

V. CONCLUSION

There are few studies which have demonstrated starch effects from phytase addition to diets in broilers, however the current study provides compelling evidence that phytase influences starch digestion dynamics where responses in starch digestion and starch disappearance rate where observed in phytase addition to wheat- and maize-based diets.

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EFFECTS OF A NOVEL BACTERIAL PHYTASE SUPPLEMENTATION ON PERFORMANCE, BONE MINERALIZATION AND NUTRIENT UTILIZATION OF BROILERS FED DIETS CONTAINING RICE BRAN AND MBM WITH DIFFERENT LEVELS OF PHOSPHORUS

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A study was conducted to evaluate the growth response, phosphorus (P) retention, bone mineralization and plasma calcium (Ca) and P of broilers fed corn-soy basal diets containing fixed levels of rice bran (RB) and meat bone meal (MBM) supplemented with a 6-bacterial origin phytase containing 1000 FYT (RONOZYME HiPhos (GT) from DSM nutritional products). Eight hundred day-old male broilers (Ross 308) were placed in 40 floor pens with 20 birds per pen. The treatments were arranged as 2 x 2 factorial with 2 levels of avP (control and 0.15% avP reduction) and 2 levels of phytase (0 and 100 g/t). The avP in the control and experimental diets were 0.45 and 0.30% during 0-17 d of age and 0.36 and 0.21% during 18-35 d of age, respectively. The dietary treatments were 1) PC; normal avP with no phytase, 2) PC plus phytase 100g/t, 3) NC; 0.15% avP reduction with no phytase and 4) NC plus phytase 100g/t. At 15 and 31 DOA, 3 birds from each replication were randomly selected for blood Ca and P assay. At the end of the experiment, 3 birds from each replication were randomly selected, left tibia were collected for bone breaking strength, % tibia ash and mineral content (Ca and P) analysis.

Throughout the experiment (0-35 DOA), an interaction between avP level and phytase supplementation was significant on feed intake (P=0.0236) and body weight gain (P=0.0364). Broilers fed diets containing reduced avP had a reduction in feed intake and body weight gain. However, feed intake and body weight gain of chicks fed reduced levels of avP was improved with phytase supplementation. FCR was not affected by phytase supplementation.

Phytase supplementation increased P retention by 24.1% (P<0.0256) over the NC diets. Interaction between avP levels x phytase were significant on tibia ash but not tibia Ca and P, in which the tibia ash was improved by 4.5% (P=0.0021) in birds fed low avP levels with phytase supplementation. Phytase supplementation increased tibia Ca (P=0.0097). Birds fed low avP diet tended to have lower bone breaking strength when compare to that of the control group (P=0.0820). No significant difference in tibia P was observed among dietary treatments. Interaction between avP levels x phytase was significant on Serum P but not serum Ca. Low P levels increased serum Ca concentrations by 8.2% (P=0.0018) and 12.2% (P<0.0080) at day 15 and day 31. Serum P of birds fed low avP diet and was significantly improved by phytase supplementation (P<0.05) both at day 15 (7.71 vs 10.14 mg%) and day 31 (5.09 vs 6.76 mg%).

From the present study, the reduction of avP at 0.15% had negative effect on broiler performance, bone mineralization and P retention. The supplementation of phytase at standard dose 100 g/t was effective to improve performance, %tibia ash, % tibia Ca and serum P of broilers fed corn-soy, RB and MBM diets containing 0.15% reduction in avP.

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RESPONSE OF BROILER CHICKENS TO DIFFERENT LEVELS OF PHYTASE, CALCIUM AND AVAILABLE PHOSPHORUS

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Summary

The influence of different levels of dietary Ca and available P on phytase activity was evaluated in broiler chickens. Feed intake, weight gain and tibia ash percentage were improved by supplementation of phytase in diet, particularly with low Ca and standard available P. This result implies the existence of interactions between supplemental microbial phytase and dietary calcium and phosphorus contents.

I. INTRODUCTION

The phosphorus in most vegetable feed ingredients is bound by phytic acid and therefore not available to poultry due to inadequate levels of phytase in the digestive tract (Zanini and Sazzad, 1999). Microbial phytase is therefore routinely included in such diets to release the phosphorus, but the activity of the enzyme may be affected by a number of dietary factors, including minerals (Selle and Ravindran, 2007). The present study was aimed at assessing the effect of different levels of dietary Ca and available P, and supplementation with microbial phytase on response of broiler chickens.

II. MATERIALS AND METHODS

Five hundred and seventy-six day-old male Ross 308 broiler were fed twelve corn-soybean meal-based diets containing three levels of Ca (Low, 0.6; Mid, 0.8 or High, 1 %), two levels of available P (Low, 0.3 or Standard 0.4 %) and with or without phytase (Quantum Blue, 500 FTU/kg). Each diet was replicated six times, with eight birds per pen and fed from hatch to 35 days, on a 3-phase feeding plan – starter (1-10 days), grower (11-24 days) and finisher (25-35 days). The diets were formulated according to the breeder specifications (Aviagen, 2007). Birds had free access to feed and water.

III. RESULTS AND DISCUSSION

Feed intake (FI) and body weight gain (BWG) declined ($P < 0.001$) with increase in Ca level, especially at low avP content (Table 1). The interactions between Ca and phytase, and between avP and phytase also had a significant ($P < 0.01$) impact on FI. Feed intake was not affected by low avP level except on the high Ca diets without phytase supplementation. Tibia ash content was also reduced by rising levels of Ca but this was significant ($P < 0.04$) only on diets containing low avP, without enzyme. Dietary supplementation with phytase was generally most effective in diets containing low Ca and standard avP. This combination resulted in an increase ($P < 0.05$) in BWG and tibia ash. Feed conversion ratio was not affected by differences in Ca, avP or phytase content although the interaction between avP and phytase was significant ($P < 0.05$).

The mineral levels tested in the current study include those recommended for commercial production. Typically such diets are supplemented with inorganic sources of Ca and P, some of which is in excess of the requirement by birds. The results obtained in the current study suggest that P levels can be reduced in the diet if a microbial phytase is

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supplemented. The supplement also appears to be more effective at a low level of Ca and available P. The latter response may be due to the release of P from bound sources.

IV. CONCLUSION

The results demonstrate that high levels of Ca may be detrimental to growth if avP is in short supply, but this can be improved through supplementation with phytase.

Table 1 - Gross response (1-24d) and tibia bone qualities at 24 days of age.

			Gross response			Tibia bone (%)	
Calcium[1]	Avail. P[2]	Phytase	FI (g/bird)	BW (g)	FCR (g/g)	Tibia ash	Ca
Low	Low	None	1640.0^{ab}	1296.9^{ab}	1.26^{ab}	41.3^{a}	41.9^{a}
		Plus	1649.1^{ab}	1321.9^{a}	1.25^{ab}	42.1^{a}	41.9^{a}
	Standard	None	1592.8^{bc}	1282.5^{ab}	1.25^{ab}	40.9^{a}	33.5^{b}
		Plus	1728.2^{a}	1347.4^{a}	1.28^{a}	44.5^{a}	42.7^{a}
Mid	Low	None	1624.8^{bc}	1206.5^{b}	1.35^{a}	41.3^{a}	41.8^{a}
		Plus	1510.3^{c}	1261.1^{ab}	1.20^{b}	42.9^{a}	42.1^{a}
	Standard	None	1613.9^{abc}	1317.9^{a}	1.22^{b}	43.4^{a}	42.0^{a}
		Plus	1541.9^{bc}	1261.1^{ab}	1.21^{b}	42.6^{a}	46.2^{a}
High	Low	None	1380.3^{d}	1029.9^{c}	1.35^{a}	38.9^{b}	42.5^{a}
		Plus	1539.2^{bc}	1209.9^{b}	1.27^{ab}	40.5^{a}	42.8^{a}
	Standard	None	1591.9^{bc}	1306.6^{a}	1.22^{b}	42.2^{a}	42.1^{a}
		Plus	1620.9^{abc}	1302.8^{a}	1.24^{ab}	42.7^{a}	42.2^{a}
		SEM	58.55	46.32	0.05	1.15	2.59
Sources of variation							
Calcium			0.001	0.001	0.631	0.040	0.049
Avail. P			0.008	0.001	0.123	0.001	0.518
Phytase			0.388	0.017	0.102	0.018	0.031
Ca x Avail. P			0.057	0.001	0.105	0.229	0.087
Ca x Phytase			0.005	0.233	0.259	0.435	0.265
Avail. P x phytase			0.005	0.035	0.020	0.993	0.043
Ca x Avail. P x phytase			0.043	0.076	0.927	0.050	0.190

[1] Calcium supplemented at 0.6 (Low), 0.8 (Mid) and 1.0 % (High).
[2] Available P at 0.3 (Low) and 0.4 % (Standard).
[a,b,c] Means bearing different superscripts differ significantly at the levels indicated.

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INTERACTION BETWEEN PHYTASE AND CALCIUM SOURCE, CONCENTRATION AND PARTICLE SIZE ON BROILER PERFORMANCE AND SKELETAL INTEGRITY

E.J. BRADBURY[1], S.J. WILKINSON[1], G.M. CRONIN[1], C.L. WALK[2] and A.J. COWIESON[1]

Summary

The study reported herein investigated the use of a marine source of highly soluble calcium (HSC) contrasted with limestone and the effect of calcium concentration, particle size and phytase on broiler performance and skeletal health. Dietary treatments had no effect on feed intake, body weight or FCR during the grower period. At the conclusion of the study birds that received diets supplemented with phytase were significantly heavier than birds that received diets with no phytase. Overall birds fed HSC at low concentration in conjunction with an exogenous phytase performed comparably with limestone.

I. INTRODUCTION

Calcium (Ca) and phosphorus (P) are essential minerals for many biological processes and skeletal health, however, they have a complex interactive relationship. Phytate is the naturally occurring storage form of P in plants, with the main storage site of phytate-P being seeds (Tamim et al., 2004). As poultry diets are comprised mainly of seed based ingredients, there is a considerable amount of phytate-P present in the diets (Angel et al., 2002; Tamim and Angel 2003). However, phytate-P is relatively poorly available and consequently inorganic sources of P are used to meet bird requirements (Angel et al., 2002; Tamim and Angel 2004; Selle et al., 2009). This results in higher dietary total P than what is required by the bird and excess P is excreted (Tamim and Angel et al., 2003). Diets with lower total Ca levels are desirable to enhance P digestibility, feed conversion efficiency and weight gain, however, lower total Ca level may have a negative effect on broiler welfare, in particular adverse effects on skeletal and leg health (Shim et al., 2012).

Calcium particle size influences Ca metabolism and is correlated to gizzard retention time with longer retention times reportedly improving shell quality in laying hens. The porosity of the Ca source and *in vivo* solubility of the Ca source are also important for Ca metabolism, and skeletal health (Zhang and Coon 1997).

The ability to replace high concentrations of limestone with a HSC source at lower concentrations may be beneficial for poultry, facilitating a reduction in total dietary Ca levels, whilst retaining intestinal absorption (Walk et al., 2012). This would result in less Ca-phytate interactions and reduce excess P excretion. Both reducing the need for inorganic P to be added to the diet, in turn reducing the cost of the diet. In previous work conducted by Bradbury *et al* (2015) it was observed that HSC may be too soluble at high concentrations, especially when phytase is present. Therefore the purpose of this study was to repeat the original study, inclusive of particle size to establish whether a larger particle size would help alleviate the problematic effects of such a highly soluble Ca source.

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II. MATERIALS AND METHODS

All experimental procedures were approved by The University of Sydney Animal Ethics Committee. A total of 2400 Ross 308 day old male broiler chicks were obtained from a commercial hatchery and randomly allocated across 95 deep litter floor pens (1.5 m x 1.5 m) with 25 birds per pen and six replicate pens per treatment. Birds were maintained at 31°C for the first five days and reduced 0.5°C per day until 21°C was reached. The lighting regime was 23h:1h (light:dark) for the first five days then 18h:6h (light:dark) for the remainder of the study. Dietary treatments were arranged as a 2 x 2 x 2 x 2 factorial, comprising two sources of Ca (limestone or HSC), two dietary concentrations of Ca (6.0 or 9.0 g/kg, 5.5 and 8.0 g/kg during the starter and grower periods, respectively), two Ca particle sizes (< 0.5 mm or > 0.5 mm) and two phytase inclusions (0 or 1000 FTU/kg). Birds were fed a starter diet from d1-d14 and were fed a grower diet from d15-d28. Birds had *ad libitum* access to feed and water. On day 27 the skeletal health of six birds from two replicate pens of each dietary treatment was evaluated using the latency to lie (LTL) procedure as outlined in Bradbury *et al.,* 2014. Pen body weight and feed intake measurements were recorded on d1, d14 and d28 of the study. On day 28 four birds per pen were euthanized via injection of a lethal dose (1ml/2kg) of sodium pentobarbitone into the jugular vein. Terminal body weight was recorded for each bird and the left tibia and foot were removed and stored at -20°C for foot ash analysis.

All bird performance data were analysed using the full factorial procedure in JMP 8.0 (SAS Institute Inc. NC, USA). All results are expressed as treatment means with statistical significance defined as $P < 0.05$. If significance was determined comparison between all pairs was performed using a Tukey-Kramer HSD test. Behavioural LTL times were analysed using Cox's proportional hazard survival analysis in GenStat (14th Edition).

III. RESULTS AND DISCUSSION

Results for the starter and grower period are shown in Table 1. During the starter period a three-way interaction was observed for Ca source*Ca concentration*particle size ($P = 0.046$) on feed intake. Birds that were fed limestone to supply 6.0 g/kg dietary Ca with a mean particle size of < 0.5 consumed significantly more than birds that received HSC to supply 9.0 g/kg dietary Ca with > 0.5 mm particle size. Furthermore birds that were fed limestone to supply 9.0 g/kg dietary Ca with a mean particle size > 0.5 mm consumed significantly less than birds fed limestone to supply 9.0 g/kg dietary Ca with a mean particle size < 0.5 mm. Body weight gain was influenced by a Ca concentration*phytase interaction ($P < 0.0001$) where phytase significantly improved body weight gain at both dietary Ca concentrations compared to birds fed diets without phytase.

Birds that were fed HSC with phytase > 0.5 mm particle size had a significantly lower FCR than birds fed HSC without phytase > 0.5 mm particle size ($P < 0.05$) resulting in a three-way interaction for Ca source*particle size*phytase. Additionally birds that received limestone with phytase > 0.5 mm particle size had a significantly lower FCR than birds fed limestone without phytase > 0.5 mm particle size ($P < 0.05$).

Grower period feed intake was influenced by multiple three-way interactions. Birds fed HSC 8.0 g/kg dietary Ca with phytase consumed more fed than birds fed HSC 8.0 g/kg dietary Ca without phytase ($P < 0.05$) (between Ca source*Ca concentration*phytase ($P = 0.001$)). Birds fed HSC with phytase > 0.5 mm particle size had the highest feed intake and consumed significantly more feed than HSC without phytase > 0.5 mm particle size ($P < 0.05$), HSC with phytase < 0.5 mm particle size ($P < 0.05$) Ca source*particle size*phytase ($P = 0.001$). Ca concentration*particle size*phytase ($P = 0.034$) influenced feed intake where birds that were fed 8.0 g/kg dietary Ca with phytase and > 0.5 mm particle size consumed

significantly more feed than bird fed 8.0 g/kg dietary Ca without phytase > 0.5 mm particle size and 8.0 g/kg dietary Ca with phytase < 0.5 mm particle size.

Body weight gain at the conclusion of the grower period was not influenced by dietary treatment. Phytase inclusion significantly increased live weight gain of broilers during the grower period ($P < 0.001$). Feed conversion ratio was significantly influenced by the Ca source*phytase interaction with birds that were fed limestone with phytase had a significantly lower FCR compared to birds fed limestone without phytase and HSC with and without phytase ($P < 0.05$).

Table 1 - Effect of dietary treatment on feed intake, body weight gain and FCR during the starter and grower period.

Dietary Treatment				Starter			Grower			
Ca Source	Ca level (g/kg)	Ca particle size (mm)	Phytase (FTU/ kg)	Feed intake (g/b)	BWG (g/bird)	FCR	Feed intake (g/b)	BWG (g/bird)	FCR	Foot ash (%)
Limestone	Low	< 0.5	0	532	433.6	1.23	1971.7	1292.7	1.54	14.33
Limestone	Low	> 0.5	0	512	434.0	1.19	1969.2	1307.9	1.51	15.42
Limestone	High	< 0.5	0	495	405.7	1.22	1986.1	1316.0	1.50	15.31
Limestone	High	> 0.5	0	495	408.8	1.21	1949.4	1307.3	1.48	15.08
Limestone	Low	< 0.5	1000	534	461.2	1.16	2033.6	1370.9	1.49	15.39
Limestone	Low	> 0.5	1000	514	445.7	1.14	1915.1	1339.3	1.44	14.61
Limestone	High	< 0.5	1000	520	460.3	1.13	1928.8	1346.6	1.45	16.11
Limestone	High	> 0.5	1000	535	460.7	1.16	1933.1	1341.0	1.45	16.23
HSC	Low	< 0.5	0	517	430.4	1.21	2006.0	1297.8	1.56	14.68
HSC	Low	> 0.5	0	518	435.7	1.19	1952.9	1325.1	1.49	14.11
HSC	High	< 0.5	0	480	400.1	1.19	1952.3	1310.2	1.50	15.02
HSC	High	> 0.5	0	477	395.9	1.20	1914.3	1284.5	1.48	14.78
HSC	Low	< 0.5	1000	527	442.7	1.19	1938.5	1297.8	1.48	14.10
HSC	Low	> 0.5	1000	521	455.0	1.15	1991.0	1355.6	1.49	15.23
HSC	High	< 0.5	1000	518	441.9	1.18	1974.2	1326.5	1.49	16.07
HSC	High	> 0.5	1000	511	443.6	1.15	2080.7	1332.9	1.57	16.19
SEM				7.5	6.4	0.02	24.1	15.5	0.03	0.40
P value				*0.682*	*0.571*	*0.797*	*0.196*	*0.569*	*0.759*	*0.078*

Birds fed higher Ca concentration diets had a higher foot ash percentage ($P<0.001$). Phytase supplementation significantly increased foot ash ($P = 0.0003$) compared to birds that did not receive phytase.

Latency to lie results were significantly correlated with body weight ($P = 0.007$). For every 100g increase in body weight the chance of the bird sitting increased by 19%. In a dietary treatment comparison, birds fed HSC 5.5g/kg dietary Ca without phytase > 0.5mm particle size was the only diet to have a significantly better standing time during the LTL test ($P = 0.02$), with the birds being 21% less likely to sit at any given point during the LTL test.

There have been few studies that have reported the use of HSC on broiler performance. Walk *et al.,* (2012) reported that broilers that were fed 0.9% dietary Ca from limestone consumed more feed than birds that were fed 0.9% dietary Ca from HSC. These results are consistent with the starter period of this study, whereby birds that were fed limestone 6.0 g/kg dietary Ca with < 0.5 mm particle size consumed significantly more than birds fed HSC 6.0 g/kg with < 0.5 mm particle size. The results of the study also show that birds that were fed limestone 6.0 g/kg dietary Ca < 0.5 mm particle size consumed significantly more feed than birds fed HSC 6.0 g/kg dietary Ca > 0.5 mm particle size. Zhang and Coon (1997) reported that larger Ca particles have a greater retention time in the gizzard when compared to smaller Ca particles and could explain why birds fed the larger particle size consumed less fed.

In this study, body weight was determined to be the most correlated with standing times and this is in agreement with Kestin et al., (2001) and Sherlock *et al.,* (2010). The results of the LTL show that HSC source at the lower inclusion level (5.5 g/kg) was the only diet whereby the birds were statistically less likely to sit, implying better skeletal and leg health. However, it is interesting to note that birds that performed best in the LTL test (HSC 5.5 g/kg dietary Ca without phytase > 0.5 mm particle size) does not correlate to the greatest foot ash percentage. Foot ash responded positively with higher Ca concentrations and phytase inclusion in the diet that is consistent with Walk *et al.,* (2012) who reported main effects of Ca concentration and phytase inclusion on tibia ash.

From the available literature, studies investigating Ca particle size report conflicting results. The beneficial effects of larger Ca particle size of egg shell and bone status in layers are documented, however, more research in broilers investigating effects on mineral digestibility and Ca particle retention in the gizzard are required. The results in this paper suggest that a HSC source when used at lower concentrations and coupled with an exogenous phytase results in bird performance comparable with higher concentrations of Ca from limestone. Further research is required to determine the economic implications of using a HSC source at lower concentrations.

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MEASUREMENT OF TRUE ILEAL CALCIUM DIGESTIBILITY OF MEAT AND BONE MEAL FOR BROILER CHICKENS

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Currently, there is a move towards the use of digestible phosphorus (P) in diet formulations for poultry due to P excretion into the environment and increasing prices of inorganic phosphates. High dietary calcium (Ca) concentrations are known to reduce P availability (Tamim and Angel, 2003). Due to the close relationship between dietary Ca and P the measurement of digestible Ca content in feedstuffs may also be necessary. Meat and bone meal (MBM) is a major organic Ca source for poultry diets, but no published data are available for the digestible Ca content of MBM for poultry.

This study was designed to determine the Ca digestibility in three MBM samples, using the regression method. The MBM samples (coded as MBM-1, MBM-2 and MBM-3) were obtained from commercial rendering plants and analysed for ash, Ca and P concentrations, particle size distribution and bone to soft tissue ratio. The Ca and P concentrations of MBM-1, MBM-2 and MBM-3 were determined to be 71, 118 and 114 g/kg, and 37, 60 and 59 g/kg, respectively. The corresponding geometric mean particle diameters (GMD) were 0.866, 0.622 and 0.875 mm, respectively. The bone to soft tissue ratios were 1:1.49, 1:0.98 and 1:0.92, respectively. Four experimental diets, containing graded concentrations of Ca were formulated from each MBM sample with inclusion levels of 20, 40, 60 and 80 g/kg diet. All diets contained titanium dioxide (3 g/kg) as an indigestible marker. A total of 288 day-old male broilers (Ross 308) were raised on a commercial broiler diet from 1 to 27 days of age. Each experimental diet was then randomly allotted to four replicate cages (six birds per cage) and diets were offered from day 28 to 31 post-hatch. Digesta samples from the lower ileum were collected on day 31, processed and analysed for dry matter, Ca and marker. Apparent ileal digestibility of Ca was calculated by the indicator method and the linear regression analysis was used to determine the true Ca digestibility.

The results showed that the apparent ileal Ca digestibility coefficient of the three MBM samples was unaffected ($P > 0.05$) by increasing Ca concentrations. The average apparent Ca digestibility coefficients of MBM-1, MBM-2 and MBM-3 were determined to be 0.501, 0.436 and 0.453, respectively. A strong linear relationship ($P < 0.001$) was observed between dietary Ca intake and digesta Ca output in all MBM samples, which is a prerequisite for application of regression method for the determination of true digestibility. The true ileal digestibility coefficients of Ca in MBM-1, MBM-2 and MBM-3 were determined to be 0.600, 0.463 and 0.497, respectively. Calcium digestibility of MBM-1 was higher ($P < 0.05$) than MBM-2, but was similar ($P > 0.05$) to MBM-3. There was no difference ($P > 0.05$) between the digestibility of MBM-2 and MBM-3. The observed variability in true Ca digestibility between the MBM samples may be explained, at least in part, by the differences in ash and Ca concentrations and bone to soft tissue ratios. Calcium in MBM is generally assumed to be highly available, but the present data suggest that this may not be the case. Future studies will evaluate the Ca digestibility in limestone, the major Ca source in poultry diets.

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VISCOSITY OF *LUPINUS ANGUSTIFOLIUS* SEEDS – COMPARISON OF *IN VITRO* AND *IN VIVO* EVALUATION IN BROILER CHICKENS

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Summary

A viscosity of digesta is detrimental for digestion and absorption processes in young broilers. The viscosity generated by sweet lupin seeds (*Lupinus angustifolius*) of 5 Polish varieties - Kadryl, Regent, Dalbor, Bojar and Tango was tested *in vitro* and *in vivo.* Lupin seeds were finely ground and incubated for 30 min in sodium acetate buffer (WEV), or for 2 h 45 min in conditions imitating *in vivo* digestion process (EEV), viscosity of subsequent extracts were measured. The ground seeds were also combined with basal diet at ratios (w/w) 250:750 or 320:680 g/kg DM, diets were cold pelleted and fed to 3-week-old broilers (11 groups x 14 birds) for 6 days. The birds were culled and viscosity of ileal digesta was measured immediately after collection (IVI) or after 6 days cold storage at -18^{o}C (IVF). There were no great differences between lupin cvs within *in vitro* viscosity, the WEV values averaged 2.75 MPas·s, while EEV averages 1.90 MPas·s for 4 cvs, and was higher for Regent cv (3.37 MPas·s). The IVI was 2.6 MPas·s in control group fed basal diet, but it was higher in birds fed lupin diets ($P < 0.05$), and increased with lupin content in diets ($P < 0.01$), being highest (30.9 MPas·s) in birds fed the diet with 320 g/kg Dalbor cv. After cold storage and thawing of digesta the IVF viscosity of all samples from lupin group lowered and did not differ statistically from control. Neither correlations were found between both *in vitro* measurements nor between WEV and IVI. It seems that only *in vivo* measurements in fresh digesta can predict the nutritional value of lupin cvs for broilers.

I. INTRODUCTION

Lupin species presently produced in Poland contain low concentrations of alkaloids (100-200 mg/kg) and due to high protein content can be used as a source of protein in swine and poultry diets. However, the use of lupins in broiler diets is limited, as lupin seeds accumulate structural non-starch polysaccharides (NSPs), which are mainly cellulose, arabinoxylans and pectin polysaccharides, consisting of β-galactan and rhamnogalacturonan mainchains with arabinose and arabinan sidechains (Brillouet and Riochet,1983; Cheetham et al,. 1993, Khalil at al. 2014). Lupin NSPs are not digested by broilers, moreover, NSPs of narrow-leafed (*Lupinus angustifolius*) lupins induce high viscosity in broiler digesta (Kocher et al. 2000, Olkowski et al. 2005). The viscosity generated by narrow-leafed lupin diets is detrimental for digestion and absorption processes in young broilers and can even increase mortality (Olkowski et al. 2001). An *in vitro* method of prediction viscosity induced by lupin seeds can be useful both for breeders and for feed companies. The main objective of the study was to compare the viscosity generated by different cultivars of narrow-leafed lupin seeds evaluated *in vitro* by two methods and *in vivo.*

II. MATERIAL AND METHODS

Narrow-leafed (*L. angustifolius*) lupin seeds of 5 Polish varieties - Kadryl, Regent, Dalbor, Bojar and Tango harvested in 2013 were ground with a cyclone mill fitted with 0.5 mm screen. The viscosity was measured *in vitro* according to Smulikowska and Nguyen (2001)

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by two methods. The WEV viscosity was measured as follows: one g of sample was mixed in a tube with 5 ml of sodium acetate buffer (pH 5), then tube was sealed with a stopper, incubated for 30 min at 40°C in a shaking water bath, cooled on ice, centrifuged (10 min at 10,000 g, 4°C), and viscosity of supernatant was measured with the use of Brookfield Digital cone/plate viscometer (model LVDV II+, Brookfield Engineering Laboratories, USA). The viscometer was maintained at 40°C by connecting the sample cup to the water bath. Triplicate analyses for each sample were determined in separate runs. The EEV viscosity was measured as follows: one g of sample was mixed in a tube with 3 ml of 0.1 N HCl solution containing 2,000 U pepsin/ml, the tube was sealed with a stopper and incubated for 45 min at 40°C in a shaking water bath, thereafter 1 ml of 1 M $NaHCO_3$ solution containing 4 mg of pancreatin was added and the tube was incubated further for 2 h as before, than cooled on ice and viscosity was measured in triplicate as at the WEV. The *in vivo* viscosity (IVI) was measured as follows: finely ground lupin seeds were combined with basal wheat, corn and soybean meal-based diet at ratios (w/w) 250:750 or 320:680 g/kg DM. The basal and test diets were cold pelleted and fed to 11 groups of 14 individually kept 3-week-old broiler females for 6 days, then the birds were culled, the content of ileum was collected, pooled from 2 birds, mixed and packed into 2 tubes (samples IVI and IVF). Samples IVI were immediately centrifuged, than the viscosity was measured as at the WEV method. Samples IVF were cold stored at -18°C for 6 days, thawed, centrifuged and viscosity was measured as at the WEV method. The obtained results were subjected to one-way and two-way analysis of variance (ANOVA) and the correlation between *in vitro* and *in vivo* measurements was assessed using STATGRAPHICS® Centurion XVI ver. 16.1.03 software (Statistical Graphic Corp., 1982-2010).

III. RESULTS

The WEV values were not significantly different between lupin cultivars and averaged 2.75 MPas·s, while EEV values averaged 1.9 MPas·s for 4 cvs and was higher ($P < 0.05$) only for Regent cv (Table 1).

Table 1 - The *in vitro* viscosity of narrow-leafed lupin cvs, measured with 2 methods, MPas·s.

Method	Lupin cultivar					SEM
	Kadryl	Regent	Dalbor	Bojar	Tango	
WEV	2.87	2.77	2.78	2.78	2.59	0.117
EEV	2.00 [a]	3.37 [b]	1.83 [a]	1.79 [a]	2.00 [a]	0.092

[a,b] means in a row with different superscripts are significantly different at $P < 0.05$

Table 2 - The *in vivo* viscosity of ileal digesta from birds fed basal diet and diets differing in lupin cvs and content, MPas·s.

Method*	Basal	Lupin cultivar/content (g/kg) in diet										SEM
		Kadryl		Regent		Dalbor		Bojar		Tango		
		250	320	250	320	250	320	250	320	250	320	
IVI	2.6[a]	8.6[bc]	10.8[bc]	13.0[c]	19.9[d]	8.5[bc]	30.9[e]	6.2[ab]	10.2[bc]	10.7[bc]	20.3[d]	1.78
IVF	2.5	3.2	3.7	3.4	4.3	3.4	4.0	3.7	4.0	4.0	5.0	0.62

*IVI – measured immediately after digesta collection;
IVF – measured after 6 days of cold storage at -18°C;
[a,b] means in a row with different superscripts are significantly different at $P < 0.05$

The IVI viscosity, measured immediately after collection in ileal digesta, in control group fed the basal diet was 2.6 MPas·s, but in all groups of birds fed lupin diets, in except that fed 250 g/kg Bojar seeds, was significantly higher ($P < 0.05$) compared to control. There were no correlations between *in vitro* WEV and EEV measurements and IVI values. After

freezing and thawing of ileal digesta samples, the IVF values in samples from control group were similar as IVI, but in samples taken from birds fed lupin diets were lower and were not statistically different between treatments (Table 2). There was no correlation between IVI and IVF values.

IV.DISCUSSION

In our former study (Smulikowska and Nguyen, 2001) the EEV viscosities in samples of different rye cultivars harvested in different regions of Poland were significantly higher than WEV viscosities, measured after prolonged incubation with proteolytic enzymes. It indicated, that some rye protein were bound with dietary fibre components making the complexes insoluble. However, in the present study there were no great differences between *L. angustifolius* cultivars within *in vitro* viscosity determined by the two methods. It may indicate, that NSPs of lupins are not interconnected with proteins. Similar conclusions can be drawn from Kocher et al. (2000) study, who found that supplementation of *L. angustifolius*-based diet with enzyme which significantly increased digesta viscosity along the small intestine did not affect ileal protein digestibility in broilers. A comparison of values obtained in lupin-fed chickens by two-way analysis of variance (data not shown) revealed that viscosity of ileal digesta depends significantly ($P < 0.01$) both, on lupin content in a diet and lupin cultivar. The great differences of NSP viscosity between cultivars, observed in the present study, highlights their limitations for use in broiler diets. However, it seems, that only *in vivo* measurements done immediately after bird culling can be connected with the nutritional value of *L. angustifolius* cvs for broilers. The measurements done with cold stored digesta were not informative and this appears to be a result of the physico-chemical structure of digesta being destroyed by the treatment.

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INFLUENCE OF FEED FORM AND PARTICLE SIZE ON THE PERFORMANCE AND NUTRIENT UTILISATION OF BROILER STARTERS FED MAIZE-BASED DIETS

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Summary

The present experiment was designed to examine the influence of feed form and particle size in maize-based diets on the performance and nutrient utilisation of broiler starters. The experimental design was a 2 × 3 factorial arrangement of treatments, which included two feed forms (mash or pellet) and three particle sizes (fine, medium or coarse). Birds fed pelleted diets had higher ($P < 0.05$) weight gain and consumed more ($P < 0.05$) feed than those fed mash diets. In mash diets, fine grinding resulted in lower ($P < 0.05$) feed per gain compared to medium and coarse grinding, whereas, in pelleted diets, there was no effect ($P > 0.05$) of particle size. Compared to mash diets, pelleting reduced ($P < 0.05$) the apparent ileal nitrogen (N) digestibility but increased ($P < 0.05$) fat digestibility. Increasing maize particle size improved ($P < 0.05$) starch digestibility and apparent metabolisable energy (AME) in pelleted diets, whilst in mash diets, particle size had no effect. Coarsely ground maize-based pelleted diets may benefit growth performance and utilisation of nutrient and energy via improving gizzard development and function in broiler chickens during starter phase.

I. INTRODUCTION

Consistent and relatively high nutritional value of maize has made it the widely used cereal in the poultry industry worldwide, contributing up to 65% of the metabolisable energy and 20% of the protein in typical poultry diets. It is common practice to grind the cereal grains prior to incorporating into the diets. Published data regarding the effect of particle size on broiler performance fed maize-based diets are contradictory. Reece et al. (1985) found that the maize particle size (geometric mean diameter, 0.68 vs. 1.29 mm) had no effect on the performance of broilers fed crumbled or pelleted diets. Amerah et al. (2008) showed that coarse grinding of maize (7-mm hammer mill screen size) had positive effects on broiler performance compared with fine grinding (1-mm screen size) in pelleted diets. The physical form of feed is an important factor, which confounds the effect of particle size on growth performance and nutrient digestibility. Abdollahi et al. (2013) showed that in maize-based diets, pelleting had no effect on the ileal digestibility of starch and N but improved that of fat. Only minimal attempts have been made to investigate the interaction between feed form and particle size on the nutrient utilisation of broilers fed maize-based diets. The present experiment was designed to compare the interaction between feed form (mash and pellet) and particle size (fine, medium and coarse) on nutrient utilisation and performance of broiler chickens in starter phase.

II. MATERIALS AND METHODS

The experimental design was a 2 × 3 factorial arrangement of treatments evaluating two feed forms (mash and pellet) and three particle sizes (fine, medium and coarse). Whole maize was obtained from a commercial supplier, and ground in a hammer mill to pass through screen

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sizes of 2.0, 5.0 and 8.0 mm for fine, medium and coarse grades, respectively. A maize-soybean meal-based diet was formulated to meet the Ross 308 strain recommendations for broiler starters (Ross, 2007). Following mixing, each diet with similar maize particle size was divided into two equal batches. The first batch was retained as unprocessed mash. The second batch was steam-conditioned at 70 ºC and pelleted using a pellet mill capable of manufacturing 180 kg of feed/h and equipped with a die ring with 3-mm holes and 35-mm thickness. All diets contained titanium dioxide as an indigestible marker. Each of the six dietary treatments was offered ad libitum to six replicate cages (eight birds per cage). Body weights and feed intake were recorded at weekly intervals throughout the 21-day trial. From d 17 to 20, feed intake and excreta output were measured quantitatively per cage for the determination of AME. On d 21, ileal digesta were collected for determination of apparent ileal digestibility (CAID) of N, starch and fat. On d 21, two birds per cage, with body weights closest to the mean weight of the cage, were selected, weighed and euthanised by cervical dislocation. The empty weight of gizzard of individual birds was determined and the pH of gizzard contents was measured with a calibrated digital pH meter.

III. RESULTS AND DISCUSSION

Birds fed pelleted diets had higher ($P < 0.05$) weight gain and consumed more ($P < 0.05$) feed than those fed mash diets (Table 1). In mash diets, fine grinding resulted in lower ($P < 0.05$) feed per gain compared to medium and coarse grinding, whereas, in pelleted diets, there was no effect ($P > 0.05$) of particle size.

Pelleting reduced ($P < 0.05$) the CAID of N but increased ($P < 0. 05$) that of fat compared to mash diets. Significant feed form x particle size interactions were observed for the CAID of starch ($P < 0.001$) and AME ($P < 0.05$). In mash diets, particle size had no effect ($P > 0.05$) on the CAID of starch, but in pelleted diets, pellets made from medium and coarsely ground maize resulted in higher ($P < 0.05$) starch digestibility than those made from finely ground maize. Increasing the maize particle size from fine and medium to coarse improved ($P < 0.05$) the AME in pelleted diets, whilst in mash diets; particle size had no effect ($P > 0.05$).

The main effect of feed form was significant ($P < 0.001$) for the relative weight of gizzard; with the gizzard being heavier ($P < 0.05$) in birds fed mash diets compared to those fed pelleted diets. Regardless of feed form, medium and coarse grinding of maize increased ($P < 0.05$) the gizzard weight compared to fine grinding. Significant ($P < 0.05$) feed form x particle size interaction was observed for gizzard pH. In mash diets, gizzard pH was not influenced ($P > 0.05$) by particle size, whereas, in pelleted diets, fine grinding elevated ($P < 0.05$) the gizzard pH compared to medium and coarse grinding.

The benefits of pellet feeding on broiler performance have been extensively reported and the current work confirms the benefits in terms of higher feed intake, weight gain and feed efficiency. Abdollahi et al. (2013) reported that pelleting a maize-based broiler diet had no effect on the CAID of N and starch but improved that of fat compared to mash diets. Jimenez-Moreno et al. (2009) suggested that steam cooking might release the lipids encapsulated within the oil bodies of grain. The fact that most of the dietary fat content in the maize-based diets originated from intact fat contained within maize and not from the supplemental oil source, lends support to this suggestion.

The shorter digesta retention time and an elevated gizzard pH, due to an under-developed gizzard, are possible physiological limits to optimal digestion in pellet-fed birds (Ravindran, 2013). It has been suggested that increased retention time in the gizzard as a consequence of increased gizzard volume may increase nutrient digestibility by providing more time for the secretion of hydrochloric acid and possibly pepsin, and by increasing the

intestinal refluxes that serve to re-expose the digesta to pepsin (Gonzalez-Alvarado et al., 2008). It is noteworthy that improvements in starch digestibility and AME in pelleted diets, with increasing particle size, were associated with higher gizzard weights and reduction in gizzard pH. Overall, although pelleting has been shown in some studies to even out the differences in particle size (Engberg et al., 2002; Svihus et al., 2004; Péron et al., 2005; Amerah et al., 2007), the current findings demonstrate that coarse grinding of maize, through enhanced gizzard development and functionality, may be beneficial to nutrient and energy utilisation and growth performance in broilers fed pelleted diets.

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Table 1- Influence of feed form and particle size on weight gain (g/bird), feed intake (g/bird), feed per gain (g feed/g gain), apparent ileal digestibility coefficients (CAID) of nitrogen (N), starch and fat, apparent metabolisable energy (AME; MJ/kg dry matter), relative empty gizzard weight (g/kg body weight) and gizzard pH in broiler starters.[1]

Feed form	Particle size	Weight gain	Feed intake	Feed per gain	CAID of N	CAID of starch	CAID of fat	AME	Gizzard weight[2]	Gizzard pH[3]
Mash	Fine	911	1145	1.257b	0.785	0.976a	0.871	14.65cd	14.9	2.76b
	Medium	932	1179	1.279a	0.779	0.975a	0.850	14.58d	17.3	2.90b
	Coarse	918	1173	1.290a	0.791	0.981a	0.821	14.68cd	17.3	2.76b
Pellet	Fine	1139	1379	1.219c	0.735	0.958b	0.893	14.71bc	10.9	3.43a
	Medium	1143	1373	1.204c	0.763	0.976a	0.910	14.81b	12.4	2.92b
	Coarse	1138	1368	1.203c	0.768	0.981a	0.939	14.95a	12.7	2.90b
Pooled SEM		9.1	13.1	0.0072	0.0111	0.0023	0.0210	0.040	0.44	0.121
Main effects										
Feed form										
Mash		920b	1166b	1.275	0.785a	0.978	0.847b	14.63	16.5a	2.81
Pellet		1140a	1373a	1.209	0.755b	0.972	0.914a	14.82	12.0b	3.08
Particle Size										
Fine		1025	1262	1.238	0.760	0.967	0.882	14.68	12.9b	3.10
Medium		1037	1276	1.242	0.771	0.976	0.880	14.69	14.9a	2.91
Coarse		1028	1271	1.246	0.779	0.981	0.880	14.81	15.0a	2.83
Probabilities, P ≤										
Feed form		***	***	***	**	**	***	***	***	**
Particle size		NS	NS	NS	NS	***	NS	**	***	0.08
Feed form x Particle size		NS	NS	**	NS	***	0.09	*	NS	*

[a,b,c] Means in a column not sharing a common superscript are significantly different (P < 0.05).

NS, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.

[1] Each value represents the mean of six replicates (eight birds per replicate).

[2] Each value represents the mean of 12 birds.

[3] Each value represents the mean of 36 pH readings (12 gizzards, three pH readings per gizzard).

XYLANASES ENHANCE NUTRITIVE VALUE OF SOYBEAN MEAL

Y.G. LIU[1] and A. PREYNAT[2]

Summary

Soybean meal (SBM) is the most widely used protein ingredient in poultry diets but its high content of non-starch polysaccharides (NSP, 22% in DM) impairs its nutritive value. A series of *in vitro* and *in vivo* studies was conducted to determine the degree of nutritive enhancement of SBM by a multi-enzyme preparation (mainly xylanases, with total 19 co-occurring enzyme activities from a single fermentation of Talaromyces *versatilis,* formerly Penicillium *funiculosum*), in comparison with a preparation rich in β-mannanases specifically targeting β-mannans in SBM. The *in vitro* studies showed that the xylanases complex enhanced (P<0.05) degradability of SBM dry matter from 32.0 to 36.9%; and hydrolysed 57.0% of the NSP extracted from SBM, whilst the β-mannanases did 51.8%. A broiler bio-assay, using semi-purified diets containing SBM as the sole natural ingredient, revealed the addition of the xylanases complex improved weight gain (5.8%) and feed conversion (6.3%, P<0.05), whilst the β-mannanases failed to generate any responses. Classical broiler metabolic studies confirmed that the xylanases complex can enhance the AMEn value of SBMby 4-6% whilst the responses to β-mannanases appear to be inconsistent.

I. NUTRIENTS AND ANTI-NUTRITIVE FACTORS IN SOYBEAN MEAL

Soybean meal (SBM) is an important source of digestible amino acids (dAA) and energy (AME). A typical broiler diet would have approximately 70% of digestible lysine (Table 1; Adisseo NIR survey, 2013) and 25% of the ME coming from SBM.

Table 1 - Range of protein and lysine contents in soybean meal (Adisseo Survey, 2013).

	Protein	Lysine	Lys Dig.	Dig. Lys
No. of Observations	161	161	161	161
Minimum, %	43.6	2.54	85.7	2.24
Maximum, %	50.9	3.01	90.5	2.66
1st Quartile, %	46.8	2.77	87.4	2.43
Median, %	47.8	2.83	88.0	2.50
3rd Quartile, %	48.5	2.89	88.8	2.55
Mean, %	47.6	2.83	88.1	2.49
S. D., %	1.39	0.08	1.02	0.08
C. V., %	2.9	2.9	1.2	3.1

On the other hand, SBM contains various anti-nutritional factors such as non-starch polysaccharides (NSP) that impair digestion and utilization of its nutrients. The NSP in SBM are complex oligomers and polymers representing 22% of dry matter, and composed of sucrose, raffinose, stachose, verbascose, pectin, cellulose and hemicellulose (Figure 1, Choct et al., 2010). These NSP can act as anti-nutrient in several ways (Fourie, 2007) by:

- increasing digesta viscosity -> reduce diffusion rate of nutrients and endogenous enzymes

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- increasing the size and solidity of the unstirred layer on the mucosal surface -> limited contact between digestive enzymes and their substrates
- encapsulating nutrients -> physical barrier between digestive enzymes and their substrates
- altering intestinal morphology
- buffering capacity of fibre -> reduction of gastric secretion

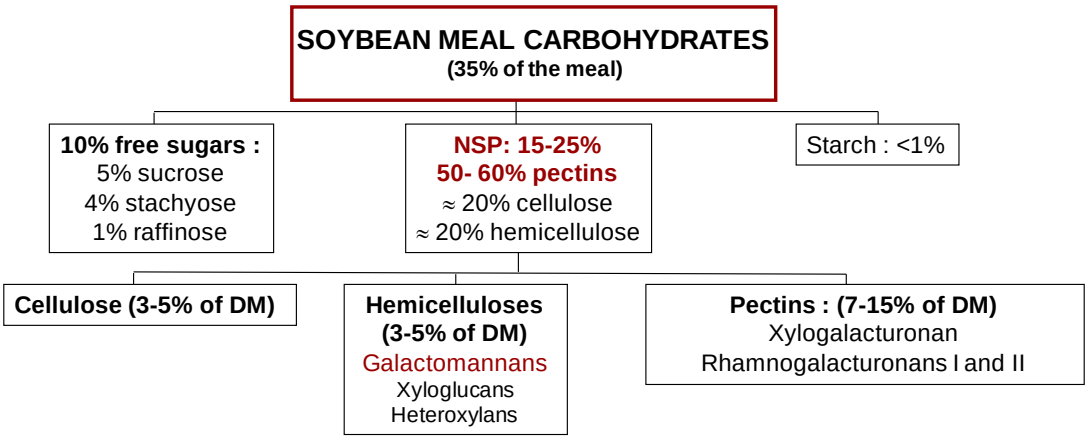

Figure 1 - Carbohydrates in soybean meal

II. MICRO-STRUCTURE OF SOYBEAN MEAL BEFORE AND AFTER INCUBATION WITH NSP-DEGRADING ENZYMES

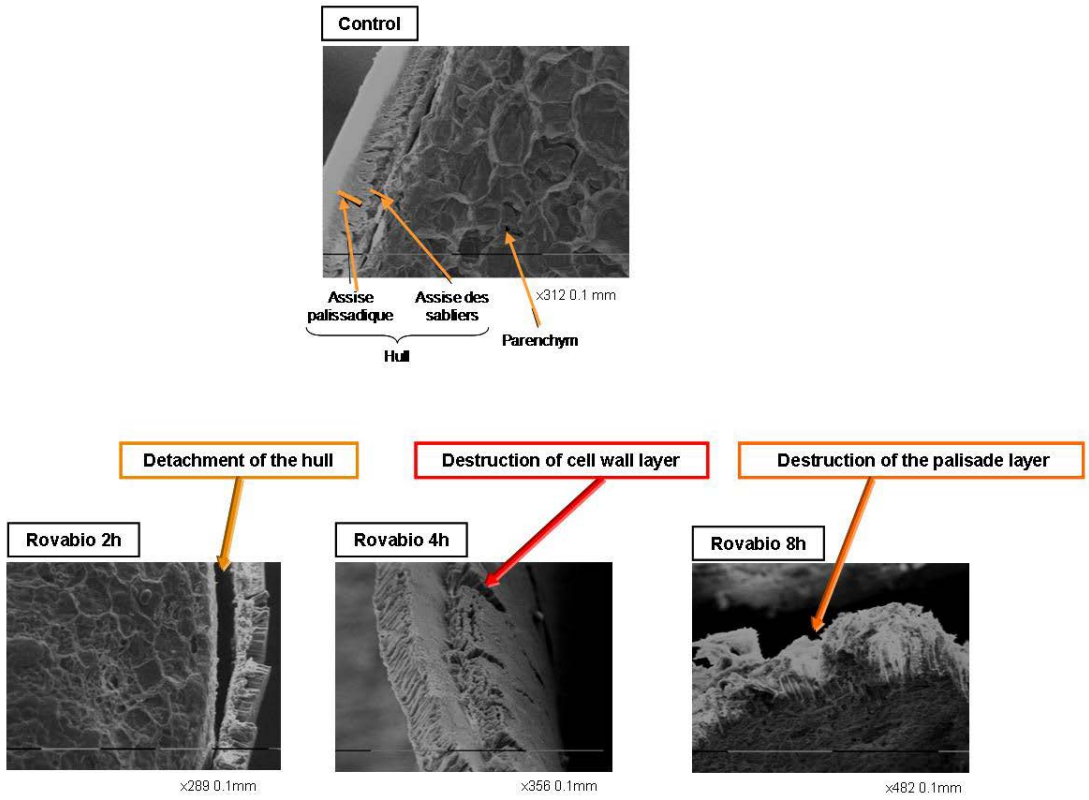

Figure 2 - Electron microscopy showing the cell walls of soybean meal and incubation with NSP-degrading enzymes (Rovabio® Excel) after 2, 4 and 8 h.

Extensive research has confirmed the efficacy of using exogenous enzymes in alleviating the negative effects of the anti-nutritional factors to enhance the utilization of both

energy and amino acids in SBM. Research also revealed that not all carbohydrate-degrading enzymes are able to break down the complex NSP in SBM effectively, yet finding an enzyme solution to match the structure of the carbohydrates remains a challenge. The authors screened a number of enzyme preparations and selected a preparation (Rovabio® Excel) from Talaromyces *versatilis* (formerly named and known as Penicillium *funiculosum*), which contains 19 co-occurring NSP-degrading enzymes, to facilitate breakdown of complex cell walls (Figure 2).

III. *IN VITRO* AND *IN VIVO* STUDIES ON NSP-DEGRADATION OF SOYBEAN MEAL

In order to enhance the nutritive value of SBM, two *in vitro* studies were conducted to investigate the degree of nutritive enhancement of SBM alone and its diets through the addition of a multiple NSP-degrading enzymes (Rovabio® Excel).

Study 1: In vitro hydrolyses of NSP extracted from SBM. Samples of defatted soybean flour were pre-treated with amylase, amyloglucosidase and acid, to remove starch and other soluble components. The portion of NSP obtained was incubated with either Rovabio® or β-mannanases according to a modified procedure of the Uppsala total dietary fibre method (Theander *et al*., 1995). The results (Table 2) suggest that both enzyme preparations hydrolysed the NSP extracts and the SBM-specific enzyme complex did not show any superiority over the xylanases.

Table 2 - Hydrolyses of soybean meal NSP by xylanases and β-mannanases.

	Hydrolysis, %	S. D., %
Xylanases	57.0	15.6
β-Mannanases	51.8	8.5

Study 2: In vitro degradability of dry matter of SBM. A total 10 SBM samples from different origins were incubated at pH 5.2, 40 °C for 2 h to compare degradability of dry matter without and with the addition of Rovabio®, with 12 replicates per treatment. The average degradability coefficient of the control was 32% while the enzyme addition enhanced degradation to 36.9% or 4.9 percentage units ($P<0.05$, Figure 3).

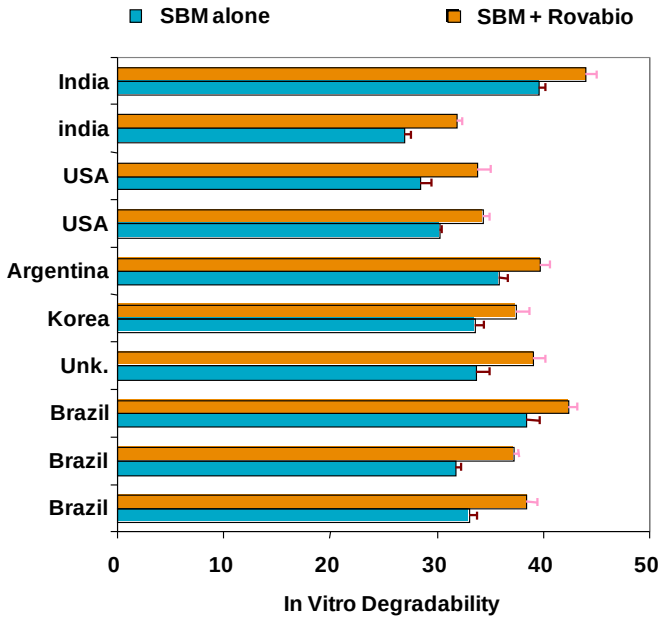

Figure 3 - Degradability (*in vitro*) of SBM before and after enzyme hydrolyses.

Study 3: In vivo ileal digestibility of SBM. Two soybean meal samples were force-fed to caecectomised cockerels to measure ileal metabolizable energy (IME), following the method described by Green et al. (1987), without and with Rovabio®. The results showed an average IME of of 2292 kcal/kg DM whilst the enzyme addition enhanced IME by 105 Kcal/kg DM or 4.5%.

Study 4: In vivo broiler bioassay of SBM. The objective of this study was to measure the efficacy of the two enzyme preparations on digestibility of SBM. Male broiler birds were fed on a semi-purified diet from 7 to 17 days of age. The diet contained SBM as the sole natural ingredient, the rest were dextrose, corn starch, soy oil, vitamins and minerals; crude protein 14% and ME 3200 kcal/kg. Each treatment had 8 replicates with 10 birds per replicate. In this bioassay, comparing with the control, the addition of xylanases (Rovabio® Excel) improved weight gain by 6.8% and feed conversion by 5.3% ($P<0.05$), whilst the β-mannanases failed to improve bird performance.

Table 3 - Broiler bio-assay showing enzyme-soybean meal interaction.

	Initial Wt (g)	Wt Gain (g)	Feed Intake (g)	FCR
Control	154.7	189.7	427.6	2.254[a]
Xylanases	154.7	202.6	430.5	2.134[b]
β-mannanases	154.8	194.2	428.8	2.212[ab]

Means without common superscript differ significantly (P<0.05).

Study 5: In vivo metabolic study of SBM diet. In a classical metabolic study, broilers were fed on wheat and barley based diets, with SBM inclusion ranging from 20 to 40%, with and without xylanases complex (Rovabio® Excel). Six diets were formulated by including combinations of ingredients and using a multiple regression model. AME of each raw material such as SBM was estimated through regression analyses. The results showed that when allocating ME value to each raw material, the enzyme addition improved SBM AMEn by 127 kcal/kg or 6% (2,129 to 2,256 kcal/kg DM).

Study 6: Broiler performance. The above findings were confirmed in performance trial where broilers were fed on reduced-energy, corn-based growing and finishing diets containing 30.4 and 25.2% SBM in the respective growing phases. The results showed that the inclusion of the xylanases complex (Rovabio®) improved 35-day live weight by +3.9% and feed conversion by 2.5%.

In conclusion, the NSP in soybean meal can be partially hydrolysed by xylanases complex, not particularly by β-mannanases. The scope of enhancement ranges from 4 to 6% in terms of dry matter degradability as well as metabolizable energy (AME), and broiler performance by 4 and 2.5%, respectively for weight gain and feed conversion.

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PERFORMANCE AND NUTRIENT DIGESTIBILITY IN BROILERS IN RESPONSE TO INCREASING DOSES OF PHYTASE

Y. DERSJANT-LI[1], A.L. WEALLEANS[1] and L.F. ROMERO[1]

Summary

The benefits of high phytase doses on the growth and feed efficiency of broilers have previously been demonstrated and they appear to be dependent on the ability of phytase to degrade phytic acid rapidly in the digestive tract. This study evaluated the ability of *Buttiauxella* or *E. coli* phytase to increase the growth and feed efficiency of broilers beyond traditional dose recommendations. In the first analysis, data from seven broiler trials were used to determine dose response relationships with a *Buttiauxella* phytase at inclusion levels ranging from 500 to 2000 FTU/kg. Phytase was added to a negative control (NC) diet that was deficient in available P, Ca and energy and a nutritional adequate positive control (PC) diet was used as a reference. A strong linear response ($P \leq 0.005$) in ADG, FCR and energy conversion was observed with increasing phytase level from 0 (NC) to 2000 FTU/kg in all phases. Overall 42 day data showed that 2000 FTU/kg phytase outperformed the PC with higher ($P < 0.05$) ADG and ADFI. Ileal P digestibility plateaued at 1000 FTU/kg. Bone ash was significantly reduced in the NC diet compared to the PC and was recovered in all phytase treatment groups. The best energy conversion ratio (MJ/kg BWG) was observed at 1000 FTU/kg, being 0.6 MJ (143 kcal) and 0.8 MJ (191 kcal) lower per kg BWG compared to the PC and NC, respectively. Phytase supplementation at 2000 FTU/kg reduced the rearing period to market size (3 kg live BW) by 2.5 days compared to the PC. In the second analysis, data from four trials were analysed to compare the efficacy of *Buttiauxella* and *E. coli* phytases; each phytase was included at 250, 500 and 1000 FTU/kg, respectively, in a NC diet that was deficient in available P, Ca, and energy, with respective PC diet as a reference. Both phytases improved ($P < 0.05$) growth and feed efficiency of broilers compared to the NC. *Buttiauxella* phytase at 1000 FTU/kg improved growth and reduced body weight corrected feed conversion (FCRc) by 4 points compared to the PC, and improved efficacy compared to *E. coli* phytase. It can be concluded that high doses of phytase at 1000-2000 FTU/kg can be beneficial in broiler production.

I. INTRODUCTION

Phytase has traditionally been used in broiler feed at a standard dose of 500 FTU/kg, but recent studies have reported extra phosphoric effects of high phytase doses in broilers (Selle and Ravindran 2007; Amerah et al., 2014). It is well known that phytate, the main phosphorous source in plant ingredients, is poorly available to poultry. Phytate can bind amino acids and minerals and reduce utilization of these nutrients. High phytase doses can further reduce the anti-nutritional effect of phytate and improve digestibility of amino acids and energy, resulting in improved animal performance. The objective of this study was to evaluate: 1) dose response of a new generation phytase (derived from *Buttiauxella spp*.) on performance and nutrient digestibility in broilers; 2) compare the efficacy of *Buttiauxella* phytase with an *E. coli* phytase, based on pooled data from several trials carried out by Danisco Animal Nutrition/DuPont.

II. MATERIALS AND METHODS

In the first analysis, data from seven trials were collected in a database, including five treatments: PC, NC and NC supplemented with 500, 1000 and 2000 FTU/kg *Buttiauxella* phytase. PC diets were formulated to meet nutritional requirements. NC diets were formulated with lower levels of

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energy, available P, and Ca (average 0.2 MJ/kg ME, 0.17 % available P and 0.15 % Ca lower compared to PC). Ross broilers were used and fed diets based mainly on corn and soybean meal. Average daily feed intake (AFDI), daily weight gain (ADG) and feed conversion ratio (FCR, g feed/ g gain) were analysed for starter phase (0-21 days) and the overall period of 0-42 days. Due to a significant difference of ADG between the PC, NC, and phytase treatments, body weight corrected feed conversion (FCRc) was calculated by correction of 3 points (0.03) for each 100 g body weight difference compared to the PC, in order to standardize the data for accurate comparison of treatment means. In addition, energy conversion was calculated to compare energy efficiency between treatment groups, as phytase was supplemented to the low energy NC diet.
In the second analysis, individual data from four trials were collected in a database, including eight treatments: PC, NC and NC supplemented with *Buttiauxella* or *E. coli* phytase at 250, 500 and 1000 FTU/kg. PC diets were formulated to meet nutritional requirements. NC diets were formulated with lower levels of energy, available P, and Ca (average 0.2 MJ/kg ME, 0.17% available P and 0.16% Ca lower compared to PC).

Outlier removal was conducted and Tukey's HSD test was used for means separation in the Fit Model platform of JMP 11 (SAS Institute Inc., Cary, NC, 1989-2013). Dose response from 0 (NC) to 2000 FTU/kg phytase was analysed using linear and quadratic models. The trial code was included in the model as a random effect, as this accounted for the underlying heterogeneity between studies (Lean et al., 2009).

III. RESULTS AND DISCUSSION

a) Dose response

Results on performance parameters during starter (0-21 d) and overall (0-42 d) phases are presented in Table 1. Increasing phytase inclusion level linearly improved ADG and FCR in both starter and overall phases. Overall performance data showed that 2000 FTU/kg phytase improved ADFI and ADG compared to the PC, and reduced body weight corrected FCRc by 3.4 and 18.6 points compared to the PC and NC, respectively. The NC had lower ($P < 0.05$) bone ash (%) compared to the PC, but all phytase treatment groups recovered bone ash to a level similar to the PC. The best energy conversion ratio was with 1000 FTU/kg phytase, which was 0.6 MJ (143 kcal) and 0.8 MJ (191 kcal) lower per kg body weight gain than the PC and NC, respectively. Supplementation of 2000 FTU/kg phytase reduced rearing days to reach a market size of 3 kg live body weight by 2.5 (Table 1). Reducing available P in the NC diets resulted in a decrease ($P < 0.05$) of ileal P digestibility (%) compared to the PC. Increasing phytase dose improved ileal P digestibility in both a linear and quadratic manner. However, it seems that the ileal digestibility of P and bone ash reached a plateau at 1000 FTU/kg phytase in broilers fed mainly corn and soybean meal based diets containing 0.24-0.27 % phytate. The further improvement on performance at 2000 FTU/kg phytase may indicate some extra phosphoric effect.

b) Comparison of two phytases

The results of the comparison of *Buttiauxella* with *E. coli* phytase are presented in Figure 1. NC diets showed reduced ($P < 0.05$) ADG, bone ash and increased FCRc compared to the PC. All inclusion levels of both phytases improved ADG and FCRc compared to the NC; no differences were seen compared to the PC, except with *Buttiauxella* phytase at 1000 FTU/kg, which significantly ($P < 0.05$) improved ADG and reduced FCRc (by 4 points) compared to the PC. Phytase at 500 and 1000 FTU/kg recovered bone ash to a level similar to the PC, even though phytase supplemented diets were 0.17 % and 0.15 % lower available P and Ca, respectively. In comparison, *Buttiauxella* phytase increased ($P < 0.05$) ADG and reduced FCRc ($P < 0.05$) compared to *E. coli* phytase, but there were no differences in ADFI and bone ash.

Table 1 - Effect of increasing phytase supplementation to the NC diet (with low energy (0.2MJ/kg), available P (0.17%) and Ca (0.15%)) on performance of broilers[1].

		PC	NC	*Buttiauxella* Phytase dose, FTU/kg			P value	
				500	1000	2000	Linear	Quadratic
Starter	ADG, g	39.0a	32.3b	38.5a	39.3a	40.0a	0.0009	0.1300
	ADFI, g	52.6a	46.6b	52.3a	52.5a	53.6a	0.1400	0.6400
	FCR	1.36b	1.47a	1.37b	1.34b	1.35b	0.0003	0.0150
	FCRc*	1.36b	1.51a	1.37b	1.34b	1.34b	<0.0001	0.0008
	E conv**	17.5b	18.7a	17.3b	17.0b	17.1b	0.0050	0.0002
	Ileal dig P, %	60.4b	53.2c	70.2a	74.0a	73.6a	<0.0001	<0.0001
Overall	ADG, g	71.3b	63.9c	72.9b	73.7ab	75.9a	<0.0001	<0.0001
	ADFI, g	120.9b	112.0c	125.2b	124.8b	130.2a	<0.0001	0.0009
	FCR	1.694b	1.753a	1.718b	1.694b	1.716b	0.0700	0.0050
	FCRc*	1.694b	1.846a	1.700b	1.665b	1.660b	<0.0001	<0.0001
	E conv**	22.6ab	22.8a	22.3ab	22.0b	22.3b	0.0700	0.0044
	Bone Ash, %	35.6a	31.0b	34.5a	34.7a	34.4a	0.3230	0.3810
	Prod day***	42.0	46.9	41.2	40.7	39.5		

[1] Starter performance: 7 trials; overall performance: one trial; ileal P digestibility: 3 trials, bone ash: 5 trials
[a,b,c] Values within rows with the same superscript are not significantly different (P < 0.05)
* FCRc: correction of 3 points for each 100g body weight difference from PC.
** Energy conversion: MJ/ kg body weight gain
***Production day: days needed to produce 3000g live BW broilers estimated based on ADG

The data from both analyses demonstrated a clear benefit of using increasing doses of phytase in broiler feed. The improved performance and feed/energy efficiency at the high doses indicated an extra phosphoric effect, for which there are several possible mechanisms of action. First, increasing phytase level can increase the degradation of phytate and reduce its anti-nutritional effects, including an increase in amino acid digestibility (Amerah et al., 2014). Secondly, phytase can reduce endogenous nutrient losses (Cowieson et al., 2008), which may also contribute to improved nutrient efficiency. In addition, phytase may have an impact on the ratio of protein and starch digestion rate, which may influence passage rate and feed efficiency (Selle, personal communication). Another factor that may be involved is the effect of phytase on sodium and glucose metabolism (Truong et al., 2014). Recently it has also been suggested that inositol released from phytate degradation may contribute to the extra phosphoric effect; however, the extent to which released inositol may influence a broiler's performance needs further evaluation. The high efficacy of *Buttiauxella* phytase compared to *E. coli* phytase is in line with other publication (Plumstead et al., 2012). This may be explained by the fact that *Buttiauxella* phytase is more effective at a lower, broader pH range, resulting in a high degree of phytate degradation in the upper GI tract of animals. A recent study showed that phytate degradation was up to 88 % in broilers fed corn and soybean meal based diets supplemented with 1000 FTU/kg *Buttiauxella* phytase (Amerah et al., 2014).

IV. CONCLUSION

Buttiauxella or *E. coli* phytase at a level equal or above 500 FTU/kg replaced 0.17 % available P, 0.15 % total Ca and 0.2 MJ/kg ME in broiler diets and maintained performance similar to the PC. Phytase supplementation at 1000-2000 FTU/kg can further improve ADFI, ADG, feed and energy efficiency, and reduce rearing days to reach market size in broilers fed a low energy, available P and Ca diet. This indicates an extra phosphoric effect which provides potentially increased economic benefits. Both phytases were effective in improving performance of broilers;

however, *Buttiauxella* phytase had a significantly higher efficacy (FCRc, $P < 0.05$) than *E. coli* phytase at 500-1000 FTU/kg.

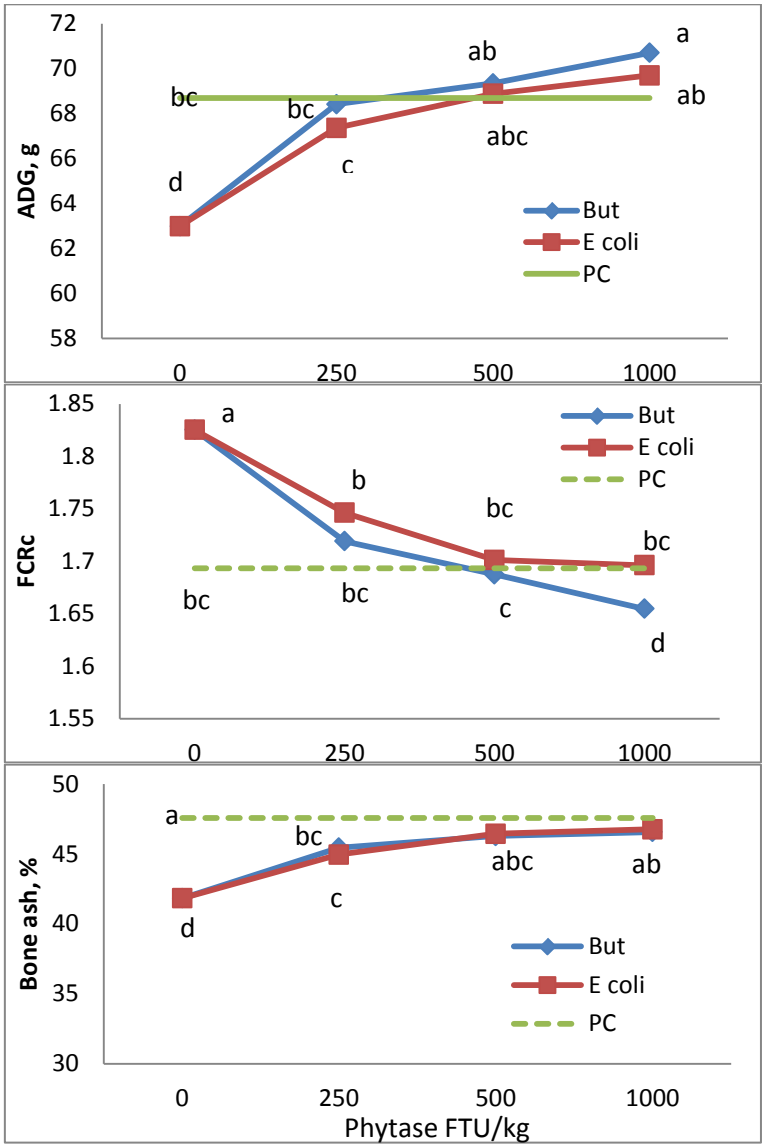

Figure 1 - Comparison of Buttiauxella (But) and E. coli phytases on performance and bone ash in broilers fed test diets for 42 days, based on statistical analysis of data from 4 trials.

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IMPACT OF PROCESSING CONDITIONS ON AMINO ACID DIGESTIBILITY OF EXPELLER-EXTRACTED CANOLA MEAL FOR BROILER CHICKENS

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Canola meal (CM) is reported to have lower crude protein (CP) and lower and more variable amino acids (AA) and digestibility than soybean meal (Khajali and Slominski, 2012). Chemical composition and nutrient digestibility of expeller-extracted canola meal (ECM) for broiler chickens has previously been characterized and compared with solvent extracted canola meal (Woyengo et al., 2010), but the possible impact of processing conditions has not been verified. The present study was conducted to characterize the effect of canola processing conditions on CP and AA digestibility of ECM for broiler chickens. Six ECM samples produced under different seed conditioning temperature (90, 95 or 100 °C) and second press screw torque (low or high) conditions were evaluated in a 3 × 2 factorial arrangement of treatments to determine the effect of processing on apparent and standardised CP and ileal AA digestibility in 24 d-old broilers. The ECM samples were incorporated into semi-purified diets to provide 200 g/kg CP, with each of the 6 canola meal samples supplying the entire CP fraction of the diet. A nitrogen free diet was also fed to determine the endogenous AA flow. Each diet was fed for 5 d to 6 replicate cages of 7 chicks and ileal samples were collected at 24 d.

Conditioning temperature by screw torque interactions were detected ($P < 0.05$) for apparent ileal digestibility (AID) of CP, Arg, Lys, Thr, Asp, Glu and Ser. Meals subjected to 95 °C conditioning temperature, irrespective of screw torque had the greatest ($P < 0.05$) AID of CP and total AA. The standardised ileal digestibility (SID) values followed a similar pattern as AID; however, when corrected for endogenous losses, the average AID coefficients of total AA improved by approximately 2.0%. The highest AID was observed for Arg, His, Met and Glu while Thr, Cys and Pro had the lowest AID. The ranking order of AA digestibility for SID estimates followed the same pattern as AID with Arg, His, Met and Glu having the highest and Thr, Cys and Pro representing the lowest digestible AA. The largest increase in AID when standardised was observed for Phe (3.7 %), Thr (3.6 %), Asp (3.1 %) and Ser (3.2 %). A negative correlation was detected between NDF (neutral detergent fibre) and NDIN (neutral detergent insoluble nitrogen) content of the meals and SID estimates of Lys (r = -0.79, r = -0.76; P = 0.001, respectively).

These results indicate that processing affects CP and AA digestibility of ECM, likely because of alterations to the chemical composition and formation of indigestible complexes of AA with fibre. Additionally, with regard to the processing conditions, canola seed is subjected to for oil extraction, pre-press conditioning temperature has a greater impact on ECM amino acid digestibility than expeller screw force.

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RESPONSE OF BROILER CHICKENS TO RISING LEVELS OF SPRAY-DRIED PORCINE PLASMA

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Broiler chicks have been shown to benefit from immediate access to feed. Although the focus of early nutrition has been on provision of energy, chicks would benefit from a more balanced nutrient profile, particularly amino acids. Spray-dried porcine plasma (SDPP) and similar products have been used by the pig industry to support piglets prior to and after weaning (Tucker *et al*., 2011). The objective of the present study was to assess SDPP as a supplement for broiler chicks at the starter phase and measure subsequent performance. Four levels of SDPP inclusion - 0, 5, 10 or 20 g/kg diet were tested in starter diets based either on maize or wheat diets. The diets were identical in nutrient profiles and formulated to meet breeder specifications. Four hundred and eighty Ross 308 day-old male chicks (initial weight, 41.0±0.92 g) were randomly allocated to the diets, which were fed from hatch to 10 days, after which the chicks were transferred to commercial-type grower (11-24d) and finisher (25-35d) diets. Data were regressed, with SDPP level as factor.

Table 1 - Feed intake (g/bird), live weight (g) and FCR (g feed/g weight gain) of birds between hatch and 35d of age after placement on starter diets containing different levels of SDPPP.

		Age	SDPP levels %				
Cereal		(days)	0.0	0.5	1.0	2.0	SEM
Maize	Feed intake	1-10	337.4	327.7	327.8	334.4	3.18
		1-35	4144.0	3957.8	3923.6	4029.0	41.49
	Body weight	10	320.6^{b}	332.0^{ab}	341.2^{a}	345.6^{a}	2.72**
		35	2608.7^{ab}	2544.4^{b}	2629.4^{ab}	2676.0^{a}	15.28*
	FCR	1-10	1.20^{a}	1.13^{b}	1.10^{b}	1.09^{b}	0.009**
		1-35	1.61^{a}	1.58^{ab}	1.52^{b}	1.53^{b}	0.012*
Wheat	Feed intake	1-10	329.5	343.5	326.0	332.4	3.13
		1-35	4079.3	4039.1	3940.8	3923.3	45.27
	Live weight	10	316.1^{b}	347.8^{a}	344.4^{a}	352.5^{a}	2.24***
		35	2538.1^{b}	2578.4^{ab}	2567.9^{b}	2735.4^{a}	27.61*
	FCR	1-10	1.20^{a}	1.12^{b}	1.07^{c}	1.07^{c}	0.008***
		1-35	1.64^{a}	1.60^{a}	1.56^{a}	1.46^{b}	0.014***

a,b – Mean values on the same row not sharing a superscript are significantly different (*P < 0.05; **P < 0.01; ***P < 0.001).

Feed intake to 10 or 35 days, on either diet type was not affected by starter level of SDPP (Table 1). Rising levels of SDPP resulted in increased body weight at 10 ($P < 0.01$) and 35 ($P < 0.05$) days in birds fed on the maize-based. Body weight was also increased by the supplement in weight-based diets, and this was significant for 1-10 days ($P < 0.001$) and 1-35 days. On the wheat-based diet, SDPP reduced ($P<0.001$) FCR for the two periods assessed while on the maize-based diet, there was a reduction in FCR between hatch and 10 days ($P < 0.01$) and between hatch and 35 days ($P < 0.05$). There were no significant effects of treatments on visceral organ weights at 24 days or carcass yield at 35 days of age. The results demonstrate that the supplement could be included at between 5 and 20 g/kg of starter diets, depending on the diet cost and rate of return. The mechanisms behind its gross effects are currently being investigated.

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XYLANASE CAN IMPROVE PERFORMANCE OF BOTH A SIMPLE WHEAT/SOYA DIET AND A MORE COMPLEX DIET IN BROILERS

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Whilst xylanase rich NSP-enzymes are used consistently in wheat based diets the question remains whether the positive response seen can be ascribed solely to the xylanase component or whether other enzyme activities are involved. This question becomes more pertinent when a variety of materials is used in the compound diet, potentially requiring a variety of enzyme activities. A trial was therefore set up to investigate whether five commercial xylanase based enzyme products, based on different source and/or production organisms and differing in declared main component activities, would give different rankings in two different wheat based diets.

2112 male Ross 308 day old broiler chicks were weighed and 22 per floor pen (2.2 m^2) were assigned to one of 8 replicates of 12 treatments according to a 2*6 factorial design. Two wheat based diets were formulated, one was a simple diet (simple) based on soybean meal and the second was a complex diet (complex) with a variety of feed ingredients (peas, distillers dried grains, sunflower meal, rapeseed meal) as well as soybean meal. Typical nutrient levels according to Schothorst recommendations were used, with the AME level reduced to allow the usage of the lower energy by-product materials. All diets contained phytase (500 FTU/kg, Quantum, AB Vista) and coccidiostats (Nicarbazin/Narasin in starter and Narasin in grower and finisher). Diets were supplemented with either no NSP enzyme or a standard dose of one of the five xylanase based enzyme products.

Birds were fed a pelleted (2.3 mm) starter (0-14d of age), pelleted (3.0mm) grower (14-35d) and finisher (35-42d) diet; both feed and water were offered *ad-libitum*. Weight and feed intake were determined and FCR calculated at 35 and 42 days of age. At 25 d of age ileal viscosity was measured in two birds per pen and at 28d of age the condition of the foot pads (0-4, 0=no lesions and 4=severe lesions) was assessed in 10 birds per pen. Litter quality (0-10, 0=wet and 10=dry and friable) was assessed at day 21 and day 35. Data were analysed by ANOVA with main effects of diet and NSP enzyme and the interaction tested.

Dietary nutrient contents and in feed enzyme assays were within acceptable limits of the targets. *In vivo* viscosity measurements showed no effect of enzyme addition, with all measured values being low (2.81 cps averaged across all treatments) indicating that the wheat used in the basal diets was a low viscosity wheat. At 35 days of age FCR was improved by only one of the enzyme products, a single xylanase product (1.483 cf 1.517 for the control; P=0.005). At 42 days of age there was an interaction between diet type and enzyme product (P<0.001) for FCR. One single xylanase product gave a significant improved FCR in both diet types (simple: 1.600 cf 1.642; complex: 1.618 cf 1.643) whilst in the simple diet three of the products showed no significant improvement and in the complex diet one of the other products showed no significant improvement. Litter quality was significantly (P<0.001) affected by diet type at both 21 and 35 days of age, with quality being higher for the simple diet at 21 days (7.0 cf 5.9) whilst at 35 days this was reversed (5.9 cf 6.7), but there was no effect of enzyme addition. Neither diet type nor enzyme treatment affected foot pad scores.

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EFFECT OF NUTRITIONAL EMULSIFIER ON FEED EFFICIENCY IN BROILERS FED DIETS BASED ON TWO DIFFERENT FAT COMPOSITIONS

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Energy is a major cost component in broiler diets and fats and oils are important energy sources. The digestibility of fat depends on different characteristics of the fat, like ratio unsaturated/saturated (U/S) fatty acids and level of free fatty acids (FFA) (Gu and Li, 2003). Nutritional emulsifiers can be used to improve fat digestibility and thus improve the energy efficiency and lower feed costs. It has been shown that a nutritional emulsifier with a very high hydrophilic-lypophilic balance (HLB) can improve fat digestibility and improve AME values in European diets (Maertens et al, 2013). A trial was designed to evaluate if this effect can be confirmed in diets with different fat sources, commonly used in Latin American diets.

The study was conducted at the experimental poultry farm of Integracion y Desarrollo Agropecuario, Michoacan, Mexico. The effect of a nutritional emulsifier was tested in two different diets. The ingredient composition of both diets was similar, except for the fat source. Diet 1 was based on yellow grease (blend of vegetable oils) with a U/S ratio of 2.1 and 12% FFA. Diet 2 was based on a mix of 20% palm and 80% acidulated soya oil with a U/S ratio of 3.0 and 48% FFA. The added fat was 3.9%, 5.5% and 5.15% of the diet and the calculated metabolizable energy was 12.34MJ, 12.87MJ and 13.08MJ in starter, grower and finisher diets respectively. Both diets were formulated with or without a nutritional emulsifier (Excential Energy Plus) at a dosing of 350 grams/MT in each phase. Each treatment had 450 birds (Ross 308) with 9 replicates of 50 birds per pen. The results were statistically analysed by factorial 2 x 2 ANOVA.

Table 1 - Bodyweight (BW), Feed intake (FI) and Feed conversion ratio (FCR) for the period (d1-42).

	Emulsifier	BW	FI	FCR
Diet 1	No	2785	4796	1.748
Diet 1	Yes	2828	4744	1.702
Diet 2	No	2727	4671	1.738
Diet 2	yes	2753	4640	1.710
SEM		8	15	0.005
P values				
Diet type		<0.001	<0.001	0.949
Emulsifier		0.003	0.070	<0.001
Interaction		0.413	0.647	0.275

Dietary supplementation of the emulsifier improved significantly bodyweight ($p = 0.003$) and feed conversion ($p < 0.001$) in both diet types. The improvement in feed conversion was 2.6% and 1.6% in diet 1 and diet 2, respectively.

These results support the hypothesis that a nutritional emulsifier may improve feed efficiency in broilers, moreover this was observed under different diet compositions. The practical application of this trial is that it shows a tool to improve feed efficiency that may lead to lower feed costs and more sustainable broiler production.

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THE *SALMONELLA* INITIATIVE

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An enduring challenge and reputational issue for the egg industry is caused by the presence of *Salmonella enterica* serovars (particularly some Typhimurium serotypes), which can cause salmonellosis in humans, throughout the supply chain. The presence and spread of *Salmonella* depends on numerous variables, and as such, there is no single effective control measure. Therefore, the presence and control of *Salmonella* are complex issues that are subject to a combination of both real and perceived risks.

There is an abundance of peer-reviewed and grey-literature (related to IP) regarding many aspects of *Salmonella*, however the type and availability of this information varies within egg production and consultancy companies. The phrases "everything is known and we don't need to fund more work" or "we did this work 30 years ago" are heard often during industry meetings to discuss *Salmonella* project proposals. Yet the challenge of *Salmonella* remains on-farm and in the market place. Therefore, there is an obvious need to collate readily available information and make it more accessible to the entire industry to provide a framework for the application of knowledge.

Currently, the level of understanding of the issue (i.e. the cause as well as the effectiveness of various controls) is variable within industry, government regulators, the food service sector, and the consumer community. Recent R,D&E activity and literature reviews in preparation by the AECL Council for Sustainable Egg Farming (CSEF) are expected to provide a robust body of knowledge and a clear description of the risks and possible management options of egg related salmonellosis illness in Australia.

As elimination of *Salmonella* on egg farms is impossible, the risk of human illness needs to be appropriately managed. However, as this is a technically complex issue with varying risks (and varying understandings of these risks) between on-farm and in food service, an effective management strategy will need to be a collaborative effort that relies on the development of good relationships between industry and government agents / regulators, be founded on robust information and involve a system (or systems) to control highest risk S*almonella enterica* serovars at various stages in the supply chain.

Key stakeholders, including state regulators/health departments, industry associations, egg producers, researchers and technical consultants (including veterinarians) are involved in the process with the aim to build relationships and guide program development. Coordination, review and dissemination of comprehensive, yet coherent, information regarding *Salmonella* is currently underway with the aim to draft a risk management plan for the control of *Salmonella* through the entire egg supply chain. This management plan needs to be robust and measurable (i.e. it relies on data and can be audited if required), able to be updated easily to include new information as it becomes available and also include options for industry uptake and development. Outcomes will also include the development of training materials and control documents for various stages of the supply chain which focus on key issues and support a management program that is both applicable to, and comprehensible by, regulators and producers.

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SALMONELLA TYPHIMURIUM INFECTION IN LAYING HENS

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Summary

The experiment was conducted to study the prolong shedding of *Salmonella* Typhimurium DT 9 in laying hens after infection. The influence of co infection with S. Mbandaka on shedding of S. Typhimurium DT 9 was also investigated. Birds were raised from day old in Salmonella free environment by maintaining strict biosecurity standards. *Salmonella* shedding was detected until 5 weeks post infection. Further work to investigate vertical transmission of *Salmonella* Typhimurium DT 9 in laying hens is in progress.

I. INTRODUCTION

Salmonella a major zoonotic foodborne pathogen causing gastric illness worldwide. Egg or egg product related *Salmonella* poisoning is a major concern for the egg industry. *Salmonella* Typhimurium (S. Typhimurium) is the most frequently reported serovar in egg related food poisoning outbreaks in Australia (The OzFoodNet Working Group, 2013). It has been studied that *Salmonella* serovars such as Enteritidis has the capacity to contaminate developing eggs within the oviduct; however the vertical transmission ability of Australian S. Typhimurium strains has not been investigated. Older birds are also considerably more resistant to *Salmonella*e than are young chicks. Shedding of *Salmonella*e in chicken faeces can be intermittent and may continue for many months (Lister and Barrow, 2008). *Salmonella* infection has been studied in laying hens, however low level of *Salmonella* colonization in adult birds has been reported (Deguchi *et al*. 2009). In Australia egg product related cases of food borne poisoning are associated with S. Typhimurium definitive type (DT) 9. Persistence of S. Typhimurium DT 9 in the poultry shed environment could cause egg shell contamination (Gole et al., 2014). The objective of this study was to study the prolong shedding of S. Typhimurium DT 9 in laying hens after infection. The influence of co infection with S. Mbandaka on shedding of S. Typhimurium DT 9 was also investigated.

II. MATERIALS AND METHODS

Fertile eggs were obtained from commercial Hyline brown layer parent flocks. Eggs were fumigated and incubated in the lab over the period of 21 days at 100.4 ^{0}F with relative humidity of 45-55 % up to day 18 and then 55-65% up to hatching. The chicks (n=32) were hatched at day 21 and housed in positive pressure rooms at Roseworthy campus, at the University of Adelaide. These rooms were previously decontaminated twice with F10 and then fumigated with formaldehyde. All animal pens, cages, trays, feeders, equipment, floor and walls of the rooms were extensively cleaned with FoamCleanS (Chemtel, Australia) followed by a wash in SaniGuard (Chemtel, Australia). Waterlines were washed using acid solution. All equipment was then moved into each of two rooms and then rooms were again fumigated with SaniGuard (Chemtel, Australia). Feed was sterilised by either gamma irradiation by SteriTech or by fumigation with formaldehyde. Water was sterilised by the addition of water purification tablets (Aquatabs, Irelands). *Salmonella* positive or negative status of the birds was monitored by testing faecal, water and feed samples at fortnightly interval before *Salmonella* challenge. All samples were processed for *Salmonella* isolation by

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culture method as described previously (Gole et al 2014). All birds were raised in pens up to week 10. At week 10, birds were divided in three groups and transferred in to cages in three different rooms. At week 14, First group (T) received 10^9 CFU of *S*. Typhimurium DT 9, second group (MT) received 10^9 CFU of S. Mbandaka and *S*. Typhimurium DT 9 and control group (C) received sterile broth. Faecal samples were collected at day 0, 1, 3, 6, 9 and 12days post infection (p.i.) and then followed byweeks 3 and 5 p.i. Faecal samples were processed for enumeration of *Salmonella* by three tube most probable number (MPN) method as described by Pavic *et al*. (2010). The susceptible *Salmonella* colonies were streaked onto Xylose lysine deoxycholate agar (Oxoid, Australia) or *Salmonella* brilliance agar (Oxoid, Australia). MPN data was analysed by ANOVA. Over the experimental period, workers used sterilised overalls, head-covers, shoe covers, masks, and gloves while working with the chickens. All housing procedures and experiments were performed according to animal ethics protocol approved by the University of Adelaide (UA) Animal Care and Use Committee.

III. RESULTS AND DISCUSSION

All birds tested negative for *Salmonella* spp prior to infection. During first week p.i., blood tinged mucous was recorded in faeces of some infected birds from both T and MT groups. This clinical sign was not recorded from two week p.i. and onwards. Birds from control group did not show any clinical signs. Mortality was not recorded in any of the infected groups. A significant difference in Salmonella shedding was observed between treatment groups, however there was no significant differences between days p.i.. Also significant interaction was not recorded (Figure 1).

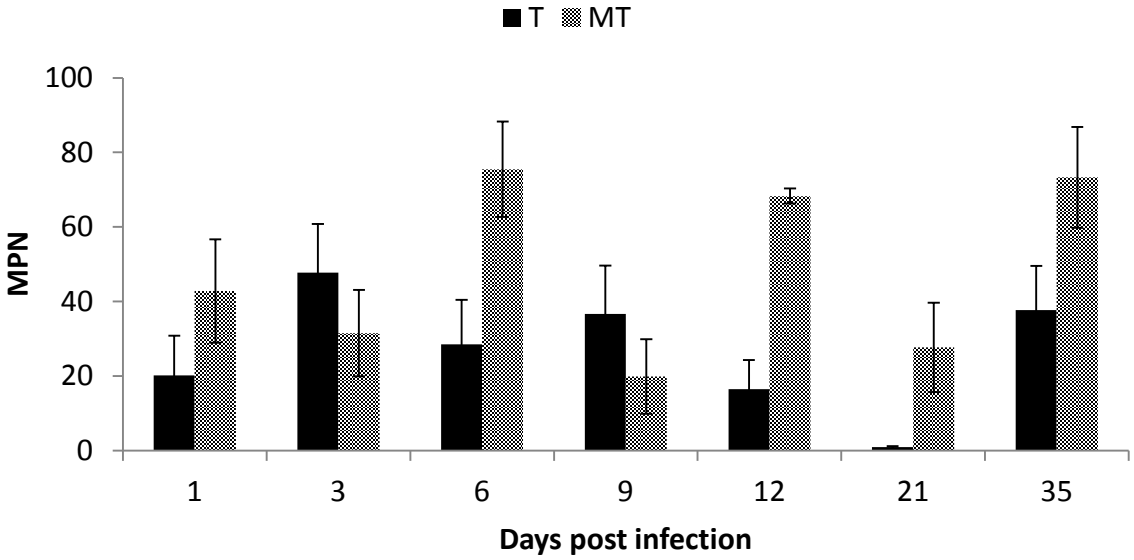

Figure 1- Salmonella shedding over 5 weeks post infection**.**

Our findings regarding the intermittent and prolong shedding of *Salmonella* are in agreement with Lister and Barrow (2008) and Gole *et al*. (2014). The experimental trial is still in progress and further work is aimed to study the salmonella shedding and ability to cause horizontal and vertical transmission by Salmonella up to week 16 p.i. To the authors knowledge this is the first Australian study examining the prolong shedding of S.Typhimurium DT 9 in laying hens.

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AN EXAMINATION OF EGGSHELL PORE STRUCTURE AND PENETRATION BY *SALMONELLA* TYPHIMURIUM

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The horizontal infection of table eggs by food-poisoning causative agents such as *Salmonella* is a serious concern for consumers and industry. While there are some factors that are associated with increased rates of infection the mechanism of bacterial entry remains unknown. The use of Computed Tomography (CT) enables the production of transverse images of the shell's interior. This imaging has allowed for identification of alternative pore structures, branching pores have been found, and both internally and externally branching pores are present in egg shells of laying hens.

Using the agar egg penetration method first described by Board and Board (1967), the contents of 208 eggs were removed and the shells filled with molten XLD agar. The eggs were then dipped in a 10^6 CFU/mL solution of *Salmonella* Typhimurium and allowed to incubate at 37ºC for 3 or 6 days. Eggshell samples from penetrated, non-penetrated and control eggs were prepared, broken into small samples (10 mm by 10 mm) and dried in containers containing silica gel desiccant. The samples were then scanned by a GE Phoenix V-Tomex micro CT scanner using the 'micro' tube at 80kV, 180mA and 70x magnification. Scans were then reconstructed with GE Phoenix datos X reconstruction software and images were analysed with VG Studio Max (2.0.5) software. To insure uniformity, the sample regions were isolated to a 1 mm radius sphere (4.19 mm^3). Transverse images were scored from two perpendicularly opposed angles.

Table 1 - Types of shell pores present in penetrated and non-penetrated shells.

	Average No. of Pores per sample area (4.19 mm^3)				
Sample Result	Straight	Internally Branching	Externally Branching	Total Pores	CT Measured Shell Thickness
Penetrated	2.49	0.08	1.36	3.9	0.37
Non-Penetrated	2.33	0.02	1.54	3.93	0.37

No significant differences in the incidence of types of pore structure or CT measured shell thicknesses were found in the penetrated and non-penetrated shell samples. All measures were very similar except for the number of internally branching pores (0.08 and 0.02).

These results indicate that different pore structures, the total number of pores and the shell thickness do not appear to play a role in the horizontal infection of eggs by *Salmonella* Typhimurium. Messens et al. (2005) using a similar method found a trend of increasing shell penetration with decreasing shell thickness (P=0.063). A number of shell structures including shell pores and mammillary shell defects have been related to the appearance of shell translucency. Eggshell translucency has also been related to increased rates of bacterial penetration; these results indicate that if translucency has a role in bacterial entry, it may be due to the structural abnormalities in the shells mammillary layer (Chousalkar et al., 2010).

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PRELIMINARY ANALYSIS OF THE DURATION OF PROTECTION OF VAXSAFE® ST VACCINE AGAINST SALMONELLA SHEDDING IN LAYERS.

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Summary

Vaxsafe® ST (Bioproperties Pty Ltd) is an *aro*A deletion mutant vaccine for the control of *Salmonella* Typhimurium in chickens. It is registered for spray and drinking water applications and has been shown to aid in the control of Salmonella in short lived birds (such as broilers). The main objective of this study was to test efficacy of Vaxsafe® ST with a new vaccination program aimed at lengthening the duration of immunity for the full productive life of layers. The first dose was delivered by spray on chicks at one day of age followed by two doses administered orally at 2 and 6 weeks of age (woa) respectively, and a fourth dose administered by intramuscular (IM) injection at 10 woa. All vaccination doses were administered at 10^7 cfu/dose. For the fourth IM dose, the vaccine was delivered in two different formats, mixed in diluent or in a mixture with Nobilis® EDS+ND vaccine (Intervet). At 16, 30, 45 and 65 woa respectively (that is at 6, 20, 35 and 55 weeks post IM vaccination) a sample of the vaccinated birds, was transported to a research facility and challenged with wild type *S.* Typhimurium (ST) or *S.* Infantis (SI). Salmonella shedding of vaccinated birds were compared with a control unvaccinated (challenged) group in order to assess efficacy of the vaccine. Vaxsafe® ST delivered in either diluent or with Nobilis® EDS+ND killed vaccine, provided protection against strong homologous (ST) and heterologous (SI) challenges compared to the unvaccinated birds.

I. INTRODUCTION

Vaxsafe® ST is a registered vaccine produced by Bioproperties Pty Ltd. The statement of claims for this vaccine is as "An aid in the control of colonisation by *S.* Typhimurium. The vaccine has been shown to reduce the excretion of virulent *S.* Typhimurium and provide chickens with an aid in protection against challenge by this strain". The registered method of administration is by spray on chickens at one day of age followed by an oral administration at 2 woa. This regimen was designed to protect short-lived birds (broilers) against *S.* Typhimurium challenge, but to date has not been widely adopted. Recently it has been used by injection in broiler breeder salmonella control programmes in Australia, combined with killed vaccines with great success. In order to extend the duration of immunity till the end of life for layers, a new vaccination program needs to be developed and tested.

Previous studies have shown that Vaxsafe® ST when given with regimens including an intra-muscular injection can provide useful protection against *S.* Typhimurium challenge and heterologous challenge with *S.* Infantis, and to a lesser degree *S.* Virchow challenge (Sharp *et al*. 2012). These experiments did not define the duration of this immunity and the cost of administration would be an impediment for general adoption in the layer industries.

In this study, the current vaccination program for Vaxsafe® ST was modified to include a third dose delivered by drinking water at 6 woa followed by a fourth dose administered by intramuscular (IM) injection at 10 woa. For the first three vaccinations, Vaxsafe® ST was delivered at a dose of 10^7 cfu in water. For the fourth vaccination, it was tested at a dose of 10^7 delivered in diluent, or mixed with Nobilis® EDS+ND killed vaccine. Nobilis® EDS+ND is a combined vaccine for the immunisation of chickens against Newcastle Disease and Egg Drop Syndrome '76. The Nobilis® EDS+ND killed vaccine consists of an inactivated antigens prepared as a water in oil emulsion.

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II. METHODS

A total of 26,000 Salmonella free commercial Hy-Line layer birds were vaccinated at the hatchery by coarse spray at day of age. The birds were transported to a commercial farm and reared in cages in shed A as per the normal farm management procedures. A group of 26,000 birds were left unvaccinated and were transported separately to the same farm and reared in cages in shed B. At 2 and 6 weeks of age all birds in shed A were vaccinated with Vaxsafe® ST (10^7 cfu/dose) in drinking water as per manufactures instructions. At 10 weeks of age, two groups of 125 birds were selected from shed A and vaccinated by IM injection (0.5mL/dose/bird) into the pectoral muscle with one of two Vaxsafe® ST formulations (in Marek's diluent or mixed with Nobilis® ND/EDS killed vaccine). A vial of freeze dried Vaxsafe® ST vaccine (1000 doses/vial) was resuspended in 3 mL of Marek's diluent and then added into 1000 doses of the killed vaccine and mixed by shaking.

Birds in shed B remained unvaccinated against Salmonella. At each of 6, 20, 35 and 55 weeks post IM vaccination, ten birds from each of the vaccinated groups and ten birds from shed B (control shed) were transported to a research facility, and placed into isolators. At 48 and 24 hours before the administration of the challenge, all chickens from all groups received vancomycin (60 mg per bird *per os* in 0.6mL). (Modified from Marcq *et al*., 2011). The challenged consisted of freshly cultured 10^9 cfu/dose of wild-type ST or SI. Chickens were monitored for clinical signs throughout the study and screened for Salmonella shedding with cloacal swabs being taken on days 0, 2, 7 and 14 post challenges. The presence and concentration of live Salmonella in swabs was determined by titration in Rappaport-Vassiliadis (RV) media (after resuscitation in Peptone broth) and identity confirmation on XLD and SMID plates followed by specific wild-type ST or SI PCR based methods. All data collected from the vaccinated and challenged birds were compared with those collected from the control groups in order to assess efficacy of the vaccine.

III. RESULTS

No clinical signs were observed in any of the vaccinated groups immediately after vaccination, or throughout the study. Prior to each challenge time-point, cloacal swabs were collected from chickens on the farm from both sheds A and B and screened to ensure that the birds were not infected with Salmonella. Also just prior to challenge, cloacal swabs were collected from birds in all groups. All these results were negative for Salmonella. In the positive control groups, the infection profile for ST was quite different to that of SI in terms of the number of organisms shedding, as well as the duration of the infection. Nonetheless, shedding from the positive control ST and SI groups was relatively high, indicating a good rate of challenge.

a) Shedding of *S.* Typhimurium and *S.* Infantis

There was a reduction in mean shedding of ST (see Figure 1. Panel a) in vaccinated chickens at most time points. Chickens challenged at 6 weeks after the IM vaccination with Vaxsafe® ST combined with Nobilis® EDS+ND showed a significant reduction the amount of ST shedding by day 14 after challenge. However, in chickens challenged at 20, 35 and 55 weeks after the IM vaccination, Vaxsafe® ST delivered in either diluent or combined with Nobilis® EDS+ND showed reduction in ST shedding by day 14 after challenge.

There was a reduction in shedding of SI in all vaccinated groups over the 3 sampling time points (days 2, 7 and 14) when compared with the unvaccinated positive control group. Shedding from the positive control remained high even at 14 days post challenge (see Figure 1. Panel b). Significant reduction ($p<0.05$) in SI shedding in vaccinated groups compared with the unvaccinated groups (positive control) was obtained when Vaxsafe® ST was delivered in diluent for challenges at 6 and 20 weeks after vaccination. Interestingly, a significant reduction ($p<0.05$)

in SI shedding compared with the positive control groups was obtained for both injected presentations of Vaxsafe® ST (in diluent and mixed with Nobilis® EDS+ND) for the challenge at 35 and 55 weeks after vaccination.

IV. DISCUSSION

This experiment lacked the power to cope with the stochastic nature of the Salmonella challenge shedding data, but trends were consistent and the demonstration of significant effects at later time points (in the case of the heterologous SI challenge) would allow the reasonable conclusion that some protection existed at earlier points. This problem may be overcome by Bayesian analysis of the data similar to a recent paper by Arnold *et al* (2014). These trends were a decrease in excretion in day 2 swabs in ST challenge in vaccinated groups and then all groups had similar rates of clearance. The SI challenge organism appears to be able to colonize the hens with cloacal shedding results from day 7 and day 14 being similar while in vaccinated groups these usually appeared to be being cleared. This suggests that the immunity from the Vaxsafe® ST vaccine primes the hen and this broad immunity can be rapidly mobilised in the gut of the chicken to reduce excretion of heterologous serovars. By day 14 the rates of shedding were similar in most cases whether the vaccine had been mixed with the killed NDV/EDS or just administered separately in diluent (Figure 1).

The vaccination regimen described illustrates potential for protection against ST egg contamination, and may provide a broad general Salmonella protection. Large numbers of layer hens in the field have now been vaccinated with similar IM regimens and it is well tolerated and rearing is reported as unaffected. Transient depression after vaccination (Groves and Sharp 2012) has not been observed in this experiment and has not a problem in the field to date. The Egg Layer industry should have the confidence from this work, previous work (Sharpe *et al* 2012), and positive experiences with similar vaccination regimens using Vaxsafe® ST in the broiler breeder industry, to begin investigating the advantages of this technology.

ACKNOWLEDGEMENTS: This research was conducted within the Poultry CRC, established and supported under the Australian Government's Cooperative Research Centres Program. We would like to thank Cheryl Colson and the team at animal housing facility at the APCAH at the University of Melbourne. Thanks also to Dr Nilhan Fernando and Dr Arif Anwar from Scolexia Pty Ltd.

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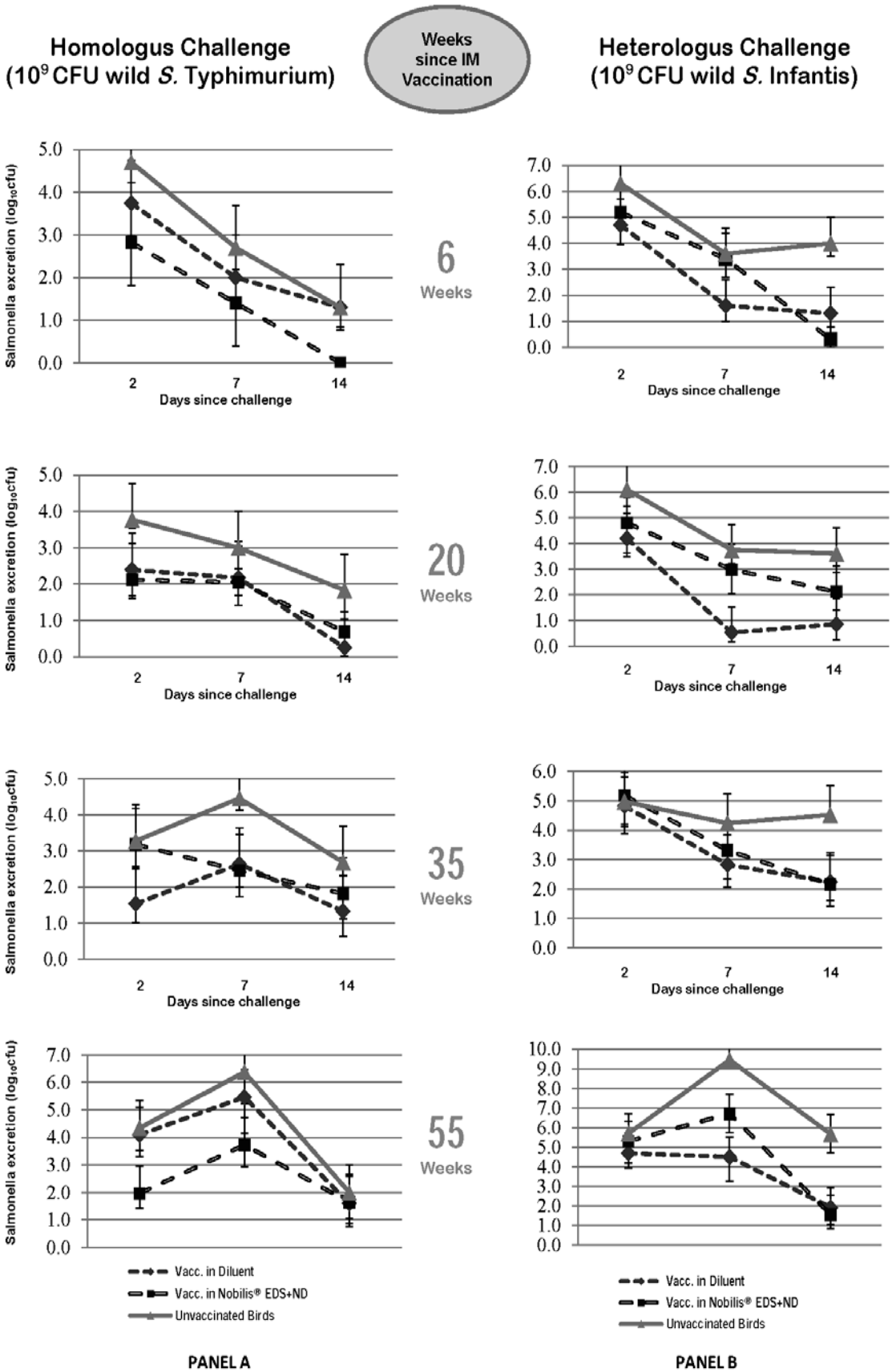

Figure 1 - Shedding of ST and SI in vaccinated and control groups challenged with wild type ST or SI at 6, 20, 35 and 55 weeks after IM vaccination with Vaxsafe® ST delivered either in diluent or mixed with Nobilis® EDS+ND killed vaccine. Means with error bars (standard error). Sampling/Data points are at 2, 7 and 14 days after challenge.

ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* IN RETAIL CHICKEN CARCASSES FROM VIETNAM

K. SARY[1], J.M. FAIRBROTHER[2], J. ARSENAULT[1] and M. BOULIANNE[1]

Summary

Vietnamese consumers are slowly turning to supermarkets as opposed to traditional markets as their first choice to buy their chicken produce. A limited number of studies have investigated chicken meat in Vietnam for potential human danger due to antimicrobial resistance in *Escherichia coli* and rarely for the presence of Extraintestinal pathogenic *E.coli* (ExPEC), a recognized cause of urinary tract infections in humans. To evaluate the safety of chicken meat in Vietnam, whole chicken carcasses, fresh or frozen, were sampled from markets and supermarkets. Primary bacterial cultures were prepared from carcasses rinsed in Vietnam and then sent to the OIE Reference Laboratory for *Escherichia coli* (EcL) in Canada for further characterization of isolates. Phenotypic antimicrobial susceptibility was determined by the automated Minimal Inhibitory Concentration (Sensititre ARIS®) system. Antimicrobial resistance of randomly selected Generic *E. coli* was observed for drugs that are classified as "very high" and "high" importance in human medicine according to Health Canada classification. Resistance was more prevalent in the Possible ExPEC subset, defined by the presence of one or more of the virulence genes *iucD, tsh, papC,* and *cnf*. One of the virulence genes, *iucD*, seemed linked to high resistance to specific antimicrobials. Presence of Extended-Spectrum β-lactamase (ESBL) producing *E. coli* was confirmed phenotypically by the Sensititre ARIS® system in the Ceftriaxone enriched (1mg/L) colonies and genotypically by the presence of the associated resistance gene family, CTX-M, verified by multiplex PCR. This is the first report of ESBL-producing *E. coli* in chicken meat from Vietnam. Multi-drug resistance to 5 or 6 classes of antimicrobial was common, being more prevalent in ExPEC isolates. Little difference was observed between retail sources, with the exception that frozen chicken carcasses demonstrated lower prevalence of putative ESBL-producing *E. coli*, and a higher multi-drug resistance (5 to 6 classes) with greater resistance to specific antimicrobials. Retail sources of fresh carcasses did not differ in antimicrobials patterns. Our results demonstrate that chicken meat in Vietnam represents a potential danger to public health, being a source of ESBL-producing and multi-drug resistant *E. coli*, including the potentially pathogenic ExPEC.

I. INTRODUCTION

In Vietnam, chicken carcasses are traditionally bought at urban open-air markets where chickens are slaughtered on-site. Since the economic revolution named *Doi moi* in late 80's, Vietnamese have developed more small businesses and a new economic class that possesses an increased purchasing power have emerged. In addition, foreign investments have allowed the implementation of new corporations, such as the introduction of supermarkets. These have restructured retail sale and accessibility to a wide variety of food products as well as other goods. With this revitalized economy and a new class of consumers, their needs and demands have also been modified. They now question quality and food safety, and as such favour supermarkets. Popularity of the traditional markets is explained by a preference for

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meat from free-range chickens (Fournier 2009) and accessibility. It is often only a short walking distance from home and food is available daily to consumers who cannot afford a fridge. However, the growing young Vietnamese middle-class is turning to another food source, supermarkets. Supermarkets have introduced self-service practices, inexpensive food products such as chicken carcasses from intensive farming, and visibility for other goods. Frozen chicken carcasses are also available in supermarkets.

Poultry meat, the second most popular meat consumed in Vietnam, after pork, has been suspected as the main carrier of Extraintestinal pathogen *Escherichia coli* (ExPEC) (Jakobsen et al., 2010). Contrary to other *Escherichia coli* pathotypes, ExPEC is asymptomatic in the digestive tract but is responsible for diseases with elevated morbidity in humans, most commonly urinary tract infections, but also newborn meningitis and septicaemia (Johnson and Russo 2002). ExPEC's site of infection varies depending on the wide array of virulence genes that it acquires and expresses. This plasticity of virulence and its ability to infect humans at many different extraintestinal sites make ExPEC a major health threat. Due to the increasing prevalence of antimicrobial resistance in clinical cases, fewer options of treatments are available. The objectives of this study are 1) to investigate antimicrobial resistance of *Escherichia coli* in chicken carcasses in Vietnam; and 2) to compare resistance from different retail sources (fresh carcasses from markets vs fresh and frozen carcasses from supermarkets).

II. MATERIAL AND METHODS

Whole chicken carcasses, fresh or frozen, were collected from markets (fresh) and supermarkets (fresh and frozen) in the centermost urban area of Hanoi over a period of six weeks in June and July 2011. Chicken carcasses were processed in Hanoi following a carcass rinse protocol from USDA Food safety and Inspection Service. The primary bacterial cultures inoculated on MacConkey plates were sent to the EcL Laboratory in Saint-Hyacinthe, Canada.

Upon reception, a total of 228 samples were recovered: 103 samples from markets (M), 99 samples of fresh carcasses from supermarkets (SM) and 26 samples of frozen carcasses from supermarkets (SMF). High level of contaminants and low presence of *Escherichia coli* in meat specimens obliged us to use an enrichment protocol inspired by Gill et al. (2012) in order to ensure maximal recuperation of *Escherichia coli* colonies. Two subsets of *E.coli* types were collected: 1) Generic, randomly selected colonies on plates and 2) Possible ExPEC positive colonies determined by PCR for selected virulence genes (*tsh, papC, iucD, cnf*). These were further tested for supplementary virulence genes (*afa*, *sfa* and *KpsMTII*) based on the Johnson definition for Human ExPEC (Johnson et al., 2003). A third subset of colonies was gathered from randomly selected samples enriched with Ceftriaxone (1mg/L) (Agerso et al., 2012). All isolates were confirmed as *E.coli* by *uidA* PCR.

III. RESULTS AND DISCUSSION

Antimicrobial resistance in *Escherichia coli* in chicken carcasses in Vietnam was investigated phenotypically by an automated standardized Minimal Inhibitory Concentration system (Sensititre ARIS®) for antimicrobial susceptibility testing. According to auto-read generated results based on the CLSI breakpoints, Generic and Possible ExPEC subsets of 82 and 64 samples, respectively, demonstrated high levels of resistance for antimicrobials classified by Health Canada as of very high importance in human medicine (Category I): Nalidixic acid (85.9%), Ciprofloxacin (37.5%); of high importance in human medicine (Category II): Ampicillin (85.9%), Gentamicin (35.9%), Streptomycin (70.3%), Trimethoprim-Sulfamethoxasole (82.8%); and of moderate importance in human medicine (Category III):

Chloramphenicol (68.8%) and Tetracycline (94%). Chloramphenicol is an antimicrobial banned from use in food animals in Vietnam, as well as in Canada. High resistance to specific antimicrobials in meat could reflect an anarchic use of antimicrobials in poultry farming in Vietnam, which could impact human health if infection occurs with ExPEC.

Interestingly, high resistance of the Possible ExPEC subset could be associated with few virulence genes. In fact, resistance to Ampicillin, Chloramphenicol, Nalidixic acid, Streptomycine, Tetracycline and Trimethoprime-Sulfamethoxasole are more often implicated with virulence gene *iucD*, which is a frequent virulence gene of ExPEC responsible for its iron-acquiring capacity. Co-selection on a same plasmid for this virulence gene and resistance genes corresponding to those previous antimicrobials could explain enhanced antimicrobial resistance in the possible ExPEC subset. Human ExPEC did not present different resistance patterns. Moreover, the possible ExPEC subset not only demonstrates high levels of resistance to individual antimicrobials but also has a majority of its samples demonstrating multi-drug resistance (MDR) (75%) of 5 to 6 classes of antimicrobials (Figure 1). Definition of multi-drug resistance and extreme drug resistance are given by Magiorakos et al. (2012). Multi-drug resistance poses an additional threat to human medicine as it considerably reduces clinical treatment options.

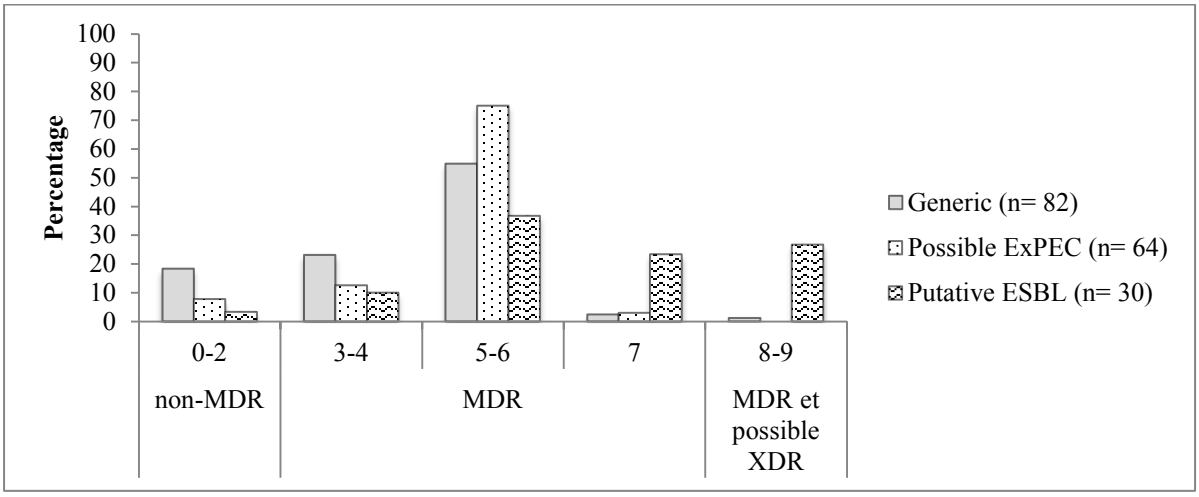

Figure 1 - Multi-drug resistance and possible presence of extreme drug resistance[a] in Generic[b], possible ExPEC[c] and putative ESBL[d] *Escherichia coli* isolates from chicken carcasses at retail in Vietnam ([a]According to definition of Magiorakos et al. 2012, Multi-Drug resistance (MDR): non-susceptible to at least one of three or more antimicrobial classes; Extreme-Drug resistance (XDR): non-susceptible to at least one of all but two antimicrobial; [b] *E. coli* isolates selected randomly; [c] Isolates positive for one or more of the virulence genes*:* iucD, tsh, cnf, papC, sfa, afa and kpsM II; [d] Putative ESBL producing isolates obtained following enrichment in Ceftriaxone (1mg\L) broth and plates.

The Ceftriaxone resistant subset allowed further investigation of resistance to one of the antimicrobial classes widely used in human medicine, β-lactamases. The resistance pattern obtained from phenotypic testing was compatible with putative Extended-Spectrum β-Lactamase (ESBL): high resistance to Ceftriaxone (≥1mg/L) and susceptible to Cefoxitin (Denisuik et al., 2013). To further confirm the presence of ESBL-producing *E.coli*, ten isolates were selected from this subset and tested phenotypically using confirmatory test plate with Cephalosporins, Cephalosporins and β-lactamase inhibitors, and Carbapenems. Eight out of ten samples were confirmed as ESBL. This is, to our knowledge, the first report of ESBL in chicken meat in Vietnam. No resistance was found for last resort antimicrobials, 4th generation cephalosporins, or Carbapenems. ESBL profiles were verified genotypically by PCR multiplex for the detection of five β-lactamase resistance genes (SHV, TEM, CMY, OXA, CTX-M). Tested isolates were associated with CTX-M resistance gene alone or in

combination; CTX-M is the most widespread resistance gene for 3rd and 4th generation Cephalosporins.

When comparing antimicrobial resistance between retail sources for each subset, frozen carcasses from supermarkets from the generic subset showed a centered distribution to MDR of 5 to 6 classes of antimicrobial. The percentage resistance per antimicrobial reveals the highest levels of resistance for the following antimicrobials: Ampicillin, Sulfisoxazole, Trimethoprim-Sulfamethoxazole, and Tetracycline. This retail source also has the lowest proportion of samples that had growth of *E.coli* on Ceftriaxone enriched media (27.2% compared to 54.5% for market and 48.6% fresh of supermarket). These findings can lead to our better understanding of the specific use of certain antimicrobials or a combination of selected antimicrobials on the farm, which results in elevated multi-drug resistance without selecting for Ceftriaxone resistance. A common supplier provided most of the sampled frozen chicken carcasses from supermarkets. This could indicate a more harmonized husbandry and antimicrobials management by the provider based on the results in frozen chicken meat.

In conclusion, chicken meat in Vietnam represents health risks to its consumers due to the presence of ESBL-producing *E.coli*, elevated resistance to antimicrobials of very high importance in human medicine, and multi-drug resistant *E.coli*. Results by retail sources did not provide any differences, except for frozen chicken carcasses.

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A LOOP ISOTHERMAL AMPLIFICATION ASSAY TO DETECT FOWL ADENOVIRUS – 8 IN A VARIETY OF POULTRY MATERIALS

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Summary

A variety of molecular tests to detect avian adenoviruses are available, but they are either time-consuming or not specific to Fowl adenovirus serotype 8 (FAdV-8). Therefore, the aim was to develop a loop isothermal amplification (LAMP) assay to specifically detect FAdV-8 in a variety of poultry samples, particularly dust, faeces and litter. The LAMP assay is a particularly rapid, robust and a low cost molecular test yet with a sensitivity comparable with the real-time PCR technique. Samples were analysed from chickens experimentally infected with FAdV-8 and derived from both tissues and environmental samples, including dust, litter and faeces. The LAMP results matched with those obtained from a generic FAdV real-time PCR test and the LAMP test has shown no cross-reactivity with FAdV serotypes 2, 9, or 11.

I. INTRODUCTION

Adenovirus infections are ubiquitous in commercially farmed birds, and probably in all avian species. The viruses belong to the family *Adenoviridae*, genus *Aviadenovirus*. Certain serotypes and species are known to be associated with primary diseases, such as inclusion body hepatitis (IBH) (Ojkić et al., 2008). Commonly involved in IBH is Fowl adenovirus serotype 8 (FAdV-8) belonging to the species Fowl adenovirus E. Signs of FAdV-8 infection typically include acute mortality induced by hepatitis in birds 3-7 weeks of age. Diagnosis can often be made based on post-mortem lesions, particularly in the liver which appears swollen, yellow, mottled with petechiae and ecchymoses. The duration of the infection usually ranges from 5–14 days with morbidity of 10–30% and a daily mortality of 3–5%.

A range of generic and serotype-specific tests to detect FAdV are available including ELISA (Calnek et al., 1982; Saifuddin et al., 1990), real-time PCR (Romanova et al., 2009; Günes et al., 2012) and PCR followed by restriction enzyme analysis (Zsak and Kisary, 1984; Meulemans et al., 2010). These tests, however, are either not FAdV-8 specific or expensive and time-consuming, so the aim of this study was to develop a rapid, low-cost and robust molecular test to specifically detect FAdV-8 in a variety of poultry sample materials, with a particular focus on environmental samples such as dust and litter or faeces.

The loop isothermal amplification (LAMP) method is not very recent (first reports from Notomi et al. (2000)), but it is a rapid and a low cost yet sensitive method for detection of both DNA and RNA. This method does not require a thermocycler as the target sequence is amplified at a constant temperature of 60-65 °C using polymerases with DNA strand displacement activity. The method usually uses 4-6 specific primers resulting in fragmentation of the target sequence and relies on DNA/cDNA strand displacement and autocycling performed by specific DNA polymerases. Due to the specific nature of the action of these primers, the amount of DNA produced in LAMP is considerably higher than PCR based amplification. The final amplification products are cauliflower-like structures with multiple loops, which result in visible turbidity due to precipitation and allow visualization via simple detection approaches (spectrophotometer, UV light). This method can deliver results that have similar sensitivity and specificity as real-time PCR assays.

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For the LAMP assay, the aim was to develop a qualitative assay, without absolute quantification.

II. MATERIAL AND METHODS

Primers were designed based on the FAdV-8 sequence, GenBank accession no. GU734104, targeting the open reading frame (ORF) 33a, which is unique for the FAdV-8 serotype (Grgic et al., 2011). The primers were designed using LAMP designer 1.10 (Premier Biosoft, USA) and are given in Table 1.

Table 1 - Primers used for the FAdV-8 LAMP assay.

Primer name	Primer sequence (5'-3')	Primer location in GenBank acc. no. GU734104
F3	GGCAACTCCGAAGATCAC	40123-40140
B3	GGCGTGGTAGCAATAAAGA	40334-40352
FIP	TCCGTAAGTGGAGCGTTGCGGTACTGTGACACCTTGC	40176-40244
BIP	GCGAGCGAACAATAAATCTGCGAAGAGTCGGCTTATATATTCCC	40263-40332
LOOPF	GGGTCGGGTTCCCTTTTC	40202-40220
LOOPB	GTGTGCTCTAGGCGGAAG	40289-40307

FAdV-8 standard material was prepared from a serotyped cell cultured virus kindly provided by Dr. Edla Arzey, of the Elizabeth MacArthur Agricultural Institute, Camden, NSW, Australia. Genomic DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Australia) following the manufacturer's instructions. The DNA was quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA). The absorbance ratio of the sample at 260 and 280 nm was assessed as a measurement of DNA quality. In order to determine the sensitivity, tenfold serial dilutions of FAdV-8 standard material starting from 5 ng/µl per reaction were prepared for standard serial dilutions. To confirm the specificity of the LAMP assay, purified standard PCR product using the F3 and B3 primer were sequenced. The FAdV-8 LAMP was also performed using FAdV-2, FAdV-9 and FAdV-11 DNA as a sample that was kindly provided from Dr. Eva Nagy, Guelph, Canada.

The mastermix per reaction consisted of 9 µl Isothermal mastermix (Optigene, UK), 1.8 µl molecular grade water, 3 pmoles of each F3 and B3, 12 pmoles of each FIP and BIP, 6 pmoles of each LOOPF and LOOPB in a total volume of 15 µl. The unknown DNA samples were diluted 1:10 and 2 µl of diluted sample was added to the reaction. The LAMP assay was performed on a Genie II instrument (Optigene, UK) at 65 ^{o}C for 45 min. The amplification plots and annealing peaks were analysed using the Genie II software supplied with the instrument using the default settings.

The 20 samples tested with the FAdV-8 assay were obtained from specific pathogen free chickens that were experimentally infected with FAdV-8. The DNA from tissue and environmental samples were extracted using a GeneJet DNA and RNA extraction kit

(ThermoScientific, Australia) and also analysed using a generic FAdV real-time PCR assay adopted from Günes et al. (2012).

III. RESULTS

All primers for the LAMP assay were designed successfully. Standard PCR products using the outer primer set F3 and B3 were sequenced and a BLAST search revealed 100% specificity for FAdV-8. Based on gel electrophoresis of FAdV-8 standard PCR and FAdV-8 LAMP products, the FAdV-8 LAMP test has shown no cross-reactivity with DNA from FAdV-2, FAdV-9 and FAdV-11.

Tenfold serial dilutions of the standards were performed amplification of samples down to a dilution of 1:1000, achieving a sensitivity similar to that of a generic FAdV real-time PCR (qPCR) assay (230 viral copy numbers per reaction), adopted from Günes et al. (2012).

A total of 18 samples originated from faeces, litter and dust from SPF chickens with known FAdV-8 infection were analysed using the FAdV-8 LAMP and the qPCR tests (Table 2). The FAdV-8 LAMP results matched the qPCR test perfectly.

Table 2 - Comparison of FAdV-specific qPCR results and those obtained with the FAdV-8 specific LAMP assay on 18 samples of different types.

Sample ID	Sample type	Days post infection	Age of birds (days)	qPCR result (Log_{10} VCN/ mg or 10^6 cells)	LAMP result (+positive -negative)
A	Litter	28	29	7.8	+
B	Litter	28	29	8.7	+
C	Litter	19	19 and 35	0.0	-
D	Dust	14	15	0.0	-
E	Dust	21	22	0.0	-
F	Dust	28	29	0.0	-
G	Dust	14	15	8.5	+
H	Dust	21	22	8.1	+
I	Dust	28	29	8.0	+
J	Dust	14	15	8.2	+
K	Dust	21	22	8.3	+
L	Dust	28	29	8.3	+
M	Dust	14	30	7.3	+
N	Dust	21	37	7.2	+
O	Dust	14	30	7.0	+
P	Dust	21	37	0.0	-
Q	Faeces	9	25	3.4	+
R	Faeces	12	28	1.0	+

IV. DISCUSSION

The majority of Adenovirus PCR tests and sequencing data published to date relate to the hexon and fibre gene. These PCR assays detect a wide range of FAdV, and have to be used in combination with sequencing, restriction enzyme analysis or high resolution melt curve analysis to differentiate serotypes. Many available serological tests such as ELISAs (Calnek et al., 1982; Saifuddin et al., 1990) also do not differentiate between serotypes of FAdV although some more recent commercial ELISA kits do. For this study, FAdV-8 specific

primers were designed based on the ORF 33a. This was identified to be unique for FAdV-8 viruses (Grgic et al., 2011), and our findings support this as the specific FAdV-8 LAMP assay has to date not amplified FAdV-2, -9 or -11.

The LAMP technique is by nature highly specific to its target sequence, but potentially less sensitive than real-time PCR assays. However, there are several publications reporting LAMP tests as sensitive as real-time PCR assays (Notomi et al., 2000; Huang et al., 2010). The FAdV-8 LAMP test that we have developed has also shown similar sensitivity to a FAdV real-time PCR. Another major advantage of the LAMP technique is its robustness and speed. Our FAdV-8 LAMP assay has been shown to reliably amplify the target sequence from poultry samples (faeces, dust, litter). None of these samples were extracted with a commercial kit or any other laboratory method, but only treated with an alkali lysis buffer at room temperature for up to 40min. Combined with the GenieII instrument (Optigene, UK) which can operate on battery power for up to one day, this would potentially allow to perform LAMP assays on poultry farms, with a molecular test result available within less than 2 hours and approximately 20-30% lower reagent costs compared to a real-time PCR test.

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INFECTIOUS BURSAL DISEASE ANTIBODY LEVELS AND VIRAL LOAD IN BURSA, FAECES, LITTER AND DUST FOLLOWING INFECTION OF COMMERCIAL BROILER CHICKENS AT 0 AND 14 DAYS OF AGE

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Summary

Australian endemic IBDV serotype 1 variant strain 02/95 was quantified using qRT-PCR in bursal tissues of commercial Ross broiler chickens inoculated orally at days zero and 14 of age and maintained to 28 days post infection (dpi). High levels of viral RNA were detected at 7, 14, 21 and 28 dpi in birds challenged at day 14 but only at 21and 28 dpi in birds challenged at day zero. Challenge at both ages induced a significant antibody response relative to unchallenged controls. Virus was detected in faeces prior to day 7 and again at 28 dpi in both challenge groups, but not during the intervening period. IBDV was also successfully quantified by qRT-PCR in poultry dust and litter from the infected groups.

I. INTRODUCTION

Infectious bursal disease (IBD) disease is caused by infectious bursal disease virus (IBDV) of the genus *Avibirna-viridae* and family *Birna-viridae*. IBDV is found worldwide and, depending on the virulence of the strain causes mortality in chickens three weeks or older and prolonged immunosuppression if birds are infected in early life (Eterradossi and Saif, 2008). IBDV was first reported in Australia in 1974 and has been the subject of considerable research conducted since then (Ignjatovic and Sapats, 2002). All Australian strains are classified as IBDV classical or variant strains and are genetically different from overseas strains (Sapats and Ignjatovic, 2002). Australian broiler chickens are protected by maternal immunity provided by breeder vaccination. As part of studies on the *in vivo* characterization of Australian IBDV strains we aimed to determine the effects of age at challenge (zero or 14 days) with IBDV Australian variant strain 02/95 in commercial broiler chickens on the persistence of IBDV in the bursa of Fabricius (the main target organ of the virus), the shedding profile of IBDV in faeces, the serum profile of anti-IBDV antibodies and the detection of IBDV in litter and dust samples collected from infected groups.

II. MATERIALS AND METHODS

The experiment was conducted in two chicken isolation sheds located at the University of New England. The experiment utilized a 2 × 2 factorial design with two levels of IBDV challenge (IBDV infection and uninfected control) and two ages of infection (days zero and 14). Two batches of 30 Ross broiler chickens (Baiada Poultry, Tamworth, and NSW) 14 days apart in age were used, with half the birds in each group challenged with IBDV and half not challenged. Challenged groups were orally infected with IBDV serotype 1, variant, Victorian-origin, Australian field strain 02/95 at a dose of10^5 CID_{50}/chick in 0.2 ml of PBS on the same day while negative controls were dosed sterile PBS. Infected and control groups were kept in separate sheds with the two age groups separated by a partition within each shed.

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Six birds from each treatment group were colour marked for a longitudinal study. Faecal samples from those colour marked birds were collected at 2, 3, 4, 5, 6, 7, 9, 12, 14, 16, 21 and 28 dpi. Blood samples were collected from the marked birds at 7, 14, 21 and 28 dpi for the detection of antibody (Ab) against IBDV in sera using ELISA (ProFLOCK® SYNBIOTICS San Diego, USA).

At 7, 14, 21 and 28 dpi three unmarked birds from each treatment group were sacrificed to collect bursa.

Dust samples were collected in settle plates from each group for quantitative reverse transcription polymerase chain reaction (qRT-PCR) quantification of IBDV at 7, 14, 21 dpi and litter samples at 7 and 14 dpi. Collected bursa, faeces and environmental samples were stored at -80°C.

Extraction of viral RNA from tissues and dust was done using the BIOLINE ISOLATE II RNA Mini Kit (Bioline Aust Pty Ltd, Alexandria, Australia) and from faecal and litter samples using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc, USA). The RNA extracted from all samples was quantified using a NanoDropH ND-1000 UV-Vis spectrophotometer (Nano-DropH Technologies, Wilmington, DE, USA) and stored at -80°C until used for IBDV qRT-PCR assay.

IBDV viral genome copy number was quantified from all extracted RNA (bursa, faeces, dust and litter) using a TaqMan® IBDV specific qRT-PCR assay developed and validated by authors based on the sequence data for IBDV VP2 of Ignjatovic and Sapats (Ignjatovic and Sapats, 2002) and carried out in a Rotor Gene 3000 Real-time PCR instrument (Corbett Research, Mortlake, NSW, Australia). A standard curve based on plasmid standards of known genome copy number was used for absolute quantification.

Appropriate statistical analyses were carried out using JMP ® 10 statistical software (SAS Institute Inc, NC) with results presented as least squares means ± SE with a significance of level $P < 0.05$.

III. RESULTS

Clinical observation of all challenged and control birds were made twice daily. No deaths were reported during the experiment and birds were clinically normal.

Repeated measures analysis of Log_{10} anti-IBDV titre revealed that challenged birds had significantly higher titres than unchallenged birds (2.52±0.14v. 1.73±0.14, P=0.0004) and there was a non-significant trend (P=0.07) towards a higher level of antibody in birds challenged at day 14 (2.30±0.12) than those challenged at day 0 (1.94±0.15). There was significant interaction between the effects of challenge and time after challenge (P=0.02) reflecting decreasing antibody titres in control birds and increasing or high levels in challenged birds over time (Figure 1).

Analysis of IBDV viral copy number (VCN) in bursal tissue revealed significantly higher levels in birds challenged at day 14 than day zero (P= 0.022), a significant increase in load over time following challenge (P=0.031) and a significant interaction between these effects (P=0.015, Figure 2). In chicks challenged at day zero viral load was very low at 7 and 14 dpi, increasing thereafter. In chicks challenged at day 14 load was highest at 7 dpi and declined slightly at each sampling up to 28 dpi. No virus was detected in control birds of both age groups.

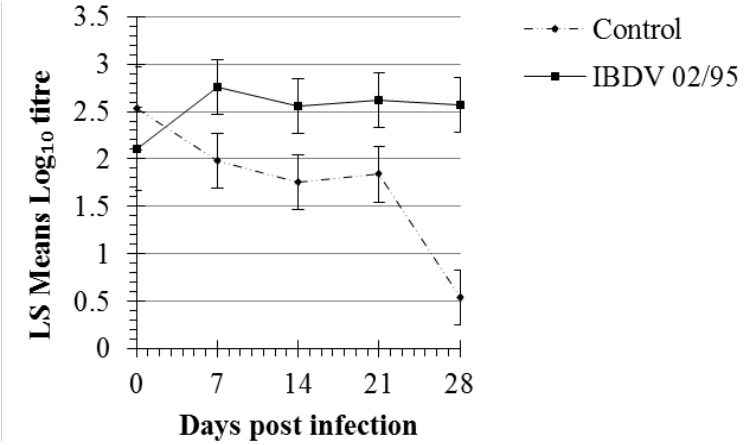

Figure 1 - Least squares mean (±SEM) log_{10} antibody titre showing interaction between the effects of challenge with IBDV variant stain 02/95 and time after challenge in commercial broilers.

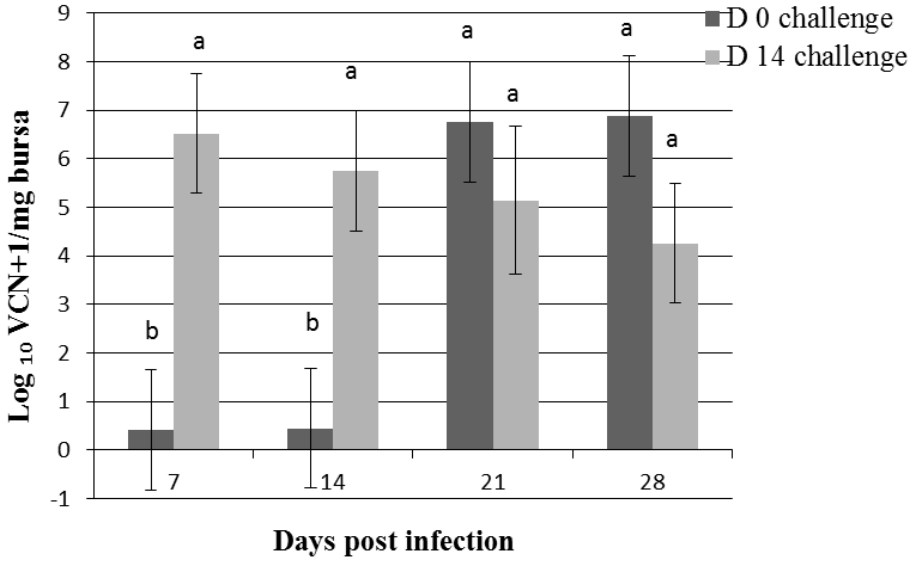

Figure 2 - Least squares mean Log ₁₀ (viral copy number/ mg bursal tissue+1) (±SEM) by time after infection in chickens inoculated with IBDV 02/95 strain at ages zero and 14 days. Different letters indicate significant differences between columns.

Analysis of Log_{10} IBDV VCN in faeces revealed no overall difference between birds challenged at days zero and 14 (P=0.75), but a significant effect of time following challenge (P=0.004) and a significant interaction between these effects (P=0.040). Both groups of chickens shed detectable virus in faeces prior to 7 dpi but not from 7 - 21 dpi. At 28 dpi IBDV was again detected, with significantly higher levels in day 14 challenge group.

IBDV was detected in dust samples from the challenged shed with mean log_{10} (VCN/mg dust+1) of 4.36, 2.96 and 2.17 (SEM = 0.35) at 7, 14 and 21dpi respectively.

Litter samples collected from the 14 day-old challenged birds contained 5.83 and 3.54 x 10^3 VCN/g litter respectively. The equivalent values for samples from the day zero challenged group were 525 and 0 VCN/g litter.

IV. DISCUSSION

Replication of IBDV in the bursa was inhibited for at least two weeks in chickens challenged at hatch, relative to those challenged at day 14. This is most likely due to the presence of maternal antibody directed against IBDV. Antibody titres in control birds fell by more than one log between days 0 and 14 and appeared insufficient at day 14 to prevent rapid viral

replication. Viral load profiles following challenge in the day 14 challenged broilers were similar to those in maternal antibody negative specific pathogen free (SPF) chicks challenged at day 0 in a previous study (Jayasundara *et al.*, 2014). Interestingly, in chicks challenged at hatch, viral load levels in the bursa by day 21 reached levels as high or higher than those observed in the birds challenged at day 14, suggesting that the effects of maternal antibody are to delay rather than completely inhibit viral replication in the bursa.

In the present study IBDV was detected in bursa up to the end of the experiment at 28 dpi. This is longer than the detection periods of 14 (Abdel-Alim and Saif, 2001) and 21 dpi (Abdul *et al.*, 2013) in commercial broilers challenged with variant IBDV, but shorter than the 42 dpi reported by (Elankumaran *et al.*, 2002).

Early shedding of IBDV in faeces as observed in the present experiment has been reported in infected SPF chickens (Takase *et al.*, 1982; Zhao *et al.*, 2013; Jayasundara *et al.*, 2014) but these the detection of virus at 28 dpi after absence for the previous 3 weeks is a novel finding.

The significant viral loads of IBDV found in dust collected at seven, 14 and 21 dpi from infected shed is in agreement with earlier reports of IBDV detection in dust (Zhao *et al.*, 2013; Jayasundara *et al.*, 2014) and suggest that dust could be an alternate sample for disease monitoring as applied in Marek's disease surveillance (Walkden-Brown *et al.*, 2013). Significant amounts of IBDV RNA were also detected at seven and 14 dpi in litter samples from the day 14 challenged group, but little or none from the than day zero challenged group.

Higher anti-IBDV antibody titres were detected in both challenged groups than their controls indicating an active immune response to challenge in both groups.

We conclude that delaying IBDV challenge from hatch to 14 days of age significantly enhanced viral replication in the bursa post challenge, and shedding in faeces, but did not alter the development of an active immune response or peak levels of IBDV in the bursa. These effects are more probably mediated by a decline in passive immunity due to maternal antibody, than by a true age effect. The latter is currently under investigation. We also conclude that the presence of IBDV can also be monitored in environmental samples such as dust and litter using RT-qPCR methods.

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TISSUE DISTRIBUTION AND SHEDDING PROFILES OF CHICKEN ANAEMIA VIRUS IN SPECIFIC PATHOGEN-FREE AND COMMERCIAL BROILER CHICKENS

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Summary

Chicken infectious anaemia is caused by a circovirus called chicken anaemia virus (CAV), and it has become an emerging threat to the poultry industry worldwide. CAV may cause clinical symptoms in young chickens. At the same time, it can maintain subclinical infection in all chickens, causing immunosuppression resulting in vaccination failure and facilitating other diseases. In the absence of clinical manifestation, a reliable method for monitoring of CAV infection is needed for the poultry industry. Two experiments were conducted, one in specific-pathogen-free-layer chickens and the other in commercial broiler chickens, to detect and quantify the viral genome in various tissues and environmental samples. CAV was detectable in a number of tissues with a high titre in thymus and bone marrow; therefore, these two tissues will be preferred samples for molecular diagnosis. The virus was detected in dust and litter samples, although at a low level. The monitoring of CAV infection using environmental samples such as dust and litter has potential, but needs further optimization.

I. INTRODUCTION

Chicken infectious anaemia is a relatively new disease of chickens, first reported in Japan (Yuasa et al., 1979), that is caused by a coronavirus called chicken anaemia virus (CAV). This virus may cause clinical manifestation only in young chickens free of anti-CAV antibody, but it can maintain subclinical infection in antibody positive commercial chickens and cause immunosuppression. Vertical transmission of the virus is well documented (Cardona et al., 2000), and it is also believed to transmit laterally to flockmates, presumably via the faecal-oral route (Davidson et al., 2008).

Commercial broiler chickens are not usually vaccinated against CAV, but the parent stock are vaccinated to protect their progeny through passive immunity. A cost effective method of monitoring of CAV levels in commercial poultry would provide an additional strategy around which to base control strategies. The detection of the viral nucleic acid in various tissues using molecular techniques is commonly used. However, the virus may not be present in all organs at all stages of infection. In addition, the tissue distribution may depend on the antibody status of the host. Therefore, the current study aimed to determine the most sensitive tissues and the timing of tissue collection for the molecular detection of CAV in antibody-free and commercial chickens. This study also aimed to investigate whether CAV can be detected in poultry house environmental samples such as litter and dust, which can be useful to monitor the infection in a manner similar to some other viruses (Walkden-Brown and Islam, 2013).

II. MATERIALS AND METHODS

Two experiments were conducted. Experiment 1 utilized a completely randomized design with three treatments. Forty-five 1-day-old pathogen-free (SPF) chicks were randomly allocated to three positive pressure isolators, with 15 chicks in each group. Chickens in two isolators were infected with two strains of CAV, a vaccinal strain CAV 3311 and a

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pathogenic strain CAV 269/7, and the chickens in the third isolator were kept as negative controls. CAV infection occurred at day-old (Day 0) orally at a rate of $10^{5.9}$ TCID50 per chicken. Six chickens from each isolator were individually marked for a longitudinal study. Faecal and blood samples were collected from these individually identified chickens for up to 4 weeks. Three chickens from each isolator from the unmarked chickens were humanely killed at 6, 13, and 28 day post-infection (DPI) for various tissue collections. Dust and litter samples were also collected from each isolator at various intervals. Experiment 2 was a repeat of the first experiment in commercial broiler chickens where only pathogenic CAV strain (CAV 269/7) was used, and the chickens were infected at two ages, day 0 and day 14. A similar sampling protocol was used. DNA was extracted from tissue, dust, and faecal samples using commercial kits (Bioline Isolate II kit), and the CAV genome was quantified using a TaqMan® real-time PCR developed and validated by our group, primers, and probes taken from Zhang et al. (2009). CAV antibody titre was measured using a commercial ELISA kit ProFlock CAV Plus (Symbiotics Corporation, Kansas City, MO, USA). The packed cell volume (PCV) was measured from blood collected in experiment 1. Data were analysed using ANOVA for the effects of DPI, treatment, and their interactions for experiment 1 and DPI, treatment, age of infection (AOI), and their interactions for experiment 2.

III. RESULTS

In experiment 1, there was a significant effect of DPI ($P < 0.006$) and treatment ($P < 0.05$) on the PCV of chickens without any significant interaction ($P = 0.09$) between the two main effects. In general, there was a higher PCV in the negative control chickens than the pathogenic CAV (CAV 269/7) infected chicken, but the PCV of chickens infected with the vaccinal CAV strain (CAV 3311) was not significantly different from that of the negative control and the pathogenic CAV strain birds. The vaccinal strain of CAV infection reduced the PCV only at Day 21 DPI.

CAV load was quantified in the thymus, bursa, spleen, and bone marrow. The viral genome was detectable in all tissues at 6 DPI (first of day of sample collection) and was present up to 28 DPI (last day of sample collection). There was a significant effect of DPI ($P < 0.0001$), tissue ($P < 0.0001$), and treatment (($P < 0.0001$) on the viral load expressed as log10 viral copy number (VCN) per mg of tissue. There was a significant interaction between the effect of treatment and DPI ($P < 0.009$). The viral load was highest in the thymus followed by the spleen and bone marrow, with the lowest viral load in the bursa (Figure 1A). In general, the genome copy of the pathogenic CAV was higher than the vaccinal strain in all tissues except the bursa. As expected, the viral load was very low at 6 DPI but increased significantly by day 13 in all organs. However, the viral load did not increase in the bursa and bone marrow after day 13. The CAV load increased in both the thymus and the spleen up to 28 DPI, the last sampling day (Figure 1B).

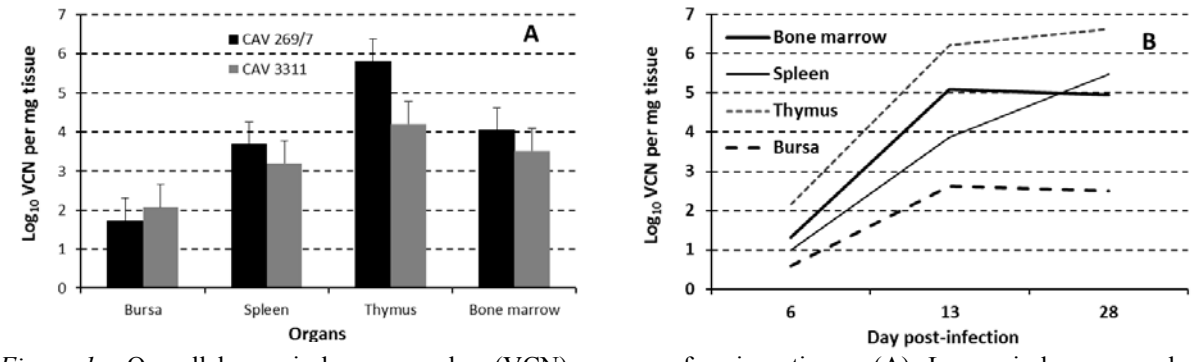

Figure 1 - Overall $\log_{10}$ viral copy number (VCN) per mg of various tissues (A). Log_{10} viral copy number (VCN) per mg of various tissues over time (B).

Most faecal samples were negative for CAV, only two faecal samples were positive at 18 DPI (one from the vaccinal and one from the pathogenic strain), and one faecal sample collected at 3 DPI was positive for the vaccinal strain of CAV. CAV was detected in a dust sample collected at 7 DPI from isolator exhaust pipes with a titre of $10^{4.1}$ VCN per mg of dust. Only two out of four litter samples were positive for CAV.

There was a significant effect of DPI ($P < 0.001$) but not treatment ($P = 0.24$) with a significant interaction between the effect of treatment and DPI on the CAV antibody titres. The CAV antibody titres for the pathogenic strain (CAV 269/7) started higher and stayed at the same level, but the vaccinal strain (CAV 3311) started very low (almost undetectable at 6 DPI) and then increased slowly up to the end of the experiment (28 DPI).

In experiment 2, CAV was detectable only in a fraction of tissue samples. The virus was not detectable in the spleen at any time point, but other organs were positive for the virus at least at one sampling time. Chi-square analysis showed that a higher number of organs of the chickens infected at day 14 were CAV positive than of the chickens infected at day-old ($P < 0.002$). Bone marrow was positive in five samples at three different time points. Three dust samples collected at 7, 14, and 21 DPI were positive for the CAV genome (Figure 2).

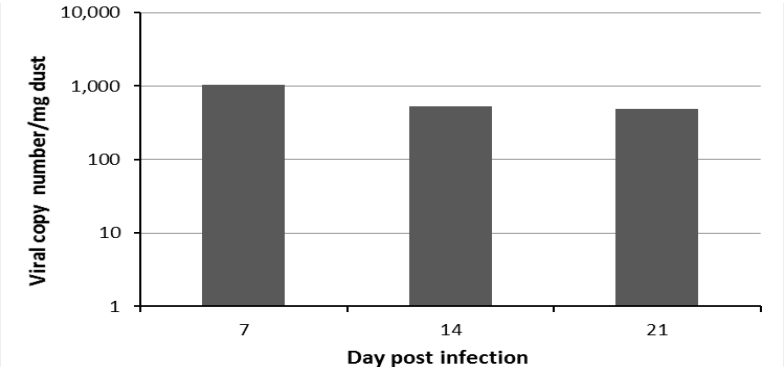

Figure 2 - Chicken anaemia virus genome copy number in the dust samples

IV. DISCUSSION

The study showed that the CAV genome can be detected in lymphoid organs and bone marrow as early as 6 DPI in maternal-antibody-free chickens but not in commercial broiler chickens. This is the first study to demonstrate that CAV can be detected in dust and litter samples in both SPF and commercial chickens. This has implications for the poultry industry, as environmental monitoring can be used for disease screening (Islam and Walkden-Brown, 2007).

Among the four organs tested, we found that the thymus had the highest viral load. This finding is in agreement with Tan and Tannock (2005). The viral copies increased up to 13 DPI, following which they either stayed at similar levels (bursa and bone marrow) or increased (thymus and spleen) up to 28 DPI in this study, whereas only a limited number of chickens were reported to be CAV positive in the thymus and spleen and none in the bursa and caecal tonsil following infection with a vaccinal virus (Vaziry *et al.*, 2011).

The virus was detectable in the faeces, but at a very low level. This could be due to inhibition of the qPCR reaction (as discussed below) or low shedding in faeces following a high dose of viral infection, as reported by others (Dren *et al.*, 2000). Therefore, the shedding of CAV through faeces and the faecal-oral route of lateral infection remain to be determined. The most significant finding of this study is the detection of the virus in chicken dust and litter. However, the viral load in these environmental samples was not very high ($10^{3.9}$–$10^{2.6}$ per mg). This could be attributable to a number of reasons. There could be some inhibition of the PCR reaction, or, perhaps, a different DNA extraction procedure is required. Before DNA

extraction from the main samples, we conducted an experiment to compare the two extraction methods for extracting nucleic acid from faecal samples, Bioline Isolate Faecal DNA kit (Bioline Reagents Limited, UK) and GenJet Viral DNA and RNA Purification Kit (Thermoline Scientific Limited, USA). There was no significant difference between the two extraction methods, although the Bioline kit gave slightly better performance. Therefore, we used Bioline kits for analysing the experimental samples. Diluting the extracted DNA up to 1:1000 did not reveal any visible inhibition of the PCR reaction.

The antibody response was quicker in the case of the pathogenic viral infection compared to the vaccinal strain, which suggests more rapid replication and synthesis of viral protein in the case of the pathogenic virus. Slow or undetectable antibody titres have been reported previously following vaccination (Vaziry *et al.*, 2011).

The second experiment has demonstrated that a low level of CAV infection could be established in antibody positive commercial chickens. The virus was detected in some tissues and dust but not in litter. The presence of the CAV genome in various tissues reached its highest level in the bone marrow; therefore, bone marrow should be the best sample for diagnostic purposes followed by the thymus and bursa, but not the spleen. Dust also has potential for use in monitoring the disease, but not litter. The inhibition of the PCR reaction in litter material is not uncommon (Dye et al., 2008), and this issue needs further investigation.

CAV infection in chickens becomes particularly complex when multiple viral infections occur along with CAV. The successful detection of multiple viruses from a single sample has been demonstrated from artificially infected SPF chickens showing clinical signs using tissue samples (Davidson *et al.*, 2008). Although this study successfully demonstrated the ability to detect the CAV genome in the dust and litter both from SPF and broiler chickens that had no clinical manifestation of the disease, these methods need further validation before industry recommendations can be made. The industry would ideally like one method for the detection of all potential viral diseases from a single sample. This may be best be achieved by applying two different nucleic acid extraction methods, one for DNA viruses and one for RNA viruses. The UNE Poultry Health Research Group is working towards this.

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KINETICS OF RISPENS CVI988 VACCINE VIRUS IN SINGLE AND MIXED INFECTIONS WITH VERY VIRULENT MAREK'S DISEASE VIRUS IN ISA BROWN CHICKENS

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Summary

We report the kinetics of infections with serotype 1 pathogenic Marek's Disease virus (MDV) and Rispens CVI988 (Rispens) vaccine virus in the same host. We conducted a 2x3 factorial experiment with two replicates testing the effects of vaccination with Rispens (vaccinated, unvaccinated) and challenge MDV (isolates MPF57, FT158 or unchallenged) using 236 female ISA Brown chickens. The chickens were vaccinated at hatch and challenged at day 5 of age, and the experimental duration was to 56 days post challenge. Peripheral blood lymphocytes (PBL) and feather samples were collected at regular intervals during the experiment. Extracted DNA was subjected to two quantitative polymerase chain reaction (qPCR) tests specific for either pathogenic MDV1 or Rispens viruses. The results showed that the Rispens vaccination reduced the pathogenic viral load in PBL by 1.4 logs and feathers by 1.2 logs. The viral load of pathogenic MDV was significantly higher in both PBL (1 log) and feather (1.3 logs) than that of Rispens in co infected hosts. The more virulent isolate FT158 had a significantly higher viral load than MPF57 in PBL (1.1 logs), but not in feather.

I. INTRODUCTION

Marek's disease (MD) is a highly contagious neoplastic disease of poultry caused by an alphaherpesvirus known as MDV. MD is estimated to cost the worldwide poultry industry US$ 1-2 billion annually. MD has been controlled by vaccination since 1970; however these vaccines do not provide a sterile immunity against MD. MD vaccines protect against disease, but do not prevent infection and replication of MDV, which has likely led to evolution of more virulent strains of MDV (Atkins et al., 2013). Several Australian isolates of MDV have been pathotyped according to the standard tests and classified into mild (m), virulent (v) and very virulent (vv) pathotypes. Co-infection studies in which the chickens are infected with both MD vaccine virus and the pathogenic virus are essential to understand the effect of vaccine virus on reproductive success of pathogenic virus. Most of these studies have been carried out using HVT and HVT/MDV2 vaccines which are readily differentiated from pathogenic MDV. Few studies have investigated co-infections with Rispens vaccine due to the difficulty in distinguishing the Rispens virus from pathogenic MDV as they are both serotype 1 MDV. Recently a method was developed by our group that distinguishes Rispens from the Australian MD field isolates (Renz et al., 2013), which we have used in this study and a companion study (Islam et al., 2014) to investigate the viral kinetics of Rispens vaccine virus and the pathogenic MDV in the same host.

The broad hypotheses under test in this experiment were that viral load as a measure of replication rate would be positively associated with virulence (FT158>MPF57>Rispens) and that vaccination would significantly reduce, but not prevent, replication and shedding of MDV with greater reductions for the less virulent MDV strain. The vaccinal protection aspect of this experiment has been reported earlier (Ralpanawe *et al.*, 2013).

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II. MATERIALS AND METHODS

The experiment had a 3 x 2 factorial design with two replicates at the isolator level. The experimental factors were: 1) Challenge virus (3 levels) - MDV isolates MPF57 and FT158, 500 pfu/chick or unchallenged, (diluent only) administered in 0.2 mL, subcutaneously (sc.) at 5 days of age; 2) Vaccine (2 levels) - Rispens vaccine (Vaxsafe® RIS Vaccine, Bioproperties, Ringwood, Vic), 4000 pfu/chick or unvaccinated (diluent only) administered in 0.2 ml, injected sc. at the day of hatch (day 0).

The full protection experiment utilised 236 female commercial ISA Brown chickens in 12 isolators, while the viral kinetics component of the study used five individually identified chickens per isolator, serially sampled for blood and feathers. Chickens were not vaccinated at the hatchery, but came from parents vaccinated against MD with the Rispens vaccine so contained heterologous maternal antibodies against MDV. The experiment started on the day of hatch (day 0) and was terminated at 61 days of age (56 days post challenge, dpc). The challenge viruses were grown and titrated at UNE in chick embryo fibroblasts (CEF). Challenge virus details are reported by Renz et al. (2012). FT158 and MPF57 were pathotyped by these authors as vv and v MDV respectively.

Blood and feather samples were collected weekly from the five wing tagged birds in each isolator commencing at 7 days post infection (dpi). Where chickens were infected with both Rispens and MDV, sampling was weekly from 7 dpi of each of the viruses, so comparisons were for the same dpi for each virus. Extraction of DNA from PBL and feathers was carried out using the automated X-tractor Gene (Corbett Robotics, Australia). DNA from dust was extracted using the ISOLATE genomic DNA kit (Bioline, Australia).

To test the hypotheses three separate statistical analyses were performed using JMP 10 (SAS Institute Inc., Cary NC, USA) as outlined below:

a) Analysis 1 tested the effects of Rispens vaccination on MDV load in chickens challenged with pathogenic MDV strains MPF57 and FT158 on day 5 with or without vaccination with Rispens on day 0. Effects fitted included chicken as a random effect, vaccination status, challenge virus, day post challenge (dpc) and the interactions between these effects.
b) Analysis 2 tested the effects of MDV challenge on Rispens viral load in chickens vaccinated with Rispens on day 0 with no challenge or challenge with MPF57 or FT158 on day 5. Effects fitted included chicken as a random effect, challenge virus, day post vaccination (dpv) and the interactions between these effects.
c) Analysis 3 tested the effects of challenge virus on the viral load of Rispens and MDV at the same day post infection (dpi) in chickens co-infected with the two viruses, Ie. vaccinated with Rispens on day 0 and challenged with MPF57 or FT158 on day 5. Effects fitted were chicken as a random effect, challenge virus, virus type, day post infection (dpi), and the interactions between these effects.

III. RESULTS

a) MDV load in vaccinated and unvaccinated chickens

Analysis one showed that MDV viral load was significantly reduced by vaccination with Rispens. MDV load in PBL of the vaccinated birds was significantly lower (1.4 logs) than that of unvaccinated birds (Figure 1-Left). The pathogenic viral load of feathers was also significantly lower (1.2 logs) in Rispens vaccinated birds when compared with unvaccinated birds. The MDV load in birds challenged with the vv pathotype FT158 was significantly higher than in those challenged with the v pathotype MPF57 for PBL ($P = 0.02$), but not for

feather cells (P = 0.06). The dpc had a significant effect of the MDV load of both vaccinated and unvaccinated birds (P < 0.0001) in PBL and feathers (Figure 1-Right).

b) Rispens viral load in challenged and unchallenged chickens

Analysis two revealed that day post vaccination (dpv) but not challenge with MDV had a significant effect on Rispens load in PBL and feather samples (P < 0.0001).

c) MDV and Rispens viral load in chickens co-infected with the two viruses.

Chickens co-infected with both viruses had significantly higher viral load of MDV than Rispens in both PBL (P = 0.0003) and feathers (P < 0.0001). Viral load was significantly influenced by day post infection (dpi) (P = < 0.0001) and was significantly higher in feathers than PBL (10^4.51 v. 10^3.2 viral copies, P < 0.0001). There was a significant interaction between the effects of challenge MDV isolate and type of virus detected (P < 0.0001) due to challenge with FT158 resulting in higher MDV1 load but lower Rispens load relative to challenge with MPF57.

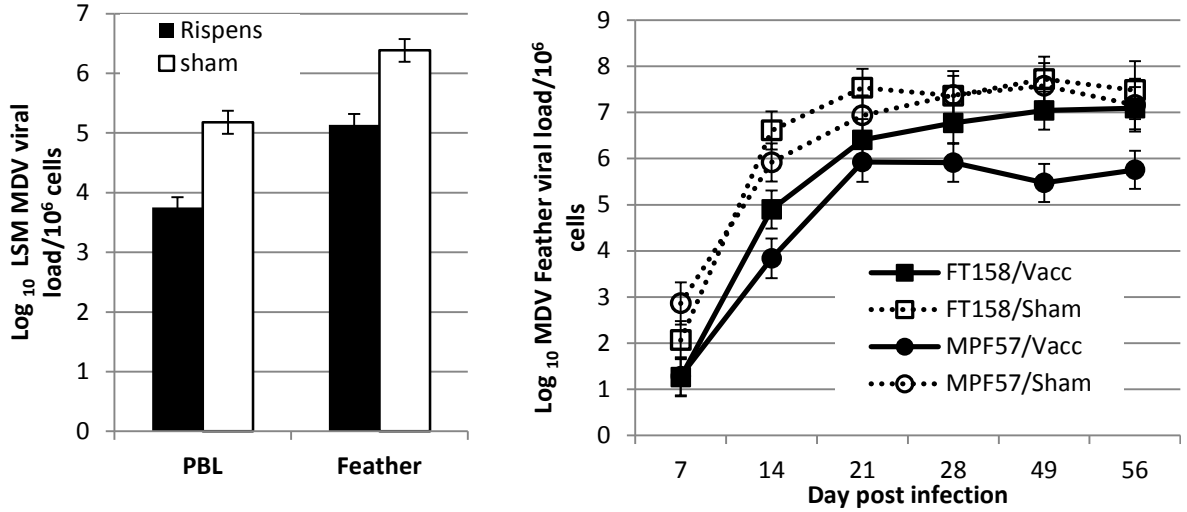

Figure 1 - The effect of Rispens vaccination on overall MDV load in PBL and feathers (left, P < 0.0001), and effects of vaccination, challenge and dpc on MDV load in feathers (Right).

IV. DISCUSSION

MDV load was significantly reduced by vaccination in both PBL and feather samples. This confirms that while vaccination did not prevent infection with MDV it did reduce viral replication significantly so has an anti-viral in addition to an anti-Marek's disease component to its action. These findings are consistent with those of a recent co-infection study with Rispens which used another Australian vvMDV isolate 02LAR (Islam et al., 2014). The higher MDV load observed following challenge with the vvMDV isolate FT158 than the vMDV isolate MPF57 is also consistent with the hypothesis that virulence and viral replication rate are positively associated for MDV as has previously been reported in splenocytes (Yunis *et al.*, 2004) and dust (Atkins et al., 2011) and that more virulent isolates are able to replicate to a greater extent in vaccinated hosts than less virulent isolates.

When chickens were co-infected with MDV and the Rispens virus the MDV load was significantly higher than Rispens viral load by approximately one log in PBL and 1.3 logs in feathers. These findings suggest that avirulent or attenuated vaccine strains replicate more slowly than virulent strains, again consistent with the hypothesis that virulence and viral

replication rate are positively associated for MDV. This has been shown in a similar study with the Rispens vaccine (Islam et al., 2014) and in splenocytes in an earlier study with HVT and MDV2 as the vaccine strains (Walkden-Brown et al., 2013).

Challenge with MDV in this study did not reduce Rispens load in vaccinated hosts (Analysis 2), unlike the finding of Islam et al., (2014). This is probably due to the fact that in the present study, challenge followed vaccination by 5 days, whereas in the Islam et al., study both viruses were administered at equivalent intervals from each other. Nevertheless, the present study revealed an interesting interaction between the viruses in the experiment. In vaccinated chickens challenged with vvFT158, significantly more of the virus detected was MDV and less Rispens, than when the birds were challenged with the less virulent MPF57. This suggests that FT158 had both a higher replication rate than MPF57 (as shown in analysis 1) and also had a greater inhibitory effect on the vaccine virus replication than MPF57. The latter was a non-significant trend in analysis 2 ($P = 0.09$ in feather and 0.23 in PBL).

These results confirm the complex interactions that occur between vaccinal MDV viruses and MDV challenge viruses of different virulence and are broadly supportive of the newer models of MD virulence and the effects of vaccination (Atkins et al., 2013).

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EARLY FEEDING AND HIGH AMINO ACID LEVEL ON PERFORMANCE OF BROILERS UNDER SUBCLINICAL NE CHALLENGE

C. KEERQIN[1], S.B. WU[1] and M. CHOCT[2]

Ao et al. (2012) showed that broilers with early access to dietary additives or specific nutrients have enhanced immune system and gut development. Furthermore, early feeding provided the birds with extra resilience against necrotic enteritis (NE).

Therefore, the present study examined the effect of early feeding a high amino acid density diet on performance of broilers under a sub-clinical NE challenge model. A total of 630 male Ross 308 broiler chickens were assigned to a 2 x 2 x 2 factorial design with two feeding regimes (FED vs HELD), two diets (control vs high amino acid) and two challenges (challenged vs non-challenged). Each treatment had 6 replicates of 12 birds. Starter diets, with or without an extra 10% of digestible amino acids, were fed to d 13, then grower and finisher diets fed to all birds from d 13 to 23 and d 23 to 35, respectively. Feeding regimes included birds given feed and water within 6h of hatch (FED) or those fed 48h after hatch (HELD). Birds were challenged on d 14 and 15 with *C. perfringens* type A strain EHE-NE18 (CSIRO Animal, AU) by oral gavage and performance data collected on d 13, 23 and 35.

The results suggested that the NE challenge, albeit at a subclinical level with no mortality, supressed FCR, with a 23 FCR point difference still remaining between the control and challenged birds at d 35. There was no significant interaction of feeding regime and NE challenge ($P>0.05$). However, diet tended to have an effect by d 35 ($P=0.081$), suggesting that a high amino acid density possibly enabled the birds to recover faster from an NE challenge compared with the control. Further investigation will examine the influence of the gut microflora and the molecular basis of NE on bird performance and compensatory growth.

Table 1 - Feed conversion ratio (FCR) of male broilers with or without extra amino acids under two regimes (FED or HELD) and NE challenge.

Factor		d13 FCR	d23 FCR	d35 FCR
Main effects				
Challenge	-	1.084	1.441	1.471
	+	1.030	1.766	1.704
	p value	0.003	< 0.001	< 0.001
Feeding	HELD	1.044	1.571	1.573
	FED	1.070	1.637	1.602
	p value	0.142	0.013	0.107
Diet	Control	1.084	1.595	1.590
	10% extra aa	1.030	1.612	1.585
	p value	0.003	0.521	0.819
Interactions (P values)				
Challenge*Feeding		0.001	0.288	0.246
Challenge*Diet		0.460	0.833	0.081
Feeding*Diet		0.775	0.035	0.579
Challenge*Feeding*Diet		0.702	0.084	0.960

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THE ROLE OF SUPPLEMENTAL GLYCINE ON BROILERS PERFORMANCE UNDER SUBCLINICAL NECROTIC ENTERITIS

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Coccidial co-infection, dietary inclusion of viscous grains and ingredients of animal origin protein are considered important predisposing factors for onset of necrotic enteritis (NE). High dietary glycine levels has been shown to increase the intestinal *C.perfringens* population in broilers (Dahiya et al. 2005). As the causative agent for NE, the spore germination of *C. perfringens* can be activated by the spore cortex-lytic enzyme (SCLE) coded by *SleC* thus to cause gastrointestinal disease. Investigations have shown that glycine forms a conjugated compound with bile salts playing a critical role in Clostridial germination (Sorg and Sonenshein 2008). This compound is recognized by the germinant receptor, CspC and is responsible for cleaving the N-terminal propeptide of *SleC*, expressing mature SCLE to activate its hydrolase activity.

The current study assessed the impact of a high dietary glycine inclusion on performance of broilers under subclinical necrotic enteritis challenge. Ross 308 male broilers (n = 720) were allocated to a 2 x 2 x 2 factorial arrangement of treatments. Factors were: *Eimeria* inoculation on d 9 – no or yes; *C.perfringens* challenge on d 14 and 15 - no or yes; dietary glycine, none or 10 g/kg from d 9 to 24. Wheat-soybean based diets were fed. Live weight, feed intake and FCR were determined on d 9 and 24.

In this study, the impact of additional glycine supplement on broiler performance under subclinical necrotic enteritis (lesion score less than 2) was demonstrated. Glycine supplement did not significantly affect the severity of NE nor did it affect weight gain, however, it improved FCR ($P < 0.05$) and decreased feed intake ($P < 0.01$) from d 9 to 24. The result suggests that dietary glycine may offer some protection against NE. The mode of action may be related to the reduction of feed intake during NE challenge or a possible interruption of *C. perfringens* sporulation. Indeed, temporary feed restriction has been shown to protect broilers from NE (Tsiouris and Georgopoulou et al. 2014).

Table 1 – Performance of broilers upon the addition of glycine at d 24 of age.

	Intake g/bird	Weight gain g/bird	FCR
Gly-	1445[a]	1056	1.369[a]
Gly+	1397[b]	1049	1.331[b]
SEM	11.8	7.8	0.011
P-value	0.007	0.554	0.017

[a,b,c]Means within columns not sharing common superscripts are significantly different($P < 0.05$).

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ROLE OF DIET ON ODOUR EMISSIONS FROM MEAT CHICKENS

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Summary

Abatement of odour emissions has become an important consideration to agricultural industries, including poultry production. In order to study the link between diet and odour emissions, an experiment was conducted using twelve Ross 308 broiler chickens. At the age of 22 days, birds of uniform body weight were selected from a total of 288 male birds, adapted to metabolic chambers for six days and fed their respective diets for 15 days. Two treatments were compared using three replicates of two birds per chamber. The two wheat-soy diets were formulated according to the 2007 Ross 308 nutrient specifications for digestible amino acids but they differed in ingredient composition and metabolisable energy content. Thus, Diet A had 13.39 MJ/kg ME and used 60g/kg canola but no corn whereas Diet B contained 12.90 MJ/kg ME and used 150g/kg corn but no canola. The odorous emissions were measured using a Fourier transform infrared spectroscopy. A total of 24 volatile organic compounds were detected and quantified; eight being the major odorous ones: 2,3-butanedione, 2-butanone, dimethyldisulfide, methylmercaptan, 2-butanol, 3-methyl-butanal, phenol and m-cresol. From this pilot study it appears that there is a strong link between diet and odour emissions from broiler chickens.

I. INTRODUCTION

Odours are generated in meat chicken houses primarily from the microbial decomposition of faecal matter in chicken litter (Jiang and Sands, 2000) as well as directly from the birds (Lacey et al., 2004). In recent years, odour emissions have become a growing concern to poultry producers. Investigations by Murphy et al. (2014) and Dunlop et al. (2011) resulted in a comprehensive list of odorous chemical compounds that are of interest to the poultry industry. Murphy et al. (2014) identified eight major volatile organic compounds from tunnel ventilated meat chicken sheds that were considered as important predictors of odour. These were dimethyl sulphide (DMS), dimethyl trisulphide (DMTS), 2-3 butanedione, 3-methyl-butanal, 1-butanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone (acetoin), and 2-butanone. In an effort to address odour issues from poultry farms, there have been attempts to develop mitigation strategies including litter treatments, biofilters, neutralising agents, air scrubbers, ozone treatment, windbreak walls and short stacks but these techniques are generally costly or impractical due to the required high ventilation rates in poultry farms (Dunlop et al., 2011). There is little information available linking diet composition to odour emissions.

Diets can be formulated to more closely meet the bird's nutritional requirements to avoid overfeeding and to reduce excretion of undigested components. This will decrease the available substrates that the microbes metabolise to odour compounds (Mackie et al. 1998). A real time odour measuring device, such as the Fourier transform infrared (FTIR) spectrometer, is feasible to measure odorous gases and quantify their individual constituents. Van Kempen et al. (2002) and Witkowska (2013) successfully used FTIR to detect and quantify odours from swine and turkey houses respectively. The objective of this study was to use FTIR to compare odour emissions from broilers fed two diets differing in ingredient and nutrient composition.

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II. MATERIALS AND METHODS

At the age of 22 days, 12 birds of uniform body weight were selected from a pool of 288 Ross 308 male broilers. The birds were adapted to the metabolic chambers (Swick et al., 2013) for six days in a climate controlled room and fed their respective test diets. The experiment started when the birds were 28 days of age and finished on day 42. Feed and water were provided ad libitum. Each diet was replicated three times with two birds per chamber. Two wheat-soy diets were formulated according to the Ross 308 nutrient specifications for digestible amino acids (Aviagen, 2007). Diets were isonitrogenous but differed in ingredient composition and ME (Diet A with 60g/kg canola and no corn, 13.39 MJ/kg ME; Diet B with 150g/kg corn and no canola, 12.90 MJ/kg ME). Diets were analysed for nutrients including sulphur content.

A Fourier transform infrared (FTIR) spectrometer (Gasmet^{TM} Model DX-4015, Gasmet Technologies, Finland) was calibrated for 30 compounds previously reported as odorants or volatile compounds from poultry production facilities. Gaseous samples were collected at day 42 from the excreta in the presence of birds. Chamber lids were closed for approximately 20 min before sample collection. Water was used to seal the chambers. At that time there was zero air exchange and odorants were allowed to concentrate prior to sampling. Carbon dioxide and oxygen levels inside the chambers were recorded during the period of closure. The emissions from the chambers were analysed using FTIR with the following set-up: flushing time- 30s, pumping time-1 min, measuring time-3 min. The gas samples were drawn at a flow rate of 2 L/min with the in-built FTIR instrument pump (i.e. 2 liters of gases were measured from each chamber). After sampling, the FTIR was flushed with pure nitrogen for 15 min before taking measurements for the next group. After the measurements, quantitative analysis was conducted in a laboratory with the use of Calcmet Professional software with a library of reference spectra for 30 gases.

The data were analysed by one-way ANOVA using the GLM procedure (SAS Institute Inc., Cary, NC). Differences were determined using the t-test. Variability in the data is expressed as the standard error means (SEM) and a probability level of $P < 0.05$ was considered to be statistically significant.

III. RESULTS AND DISCUSSION

The total duration allocated for concentration and measurement of odour (i.e. 25 min) was sufficient to capture the volatiles with accetable levels of CO_2 and O_2 (< 2% and > 18%, respectively) inside the chambers. The temperature and humidity at the time of sampling were recorded as 22-23°C and 75%, respectively. Since FTIR provides results in parts per million (ppm), slight changes in temperature or humidity at sampling would not affect results. Altogether 24 volatile organic compounds were detected and quantified. Out of these, the eight considered as key odorants are listed in Table 1. The first six compounds are considered to be important poultry odorants (Murphy et al., 2014). Two important odour predictors not detected by FTIR were dimethylsulfide and dimethyltrisulfide but methylmercaptan was detected. The mercaptans are unstable and can be oxidized easily to other sulphur compounds upon storage or during carbon capture sampling techniques using a thermal desorption analysis process (Parcsi, personal communication). Murphy et al. (2014) used a thermal desorption process to quantify the volatiles and it is possible that the mercaptans were oxidized to dimethylsulfide and dimethyltrisulfide in those studies. In the current study, very short sampling intervals were used, thus it could be stated with reasonable confidence that the mercaptans did not have time to oxidise into other sulphur compounds. The concentration of methylmercaptan therefore might have been higher and oxidised products

lower in this study than previously reported. Mercaptans and thiols are considered to be important odorants in animal production facilities (Trabue et al., 2011).

Table 1 - Odorous compounds emitted from meat chickens fed two commercial diets.

Compounds	Diet A, ppm (+canola seed)	Diet B, ppm (+corn)	SEM	P-value
2, 3-butanedione/diacetyl	1.099[b]	2.307[a]	0.286	0.005
2-butanone	0.923	0.704	0.157	0.548
Dimethyldisulfide	3.242	3.079	0.154	0.651
Methanethiol/methylmercaptan	19.393[a]	15.607[b]	0.940	0.014
2-butanol	0.000	0.344	0.109	0.116
3-methyl-butanal	0.317	0.496	0.166	0.645
Phenol	0.880[b]	0.981[a]	0.026	0.027
m-cresol	0.582[b]	1.051[a]	0.112	0.006
Excreta moisture, %	76.20[a]	68.25[b]	1.530	0.035

[a,b] Means in the same row with different superscripts differ ($P < 0.05$) or ($P < 0.01$).

Methylmercaptan, dimethyldisulfide, 2,3-butanedione, phenol and m-cresol were measured at higher concentrations than the odour detection threshold (Schiffman *et al*., 2001). Methylmercaptan has a rotten cabbage smell (Merck Index, 1968) and was produced at higher levels ($P < 0.05$) from broilers fed Diet A. Dimethyldisulfide was also detected in chamber air from both diets but the concentration of the total sulphur compounds was higher with Diet A. These results suggest that the use of 60g/kg canola seed led to a higher level of sulphur containing odorants compared to Diet B that did not have canola seed. The calculated digestible methionine plus cysteine were similar in both diets (7.3 g/kg vs. 7.0 g/kg). This small difference in dietary sulfur amino acid levels is unlikely to produce differences in odour among the treatments. However, a higher excreta moisture content was observed in chambers from birds fed Diet A ($P<0.05$). The litter moisture content does not correlate with odour emissions but correlates with odorant characteristics. Increased litter moisture is associated with higher concentrations of organosulfides, aldehydes, and alcohols (Murphy et al., 2014) due to increased anaerobic degradation (Jiang and Sands, 2000). Therefore, the higher organosulfide emission from Diet A in this study may be related to a higher excreta moisture content.

Diacetyl (2, 3-butanedione) has a rancid butter smell (Dunlop et al., 2011). This compound was produced at higher levels ($P < 0.01$) in chambers from broiler fed Diet B than those fed Diet A. Diacetyl is considered an important chicken odorant due to its relatively low human detection threshold (Murphy et al., 2014). Diacetyl is a product of fermentation. Future investigations of intestinal metabolites and microbiota may help elucidate the mechanism by which diet composition influences diacetyl production. Diet B produced higher levels of phenol ($P < 0.05$) and m-cresol ($P < 0.01$) compared to Diet A. These compounds are considered to be strong odorants by some researchers (Trabue et al., 2008b) and weak odorants by others (Murphy et al., 2014). Phenol originates from the microbial degradation of tyrosine in the intestinal tract of animals and from phenolics contained in litter (Le et al., 2005).

This study clearly showed that diet has an impact on odours produced from broiler chickens. Using closed circuit metabolic chambers coupled with FTIR allowed for accurate detection and quantification of the odorous compounds that are of interest to the poultry industry. Minor changes in diet composition were found to change the relative abundance of gases associated with odours. Further investigation is warranted to more fully understand the

effect of microbial metabolism of nutrients and metabolites in the gut and litter on odour formation.

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SUPERDOSING PHYTASE IN WHEAT-BASED DIETS IMPROVES LITTER AND FOOT PAD SCORE WHILST SIMULTANEOUSLY IMPROVING PERFORMANCE

M.R. BEDFORD[1] and C.L.WALK[2]

Superdosing (SD) of phytase is becoming more commonplace as the benefits of almost complete phytate destruction significantly outweigh the costs of additional enzyme. Improvement in performance is often noted but recently some reports of wet litter, which has been associated with, but not attributed to, SD phytase have emerged. Some data suggest that increasing dosages of phytase reduce litter moisture (Bedford et al, 2007, Bedford et al., 2012) when no further adjustment of minerals is made. Such information is lacking in wheat based diets, however, particularly when the phytase is used in combination with a xylanase.

640 male Ross 308 day old broiler chicks were weighed and assigned to 4 treatments with 20 birds per floor pen ($2m^2$) and 8 replicates per pen. A basal wheat-based diet was formulated to supply all nutrients at requirement level or greater, with the exception of Ca, P and Na which were reduced by 0.165, 0.15 and 0.03% respectively. Diets were supplemented with either 500 or 1500 FTU/kg phytase (Quantum Blue, AB Vista Feed Ingredients Ltd) with and without a xylanase at 16,000 or 0 BXU/kg (Econase XT, AB Vista Feed Ingredients, Ltd). The addition of the 500FTU/kg dose resulted in Ca, P and Na being supplied at requirement whilst the 1500 FTU/kg dose (SD) more than compensated for these reductions. Birds were fed a crumbled starter (0-21d of age) and pelleted grower (21-42d) and both feed and water were offered libitum. Weight and feed intake were determined at 21 and 42d of age and FCR calculated. At 21 and 42d of age, the condition of the foot pads (1-3, 1 = no lesions and 3 = severe) and litter (1-5, 1 = fully friable and 5 = capped > 80%) were assessed. Water and feed intake were monitored by load cell in each replicate pen every 15 minutes and a plot generated for the water:feed intake for each treatment. Data were analysed by ANOVA with main effects of phytase and xylanase dose and the interaction and LSmeans separated where appropriate by the P-diff procedure of JMP 11.

Dietary nutrient contents and in feed enzyme assays were within acceptable limits of the targets. Mortality was relatively high at 9% and was related to the very heavy weights achieved (3.5kg at 42d of age) but there were no diet effects. No interactions were noted for any parameter measured. Intake increased significantly at both 21 and 42d of age with SD phytase addition as did gain, (gain $p = 0.086$ at 42d of age). Xylanase improved FCR at 42d from 1.66 to 1.60 and when FCR was corrected for body weight differences (33g=1 point), SD phytase improved FCR by 5 points. Litter and food pad data were not influenced by treatment at 21d but both improved with SD phytase a 42d. The water to feed ratio tended to be lower in the SD phytase treatments but no consistent xylanase effect was noted. These data suggest that increasing the dose of phytase without applying any further matrix results in better performance, less pressure on the litter, and fewer problems with foot pad dermatitis in wheat based diets. The addition of xylanase improved performance but had no influence on litter or foot pad dermatitis.

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ANTIOXIDANTS IN BROILER BREEDER DIETS CAN AFFECT OFFSPRING PERFORMANCE

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Summary

In a series of experiments it was shown that the antioxidant mix in broiler breeder diets can affect broiler performance trans-generationally. To study this, a polyphenol blend with high antioxidant properties was used to partially replace (50%) vitamin E in breeder diets. Chick weight was found to be significantly heavier at hatch, and offspring growth performance (average daily gain) improved significantly in young breeder offspring, while this effect was not observed when only the vitamin E level increased in the breeder diet. The study shows that partial replacement of dietary vitamin E (50%) with a polyphenol blend in breeder diets can improve offspring performance.

I. INTRODUCTION

Antioxidants are an important dietary component of animal diets. They counter oxidation and limit oxidative stress. There exists an antioxidant-oxidant balance in animal tissues. Vitamin E (Panda & Cherian, 2014), vitamin C (Whitehead & Keller, 2003) & vitamin A (Surai *et al.*, 1998) are vitamins that provide an anti-oxidative defense. Besides vitamins, also carotenoids and the enzymes glutathione peroxidase (GSH-PX), catalase (CAT) and superoxide dismutase (SOD) are important in maintaining this balance (Yigit *et al.*, 2014). Animal nutrition research has recently been focusing on the antioxidant capacity of polyphenol compounds (Liu *et al*., 2014; Surai, 2014). The avian embryo development relies on antioxidants accumulated in the egg yolk via breeder diet (Yigit *et al.* 2014). Various antioxidants that belong both to enzymatic and non-enzymatic groups can counter oxidative stress (Khan, R.U., 2011), but it is not well described how antioxidants interact with each other. Overall anti oxidation capacity might help optimal embryonic development (Yigit *et al.*, 2014), and therefore possibly also post-hatch performance.

In order to evaluate if a variation in dietary antioxidant status in breeding bird diets affects offspring performance, it was tested whether a 50% replacement of dietary vitamin E by a selected combination of polyphenols influences breeder performance, embryo development -/- hatchability and post-hatch growth performance. The results are described in this paper.

II. MATERIALS AND METHODS

Two breeder experiments were performed at the Cargill Innovation Center in Velddriel, the Netherlands. A single flock of broiler breeders was used in two periods (1st period=30-39 weeks of age, and the 2nd period=56-64 weeks of age). The eggs produced by these broiler breeders, at the end of both trial periods, were collected and incubated. Fertility and hatchability were measured for both breeder groups during both trial periods. Last, the performance of the broilers that hatched from the two breeder groups was measured. The connected experiments are described in more detail below. Proviox™, a blend of natural antioxidants that in anti-oxidative activity equals vitamin E50 (vitamin-E equivalent), was used to replace vitamin E in the diet.

a) Broiler breeders

Two experiments were performed with the same animals (1st experiment = young flock: 30-39 weeks old, and 2nd experiment = older flock: 56-64 weeks old). The flock consisted of 730 females and 92 males (Ross 308 broiler breeders). The animals were commercially obtained

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(Pluvita B.V., the Netherlands) and were randomly assigned to 24 floor pens. Each pen contained 3 males and 30 females. The animals received the same diet during the six week pre-experimental periods. During the first 9-week experimental period (30-39 weeks old) four diets were fed: two levels of vitamin E (0.008 g/kg and 0.016 g/kg) and two diets where 50% of the vitamin E was replaced with polyphenols, (0.004 g/kg vitamin E + 0.004 g/kg polyphenols and 0.008 g/kg vitamin E + 0.008 g/kg polyphenols). At 56-64 weeks old two diets were fed, a control diet, and a diet where 50% of the vitamin E was replaced by polyphenols. The control diet contained 0.008 g/kg vitamin E. The treatment diet contained 0.004 g/kg vitamin E and 0.004 g/kg polyphenols (a 50% replacement of vitamin E). In the second experiment two other treatments were evaluated, but due to the scope of this paper not described here. Female and male diets were isoenergetic, containing respectively 145 and 130 g/kg crude protein. The diets were corn, wheat, soybean meal based. No differences occurred in crude fat, crude fiber, dry matter, amino acid levels, minerals or vitamin levels between the two treatment groups. Diets were produced by Research Diet Services BV (RDS), Wijk bij Duurstede, the Netherlands.

b) Incubation, fertility & hatchability

Eggs from both breeder groups were collected after feeding the experimental diets for 6 weeks, and were incubated in the same incubators. Eggs were incubated at the In Ovo Bioassay research facilities of the Cargill Innovation Center in Velddriel, the Netherlands. For a 6 day period (young flock) and 11 day period (older flock), eggs were collected from the two groups of broiler breeders. Eggs were stored and incubated in blocks based on breeder pen and storage day. Only intact, clean eggs were included. The eggs were placed in a NatureForm NMC 2340 incubator with automatic temperature and relative humidity controls. The temperature was initially set at 37.5°C (E0), and was gradually decreased to 36.7°C (E12 until E18). At E7 and E18 all eggs were candled and empty eggs or eggs containing dead embryos were recorded and removed. Eggs were transferred to hatching baskets at E18, kept in the same groups as during setting. At hatch (E21) the number of dead chickens, late dead embryos (E19-E21) and pipped eggs were recorded. The number of hatched chickens was recorded per replicate, as well as average chicken weight. Hatchability was calculated as the percentage of total eggs set.

c) Offspring

Growth performance was measured twice (once with hatchlings from the eggs laid at 38-39 weeks of age, and once with the hatchlings from the eggs laid at 63-64 weeks). The first experiment lasted until 35 days and the second until 34 days. All the chickens were produced by the two dietary groups of broiler breeders. Chickens were sexed at hatch. 504 males were included in the growth performance trials. Only completely dry chickens, showing active movement and with a good navel score were included. Floor pens were used with 90 x 225 cm floor area per pen. One feed was fed to the offspring. Water and feed were provided *ad libitum*.

d) Statistical analysis

Performance parameters (normal distribution), hatchability and embryonic mortality (binomial distribution) were subjected to mixed model analysis using the PROC MIXED procedure in SAS (version 9.3, 2011, SAS Institute Inc., Cary, NC) using breeder pen as the experimental unit. For experiment 1, contrasts were used to determine significant relationships for 1) the effect of partially replacement by polyphenols, and 2) the effect of vitamin E. For experiment 2, again contrast 1 was used to determine significant relationships. Data are expressed as least square means. Effects were considered to be significant when $P \leq 0.05$.

III. RESULTS AND DISCUSSION

a) Broiler breeder performance

Breeder performance results are presented in Table 1. In young breeders the partial replacement (50%) of vitamin E with polyphenols decreased breeder laying percentage. This effect was not found in older breeders. Polyphenols reduced the amount of broken egg shells in young breeders.

Table 1 - Broiler breeder (Ross 308) performance during two production periods of the same flock (30-39 & 56-64 weeks of age). From 30-39 weeks 4 groups were fed two levels of vitamin E (0.008 and 0.016 g/kg), and two partial (50%) replacement diets (0.004+0.004 g/kg vitamin E and polyphenols, and 0.008 g/kg Vitamin E + 0.008 g/kg polyphenols). In the 56-64 week old flock the 0.008 g/kg vitamin E level and the partial 50% replacement with polyphenols was tested again.

Breeder diet:	Young breeder flock (30-39 weeks)						Older breeder flock (56-64 weeks)		
Vitamin E (g/kg)	0.008	0.016	0.004	0.008	Replace-ment Effect (*P*)	Vitamin E Effect (*P*)	0.008	0.004	*P-value*
polyphenols (g/kg)			0.004	0.008				0.004	
Laying %	84.3	85.0	80.4	82.7	*0.015*	*0.689*	50.4	54.8	*0.193*
Av. egg weight (g)	61.2	61.6	61.7	61.0	*0.803*	*0.472*	71.3	71.9	*0.294*
Av. egg mass (g/h/d)	51.6	52.4	49.5	50.4	*0.020*	*0.519*	35.9	39.4	*0.134*
BW at start exp. (kg)	3.51	3.48	3.51	3.51	*0.476*	*0.192*	4.29	4.25	*0.433*
Final BW (kg)	3.88	3.83	3.85	3.81	*0.348*	*0.176*	4.45	4.37	*0.237*
Broken eggs (%)	1.2	1.3	0.4	0.9	*0.025*	*0.795*	1.5	0.8	*0.277*
Dirty eggs (%)	1.7	1.8	1.6	1.5	*0.618*	*0.821*	2.1	1.8	*0.551*

n= 6 pens per treatment

b) Incubation, fertility & hatchability

Egg weight, fertility, hatchability (%), yolk free body mass and chick length were not significantly different between the two groups in both periods (P>0.05). Higher average chick weight (*P*=0.018) and higher relative chick weight (*P*=0.012) were found in the polyphenol replacement group in hatchlings from the older brood stock.

c) Offspring performance

Offspring performance is shown in Table 2. Body weight at day 1 did not differ in the two trials between the two groups included in the grow-out trial (*P*=0.518 in the offspring of the young flock & *P*=0.790 in the offspring of the older flock respectively). Mortality did not differ in the two trials between the two groups.

In offspring of young broiler breeders, a trans-generation effect of vitamin E supplementation by polyphenols resulted in significant improvement in broiler performance, while a higher vitamin E level decreased offspring performance. When 43 week old Indian River broiler breeders were fed a diet supplemented with 0.3g/kg vitamin E, it did not have any effects on offspring weight gain, (Haq *et al.*, 1996). A dose response study of vitamin E (0.025, 0.050, 0.075 and 0.1 g/kg) using 24-54 wk old Ross broiler breeders also showed no difference in laying percentage, egg production, hatchability, or offspring chick weight. Vitamin E was transferred from hen diet to egg up to 0.075 g/kg, but offspring weight gain, feed conversion ratio, or livability were not significantly affected by the dietary vitamin E level (Hossain *et al.*, 1998). A

single elevation of vitamin E has then the desired effect of increasing antioxidant level of the offspring, but this does not result in higher offspring performance.

Table 2 - Offspring growth results (Ross 308), from broilers produced by one breeder flock during two periods (30-39 & 56-64 weeks of age). From 30-39 weeks the breeders received four diets, two vitamin E diets (0.008 and 0.016 g/kg), and two partial (50%) replacement diets (0.004+0.004 g/kg vitamin E and polyphenols, and 0.008 g/kg Vitamin E + 0.008 g/kg polyphenols). From 56-64 weeks the breeders were fed two diets: a control diet (vitamin E = 0.008 g/kg), and a diet where 50% of the vitamin E is replaced by polyphenols.

Breeder diet:	Offspring performance of young breeder flock (30-39 weeks)				Offspring performance of older breeder flock (56-64 weeks)				
Vitamin E (g/kg)	0.008	0.016	0.004	0.008	Replace-ment effect (*P*)	Vitam in E Effect (*P*)	0.008	0.004	*P-value*
Polyphenols (g/kg)			0.004	0.008				0.004	
ADG[1] (g)	62.3	59.7	63.8	64.1	0.008	0.064	71.8	72.8	*0.509*
ADFI[2] (g)	94.3	91.8	97.2	96	0.011	0.167	106.3	107.9	*0.463*
F:G[3]	1.517	1.537	1.524	1.508	0.078	0.020	1.479	1.482	*0.818*
F:G corrected for BW[4]	1.507	1.546	1.504	1.485	0.008	0.015	1.479	1.476	*0.828*

n= 6 pens per treatment
[1] ADG=average daily gain for the period (g)
[2] ADFI= average daily feed intake for the period (g)
[3] F:G=Feed to gain ratio, mortality corrected.
[4] F:G corrected= corrected to lowest body weight group with -0.02 per 100 gram extra weight.

In this study, the effect of the presence of a mix of antioxidants (presence of both vitamin E and polyphenols) was measured in the production of heavier chicks at hatch, and in improved offspring performance in young breeders. It is likely that interaction between vitamin E and polyphenols causes the significant improvement in offspring performance.

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IMMUNOGENIC INGREDIENTS IN POULTRY: APPLICATION OF INNOVATIVE CONCEPTS LEADING TO SUSTAINABLE SOLUTIONS FOR IMPROVED PRODUCTIVITY

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Summary

Recently, research has focused on the impact of immunogenic factors present in certain ingredients widely used in the poultry industry. These immunogenic factors cause an unnecessary and counter-productive immune reaction in the animal that can potentially lead to reduced productivity. In poultry feeds, β-galactomannans are the immunogenic substance which has been most studied. These molecules occur naturally in ingredients such as soybean, rapeseed, sunflower and palm kernel meals. Recent research shows that the enzymatic hydrolysis of β-galactomannans can benefit poultry production efficiency.

I. INTRODUCTION

Feed Induced Immune Response (FIIR) as a response to β-galactomannans is a potential threat to broiler performance and uniformity (Anderson and Hsiao, 2006). β-galactomannans can be considered as the leading molecules and are most prevalent in a wide variety of feed ingredients including soybean meal (SBM), sunflower meal, palm kernel meal, copra meal, and sesame meal. Since soybean meal is a major protein source in feeds produced around the world, β-galactomannans are present in most feeds (Hsiao et al., 2006). β-galactomannans in SBM are linear polysaccharides composed of repeating β - (1-4) mannose β -(1-6) galactose and/or glucose units attached to the β -mannan backbone. They are highly viscous, water soluble, heat-resistant compounds that survive the drying/toasting phase of soybean processing (Hsiao et al., 2006).

β-galactomannans can be recognized by the innate system and considered by the intestinal mucosa as Pathogen Associated Molecular Patterns (PAMP) by several Pattern Recognition Receptors (PRR), including the serum protein mannose binding lectin MBL (Figure 1) as well as several cell surface receptors on key immune system cells including mannose receptor (MR) and others (DC-SIGN) on key immune system cells (Stahl and Ezekowitz, 1998.). Consequently, they provoke an intestinal micellimmune response which is energy consuming and wasteful . The association of mannan with the surface of numerous pathogens has likely led to β -galactomannan's conserved recognition by the innate immune system (Stahl and Ezekowitz, 1998).

As illustrated in Figure 1, the Innate Immune system can also be activated when molecular patterns also found in high molecular weight mannan present in leguminous feedstuffs such as soybeans are detected by the mannose binding lectin thus associating into structures that have multiple CRD (carbohydrate binding domains). This can potentially induce a strong and costly feed induced immune response (Anderson, 2009).

Recently β-mannanase has been used to hydrolyze β-galactomannans into mannan oligosaccharides fragments that can no longer be recognized by toll like receptors (MBL) (Figure 2). Thus, by negating FIIR, beta mannanase can potentially help conserve valuable energy and that can be used towards improved growth and performance.

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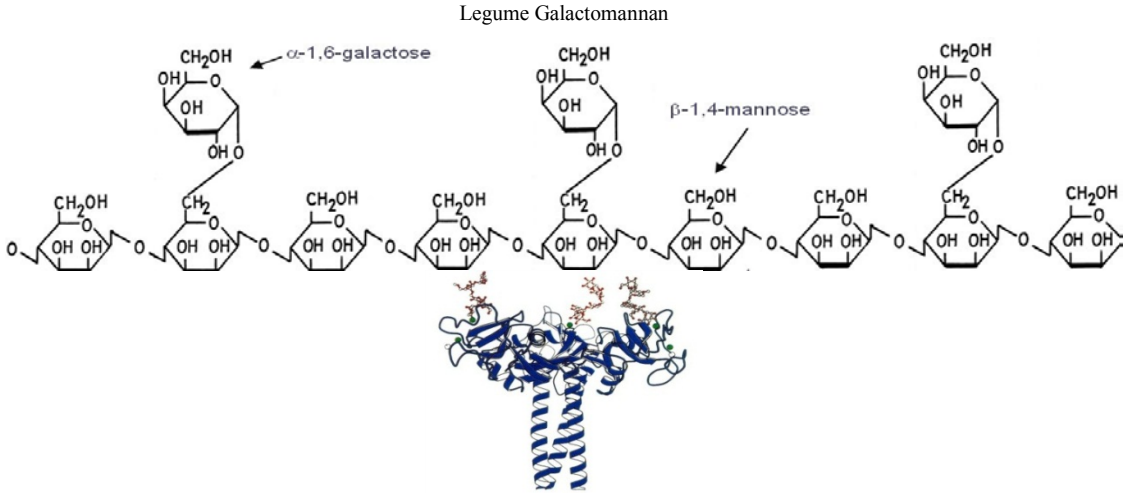

Figure 1 - β-galactomannan can be detected by multi-CRD binding site structure of Mannose Binding Lectin (Weis & Drickaner K, 1994).

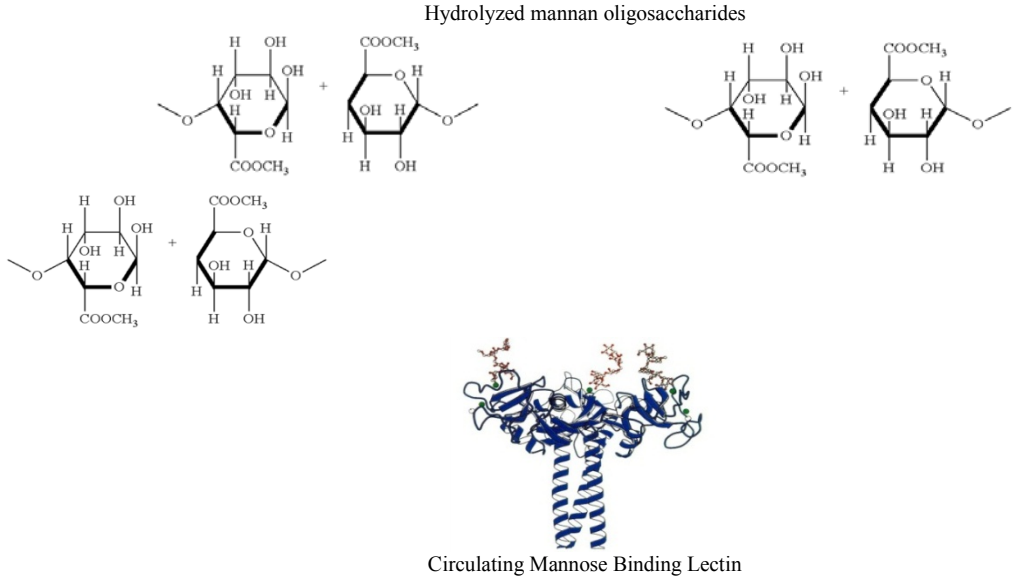

Figure 2 - Hydrolyzed mannan oligosaccharides are no longer detected by multi-CRD binding site structure of Mannose Binding Lectin (Weis & Drickaner K, 1994).

II. MATERIALS AND METHODS

A broiler experiment was conducted to evaluate the effects of β-mannanase on live performance and uniformity at market age (Table 1). For this trial, birds were grown using three feeding phases for each treatment to 43 days using 5 treatments and 8 replications with 50 birds placed per pen. Treatments consisted of: 1. Positive control diets ranging from 3217 to 3086 Kcal/kg and 21.75 to 18.00 % Crude Protein, 2. As 1 minus 42 Kcal/kg in each phase, 3. As 1 minus 77 Kcal/kg in each phase, 4. As 2 + 225 g of β-mannanase/metric ton of feed in each phase, and 5. As 3 + 400 g of β-mannanase/metric ton of feed in each phase.

III. RESULTS AND DISCUSSION

Results from the present study showed that adding β-mannanase at 225 g/metric ton of feed significantly improved weight gain and live weight uniformity in energy reduced diets (Table 1). The live performance benefit of the energy reduced feed was restored to that of the positive control which contained an additional 42 Kcal/kg of ME. β-mannanase when added at 400 g/metric ton of feed significantly improved final weight, FCR, and uniformity when compared to treatment 3. Weight adjusted feed conversion (WAFC) benefit was superior to that of the positive control which contained an additional 77 Kcal/kg ME. β-mannanase significantly improved live weight uniformity compared to the energy reduced ration by lowering the coefficient of variation by approximately 2 percentage points.

Table 1 - The effect of β-manannase[2] on broiler productivity, and weight uniformity (% coefficient of variation) in commercial broilers (Knox, 2009).

Treatment	BW (g)	FCR	WAFC*	Mortality %	Uniformity (CV**)
Positive Control (PC)	2046[a]	1.897[bc]	1.874[b]	4.81[a]	9.98[ab]
PC – 42 Kcal/Kg ME	1969[bc]	1.886[b]	1.892[b]	4.81[a]	11.17[bc]
PC – 77 Kcal/Kg ME	1923[c]	1.966[d]	1.988[a]	3.61[a]	12.19[c]
PC – 42 Kcal/Kg ME + 225 g β- mannanase /metric ton of feed	2059[a]	1.894[bc]	1.867[b]	3.85[a]	9.81[a]
PC – 77 Kcal/Kg ME + 400 g β- mannanase /metric ton of feed	2091[a]	1.807[a]	1.768[c]	4.57[a]	9.47[a]

[a-e] P < 0.05.* Each 23 g in weight is converted to 0.01 of feed conversion.
*Weight adjusted feed conversion
** Individual weights determined at 42 days
[2]Elanco Animal Health, minimum 160 Million units/kg

IV. CONCLUSIONS

β-mannanase can improve live performance and uniformity in broilers. Further studies are required to confirm these findings since improvements in live weight uniformity in grow-out barns will translate to an improvement in the consistency of processed poultry products (Anderson, 2001, Knox, 2009).

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THE IMPACT OF BEDDING MATERIALS ON BROILER PERFORMANCE

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The importance of litter quality for rearing broilers is well recognised (Anisuzzaman and Chowdhury, 1996). Broilers do not reach their genetic potential for weight gain and feed conversion ratio when there is a poor environment including suboptimal litter. Although a variety of products have been used as bedding materials for poultry, the supply and availability of bedding litter is becoming scarce and more expensive. Therefore, alternative sources of litter are of interest to the industry. The objective of the present study was to assess broiler performance housed on pelleted wheat straw as an alternative litter source to commonly used bedding materials including rice hulls, wood shavings, chopped straw and shredded paper. Three hundred and sixty Ross 308 d-old male chicks were used. There were 5 treatments with 6 replicate pens each with 12 birds. Wheat-sorghum-soybean meal diets were formulated to meet the Ross 308 specifications and were fed as starter (0-9 day), grower (10-24 day) and finisher phases (25-35 days). All birds received the same diet in each phase.

Growth performance of broilers was affected by types of litter at day 10 but not thereafter (Table 1). The feed conversion ratio (FCR) of chicks reared on pelleted straw was improved compared ($P < 0.05$) to birds reared on chopped straw and rice hulls whereas there were no differences with wood shavings and shredded paper ($P > 0.05$). On day 25, birds reared on wood shavings tended ($P < 0.054$) to have improved FCR when compared to those reared on shredded paper and rice hulls. The results demonstrate potential benefits of using pelleted wheat as a bedding material. Bird performance improvements will need to be assessed against cost and availability of this material.

Table1 - Performance in response to five types of bedding materials.

Period	Rice hulls	Wood shavings	Pelleted straw	Chopped straw	Shredded paper	P value
Body weight gain (g/bird)						
1-10d	273	269	279	266	272	0.332
1-24d	1293	1322	1359	1296	1331	0.218
1-35d	2485	2521	2523	2472	2602	0.064
Feed intake (g/bird)						
1-10d	329.94	313.92	317.85	317.83	316.87	0.304
1-24d	1705.77	1677.8	1750.45	1702.48	1767.33	0.129
1-35d	3573.12	3665.88	3688.90	3586.42	3843.88	0.071
Feed conversion ratio (FCR)						
1-10d	1.209[a]	1.167 [ab]	1.137[b]	1.193[a]	1.164[ab]	0.026
1-24d	1.32	1.27	1.28	1.31	1.32	0.054
1-35d	1.43	1.45	1.46	1.45	1.47	0.614
Livability %						
1-10d	100	98.61	98.61	98.61	100	0.735
1-24d	98.61	98.61	95.83	98.61	97.22	0.768
1-35d	98.61	94.44	95.83	97.22	95.83	0.640

[ab] Means sharing the same superscripts are not significantly different from each other at $P < 0.05$.

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FEATHER PECKING IN LAYERS - STATE OF RESEARCH AND IMPLICATIONS

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Summary

Feather pecking and cannibalism are considered the most important problems in egg producing flocks. Despite extensive research during the last five decades the causes of these damaging behaviors have not been identified and the problem still persist. According to the prevailing theory feather pecking is considered a misdirected food pecking or foraging behavior. It is assumed that food pecking/foraging is independent of satiation. Under intensive feeding conditions satiation may occur before the drive of feather pecking is consumed. In the absence of suitable foraging opportunities the food pecking activity is directed towards the feathers. Consequently the strategies to control feather pecking have been focused on avoiding early satiation (e.g. pelleted feed), increase of time spent feeding (fiber diluted diets) and the presentation of foraging opportunities (e.g. deep litter, free range). A favorable influence of high fiber diets, litter on other enrichment devices on feather pecking has been reported in numerous publications. However, the reduction of feather pecking was only gradual and in some studies there was no effect at all. In addition the risk of feather pecking and cannibalism is higher in deep litter systems and free range is obviously very high. Therefore the foraging theory needs to be revised. Recent studies using lines selected for high and low feather pecking have shown, that high feather pecking birds have a particular preference of eating feathers and fiber and prefer feathers over fiber when given the choice. It has further been shown that high feather pecking birds differ in their intestinal microbiota from low feather pecking birds. It is therefore assumed that feather eating is the primary motivation for feather pecking. The specific microbiota of high feather pecking birds may be the cause of their appetite for feathers. Although the incidence of such primary high feather pecking birds may be low in commercial flocks, their pecking activity may be transferred to flock mates through imitation and learning. This explains the deleterious effect of group size on feather pecking. High light intensity stimulates feather pecking through increasing the general activity. A similar effect is attributed to the generally known effect of nutritional deficiencies. The conventional environmental measures against feather pecking and cannibalism can delay or attenuate feather pecking, but they do not prevent it. There is a need to unravel the genetic basis of this behavior. Quantitative genetic studies have proved sufficient genetic variation for severe feather pecking. However, the identification of primary feather peckers in commercial breeding stocks is too difficult. Therefore selection against feather pecking was not successful so far. Using modern tools of molecular genetics will allow the identification of genetic markers, which can be applied in practical breeding programs.

I. INTRODUCTION

Feather pecking and cannibalism have been reported as undesirable or vicious behavior in poultry and more than 120 years (Oettel, 1873). Despite intensive research the causes of these particular behaviors are still unknown. Following the publications of this topic throughout the last century it is obvious that changes in the management or feeding systems where suspected to cause feather pecking and cannibalism. Transition from free range towards indoor deep

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litter and cages systems have also been reported as influencing factors. Similarly the changes in feed formulation, e.g. replacement or fish meal and meat meal by soybean meal or other protein sources of plant origin have been considered a major cause of feather pecking and cannibalism (Walser, 1997). Finally selection for high egg production and feed conversion rate is being discussed as potential influencing factor. Despite intensive research on this topic and improvements in feeding and husbandry the causes of feather pecking and cannibalism have not been identified and the problem persists. Ironically the return from conventional cages towards deep litter and free range systems has increased the problems related with feather pecking and cannibalism (Sherwin et al., 2010; van de Weerd and Elson, 2006). The problem will become more important when beak trimming is prohibited. A library search in CAB showed that more than 800 articles have been published in academic journals from 1970 onwards. It is assumed that much more research results exist, which have not been published because they did not show the expected results. The published literature is probably biased in this regard. Another risk of bias is intended or unconscious selection of information which fit a given hypothesis. With regard of the huge amount of publications it is not possible to present a complete survey of all literature sources. However, our selection has been carried out with special reference to contrasting results and conclusions.

II. THE UNDERLYING MOTIVATIONS OF FEATHER PECKING

The most widespread theoretical explanation of feather pecking and cannibalism is based on the assumption that feather pecking is misdirected food pecking and food searching (foraging) behavior (Blokhuis, 1986). Indeed the pattern of feather pecking is similar to feed pecking (Dixon et al., 2008). It is further assumed that the motivation of food pecking and foraging is independent of satiation, and that under intensive feeding conditions satiation occurs before the drive for pecking is consumed. In the absence of suitable materials the birds would address their activity towards the feathers of group mates. This theory is supported by a large number of different experiments. Reducing the time spent feeding through pelleted or crumbled feed increased feather pecking and cannibalism (Jensen et al., 1962; Savory et al., 1999). Time spent feeding is even more severely reduced under restricted feeding schedules and the risk of feather pecking and cannibalism is increased under these conditions (Blokhuis, 1986). High fiber diets showed a positive effect on feather pecking, feather score and cannibalism (Bearse et al., 1940; Scott et al., 1954; Savory, 1980; Hartini et al., 2002; van Krimpen et al., 2008; Kriegseis et al., 2012). This has been explained by the extension of time spent feeding. The effect of dilution on damaging pecking was, however marginal in many of the above cited experiments. It not only depended on the level and material of dilution, but also on the breed used. In some cases there was no response to fiber even though 10 percent were supplied (Savory et al., 1999). Some authors consider food pecking not as an isolated element of behavior but as component of a more general motivation of foraging, which comprises food searching, scratching and ground or litter pecking (Blokhuis, 1986). There is abundant information that feather pecking increases in non-litter management systems. Blokhuis and van der Haar (1989) and Huber-Eicher and Wechsler (1997) found less feather pecking when the birds were kept on litter. However, the effect of litter vs. slats was only significant when the hens were fed a pelleted diet (El-Lethey et al., 2000). While the above cited references stressed the importance of foraging, e.g. feed-related activities, Channing et al. (1999) and Sherwin et al., (1999) suggested that feather pecking was related to exploratory behavior in general. This comprises not only edible but also other objects. The effect of such objects on feather pecking, however, has shown to be highly variable and they have not been introduced in practical layer farms. Edible materials seem to be more efficient in reducing feather pecking than non-edible objects. At present special pecking blocks on

mineral basis are being used in layers and broilers as occupation material. Holcman et al. (2008) found an increase of behavioral activities in response to pecking blocks. But there was no information on feather pecking. Unpublished reports from commercial farms have shown positive effects on the plumage conditions. Whether this effect is due to reduced feather pecking or blunting of the beaks has still to be elucidated.

In summary, most experiments confirmed a close relationship between feather pecking and cannibalism and food pecking. Providing litter material stimulates pecking and foraging behavior and reduces feather pecking and pecking related damages under experimental conditions. These results obviously support the hypothesis that feather pecking is misdirected food pecking or foraging. There are, however doubts on this hypothesis. It is generally known that severe cases of feather pecking and cannibalism occur under deep litter management systems and even in free range where there exist foraging opportunities in abundance. It is concluded that feather pecking does not substitute exploratory pecking.

III. NUTRITION AND FEATHER PECKING

It is well documented that nutrient deficiencies increase the risk of feather pecking and cannibalism in poultry. This effect has been reported for total protein, amino acids and various minerals (see for review Kjaer and Bessei, 2013). The effect of deficiencies can be explained by an increase of exploratory behavior in general (Wood-Gush, 1971). Since feather pecking also occurs when the birds are fed diets which, according to the current knowledge, fully meet their requirement the question remains whether hidden deficiencies exist. Commercial diets are usually based on the mean performance of flocks and the birds with the highest production may not receive adequate amounts of nutrients. Jensen et al. (2005) reported that feather pecking hens grew faster, came earlier into lay and were more active. However, experiments have shown that the risk of feather pecking only increases when the known standards for energy, protein, specific amino acids and minerals are not fulfilled. Higher levels of the essential nutrients did not show positive effects on feather pecking (Kjaer and Bessei, 2013). Therefore it is not likely that feather pecking is caused by hidden nutrient deficiencies. But it cannot be excluded that feather pecking birds are not able to adjust their nutrient intake according to their requirement, or that feather pecking birds differ from the non-feather pecking birds in their nutrient selection behavior. In a choice feeding experiment using high and low feather pecking birds, high feather pecking birds consumed more protein than required when soybean meal was fed separately from the rest of the diet (Kircher et al., 2012). High feather pecking birds also showed a high preference for fiber when given a high-fiber and a low-fiber diet in a choice feeding experiment (Kalmendal et al., 2012). The differing response of high feather pecking birds in choice feeding experiments indicate differences in the metabolism and confirms the assumption of Jensen et al., (2005) that feather peckers differ from non- feather peckers in their resource allocation. As described above, the intake of indigestible fiber has obviously a positive effect on feather pecking and cannibalism. Under the foraging theory this effect has mainly been discussed under the aspect of increased time spent feeding and pecking. There are, however, various other effects of fiber which need to be considered. Dietary fiber stimulates the activity of the gizzard and the gut motility. Feed retention time is increased in the foregut, but decreased in the hind gut. It also stimulates the excretion of bile acid and enhances the gastrointestinal reflux (Hetland and Choct, 2003). Since chickens eat considerable amounts of litter (Hetland and Svihus, 2007), the above mentioned positive effect of litter on feather pecking may also be attributed to the intake of fiber. This aspect is confirmed by the finding that sand as litter had no influence on the development of feather pecking (Huber-Eicher and Wechsler, 1997). Dietary fiber plays also an important role through its influence on in the composition and

activity of the intestinal microbiota (Hartini et al., 2003). The potential role of the microbiota in feather pecking will be dealt with below.

It has been observed that feathers cast during moulting are frequently eaten and the absence of feathers on the floor of pullet houses is taken as an indicator of later feather pecking. Ramadan and v. Borell (2008) could reduce feather pecking when the feathers cast during rearing were frequently removed from the litter. This led to the speculation that eating cast feathers in early live may facilitate feather pecking in later periods. Benda (2008) raised commercial layer hybrids with diets containing 10 % of shredded raw feathers. These birds showed a lower feather pecking activity during the rearing period than birds receiving a conventional diet containing the same levels of nutrients. When the birds were switched to the conventional diet they performed significantly more severe feather pecking than the control birds. When the birds receiving the high feather diet during rearing were given the choice between the conventional and the high feather diet, they ate significantly more of the high feather diet than the birds reared on the conventional diet. This confirms the assumption that feather eating in young birds generates a preference for feathers in adults. The close relationship of feather pecking and feather eating has been shown in various experiments (Bögelein et al., 2010; Häusler, 2007; Harlander et al., 2007). This raises the question on the role of feathers as dietary component. Since the digestibility of protein in raw feathers is low (McCasland and Richardson, 1966) and the birds are usually fed diets which fully cover the nutrient requirement of the birds, other causal factors are sought.

Eating whole feathers (Harlander et al., 2006) or coarsely chopped feathers included in the feed (Benda, 2008) reduced the passage time of the digesta in the digestive tract. In this regard feathers show a similar effect as insoluble crude fiber. Birds from the high feather pecking line clearly distinguished between feathers and fiber, and consistently preferred feathers over fiber when given the choice (Häusler, 2007). This shows that dietary fiber does not substitute feathers in these birds.

Assuming that feather eating is a main motivation for feather pecking attempts have been made to control it through repellents (Anonymus, 2009; Harlander-Matauschek et al., 2009). A wide range of bitter or irritant substances applied on feather have been identified which reduced the acceptance of feathers. Methods of application however have to be developed for the application under practical conditions.

IV. THE ROLE OF MICROBIOTA

The special preference of high feather pecking hens for feathers raises the question of the underlying mechanisms. One of the potential influencing factors is the development of a different intestinal microbiota. The first study of the role of the microbiota on feather pecking has been carried out by Meyer et al. (2012). The inclusion of 5 percent raw feathers in the diet of commercial layer chicks increased the keratinolytic bacteria in the digestive tract of chicks. DNA extracts of the caecal microflora showed a reduced diversity and also significant changes in in the microbial metabolites, such as short chained fatty acids, ammonia and lactate. Comparisons of the microbiota of birds of a high and low feather pecking line confirmed differences in the metabolites (Meyer et al., 2013). Besides the above mentioned components biogenic amines showed different levels in the high and low feather pecking lines. It is known that both, the microbiom itself as well as its metabolites influence the mental state and play an important role in the regulation of appetite in humans (Fiori and Turechi, 2008; Grenham et al., 2011). The modification of the microbial activity through the ingestion of feathers indicates the potential connection between feather pecking behavior and gut function. It is known that the intestinal microbiota can be determined by genetic factors. The microbiome of chicken lines selected for high and low body mass differed significantly

by line and gender (Zhao et al., 2013). On the basis of the above results it can be hypothesised that high feather pecking birds have a specific microbiome, consisting of keratinolytic bacteria, such as Enterococcus faecium and Lactobacillus strains, and that this microbiome generates the specific preference for feathers in high feather pecking birds.

V. OTHER ENVIRONMENTAL FACTORS RELATED TO FEATHER PECKING

Besides feed and litter a multitude of other environmental factors have been identified as factors influencing feather pecking, e.g. light, group size and stocking density, temperature, noxious gases, use of free range, use of perches. On the basis of this information multidisciplinary management packages have been developed. The most complex consists of 46 potentially protective management strategies, which have been applied in a large scale experiment under practical conditions (Lambton et al., 2013). There was a gradual decrease of severe feather pecking, feather damages and mortality with increasing number of applied management strategies. However, it has been noticed that in spite of the interventions, the incidence of injurious pecking and damages caused by feather pecking were high. In conclusion genetic selection would be needed to minimize damages caused by feather pecking and cannibalism.

VI. GENETIC FACTORS OF FEATHER PECKING

First information on the genetic background of feather pecking and cannibalism stems from differences between lines and breeds. There are numerous studies which report variation among different pure lines or commercial hybrids for feather pecking behavior, pecking related feather damages or mortality through cannibalism (Ambrosen and Petersen, 1997). Heritability estimates reported by different research groups showed low heritability coefficients from .06 to .12 in pullets (Bessei, 1984; Kjaer and Soerensen, 1997; Rodenburg et al., 2003) and higher values in laying hens (Kjaer and Soerensen, 1997; Kjaer et al., 2001; Rodenburg et al., 2003). There is obviously sufficient genetic variation to select against feather pecking. This has been confirmed in short term experiments (Bessei, 1995; Kjaer et al., 2001). Selection against feather pecking behavior has not been implemented in commercial breeding programs so far. The behavioral observations are considered too difficult and time consuming. However, there exist several experimental lines which differ in their propensity for feather pecking and cannibalism (Muir and Craig, 1998; Su et al., 2006). These lines are now being used for genomic studies on the level of QTL, gene expression (micro arrays) and SNPs. It is expected that these studies not only detect genetic markers related to feather pecking, but also unravel the underlying behavioral and physiological mechanisms. Buitenhuis et al. (2003) found significant QTLs in F2-crosses of commercial lines for gentle feather pecking, but not for severe feather pecking. Similarly Jensen et al. (2005) detected one suggested QTL with low explanatory value for severe feather pecking in F2-crosses of Jungle fowl and White Leghorn. Keeling et al. (2004) found a significant QTL for feather damage which was related with white feather colour. Further QTLs related to feather pecking have been reported by Wysocki (2006). SNP studies indicated that feather pecking is related to the dopamine and serotonin pathways (Flisikowski et al., 2009; Biscarini et al., 2010). Recently, Wysocki et al. (2013) identified 4 potential candidate gens for severe feather pecking.

It is expected that information on feather pecking on the molecular genomic level will provide tools to identify the birds performing feather pecking and cannibalism and this can be used for selection in commercial breeding programs.

VII. CONCLUSIONS

The prevailing explanation of the causes of feather pecking and cannibalism is the assumption that chickens have a natural drive for food pecking and food searching (foraging) which is independent of satiation. Consequently the main strategy to control this behaviour is based on feeding strategies which increase the time required for food pecking and the use of foraging and occupation material. However, it is well documented that severe feather damages and cannibalism occur even under optimal nutrient supply and rich environmental conditions. Recent results have shown that the theory of foraging needs a major revision. On the basis of information from practical poultry production and experiments food pecking and scavenging cannot be considered as the primary cause of damaging pecking. Instead we propose the following theoretical model: There exist birds with a genetically determined high feather pecking activity (primary feather peckers) related to feather eating. These birds eat more protein than required for production and maintenance and show a high preference for both fiber and feathers. They prefer feathers when they are given the choice between fibre and feathers. They also differ from low feather pecking birds in the composition of the intestinal microbiota. Both, fiber and feathers have similar effects in the digestive tract insofar as they increase the activity of the gizzard and reduce the retention time of the digesta. The special microbiota of the high feather pecking birds may be the cause of their high preference for feathers as compared to fibre. This explains why dietary fibre and the presence of litter attenuate or delay the incidence of feather pecking, but do not prevent it. Group size is an important factor for spreading feather pecking from the primary feather peckers to other group mates. The foraging theory has led to an overestimation of the importance of litter, free range and enrichment devices for the control of the damaging behaviour. Similarly to high fibre diets litter and other means of environmental enrichment attenuate feather pecking, but do not prevent it. Provided that feather eating is the primary motivation of feather pecking the strategies of controlling this behaviour should focus on the application of repellents on the feathers. In the long-term genomic studies are expected to identify primary feather peckers through genetic markers. These will enable commercial breeders to control feather pecking and cannibalism through selection.

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VISUAL IDENTIFICATION OF POTENTIAL FEATHER PECKERS BY USING PLUMAGE COVER METRICS AND FEATHER CONSUMPTION IN LAYING HENS

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It is difficult to differentiate feather pecking birds within a flock. Using a simple method of identifying feather peckers could be an important tool for feather pecking studies. Feather eating behaviour is common in floor-housed laying hens, a behavior which is positively associated with developing feather pecking habits. It has been suggested that feather eating is redirected as feather pecking towards other birds when loose feathers are no longer available in the litter to be consumed (McKeegan and Savory, 1999). On the other hand the feather coverage can be easily evaluated and can be potentially used as a measure of welfare in laying hens. Our research objective was to examine if there was a positive correlation between feather pecking hens and their plumage cover. This study aimed to determine if plumage condition in birds might be related to feather eating and presumably feather pecking in independently the housing and the breed.

The trial involved commercial 30 ISA and 37 HLY laying hens, respectively average 75 and 64week-old birds. We classified birds into two groups based on simple subjective visual perception of their feather coverage: Dense Feathered (DF) and Feather Poor (FP) groups. In order to mathematically identify differences between DF and FP groups, plumage density was measured by using a square screen (1 X 1 cm) as a template over three defined areas to determine feather density (number of feathers/cm^2). The denuded area was measured by placing a clear quadrant (1 cm^2) on top of the denuded area. The four areas measured were; the back of the birds' neck, the tail, back and vent which are known to be highly pecked areas (Bilcik and Keeling, 1999). Shank length, body weight, and lower beak length were also measured as a potential indicator of feather eating birds. Feathers consumed was assessed following routine veterinary surveillances on the farms, in order to study the relationship between plumage condition and feather eating.

The number of feathers in the gastro-intestinal tract (GIT) of laying hens (particularly in the crop and gizzard) were recorded. The total number of feathers in the GIT was not significantly different between DF and FP groups averaging 2.7 and 2.9 feathers/bird, respectively. However, 78% of the birds in the FP group were shown to be feather eaters as opposed to only 51% of the birds birds in the DF group. In addition, the birds with presence of feathers in the GIT showed higher ($P<0.01$) feather density in the area of the neck and tail than the group of birds not consuming feathers. However, no relationship was found between feather consumption and any of the denuded areas. Therefore, according to our study, feather appetite is significantly related to poor feather coverage. Furthermore, the feather eating group had a shorter beak length ($P<0.05$). It is noted that both feathers and the outer shell of the beak are composed of keratin (Frenkel and Gillespie,1976). Therefore, it is tempting to speculate that the feather eating behavior may be related to marginal nutritional deficiencies which, in turn, could affect full beak development and result in the development of specific appetites such as for feathers. The findings in the current study may contribute to the behavioural and phenotypic characterization of feather pecking birds and warrant further investigation to study a potential link between feather pecking and the specific appetites study, related to incidence of feather pecking such as amino acid.

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NEST-BOX USE BY YOUNG FREE RANGE HENS MAY BE INFLUENCED BY PERCHING LOCATION RESULTING FROM SOCIAL AVOIDANCE

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Summary

Egg laying data were analysed from an experiment involving 800 ISA Brown hens (16 pens of 50 birds) housed on litter. The birds were reared as part of a larger experiment investigating the development of feather pecking behaviour by free range hens. Two main effects were investigated: (1) provision of straw to increase foraging behaviour (FORAGE), and (2) transport, relocation and mixing (TRM) at 16 weeks to simulate bird transfer from the rearing to the laying farm. Nest-boxes were open from 16 weeks of age and hens could access the outdoor range area from 21 weeks. The number of nest-box, floor and range eggs per pen were counted daily until 32 weeks. While there were no effects of treatment on hen-day egg production, there were proportionally more nest-box eggs in the TRM compared to the Non-TRM treatment (66.4% vs 52.2%; P = 0.009), but no effect of FORAGE compared to No FORAGE (56.9% vs 61.8%; P = 0.292) and no interaction between the main effects TRM and FORAGE. Video observations revealed in the first week after TRM, when nest-boxes were present, more TRM than Non-TRM treatment hens roosted overnight on the perches in front of the nest boxes (8.1 and 5.4 hens per pen; P = 0.002). This may have familiarised the TRM hens with the nest-boxes, once egg laying commenced. The higher proportion of nest-box eggs, and correspondingly lower (P = 0.012) proportion of floor eggs, in the TRM pens may represent social avoidance behaviour by TRM hens associated with being mixed with unfamiliar pullets at 16 weeks. However, the finding requires further investigation.

I. INTRODUCTION

Commercial egg production in Australia is moving away from caged housing toward non-caged-housing of hens (AECL, 2013). Non-caged systems are presumed to improve hen welfare, for example hens being able to perform more natural behaviours, like foraging and nesting (see Cronin et al., 2014). Non-caged systems can allow the inclusion of environmental enrichment to encourage foraging behaviour, while nest-boxes provide a preferred location for oviposition to facilitate egg collection, while minimising floor eggs. Nest-boxes should reduce disturbance on hens at egg laying, and Cronin et al. (2012) reported hens had lower stress hormone concentrations if disturbance was reduced at oviposition. While non-caged systems increase other risks to hen welfare from inappropriate behaviours such as feather pecking, injurious pecking and cannibalism (Rodenburg et al., 2008; Fossum et al., 2009), egg producers are also aware of economic issues such as floor eggs (Nicol et al., 2003). Floor eggs require manual collection, are more likely to be dirty and thus down-graded and de-valued, and are possibly more prone to shell fracture and being eaten by the hens.

The present paper reports an interesting finding related to nest-box use by non-caged hens, and thus the occurrence of floor eggs. These observations were identified during an on-going experiment in which we are investigating the effects of rearing factors on the development of inappropriate pecking behaviours by free-range hens. The objective of the present paper therefore is to report on the occurrence of nest-box and floor eggs due to the effects of environmental enrichment from added straw, and simulating the transfer of pullets at 16 weeks from the rearing farm to the laying farm.

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II. MATERIALS AND METHODS

Hen-day egg production and the proportions of nest-box and floor eggs were analysed from 19 - 32 weeks of age for 16 pens of ISA Brown hens housed on litter of wood shavings in an uninsulated shed. Pens measured 1.83 m wide x 3.25 m deep and contained 50 pullets at 16 weeks. The birds were obtained at day-old from a commercial hatchery and were not beak-trimmed. The experiment was a 2 x 2 factorial arrangement with replication. The two main effects were: FORAGE - from six weeks of age 200 g of straw was provided daily in a self-dispenser to stimulate foraging behaviour. Each No FORAGE (control) pen had an empty dispenser. TRM - at 16 weeks of age pullets were transported (max 8 per crate) by vehicle for 35 min, followed by relocation to a new pen in which they were mixed 50/50 with both familiar and unfamiliar pullets. Birds in the Non-TRM treatment remained in their original (home) pens. Cameras mounted in each pen enabled continuous video recording of the birds.

The experiment was blocked according to side of the shed (north versus south) and treatments were fully randomised to pens within blocks. Feed and water were available *ad libitum*. From six weeks of age each pen contained a fiverung perch unit for perching, and from 16 weeks of age, a 10hole roll-away nest unit with plastic mesh nest inserts (SKA®) for egg laying was installed. Each nest-box unit had two rows of five singlebird nests, above and below. A perch was provided across the front of the nest-box units, at both levels of the unit. Photoperiod was increased from 12 h light (L) and 12 h dark (D) at 17 weeks, to a 15 h L and 9 h D at 23 weeks. At 21 weeks of age, each pen of birds was allowed continuous (i.e. 24 h) access to an outdoor run via a pop-hole (0.4 m high x 0.6 m wide) set into the rear wall of the pen. Light levels measured at floor level in mid-pen averaged ~35 and ~40 lux when pop-holes were closed compared to open. Outdoor runs measured 1.83 m wide x 10 m long x 2.1 m high, and were enclosed with wire mesh. The 1.4 m length closest to the shed in the outdoor runs was a metal-roofed verandah, beyond which was a 1.8 mwide section of 70% beige shade cloth overhead.

The number of eggs collected weekly per pen was recorded until week 32, along with egg location (upper and lower nests, floor and, once the pop-holes were opened and outside range). Differences due to the FORAGE and TRM main effects and AGE on hen-day egg production and the proportion of eggs laid in each location were determined using linear mixed models in REML (GenStat release 14.1, VSN International Ltd). The treatments FORAGE, TRM and AGE were treated as fixed effects, and block and pen were treated as random effects. A post-hoc analysis of the average number of birds per pen recorded at the nest-box perches at 2300 h each night was conducted in GenStat to analyse the effects of FORAGE and TRM. The experimental unit was the pen of birds.

III. RESULTS

On average, more birds roosted on the perches in front of the nest-boxes during the seven nights after TRM (8.1 and 5.4 per pen, respectively for TRM and Non-TRM; sed = 0.696, $P < 0.002$). There was no effect of FORAGE and no interactions between TRM and FORAGE. The first egg was laid on day 126 (18 wks), with birds laying eggs in all 16 pens from week 19. In weeks 21 and 24, respectively, 50% and 80% hen-day egg production was reached. Hen-day egg production between 19 and 32 weeks did not differ ($P > 0.05$) due to the FORAGE or TRM main effects, and there was no interaction between these treatments. As expected, hen-day egg production increased with age ($P < 0.001$), although there were no interactions between the main effects and age. However, the proportion of nest-box eggs was significantly greater in the TRM treatment compared to the non-TRM treatment (66.4 and 52.2%, respectively; $P = 0.009$, sed 4.457). Concomitantly, the proportion of floor eggs was lower due to the TRM main effect (33.3 and 47.1%, respectively; $P = 0.012$, sed 6.580). There was no effect of

FORAGE, and no FORAGE x TRM interaction on the incidence of either nest-box or floor eggs. The proportion of nest-box eggs increased with hen age ($P < 0.001$), but there was no treatment x age interactions. The change in the proportion of nest-box eggs over time is shown in Figure 1, according to the FORAGE x TRM treatment interactions. A low incidence of eggs laid on the outdoor range was recorded after pop-holes were opened in week 21 (~0.5% of eggs per week). Figure 2 shows the change in proportion of eggs laid in the upper and lower nest-boxes, floor and outdoor range to week 32 of age.

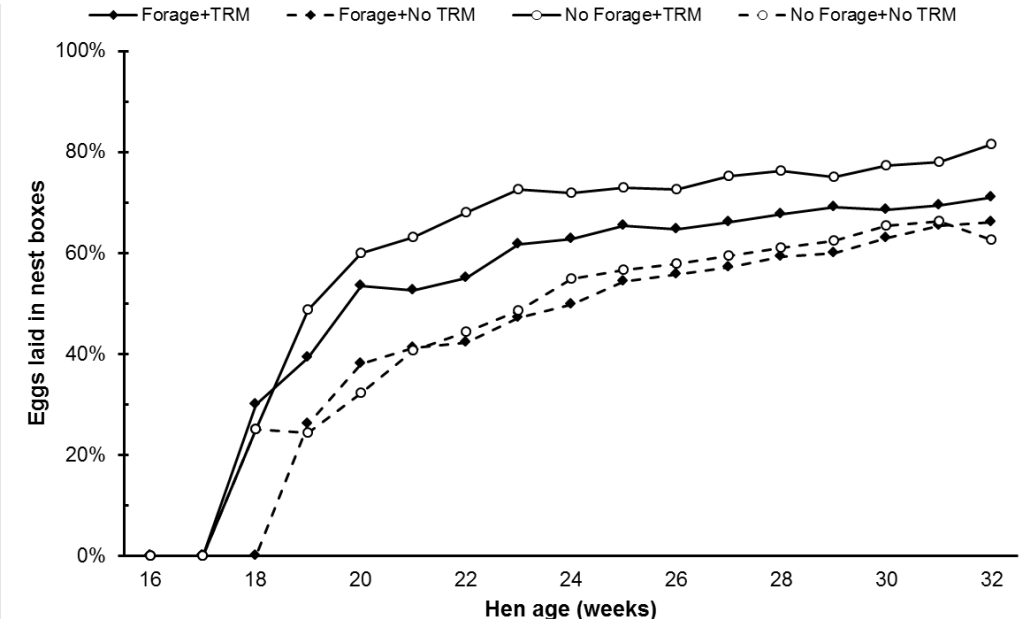

Figure 1 - The proportion of nest-box eggs to 32 weeks of age. Values shown are the Forage x Transport-Relocation-Mixing (TRM) interaction means, with four pens per treatment.

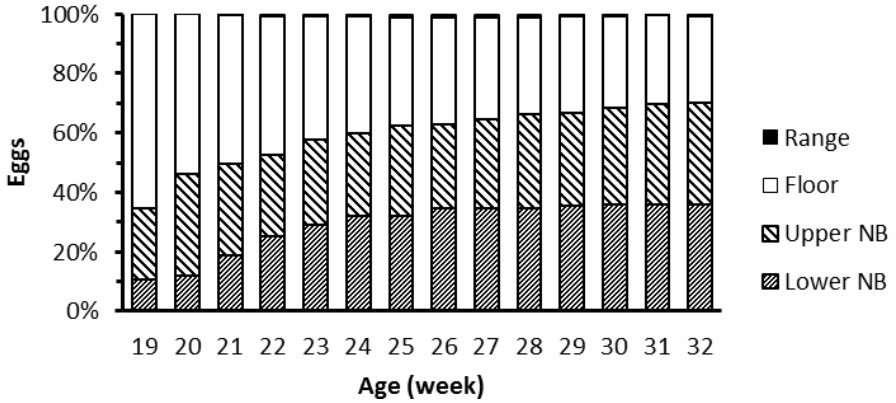

Figure 2 - The proportion of eggs recorded in different locations from 19 to 32 weeks of age. Egg locations were: the upper and lower nest-boxes (NB), floor inside the shed and outdoor range.

IV. DISCUSSION

In the present experiment we observed a higher proportion of nest-box eggs in pens of birds that had been transported, relocated and mixed at 16 weeks of age. Indeed, in the early weeks of lay the magnitude of difference in the occurrence of nest-box eggs was in the order of 20% more for pens of birds that were transported, mixed and relocated to new pens compared to non-treated pens. As expected, this difference was maintained over time, as represented in Figure 1, supporting previous research which reported that laying hens were conservative in their oviposition site (Cronin et al., 2007). It is the relatively large difference between floor

compared to nest-box laying that is interesting and deserves further investigation. Perching behaviour collated from the video records showed that more birds in the TRM pens roosted overnight on the perches attached to the front of the nest-box units during the first week that the nest-boxes were available. The motivation of birds to roost on the 'new' perches may have been related to avoidance of aggression orsocial stress following mixing, when birds would be expected to re-establish their social hierarchy. Further, the fact that more pullets in the TRM pens perched on the nest-box units (indeed some stayed in the nests overnight) may have increased their familiarity with nest-boxes as relevant future oviposition sites.

Results also suggest that the presence of forage may have ameliorated some of the adverse effects of the TRM treatment, although a statistical difference was not found between the proportion of nest-box eggs in the FORAGE + TRM compared to No FORAGE + TRM treatments. However, in our larger study (unpublished) we had recorded fewer aggressive pecks in pens of birds in the FORAGE compared to No FORAGE treatment, and this may be relevant to the recorded observation regarding the increased use of the nest-box unit perches.

While laying hens are motivated to lay in nest boxes, not all hens choose to do so, with a proportion of eggs being laid on the floor or in the outdoor range (Nicol et al., 2003). In a previous experiment (Cronin et al., 2013) we found a trend for more floor eggs if a deeper layer of wood shavings was provided during rearing. Thus, some factors applied during rearing to reduce inappropriate behaviours such as feather pecking or injurious pecking, may increase floor eggs. On the other hand, the present experiment suggests that the practice of separating the rearing and laying phases of production, and the need to transport birds between rear and lay, may benefit producers by subsequently increasing the occurrence of nest-box eggs, and thus decreasing the occurrence of floor eggs.

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FREE-RANGE BROILER CHICKEN BEHAVIOURAL TIME BUDGETS: INSIDE AND OUTSIDE OF THE SHED

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Summary

The demand for free-range chicken meat in Australia is increasing. Free-range products are usually perceived as more welfare friendly by consumers (Department for Environment Food and Rural Affairs, 2011) and particularly fulfill the belief that the ability to perform natural behaviour leads to better welfare. However, there is a lack of scientific knowledge relative to the use of the outdoor range by broilers and its implications, advantages or disadvantages, in terms of bird behaviour and welfare. Therefore this study investigated behavioural time budgets of broiler chickens on two Australian commercial farms. Behaviour was monitored inside the shed and in four range areas, differing in resource availability (tree, shade cloth or no resource present) and distance from the shed (adjacent to the shed wall or 7.5m from the shed). Results indicated that distance is a deterrent for range use, as few broiler chickens were seen in areas 7.5m from the shed. Furthermore, behavioural time budgets differed between broiler chickens observed in the shed compared to those in the range; there was more active, exploratory and vigilant behaviours seen in the range and more resting and comfort behaviors observed in the shed. However the implications of such behavioural differences remain unknown.

I. INTRODUCTION

Measuring behavior is a useful tool to determine an animal's metal well-being and thus welfare status (Dawkins, 2004). Very few studies have investigated the differences in broiler behaviours in the outdoor range as compared to indoor, or between range areas. Comparing broiler behavior inside the shed and out in the range may indicate if range access permits or encourages particular behaviours. If these behaviours have proved important to broilers one may suggest their welfare state is improved with range use. It is likely that the effects of range use will differ depending on what part of the range an individual utilizes, again these effects may be reflected in their behavior, i.e. areas far from the shed with no cover or resources may elicit a fear response, evidence of this would include less resting and more vigilant behaviours. Indeed it has been suggested that free-range broiler behaviours are related to resource availability and ranging distance (Dawkins, Cook, Whittingham, Mansell, & Harper, 2003; Jones et al., 2007; Mirabito, Joly, & Lubac, 2001; Weeks, Nicol, Sherwin, & Kestin, 1994). However the results of these studies may not reflect chicken behavior within an Australian commercial setting; primarily due to different climatic conditions but also differences in flock size, strain, growth rate, and experimental settings (i.e. noncommercial properties). Such an understanding is likely to help range design and management to encourage a more uniform distribution of birds in the range and increase the welfare of the individuals that utilize the range by providing what they want.

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II. METHODS

This study investigated broiler behaviour in two commercial free-range flocks of Ross 308 birds in Victoria in winter. Relevant characteristics of both farms are summarized in Table 1.

Table 1 - Characteristics of farm one and farm two, including variables of range access and resources.

	Farm One	Farm Two
Shed size (m^2)	116 x 11.5	60 x 15.5
Stocking density (birds/m^2)	15.7	15.8
Number of popholes	10	6
Size of popholes (mm)	1200 x 300	1070 x 320
Tree species available	Evergreen elders (*Alnus jorullensis*) (approx. 5m wide)* Lillypilly (*Acmena* smithii) (approx. 1m wide)	Various fruit and gum trees; including Apricot tree (*Prunus armeniaca*) (approx. 3m wide)*
Artificial shade	10 x triangular (3 x 3m) shade cloth	** 1 x triangular (2.5 x 3m) shade cloth
Available pasture	1200 m^2 adjacent to the shed 600 m^2 at the rear of the shed	1296 m^2

* Tree species used in current study
** Thin material encouraged movement in heavy winds

We hypothesized that there are differences between the number and frequency of behaviours in the shed compared to out in the range, and between range areas that differed in distance from the shed and resource availability. Six 2.5 × 3 m areas were studied: in the shed at the centre or along an edge, outside along the shed with or without a shade cloth cover, and 7.5 m from the shed with or without a tree. Behaviour was recorded daily via video cameras from 21 to 40 days of age during range access, 0800-1700h; every second day of video footage was analysed. The behaviour of four randomly chosen birds was observed for 30 sec in each location every hour. In addition, the number of birds in each location was noted hourly for every day of range access. Data were analysed using GLM in Minitab if considered normal, to test the effects of inside vs. outside, resource (either tree or shade cloth) and distance, with time and age as repeated measures, or with a Kruskal-Wallis test otherwise.

III. RESULTS

Little data was collected from farm one with seven days of range access due to poor weather conditions (average minimum temperature 8.6°C, average maximum temperature 12.7°C, total rainfall 89.3mm), relative to farm two (average minimum temperature 10.2°C, average maximum temperature 16.9°C, total rainfall 54.5mm) which consequently had 28 days of range access. On Farm two, there was an effect of distance ($P < 0.001$) but not resource on the number of birds in the range, such that similar numbers of birds were found close to the shed with or without a resource, and very few birds 7.5m from the shed with or without a resource. As expected weather conditions had an effect on the number of birds located in the range, however there was no effect of time of day or age. Birds performed more active (e.g. locomotion and standing) ($P < 0.001$), exploratory (e.g. pecking and foraging; $P < 0.001$) and vigilant ($P = 0.056$) behaviours outside and more resting ($P < 0.001$) and comfort behaviours (e.g. preening, dust bathing, wing flapping and stretching) inside ($P < 0.01$). The diversity of

behaviours was higher inside than outside for Farm 1, and similar for Farm 2 but with different behaviours between outside and inside.

Table 2 - Total number of chickens observed in each location; inside the shed adjacent to the wall, inside the centre of the shed, close to the shed without a resource (- distance - resource), close to the shed with a resource (- distance + resource), 7.5m from the shed without a resource (+ distance - resource), and 7.5m from the shed with a resource (+ distance + resource). Mean percentage ± S.E of behaviours. Means with differing subscripts across a row are statistically different (P≤0.01).

	Inside		Outside	
Total number of chickens	Adjacent to wall	10472[a]	- distance - resource	564[b]
			- distance + resource	448[b]
	Centre of shed	11195[a]	+ distance - resource	0[c]
			+ distance + resource	1[c]
Active (%)	10.99 ± 0.65[a]		34.55 ± 1.20[b]	
Exploratory (%)	2.95 ± 0.41[a]		23.62 ± 1.29[b]	
Vigilant (%)	0.65 ± 0.17		1.07 ±0.21	
Resting (%)	57.30 ±7.05[a]		1.43 ± 0.94[b]	
Comfort (%)	5.33 ± 0.51[a]		1.33 ± 0.27[b]	

IV. DISCUSSION

Very few birds ventured 7.5m from the shed, even with a tree; this is in accordance with the literature that states that distance is a significant deterrent, even when providing cover (Rivera-Ferre, Lantinga, & Kwakkel, 2007). The effects of resources require further research. These findings on free-range broiler behaviour in commercial Australian conditions are consistent with results overseas. A follow-up study in the summer should allow studying the effect of season on those farms. Our results suggest that the outdoor range is an environment that promotes exploration and active behaviours. However, the welfare implications of these behavioural changes require investigation, because for instance exploration and activity may improve leg health but increased vigilance could result in fear-related stress. A thorough understanding of the behaviour of free-range broilers can help to improve range design, increase and promote uniformity of range use and ideally improve broiler welfare.

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WELFARE AND EFFICIENCY IN POULTRY PRODUCTION

M.S. DAWKINS[1]

Summary

The demand for chicken meat continues to rise across the world, leading to calls for greater efficiency and sustainable intensification. This has implications for poultry welfare through potential conflicts between welfare and economics. Reducing this conflict and ensuring that good welfare is an economically viable part of the sustainable, efficient poultry production needs:

a) An agreed and workable definition of good welfare
b) Better ways of measuring and assessing welfare so that its financial advantages can be more clearly evaluated and integrated with other priorities such as human health, animal health, environmental protection, reduction in antibiotic use and financial viability.

Important new developments are use of technology that allows continuous monitoring of poultry health and welfare, higher standards of data analysis and research aimed at both finding solutions to the many pressures on the poultry industry and showing the economic benefits of good welfare.

I. INTRODUCTION

A concern over how to feed the rising human population while at the same time minimizing the effect on the environment has led to calls for agriculture to become more 'sustainably intensive' and more efficient. (Royal Society, 2009; Steinfeld *et al*., 2006; Garnett *et al*., 2013; Gerber *et al.,* 2013). The human population is projected to be at least 9 billion by 2050 (FAOStat, 2012; Godfray *et al*., 2010) and the current trend is for the consumption of meat and dairy products to keep on rising (Gerber *et al*., 2013) as people become wealthier and want what they see as a better diet. Chickens are already, at over 60 billion killed each year, the most commonly consumed animal (FAOStat, 2012) and projected to overtake pork by tonnage as well as numbers by 2020, with most of the increase is expected to occur in SE Asia and sub-Saharan Africa (USDA, 2013; Gerber *et al*., 2013).

Modern breeds of 'broilers' (meat chickens) are already highly efficient producers of protein due to a combination of diet, management and, in particular, selective breeding for high juvenile growth rate, breast meat yield and efficiency of food conversion (Flock *et al.,* 2005; Bessei, 2006; Estevez, 2007; Arnould and Leterrier, 2007). Many broilers currently convert 3kg of food into 2 kg of meat (a Feed Conversion Ratio of 1.5) and some forecasts are for chickens to achieve FCRs of 1.2, making them by far the most efficient terrestrial converters of feed to meat, far more efficient than pigs or cows. However, selective breeding for such efficient feed conversion has already had side-effects on the health and welfare of the birds including susceptibility to cardiovascular disease (Julian, 1995) and lameness (Kestin *et al.,* 1992; Rauw *et al*., 1998; Sanotra *et al.,* 2001; Bradshaw *et al.,* 2002; Burt, 2002; Knowles *et al.,* 2008). Selective breeding for fast juvenile growth rate has also had knock-on effects on the welfare of the parent birds ('breeders'). Without feed restriction, these breeder birds rapidly become obese (Dunnington and Siegel, 1985), have locomotory problems (Katanbaf *et al.,* 1989), the males have reduced fertility (McGary *et al.,* 2002). While these negative symptoms can be avoided by restricting the amount of food that the

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growing breeders receive, this is often only 25-50% of what the birds would consume if fed *ad libitum* (Savory and Maros, 1993; Ducuypere *et al.,* 2007; Renema *et al*., 2007) and raises welfare problems of its own since birds exhibit signs of chronic or metabolic hunger (Mench, 2002; de Jong *et al.,* 2002; Hocking, 2004; D'Eath *et al*., 2009). Furthermore, as broiler growth has continued to increase, the degree of feed restriction needed to keep broiler breeders on a healthy growth trajectory has also increased (Renema *et al.,* 2007).

These findings raise serious questions about what will happen to the welfare of chickens in the ever more efficient agriculture of the future. Poultry breeding programmes based on economically important production traits have already been held responsible for reduced welfare in both broilers and breeders over the last 50 years (Rauw, *et al.,* 1998; Jones and Hocking, 1999; Sandoe, *et al.,* 1999; Renema *et al.,* 2007) and so bird welfare looks to be under continuing if not increasing threat over the next 50 (Lawrence, 2008; Dawkins, 2012). As the most widely eaten animal in the world, acceptable to most cultures and widely seen as healthy, nutritious food for humans, poultry are in the front line for efforts to make them even more efficient as meat producers – to grow faster with less food, less water and less space. But what will this do for the welfare of the birds themselves? Will breeding for greater efficiency inevitably mean compromising their welfare? Are there limits as to how far we can push selective breeding, dietary improvements and intensive management before socially unacceptable limits to welfare are reached (Sandøe *et al*., 2009)?

The key question I want to address here is whether there is an inevitable conflict between increasing efficiency in poultry production and animal welfare. Or, putting the question more pessimistically: is there any place for animal welfare in a world concerned with providing enough food for humans, mitigating climate change and trying to preserve biodiversity?

II. ECONOMIC BENEFITS OF GOOD WELFARE

If poultry welfare is seen as an isolated cause, setting itself up in opposition to these other major concerns then there is a very real danger that it will have only a low priority in the more efficient, more environmentally sensitive agriculture of the future. It will be affordable by only a small minority of relatively wealthy people with particular views about animal ethics. However, if the case can be made for the economic, human health and environmental benefits of high standards of animal welfare, then welfare becomes a necessary and commercially important part of sustainable food production worldwide (Dawkins, 2012; Garnett *et al*., 2013). In the same way that ecologists increasingly make the case for conserving habitats and preventing the loss of biodiversity by putting a monetary value on the 'services' or 'natural capital' that a healthy environment provides, such as water retention, soil fertility, pollination and tourist attractions (Balmford *et al*, 2002), so the business and other benefits of animal welfare need to be drawn out far more clearly than they have been up to now.

Healthy, high welfare animals bring a range of commercial benefits such as lowered mortality, reduced food waste, higher quality products, lower costs of medication, but these benefits have not yet been sufficiently appreciated or even documented. Also, too much emphasis has been put on the willingness of consumers to pay extra for high welfare. However, consumer preferences are too fickle and too price dependent to ensure that farmers can invest in good welfare unless good welfare has other gains (Dawkins, 2012) so that producers to see the long term financial benefits of poultry welfare. Such benefits are much more likely to appeal to producers in countries struggling to feed their own human populations or in cultures where attitudes to animals are different from those in richer countries.

Making the case for good welfare as part of the sustainable, efficient poultry production needs at least two components:

a) An agreed and workable definition of good welfare
b) Better ways of measuring and assessing welfare so that its financial advantages can be more clearly evaluated in relation to other priorities such as human health, animal health, environmental protection, reduction in antibiotic use and financial gain.

a) Defining animal welfare

Although there are many different definitions of good welfare (e.g. Broom and Johnson, 1993; Fraser, 2008), the simplest and most straightforward is that animals are healthy and have what they want (Dawkins, 2008). This two-category definition has the advantage that it covers what most people mean by good welfare, it is easily understood by everyone, whether they are consumers, producers or scientists and above all, it directs attention away from what well-meaning people think is good for animal welfare towards the what we actually need to know about the animals needs. It also encompasses many other definitions (Dawkins, 2012). For example, many people have argues that good welfare must include the ability of the animal to behave 'naturally', using naturalness as a criterion of welfare (REFS). But under this definition, natural behaviour would only be included as a necessary part of good welfare if it it could be shown that the opportunity to do the natural behaviour a) improves the animal's health and/or b) is something the animal wants or chooses to do. If it is neither, it is difficult to argue that the animals welfare has been improved, however natural it is. Thus 'natural' behaviour (like 'stress' hormones or other criteria of welfare) is neither excluded or included in the definition of good welfare. It has to earn its place by showing that it fulfills the requirements of either or both of the two categories.

A further advantage of this definition is that it includes health as part of the definition and so makes it particularly easy to show the commercial benefits of good welfare as losses through disease are an obvious financial threat to the industry.

b) Technology to measure and asses welfare

Poultry producers across the world are faced with a raft of different pressures in addition to commercial ones. They have to satisfy national and international standards of food safety and environmental protection. They have to control disease but are under pressure to reduce anti-biotic use. They have to meet rising costs of feed, land and labour and still improve the welfare of their birds. An important way forward must be to find ways in which producers can, if possible, meet all these goals at the same time. We need new approaches to research that understands the multiple problems that producers face and systematically sets about finding ways of reducing all of them. For example, although there may have been conflicts between breeding birds for increased production and good welfare in the past, we now need to look to breeding programmes and management systems that lead to greater efficiency and also protect or even improve animal welfare (Jones and Hocking 1999; Arnould and Leterrier 2007; Thiruvenkdan *et al* 2011; Dawkins and Layton, 2012). For example, selection for traits such as increased disease resistance, leg strength and liveability can actually improve it (Jones and Hocking 1999; Arnould and Leterrier 2007; Aggrey, 2010; Thiruvenkdan *et al.,* 2011). By broadening the selection criteria of breeding programmes it may be possible to reduce the apparent conflict and achieve a wider range of goals (Lawrence *et al*., 2004; Beaumont and Chapuis, 2004; D'Eath *et al*., 2010; Dawkins and Layton, 2012). Indeed, breeding companies now increasingly incorporate health and welfare goals alongside economic ones into their breeding programmes and use a variety of traits such as leg health

and feather cover, as well as meat yield and feed conversion efficiency to select their breeding birds (Katanbaf and Hardiman, 2010).

While good stockmanship remains essential even to intensive poultry production (Dawkins et al, 2004), there is now an increasingly important role for automated measures of welfare that allow continuous assessment of large commercial flocks and so lead to more effective management. For example, smartphone cameras inside broiler chicken houses are able to detect flocks with a high proportion of lame birds, using simple measures of optical flow (Dawkins *et al*., 2009). Optical flow detects the movement patterns or 'flow' of chicken flocks as they move around a house. Flocks with a high incidence of birds with poor gaits (3,4,or 5 on the Bristol Gait score (Kestin *et al*., 2009)) not only have a lower mean optical flow, they also show a higher skew (deviation of the mode from the mean and a higher kurtosis (indicating odd or unusual movement). Lame flocks are not made up of birds that are uniformly lame. Rather they are contain birds with a wide range of walking abilities, some healthy (gait score 0 or 1) and others less so It is this range of walking ability that the optical flow algorithm is able to detect. The same system is able to detect flocks that are likely to end up with a high % mortality and high levels of hockburn as measured in the slaughter plant (Dawkins *et al*., 2009; 2012). The same technology even allows prediction of which flocks will have final levels of hock burn measured at the slaughter plant when the birds are as young as 3 days old (Roberts *et al*., 2012). Skewness and kurtosis (both measures of heterogeneity or lack of uniformity of movement in a flock) are particularly informative of flock health. A depressed mean rate of movement suggests that a flock may be less healthy than average but a raised kurtosis definitely indicates that the flock needs attention. Optical flow analysis is also able to predict which young flocks of egg-layers are most likely to develop serious feather damage in later life (Lee *et al*., 2011).

Good flock management is likely to be improved by having good current measures of the welfare state of a flock and even more so by being able to predict problems at a very early stage before they become serious and when interventions are still possible. However even greater improvements in flock management with consequent improvements in both efficiency and welfare are possible by improving the analysis of the data already collected by producers, such as records of mortality, culls, water use, vaccination, temperature and humidity. The collection of such data is good commercial practice for many companies but the data is often scattered (some on paper, some electronic) and data from different sources (breeding farm, hatchery, growing farms, slaughter plant) are often not brought together. Much greater use of this data, with better analysis could give valuable insight into practices that affect welfare, disease and efficiency could be a great resource, allowing producers to see what works and what does not and just what financial gains are apparent from different practices. For example, a widescale study involving over 70% of the producers in the UK showed that the % time a broiler house is outside the limits of temperature and humidity recommended by the breeder companies in the first week is a clear predictor of flock mortality, pododermatistis and hockburn in growing flocks (Dawkins *et al*., 2004). Both 'efficiency' (lower mortality, less waste) and welfare would be improved by attention to the first week environment.

III. CONCLUSIONS: CAN WE 'HAVE IT ALL'?

There are potential conflicts between standards of welfare and commercial poultry production that can best be resolved by working with the poultry industry to find solutions to the many pressures it faces across the world. These include controlling disease, responding to world-wide calls to reduce anti-biotic use, meeting national and international standards of food safety and product quality, absorbing rising feed costs, improving efficiency of production and responding to demands for higher standards of animal welfare. Before assuming that

welfare and efficiency are inevitably in conflict, we need to challenge some widely held assumptions and look for the economic gains that high standards of animal welfare can bring. As health is an important part of animal welfare, there are obvious economic gains from breeding programmes and management systems that concentrate on improving poultry health and thus have direct economic gains through more, healthier animals and losing less to waste. If animal welfare is seen as a single goal, isolated from other concerns of the poultry industry, then it is likely to lose out in the face of other priorities. But if its true economic value is appreciated, then animal welfare becomes firmly established as a key part of sustainable agriculture.

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AUTOMATED ASSESSMENT OF HEALTH AND WELFARE IN COMMERCIAL BROILER CHICKEN FLOCKS USING OPTICAL FLOW

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Assessing the welfare of growing broiler chickens is mainly done *post mortem*, using measures taken at the slaughter plant (% birds with hock burn and pododermatitits) or total pre-slaughter mortality. For a minority of flocks, the gait (walking ability) of living birds is also assessed but this process is very labour-intensive and has been criticized as subjective. We have developed a system of automatically monitoring the welfare of chickens throughout their lives, thus giving farmers a continuous readout of the state of their flocks in real time and, more importantly, allowing them to detect the early signs of problems and so to intervene before these become serious. Using video and smartphones inside commercial broiler houses, we have shown that the movements of growing broiler flocks give rise to patterns of 'optical flow' that can be analysed statistically and correlate with key welfare outcomes such as mortality, hockburn, pododermatitits and the proportion of a flock with poor gaits. Our aim is to develop am inexpensive and easy to use tool to help producers to manage their flocks for greater health, higher welfare and greater efficiency. It does not replace good stockmanship but is an aid to even better management, with the potential to target interventions and reduce total medication.

The optical flow algorithm measures the rate of change of image brightness. It does not track individual birds but gives an indication of the state of the whole flock. The algorithm is simple enough that it can be run on a smartphone, which delivers four measures every 15 minutes: mean, variance, skew (deviation of mode from mean) and kurtosis (which indicates extreme or unusual events and a lack of uniformity in the flock movement). Flocks with low mean and high kurtosis are associated with higher mortality and increased incidence of hockburn and poor gaits. In other words, such flocks move less overall and have less uniform movement (Dawkins et al., 2009; 2012). By contrast, healthy flocks (low mortality, low incidence of leg and walking disorders) have high mean and low kurtosis. By combining the four measures (mean, variance skew and kurtosis) it is possible to predict in flocks as young as three days which ones will develop high levels of hockburn at slaughter (Roberts *et al.,* 2009). The method will be described in relation to further measures of poultry health and welfare.

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IDENTIFICATION OF BIOMARKERS FOR FOOTPAD DERMATITIS DEVELOPMENT AND WOUND HEALING

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Summary

Footpad dermatitis (FPD) has gained economic importance in commercial broiler and turkey industries around the world and is used as a criterion to determine animal welfare and appropriate stocking density in many countries. Trace minerals such as Zn, Cu, and Mn are known to play a role in skin structural integrity and wound healing. To understand the biology and identify biomarkers for FPD development and wound healing, a FPD model was developed. A battery cage trial was conducted where paper sheet was placed on the bottom of cages to hold faeces and induce FPD and later removed to allow lesions to heal. Covering the bottom of cage with paper sheet induced 99% incidence of grade 3 footpad lesions on d 13. Subsequent removal of paper on d 14 allowed footpad lesions to heal by d 30. The amount of total collagen protein, and mRNA levels of COL1A1 (encode type I α1collagen), tissue inhibitor of metalloproteinase 3 (TIMP3), TNXB (encode tenascin X) and TNC (encode tenascin C) in footpad skin was related to footpad lesion scores, indicating that these parameters can be used as biomarkers for FPD development and wound healing.

I. INTRODUCTION

Foot pad dermatitis is a condition where necrotic lesions develop on the plantar surface of footpads of broilers and turkeys. Chicken feet (paws) have become the third most important economic part of chicken with breast and wing being the first and second parts (US Poultry & Egg Export Council, 2009). In addition to causing a downgrade of paw quality and economic loss, the occurrence of footpad lesions is used as an audit criterion to determine animal welfare and stocking density of poultry production systems in the EU and the USA. Footpad lesions are a route by which microorganisms from the litter can cause localised infection and septicaemia.

The identification and use of appropriate biomarkers for FPD may assist in modelling the condition and facilitate quantitative assessment of FDP development and mitigation under experimental and field conditions. Previous work (Manangi et al., 2010) has shown that the use of HMTBa chelated trace minerals reduces the severity of FPD scores in broilers, as Zn, Cu and Mn are known to be cofactors of enzyme involved in the synthesis and maintenance of connective tissues. However, subjective FPD scoring may not be adequate to determine onset of, or stage of recovery from, the condition and allow mitigating management decisions to be implemented. Quantitative analysis of the key connective tissue components and expression of genes that encode for key processes in maintaining skin integrity (connective tissues) may allow for early detection of FPD onset and assessment of FPD mitigation in the absence of a visible change in lesion FPD score. The current study was conducted to determine if FPD could be induced under experimental conditions and to assess changes in connective tissue parameters (collagen and gene expression) under the conditions of the model to ascertain their suitability as FPD biomarkers.

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II. MATERIALS AND METHODS

In a battery cage trial, day of hatch Ross 708 male broiler chicks were fed a common corn-SBM diet and assigned to 3 treatments (T1, T2 and T3) with 8 cages per treatment and 11 birds per cage. Cages without papers in T1 served as a negative control. Paper sheet was put on the bottom of cages in T2 during the entire growth period (d 0-30) to hold faeces and continually induce FPD of broilers. Paper sheet was put on the bottom of cages in T3 during d 0-13 to induce footpad lesions and removed during d 14-30 to allow lesions to heal. Representative birds with lesions most close to pen average lesion scores were chosen to collect footpad skin samples. The footpad skin samples were subjected to total collagen analysis, RNA isolation, cDNA synthesis and quantitative PCR using 7500 Fast Real-Time PCR System for quantitation of relative mRNA levels of COL1A1, TIMP3, TNXB and TNC. Actin was used as the loading control. All of the primers for qRT-PCR were verified.

III. RESULTS AND DISCUSSION

Covering the bottom of battery cages with paper sheet induced 99% incidence of grade 3 footpad lesions in both T2 and T3 on d 13. Footpad lesions became more severe in birds on T2 as contact with faeces continued post d 13. After removing the paper sheet on d 14, the footpad lesions of birds in T3 were lower compared to birds in T2 by d 19 and had attained a similar FPD score to birds in T1 by d 27 (Figure 2A). Collagen is the main component of connective tissue. The total collagen levels of grade 3-5 footpads in T2 decreased by 44%, 45% and 48% on d 14, d 22 and d 30, respectively ($P < 0.05$), compared to grade 1 footpads in T1 (Figure 2B). The total collagen levels of grade 3 footpads in T3 were decreased ($P < 0.05$) by 40% on d 14 compared to that of T1, but once paper sheet was removed on d 14, collagen levels started to recover on d 22 and returned to the similar levels of T1 on d 30 (Figure 2B). These results suggest that footpad with lesions lost collagen and collagen levels were related to footpad lesion scores.

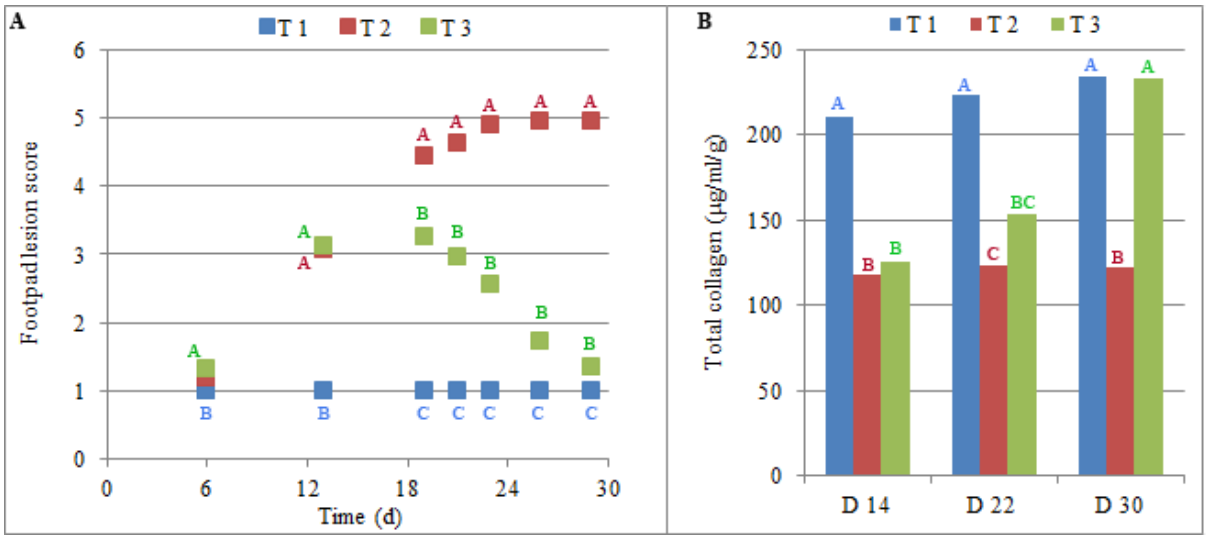

Figure 2 - Broiler footpad lesion score change over time (A) and total footpad collagen levels (B). $^{A, B, C}$ Means with different letters are significantly different ($P < 0.05$).

Consistent with the loss of collagen in grade 3-5 footpads and recovery of collagen in healed footpads, the mRNA levels of COL1A were decreased ($P < 0.05$) in grade 3 footpads on d 14 and grade 5 footpads on d 30 compared to grade 1 footpads in T1, and returned to normal levels in healed footpads in T3 on d 30 after removing paper sheet on d 14 (Table 1).

Metalloproteinase and tissue inhibitors of metalloproteinase are balanced in order to maintain structural integrity of connective tissue (Martins et al., 2013). TIMP3 gene expression was decreased by 47% and 49% in T2 and T3 on d 14, respectively, and by 37% in T2 on d 30, compared to that of T1, and returned to the same levels as T1 in healed footpads in T3 after removing paper sheet (Table 1). The decrease of TIMP3 gene expression in grade 3-5 footpads indicates insufficient amount of TIMP3 to inhibit matrix degradation leading to rapid matrix degradation. This is correlated with the presence of lesions, loss of collagen protein and reduced expression of COL1A1. The increase of TIMP3 in healed footpads in T3 is consistent with recovery of footpad lesions and an increase of collagen protein and COL1A1 gene expression.

Tenascin-X, an important regulator of collagen deposition *in vivo*, organizes collagen fibrils and regulates structure and stability of elastic fibers in the extracellular matrix (Erickson, 1993; Bristow et al., 2005). Tenascin-X deficiency is associated with an inherited connective tissue disorder in humans (Burch et al., 1997). Tenascin-X null mice had reduced density of collagen fibrils in the skin (Mao et al., 2002). TNXB gene expression was reduced by 83% and 62% in T2 and T3 on d 14, respectively, and by 86% in T2 on d 30, compared to that of T1. After removing paper sheet on d 14 in T3, TNXB gene expression in healed footpads on d 30 returned to the similar levels as healthy footpads in T1. These results suggest that the significant reduction of TNXB gene expression is an indication of necrosis in the connective tissue of footpad skin.

Tenascin-C is important for wound healing and its production increases at junction between epidermis and dermis beneath migrating and proliferating epidermis, and in granulation tissue during the wound healing process (Mackie et al., 1988). In this study, TNC expression was increased by 4.3 and 3.4 fold in T2 and T3 on d 14, respectively, and by 2.8 fold in T2 on d 30, compared to that of T1. After removing paper sheet on d 14 in T3, the TNC expression in healed footpads on d 30 returned to the similar levels as healthy footpads in T1. Wound healing is a positive feedback mechanism of animals in response to necrosis and it can happen simultaneously with development of lesions. The increase of TNC gene expression in grade 3-5 footpads suggests that wound healing was occurring during the development of FPD.

Table 1 - The relative mRNA levels of TNXB, COL1A1, TIMP3 and TNC in footpads of birds at 14 and 30 days of age.

	Day 14				Day 30			
Treatment	TNXB	COL1A1	TIMP3	TNC	TNXB	COL1A1	TIMP3	TNC
T1	0.866^{A}	1.753^{A}	0.563^{A}	0.825^{B}	0.415^{A}	1.514^{A}	0.832^{A}	2.325^{B}
T2	0.143^{B}	0.436^{B}	0.300^{B}	3.559^{A}	0.059^{B}	0.535^{B}	0.522^{B}	6.575^{A}
T3	0.329^{B}	0.613^{B}	0.286^{B}	2.784^{A}	0.327^{A}	1.576^{A}	0.838^{A}	3.245^{B}
SEM	0.1058	0.1494	0.0638	0.332	0.0686	0.1101	0.0659	0.7488
P value	0.0002	<0.0001	0.0103	<0.0001	0.0038	<0.0001	0.0035	0.002

$^{A, B}$ Means within a column with different superscripts are significantly different ($P < 0.05$).

The proposed model adequately induced FPD and could be used to assess prevention or treatment measures. The amount of total collagen protein and mRNA levels of COL1A1, TIMP3, TNXB and TNC were related to dynamic change in footpad lesion scores, suggesting that they are biomarkers for footpad lesion development and the wound healing process. The evaluation of FPD has focused on subjective scoring of lesions present on the plantar surface of footpad. The identification of these biomarkers for footpad lesion development and wound healing may allow better understanding of the pathology of FPD and formulate strategies to intervene or prevent FPD development and promote footpad lesion healing.

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INCUBATION TEMPERATURE: INFLUENCE ON CHICK HATCH TIME AND BONE ASH

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Leg abnormalities and reduced bird locomotion is a concern for the Australian broiler industry. With commercial broilers spending up to 30% of their lifespan in the egg, incubation temperature has been linked to some leg weakness issues (Yalcin, et al 2007). The recommended incubation temperature (RIT) for good chick quality at hatch is 37.8^0C (Joseph *et al,* 2006). Both higher and lower incubation temperatures have been implicated with reduced leg strength. Interestingly, incubation of a broiler parent line at temperatures below 37.8^0C resulted in higher femoral bone ash (BA) at hatch (Groves and Muir, 2014), and at 6 weeks of age, an ability to stand for longer in a latency-to-lie test compared to birds incubated at RIT. This experiment was designed to assess the impact of lower than recommended incubation temperatures on hatch time and bone ash in commercial broilers.

Fertile eggs were identified with a unique number, and incubated at either 37.8^0C (RIT) egg shell temperature (EST), or with a gradual increase from 37.4^0C at the start of incubation (sett) to 37.8^0C (Test Temp). Hatch was observed at 492hrs (identified as Early hatch (E)) and again at 516hrs (identified as Late hatch (L)) of incubation. Chicks were taken out of the incubator (take-off) either early (E=492hrs) or late (L=516 hrs). A subset of both the early and late hatched chicks were taken-off and sampled at 492 and 516 hrs respectively. These treatments were identified as EE i.e. early hatch early take-off, and LL i.e. late hatch late take off, respectively. Of the early hatched chicks, two other treatment groups were created at 492hrs. One group was immediately taken-off, given access to feed and water for 24hrs (identified as Early Fed), and then sampled at 516hrs. The other group of early hatched chicks remained in the incubator for a further 24hrs (Early hatch Late take-off – (EL)), until 516hrs, when they were sampled. At sampling chick weight, chick length and bone ash (BA) were measured.

Incubation under Test Temp resulted in a greater number of chicks hatching late compared to incubation under RIT conditions. Early hatch early take-off (EE) and late hatch late take-off (LL) chicks had similar mean bodyweight at take-off. In comparison, EL chicks were significantly lighter and Early Fed chicks were heavier at 516hrs. EE and LL chicks were of similar length, which were both significantly shorter than EL and Early Fed chicks. EE and EL chicks had significantly lower BA than LL and also Early Fed chicks.

EST can influence hatch time. Lower EST during the early stages of incubation appears to delay hatch. Late hatched chicks had higher BA than early hatched chicks. However, the provision of feed to early hatch chicks significantly increased their BA. Further investigation into the role of yolk and feed derived Ca and P during the early post-hatch period, on BA and leg strength throughout the broiler grow out is required. The influence of time spent by the chick in the incubator hatcher tray before take-off, on BA, also deserves consideration.

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GUT IMPACTION IN FREE RANGE HENS

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Summary

This case study reports the outcome of an extreme overconsumption of pasture by free-range laying birds. It outlines the consequences of malnutrition due to animal behavior and housing condition in free-range laying hens, such as increased mortality and morbidity, leading to reduced animal welfare outcomes and compromised bird performance. This paper suggests a number of practical solutions and outlines the significance of future studies on fibre intake in free range birds.

I. INTRODUCTION

Free range production is a fast growing sector of the Australian egg industry. However, exposing hens which were genetically selected for intensive in-house cage production to the outdoor environment remains a challenge in many ways. For example, range usage results in reduced laying performance and higher mortality compared to layers housed in cages (Glatz et al., 2005). Causes for reduced animal performance and increased health issues under free range conditions may include misplacement of eggs, reduced production of eggs, increased exposure to health hazards such as predators, parasites and other diseases (Kijlstra et al., 2009; Lomu et al., 2004; Vaarst et al., 2005). Also, the quality and amount of pasture intake may vary significantly among individual birds due to flock behavior and range design (Hegelund et al., 2005; Walker and Gordon, 2003). In some occasions, the curiosity of these animals in combination with the ability of feed selection may result in overconsumption of pasture by some birds severely affecting normal formulated feed intake. Although only small amounts of nutrients can be attained from grass, consumption of a large amount of grass may give a feeling of satiety and as a result the intake of balanced feed is reduced. Consequently malnutrition ensues, resulting in a severe loss of body condition. In sub-clinical cases, affected birds exhibit reduced performance and in severe cases, death.

II. MATERIALS & METHODS

A free range farm housing 5 flocks with approximately 2500 Bond Brown hens per flock was investigated. On average, one hectare range was available to 350 birds. Commercial feed designed for laying hens was offered ad libitum (Table 1). Daily feed consumption was estimated at < 100 g/hen/day. The pasture contained common grass (phalaris, coxs foot, fescue), chickory, clover, lucerne, and plantain as well as bushes native to New South Wales. This “improved pasture” was introduced decades ago when the farm was used for cattle production. The flock with the highest production rate (hens 30 weeks of age, body weight 1.75 kg, 65.3% hen housed production) experienced severe loss of body condition and 17.2% overall mortality. When examining the carcass, severely grass-impacted gizzards became obvious, with large quantities of grass also present in the crop. In many extreme cases, the proventriculus and isthmus were dilated and appeared contiguous. Excreta containing long coils of grass became noticeable. The farmer supplemented the diet with 5% meat and bone

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meal, changed from mash to pellet diets whilst at the same time increasing the nutrient density by 1.16%, calculated as from 105g/hen/day to 90g/hen/day, and used 0.1% apple cider vinegar (contains 6% acetic acid) in the drinking water. (The apple cider vinegar was used in order to support the digestion of the grass and to apply a flushing effect. The use of this organic certified apple cider vinegar was in agreement with the biodynamic philosophy of the farm.)

Table 1 - Feed composition and nutrient content of the original diet

Nutrient content	g/kg dry matter as analysed
Crude protein	189.8
Arginine	10.87
Methionine	4.42
Cysteine	2.94
Lysine	10.31
Threonine	6.91
Tryptophan	2.60
Valine	9.63
Isoleucine	7.59
Leucine	16.31
Serine	7.93
Glycine	10.08
Ash	131.7
Ether extract	40.7
Crude fiber	39.3
Calcium	44.8
Phosphorus	6.9

As analysed results, University of Missouri Ag Experiment Station Laboratories, USA.

III. RESULTS

After two weeks of treatment, body condition improved, laying performance enhanced and mortality reduced. Continuing feeding a pelleted diet, and providing apple cider vinegar, flocks at 30 weeks of age had an average body weight of 1.95 kg, 86.9% hen house production, and only 5.14% mortality while remaining on the same range. Coiled grass in the excreta is still common to date, indicating that the feeding behaviour of the animals has not changed.

IV.DISCUSSION

In general, the consumption of large amounts of fibrous fodder reduces the intake of a balanced feed, leading to undernourishment in energy and essential nutrients such as amino acids. This is particularly true in high performance animals like the modern laying hen and the mortality and morbidity concerns associated with gut impaction in free range hens cannot be ignored.

In the present report, based on feed analysis, nutrient requirements of a standard brown laying hen would only be covered by at least 105 g feed intake/hen/day. Those estimations do not take into account extra energy required for free range birds due to temperature maintenance and additional activities. Furthermore, it was estimated using crop content of dead birds that the severely affected animals ate up to 60 g grass/day. Others have

estimated that average forage consumption of free range hens is about 30-40 g dry matter/hen/day, which does not affect productivity (Singh and Cowieson, 2013). Analysis of the fibre consumption of hens in this study would have been beneficial. However, deficiency of essential amino acids could be demonstrated by performing feed analysis in combination with estimating feed intake and might be one of the reasons why the inclusion of 5 % meat and bone meal was beneficial to the animals. Due to a lack of data regarding energy intake of the chickens and energy content of the feed, the metabolisable energy available for the hens can only be speculated. However, it is very likely that the energy requirement wasnot met and the increase in nutrient density due to pelleting of the feed enabled maintenance of hen well-being and productivity up to date.

In order to minimise the intake of viscous structural materials such as long grass, range management such as mowing or grazing with cattle or sheep should be considered. The use of acidifiers, such as acetic acid, in the diet may alleviate the viscous nature of the material so passage time of compacted material through the gut is promoted. With a functioning gut, improved nutrient digestibility and feed intake can then follow.

However, little is known about the additional grass consumption of free range laying hens and its impact on nutrient density (Singh and Cowieson, 2013). Specifically, nutrient recommendations for free range birds need to be developed. Therefore, standardized estimations of the nutrient and fibre intake of pasture by free range birds are required. Future studies will have to focus on the range management effect on feeding behaviour, the physiological feedback processes that initiate, promote and sustain fibre ingestion, as well as digestive physiology of free range hens in terms of viscosity in the different parts of the gut.

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THE EFFECT OF TIME OFF WATER ON THE WELFARE OF SPENT LAYING HENS

J.-L. RAULT[1], P. SCOTT[2], A. TILBROOK[3] and P. HEMSWORTH[1]

Summary

The Code states that the maximum time off water for poultry during transport should not exceed 24 h. There is no scientific evidence to indicate the suitability of this recommendation in terms of bird welfare. This project aimed to assess the implications of water deprivation on the welfare of spent laying hens. Two experiments were conducted to investigate behavioural and physiological changes in hens maintained for 12 to 32 h off water. Hens changed their behaviour as early as 12 h after water deprivation. Physiological changes occurred by 24 h, to a similar level to what was seen at 32 h, suggesting that a plateau was reached in terms of acute physiological adaptation. The threshold indicative of acceptable welfare remains debatable depending on value-based judgements.

I. INTRODUCTION

The 'Australian Standards and Guidelines for the Welfare of Animals — Land Transport of Livestock' (Animal Health Australia, 2012) states that the maximum time off water for poultry during transport should not exceed 24 h (item SB10.1). However, there is no scientific evidence to indicate the suitability of this recommendation in terms of hen welfare.

II. MATERIALS AND METHODS

A first experiment aimed to equate physiological changes induced by water and feed withdrawal with behavioural changes. Hens first try to adjust behaviourally to challenging situations and as such, behavioural changes in situ are likely to be reliable indicators of water requirements. Behaviours (head up, head out, head in feeder, inactive or not visible) were observed through 3-min scan sampling using video recording for the last 12 h in 270, 81 week-old Hy-Line spent hens subjected to water and feed withdrawal for 12, 18, 24 or 32 h, solely after 32 h off feed, or with *ad lib* access in cages of 5 hens. At the end of treatments (1500h), blood samples were collected from 216 hens for physiological measures (osmolality, packed cell volume, corticosterone), as well as comb colour and weight loss (pre- vs. post-treatment). All birds were housed in an environmentally-controlled research shed (temperature: 19.2-24.2°C, relative humidity: 50-60%).

A second experiment employed a motivation test using the rationale that longer time off water should lead to a higher price paid to access water, in this case willingness to squeeze through a narrow opening. Twenty hens were subjected to water withdrawal for various lengths of time (0, 12, 18, 24 or 32 h) and work level with door gaps from wide to narrow (150, 135, 120 and 100 mm) following an incomplete randomised block design with 10 tests per hen across 5 weeks in an environmentally-controlled research shed setting (temperature: 18-24°C, relative humidity: 50-60%). The test cage was identical to their home cage, with two adjacent cages connected by the variable door gap. The hens were acclimatised to the testing apparatus by being placed twice a day for 15 min for 6 days prior to testing.

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Data were checked for normality and homogeneity of variance, and transformed if needed, and subsequently analysed using a mixed model (Proc Mixed, SAS).

III. RESULTS AND DISCUSSION

Table 1 - Experiment 1 summary of the behavioural and physiological changes. Squares with different colours (black, white) differ from each other (P < 0.05) except grey which do not differ from black or white (P > 0.05). The arrows indicate the direction of change. FW had access to water and feed *ad lib*, and W to water *ad lib* only and feed deprived for 32 h.

	FW	W	12h	18h	24h	32h
Behaviour:						
Head out				↑		
Head up			↑		↑	
Head in feeder			↑	↑		
Inactive		↑		↑		
Not visible		↑				
Physiology:						
Weight loss		↑	↑		↑	↑
Corticosterone			↑	↑		
Packed cell volume		↑				↑
Osmolality	↑		↑		↑	↑

Table 2 - Experiment 2 summary of the behavioural changes. Squares with different colours (black, white) differ from each other (P < 0.05) except grey which do not differ from black or white (P > 0.05).

	0h	12h	18h	24h	32h
Location:					
Control side (sec)			↓	↓	
Water Side (sec)					↑
Water Quarter (20 cm from water nipple; sec)			↑	↑	↑
Drinking behaviour:					
Drinking (sec)		↑	↑	↑	↑
Latency To Drink (sec)		↓	↓	↓	↓
Exploratory behaviours:					
Walk (sec)				↓	↓
Stand (sec)			↓	↓	↓
Peck Feeder (sec)					↑
Comfort behaviours:					
Preen (number)				↓	
Body Shake (number)				↓	
Wing Flap (number)			↓	↓	↓

Experiment 1 showed that behavioural changes occurred as early as 12 h and 18 h, suggesting that this is a period during which hens adjust their behaviour in response to the thwarting situation (Table 1). These behavioural changes preceded the physiological changes at 24 h (weight loss) and 32 h (packed cell volume, osmolality). However, the reduced activity ('lethargic state') expected as time off water and feed increased did not eventuate.

Experiment 2 showed that the use of narrow vertical door gaps had little effect as a measure of the motivation of the hens to reach the water drinker located in the adjacent side of the testing apparatus, and the interaction between door gap and treatment was never significant, which implies that the door gap was more of a physical difficulty irrespective of

the motivation to drink. Nonetheless, clear behavioural differences (all $P < 0.05$; Table 2) appeared as a result of the length of water removal, reaching a plateau at 24 h with no differences between 24 h and 32 h in most behaviours (e.g. drinking duration). However, changes were already seen in some behaviours at 18 h after water removal (e.g. location of the hen close to the drinker, reduced standing).

IV. IMPLICATIONS

Hens changed their behaviour as early as 12 h after water deprivation (first time point). Nevertheless, behavioural changes do not necessarily equate strictly to a state of compromised welfare, as behaviour is primarily a coping strategy to adapt to change. Physiological changes occurred by 24 h, to a similar level to what was seen at 32 h, which suggests that a plateau was reached in terms of acute physiological adaptation. Consequently, these findings question the welfare of hens that have water withdrawn for 24 h or longer. Nevertheless, there are no clearly defined thresholds indicative of acceptable and unacceptable welfare in the measured responses. When relying on behavioural, physiological, and fitness measures to determine welfare risks, a judgment is made about what degree of change in these indicators is likely to indicate compromised animal welfare. If one favours a conservative decision, the behavioural changes suggested that welfare starts being compromised earlier than 24 h after water removal, and probably somewhere between 18 h and 24 h. However, if one favours the physiological changes, physiological adaptation reached a plateau at 24 h, suggesting that 24 h appear as the maximum acceptable time off water and that 32 h is too long.

These experiments have been conducted under favourable handling and climatic conditions. It should be recognized that factors other than water and feed deprivation are likely to influence hen welfare during transport, such as the health status of the hens prior to loading, their body condition, stress of handling, social stress of mixing, duration of transport and the weather during transport and lairage. Further research is required to determine what factors specifically influence the welfare of spent hens during transport in field conditions.

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WELFARE PROBLEMS OF LAYING HENS IN EUROPE

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Summary

After the ban of conventional cages for laying hens in the European Union there was a rapid development of alternative systems. While furnished cages have replaced conventional cages in most EU countries, there are problems of acceptance of eggs from furnished cage in some countries, and the producers changed to non-cage systems: barns, aviaries and free range. There exist obviously different opinions among the countries on the state of welfare in furnished cages and non-cage systems. Furnished cages provide structural elements, such as perches, scratching areas and nests, which are considered essential for the behavioral wellbeing of the birds. However, these structures are presented under restricted conditions. There is also no deep litter available. The larger space for movement is mainly obtained by increasing group size and to a lesser extent to stocking density in both, non-cage systems and furnished cages. Large group size is known as risk factor for feather pecking and cannibalism. There is also a high incidence of bone breakage in non-cage systems. These aspects have to be considered when appraising the welfare situation in the different systems. The present study summarizes the state of knowledge on the most important welfare issues: Stocking density and group size, use of perches, litter, nests and free range.

I. INTRODUCTION

Keeping laying hens in conventional cages has been banned in Europe for animal welfare reasons as of the end of 2012. This then has initiated the development of highly variable alternatives in the EU countries. While furnished cages have replaced conventional cages in most European countries, loose housed systems (deep litter and aviaries) have been chosen in Austria, Germany, the Netherlands and Sweden as the main management system. The rationale for the replacement of conventional cages through floor systems in Germany was the reluctance of the main retailers to sell eggs from cages hens. The EU marketing regulation has only one code for eggs of both, enriched and conventional cages. Since eggs from conventional cages can still be imported from non-EU countries, the retailers considered it too difficult to communicate the difference between both systems. In addition, in the consumer's perception aviaries and free range systems are generally assumed to be more animal friendly than furnished cages. This opinion is not shared by scientists and veterinarians, and recent reports in public media raised concern about the state of welfare loose house systems including organic egg production. In the following I will deal with the most important welfare issues in furnished cages, deep litter indoor systems and free range.

II. STOCKING DENSITY AND GROUP SIZE

High stocking density (350 – 450 cm²/bird) in combination with the small group size (4 – 5 birds) in conventional cages represents an extreme restriction of the total space for movement. Indeed the total space available has been identified as the most important factor influencing the movement of laying hens (Mallapur et al., 2009). The EU regulation for furnished cages, which replaced conventional cages as of 2013, provide considerably more space per hen (750 cm²), and commercial furnished cages are designed for 10 to 90 birds.

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This increases the total space available from about 2000 cm² in conventional cages to 7,500 - 68,000 cm² in furnished cages. The increase of total space through increasing group size is not generally considered an improvement of the bird`s welfare. The rational of the development of furnished or enriched cages was to combine the advantages of conventional cages, such as hygienic conditions and small group size, with those of deep litter systems, namely perches, scratching area and nest sites (Elson and Tauson, 2012). The risk of aggressive encounters and feather pecking and cannibalism increases with increased group size (Robinson, 1979). The trade-off of freedom to move and the risk of damages seem to be differently appraised in different countries. In Germany for instance, the law for the protection of animals gives a minimum total space per cage of 2.5 m² and a minimum area per bird with nest area of 890 cm². This results in a minimum group size of about 28 birds. In contrast, the legislation in Sweden and Denmark fixed the maximum group size in furnished cages to 16 and 10 birds respectively. The scientific basis of a minimum total space or a maximum group size in furnished cages is rather weak. Appleby (2004) is of the opinion, that 800 cm² per bird with eight birds per group are required to allow the birds sufficient freedom to perform the essential behaviors. Guinebretrière et al. (2009) reported more visits to the nests and higher use of perches, but no effect on dust bathing behavior with 60 birds per group as compared to 40 and 20 birds per group. Keeling et al., (2003) found a curvilinear effect of group size on welfare related traits with an intermediate size of 30 birds as more problematic than smaller or larger groups. This effect however, was mainly related to the social behavior. Severe feather pecking, feather damages and fear were higher at the highest group's size in earlier studies (Bilcik et al., 1998; Bilcik and Keeling, 1999; 2000; Nicol et al., 1999). There was no cannibalism in furnished cages with groups up to 16 birds (Wall, 2003; Guedon and Faure, 2004). In beak trimmed hens the mortality was low even in groups of 60 birds (Huneau-Salaün et al., 2011). But recent large scale experiments using groups of 30 and 60 non-beak trimmed birds in different commercial furnished cages there were high levels of cannibalism (Anonymous, 2012). This has not only been attributed to high group size but also high light intensity. Group size in furnished cages is still considerably lower than in non-cage systems. It is well documented that the risk of mortality through damaging pecking is higher in barns, aviaries and free range (Blokhuis et al., 2007; Sherwin et al., 2010; Elson and Tauson, 2012) and the large groups are considered as the main causal factor.
There is a sudden change of stocking density and group size when pullets are transferred to the layer facility. Stocking density at the end of the rearing period comprises 15 to 20 birds per m² and – according to the EU regulation - 9 birds per m² in the layer house. There is a routine established that after transfer to the layer house the birds will not receive access to the total space. The litter area which represents about one third of total space will be closed. This is to ensure that all birds will find feed, water and nest sites. The stocking density in this stage is similar to the pullet rearing condition but higher than laid down in the regulation for laying hens. This practice is tolerated in the EU for the time before the onset of egg laying, but in many cases there is an extension for several weeks. There are reservations against the restriction of floor space and access to the litter and in some countries. In Sweden it is prohibited for welfare reasons. Recent unpublished experiments have shown, that the deprivation of space and litter from 16 to 18 weeks of age had no negative effects on production mortality and welfare criteria of laying hens. Further studies are needed to clarify the welfare implications of the reduced floor space and deprivation of litter in the transitional phase from the pullet to the layer housing.

III. THE IMPORTANCE OF LITTER

Litter material is considered an important stimulus for scratching and dust bathing in laying hens and lack of litter was a main reason for the ban of conventional cages. The EU regulation for furnished cages requires opportunities for scratching and pecking, but there is no specific information on size of scratching area and type and quantity of litter. Most of the furnished cages do not provide deep litter. There exist scratching areas of highly variable size, from extremely small solid plates of plastic to large areas of plastic or rubber mats. At the time being feed as pecking and scratching substrate is being distributed automatically in regular intervals. This solution is compliant with the wording of the EU regulation. The birds are using system intensively for scratching and pecking, but the question is raised, whether the need for dust bathing is satisfied as well. It is obvious, that the small amounts of feet do not allow the transport of dust into the feathers. The birds often are observed to perform dustbathing on the wire floor outside the scratching area while pecking in the delivered substrate. This type of dust bathing is being called shame dustbathing or vacuum dustbathing. This behavior not only occurs under restricted area and material in furnished cages but also when sufficient substrate and space are available (Vestergaard et al, 1990 Smith et al., 1993; Olsson et al, 2002). Dust bathing sequences in furnished cages are shorter and do not comprise all elements of a complete dust bath as in deep litter (Louton, 2014). The causes for the incomplete dust bathing sequences are either in adequate substrate or social disturbances. Cox and de Baere (2005) observed similar rates of short and incomplete dust bathes in both, deep litter boxes and on scratching mats without obvious reasons for the interruption. Feed as substrate does not contribute to remove lipids of the feathers, which is suggested a function of dust bathing (Scholz et al., 2011; 2014b). Sham dust bathing, however, also occurs independently of the lipid contents of the feathers (Norgaard-Nielsen and Vestergaard, 1981) and even in total absence of litter on wire floor (Bessei and Klinger, 1982; Lindberg and Nicol, 1997). There exist controversial opinions among scientists regarding welfare implications of substrate. While some authors consider dust bathing behavior without substrate as abnormal behavior, which does not fulfill the natural drive of the hens (Vestergard, 1980; Louton, 2014), others concluded that the performance of the behavior as such is important, regardless of the substrate (Lindberg and Nicol, 1997; Widowski and Duncan, 2000). The relative importance of litter for dust bathing has been studied in experiments using different methods. While the birds make extensive use of litter, when it is freely available, they are not prepared to work hard to get access to litter (Dawkins, 1983; Laine et al. (2007). On the basis of such experiments it has been concluded that dust bathing behavior has a low “priority” (Petherick et al., 1993; Bubier 1996). This may be the reason why there is no social competition for the dustbathing area even under crowded conditions (Olssen and Keeling, 2003). Independently of the influence of litter on the behavior of the birds, regular distribution of wheat bran on the scratching mats of furnished cages has shown positive effects on mortality, body weight and feather scores of non-beak trimmed layers (Huneau-Salaün et al., 2014).

IV. PROBLEMS RELATED TO THE NEST

According to the EU regulation 1 m² of nest area is required for 120 hens in deep litter systems. This corresponds to 83 cm² per bird. There is no EU regulation for the nest area in furnished cages. But in Germany a minimum the nest size per cage is 800 cm². This space can be shared by 10 hens. Nest area is not comprised in the figures of floor space. According to the EU regulation the nest floor should be constructed in a way that the hens are not in contact with wire mesh. Therefore the roll away wire floor is usually covered with different types of plastic material. One manufacturer provides a plastic coated wire mesh. This

complies with the wording of the regulation. It is, however, not allowed in Germany. There exist plenty of choice experiments to elucidate the preference for different types of nests, positions within the house and characteristics of the nest floor. The essential criterion for the hen to accept an area as nest is the enclosure and its accessibility (Rauch, 1995). While nests in barns and aviaries are built in separate blocks, located on the slatted floor (integrated nests), or at the walls, the nest area in furnished cages is separated by metal walls or – in most cases – by plastic curtains. The acceptance of nests is relatively high in both furnished cages and non-cage systems. Independently of the nest type more than 90 percent of the eggs are laid in the nest (Wall and Tauson, 2013). The distribution of the hens can be highly unbalanced across the nest position. Higher nests and nest in an integrated position are usually preferred (Report….). This results in overcrowding in these sections and cause mortality through overheating or suffocating. The problems can be further aggravated when not sufficient nest space is provided. The occupation of the nest area also depends on the synchronization of oviposition within a flock and the average time of the birds spent in the nests. Icken et al. (2009) recorded the presence of hens in individual nests using transponder technique. The time of maximum nest occupation varied among different flocks from 2 to 5 hours, and the mean duration of stay in the nest from about 30 to 45 minutes. This corresponds with results of Sherwin and Nicol (1993) and Petherick et al. (1993). In some studies the duration of stay in the nest was shorter (25 min; Freire et al. (1998) or even longer than 1 hour (Cooper and Appleby, 1996). Regarding the high variability of nest use it may be advised to provide more than the minimum nest space laid down in the EU directive. However, nest area has to be provided additionally to the basic floor space allowance. Therefore the farmers not only have to invest in more nest area but also in increased the total house surface.

The risk of mortality through overcrowding in the nests is rather low in furnished cages, especially when plastic curtains are used. The hens may exit the nest at any place when too many birds try to lay their eggs at the same time. There is, however the risk of cloacal cannibalism when during oviposition the vent of the birds is visible outside the curtains and attracts the attention of cage mates. It is therefore advised to increase the nest area and to keep the gap between the curtains and the floor as small as possible (Anonymous, 2012).

V. PERCHES

Chickens have a strong motivation to rest and sleep on perches. The availability of perches has therefore been considered a basic behavior need. The minimum perch space for layers housing systems in Europe is 15 cm per bird. There was, however, no difference in the percentage of hens perching whether the perch length per bird was higher (17 cm; Hergt et al, 2007) or lower (12 cm; Tauson, 1984). Similar to the nest area the perches are often not evenly occupied. Higher perches are usually preferred over lower ones. This can lead to extreme crowding on the upper tiers of aviaries (Abrahamsson and Tauson, 1993). The welfare aspects of perches not only concern resting behavior. Perches are considered to reduce aggression and feather pecking by allowing the birds to escape from aggressive or cannibalistic attacks of group mates. This has been confirmed by Donaldson and O´Connel (2012). Perches also increase bones strength, mainly of the wings (Sandilands et al., 2009). However the movement to and from aerial perches bears the risk of bone fractures. The furculum and the keel bones are mainly affected. The percentage of layers of none cage systems showing old bone fractures at the end of the laying period range from 50 to 80% (Wilkins et al., 2004; Freire et al., 2003; Scholz et al., 2008). The causes for bone breakage are manifold. Osteoporosis is considered the predisposing factor. In addition, inadequate position of perches, crowding in certain areas, low light intensity, poor flying ability and

experience, and high body weight have been identified as risk factors by Sandilands et al. (2009). According to own observations there are frequent accidents in aviaries when the birds try to descent from higher tiers of aviaries in the morning. Raised platforms with integrated perches and ladders in between the tiers may reduce the risk of accidents. However, integrated perches are not generally accepted as ”perches”. Since chickens prefer higher perches it is assumed that they do not feel save on the lower ones. Hence “aerial” perches only are considered to meet the natural needs of the birds by some authors. There is, however, no generally accepted minimum height for perches. Some definitions of perches require that the perch must not be fitted on the slats, or that the perch should be positioned in a height that other birds can pass underneath, or that the perching birds may not be reached and disturbed by birds walking on the floor. According to tests of Faure and Jones (1982a,b) laying hens perceive surfaces raised 5 cm above the floor as perch, regardless of their characteristics. This shows that from the bird´s side even integrated perches may satisfy their need of perching. The availability of perches may reduce the risk to be cannibalized (Gunnarsson et al., et al., 1999; Pötzsche et al., 2001). It is, however, known that targets of feather pecking do not try to escape and often tolerate continuous and severe feather pecks and cannibalistic pecks. This is in contrast to aggressive attacks. Therefore perches may be useful to avoid aggressive pecks but not feather pecks. Perches may even lead to more damages when they are located in a way that the birds present vulnerable body part to feather peckers (Moinard et al., 1998). Pickel et al. (2010) could show that most of the body weight rests on the keel bone while the hens are sitting on the perch, and the permanent pressure is most likely the cause of the deformation. Newly developed perches with a soft surface have shown to reduce keel bone deformation and improve the footing characteristics (Scholz et al., 2014). It is expected that improved designs of perches not only reduce keel bone damages through high pressure during resting, but also prevent bone breakage through save navigation.

VI. FREE RANGE

Free range is the considered the most welfare friendly management system where the birds enjoy more freedom to move and a wide variety of environmental stimuli. Most consumers perceive free range as adequate and natural environment for chickens. Problems arise when large groups of hens are kept under free range conditions. Domestic chickens tend to keep close contacts with their pen. The mean distance of chickens kept from the stable when their movement was not restricted by fences was 50 m, and in some cases up to 300 m (Engelmann, 1948). These results were confirmed by recent studies. 60 percent of the birds remained in a distance of 50 m from the stable, and only a few birds moved to a distance of 250 m. The percentage of birds observed in the free range was in the range of 30 to 36 (Harlander-Matauschek et al., 2001). The use of free range declined with increasing flock size but not with reduced size of the openings (Harlander-Matauschek et al, 2001). There was a high variation of the use of free range of individual birds. The mean time spent in the free range was about 4 hours per day. Rauch et al. (1999) reported a negative relationship between the number of visits to the free range and the duration of visits. The majority of the birds spent a large number of short visits (less than 5 minutes), while a few birds spend up to 4 hours continuously in the free range. The concentration of the visits on the area nearby the openings leads to rapid deterioration of the vegetation and accumulation of nutrients in the soil and ground water. Therefore the establishment of free range systems is not permitted in many areas. Various attempts have been made to ensure a more regular use of the available space through different types of natural or artificial shelter, tries, bushes china grass, huts, etc. These measures increased to some extend the distance of the birds from the poultry house, but not significantly the percentage of birds using the free range (Harlander-

Matauschek et al., 2001). The establishment of a winter garden as transition between indoor and outdoor conditions is being used as complementary element in barns, aviaries and free range systems. The birds may use the roofed area adjacent to the stable under adverse climatic conditions. This protects the vegetation of the free range and, at the same time, gives the birds the opportunity to move, scratch and dust bath in the open air. The winter garden, however, is recognized as part of the indoor surface and not as free range. Therefore the birds have to be given access to the range at any time during the light period and the area close to the outlets is becoming muddy during rainy days. The birds carry dirt inside the buildings and the nests. Since the free range has to be opened as soon as the light is switched on, there is also a risk that eggs are laid in the range area. Another problem of the free range regulation is the prohibition to use the free range for other purposes. This prevents planting maize or other cultures which remove the nutrients excreted by the birds and at the same time provides shelter against predators.

The establishment of mobile free range systems is becoming popular for small scale layer farms. There exist a wide range of commercial mobile layer houses. The constraints are supply of water and electricity, removal of manure, high labor costs and low production rates.

VII. CONCLUSIONS

After the ban of conventional cages in the European Union there was a shift in the egg production systems towards furnished cages and non-cage system, such as barns, aviaries and free range. Although furnished cages provide essential structural elements for scratching, perching and nesting behavior, there is concern about the restricted space to move. Perching and nesting behavior in furnished cages is similar to that in non-cage systems. Due to the restricted amount of litter dustbathing behavior in furnished cages differ from deep litter systems. The distribution of feed as pecking and scratching material in furnished cages does not contribute to reduce the lipid contents of the feathers. There are, however, positive effects of the distribution of substrate on other welfare related criteria. High incidence of bone breakage and cannibalism in non-cage systems raise concern about the welfare status under these conditions. Improvement of perch design and position is required to reduce the incidence of bone breakage and the problem of feather pecking and cannibalism has to be addressed so as to ensure a high standard of welfare in non-cage systems.

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A BRIEF REVIEW OF DRIVERS FOR CHANGE IN THE FREE RANGE POULTRY INDUSTRY

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Summary

The free range poultry industry in Australia has grown quickly in the past 10 years, with market share in 2013 for free range eggs and chicken meat estimated at 39% and 15% respectively and predicted to grow. A review was undertaken to identify some of the key drivers for change in the free range poultry industry. The drivers identified were food safety, biosecurity, bird health, retailer and consumer demand, global influences and sustainability, which all have implications for how the industry may change into the future.

I. INTRODUCTION

By 2050, world population is expected to exceed 9 billion; 80% will live in developing countries, with increased income and increasing demand for meat, milk and eggs. Arable land availability will decrease; moving farming toward more intensive production systems and efforts to ensure a lower environmental footprint will be a major impetus. By 2030, the global demand for chicken meat is estimated to be 39% of total meat consumed, becoming the most commonly consumed meat in the world. In Australia, chicken meat consumption is forecast to rise to 45kg per person in 2014/15 (ABARES, 2014), whilst egg consumption/person has increased 30% in 10 years from 164 eggs to 213 eggs in 2013 (AECL, 2013). The direction that poultry farming moves in the future will be driven by various factors associated with both supply and demand of these products. Therefore, an understanding of the associated drivers for change is of significance. The purpose of this paper is to outline the key drivers for change, particularly for free range poultry production and the likely implications for how the industry may change in the future.

II. FOOD SAFETY AND BIOSECURITY

A primary concern in the poultry sector is food safety particularly reducing bacterial contamination to produce high quality food that consumers want and is safe to eat. To understand contamination of both egg shells and chicken meat it is important to evaluate food safety, particularly zoonotic pathogens, such as *Salmonella* and *Campylobacter spp.* As the number of free range systems expand the opportunity for contamination of both eggs and chicken meat also has the potential to increase. In Australia, eggs (collected at various ages) from a conventionally caged flock had a significantly lower total microbial load than eggs from a free range flock although the overall bacterial load across farms in the study was also low (Samiullah et al., 2014). A larger study conducted in Sweden showed a higher risk of both parasitic and bacterial disease (and cannibalism) in alternative litter based, and free range systems, compared with cages and suggested more rigorous biosecurity was required (Fossum et al., 2009). However, the study was conducted during the change-over from cages to alternative systems, which in itself brings a need for new knowledge and understanding of how birds will adapt to new non-cage systems.

Historically, traditional methods of keeping poultry outside in moveable huts and inside barns were the most prevalent systems. The systems then changed, mainly due to

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inability to scale-up production, but also because of hygiene and disease challenges. Hence, laying birds were moved from floor based indoor and outdoor systems into cages (Tauson, 2005 & Duncan, 2001). These issues highlight the need to obtain sound, science-based evidence before implementing changes across industry as environmental complexity plays a large role in managing risk factors associated with alternative production systems.

During 2013, over 400,000 free range and cage layer birds were culled during an Avian Influenza (AI) infection in NSW where disease transmission from wild birds was suspected. This was a substantial setback, not just for the farm owners, but for consumers, retailers and the industry. A study in the Netherlands comparing different poultry production sectors concluded the risk of low pathogenic AI infection was 11 times higher in free range systems compared with indoor systems. They also suggested that seasonal differences could be expected and may be due to exposure to a contaminated environment (Gonzales et al., 2012). These situations are a warning regarding the potential risk of disease and the associated challenge of managing that risk with an increasing number of free range systems being undertaken.

In Europe, several different alternative systems have been developed to address issues such as feather pecking and cannibalism, including the Rondeel™ system. A system such as this might offer an alternative strategy as a compromise between free range and cage systems, as it has a number of different areas for the birds (e.g. roosting at night, day quarters and a wooded area), which are fully contained and offer protection from predators and provides a partial barrier to wild birds.

III. MARKET DEMAND

Another strong driver for the move toward non-cage egg systems is that being enforced by the major supermarkets in Australia. Woolworths™ stated their plan is to remove all cage eggs from their shelves by 2018, whilst both Woolworths™ and Coles™ already use eggs from non-cage systems in their own brand products. Even more recently, McDonalds™ and Subway™ have indicated that they plan to move toward non-cage eggs as part of a newly developed animal welfare initiative, with McDonalds™ stating eggs marketed in their product range will be 100% cage free by the end of 2017.

Recently several multinational corporations (Nestlé, Unilever and Heinz) have made changes to their corporate animal welfare policies, which will affect thousands of suppliers and producers on how they will adopt appropriate production systems to meet the demand on a cost effective basis. Nestlé have stipulated that their producers move towards eliminating barren battery cages, after joining with the Non-Governmental Organisation, World Animal Protection. This will have ramifications throughout the agricultural industries, and sends a strong message regarding animal welfare that changes are occurring, to which the industry needs to respond and to be at the forefront of these changes.

In 2012, Europe banned the use of barren battery cages, which supported an increase in free range, barn and aviary systems, and development of enriched or furnished cages, especially in the UK. The benefits of the furnished cage, compared with the conventional cage, revolve primarily around the ability of the layers to express a larger repertoire of behaviours. Lay et al. (2011) completed a review in the UK of hen welfare from different production systems and concluded that mortality was generally lower in furnished cages, compared with conventional cages and non-cage systems, but that hens experienced stress in all housing systems. Ultimately, no single production system ranked highly for the welfare parameters measured in the hens.

IV. FREE RANGE DEFINITION AND CERTIFICATION

Another challenge is the uncertainty of the definition of free range in Australia. There is currently a move to develop this definition, as legislation regarding outdoor stocking density varies by state. For example, Queensland has set an outdoor stocking density at 10,000 birds/ha, whilst the Australian Capital Territory has set its outdoor stocking density at 1,500 birds/ha. The higher limit in Queensland was suggested to allow the farmers in that state to compete in the national marketplace, where there may be variable stocking densities. This confusion surrounding the definition of free range, affects not just producers, but also consumers when buying free range products.

Animal welfare certification schemes have been developed to support people to make informed choices on the animal-derived food products they purchase. However, there are a large number of voluntary certification schemes with differing standards, requirements and costs, which can be confusing, both for those wanting to sell food products under these schemes, but also for consumers purchasing those products. In 2011, the Farm Animal Welfare Committee suggested that many consumers in the UK were motivated by animal welfare, but were confused by the information provided, leading to frustration when buying. In a review of Animal Welfare Quality Assurance systems in the UK and Europe, various supermarkets were addressing whether to develop their own welfare schemes. One of the stated risks was that a multitude of schemes with different (and potentially chaotic) standards could be developed, (Martin & Blache, 2014), which may confuse consumers further.

V. SUSTAINABILITY

Australia has important environmental conditions which will contribute to the direction poultry farming takes in the future. Issues such as water management, drought and high temperatures, vegetation requirements, energy use and recycling of waste materials will need to be addressed. For example, ensuring that litter remains friable for the life of the meat chicken flock is of major concern at the moment, and there is also a need to ensure that litter, once removed from the shed, is appropriate for re-use in other systems for recycling. There is also investigation into the possibility of reusing litter between flocks to ensure associated costs are kept low, but also to ensure sustainability of that litter resource in the long run.

As technology changes, the industry needs to be in a position to take advantage of potential benefits, be they related to production, animal welfare or sustainability of the industry. For example, lighting technology (which has associated cost savings) may contribute toward improved bird welfare.

VI. RESEARCH PRIORITIES

Obtaining sound, science-based evidence before implementing changes across industry is essential. Past research has shown there are challenges when moving from one system to another, especially if it involves free range, as environmental complexity brings associated risk. Additionally, understanding how the modern bird breeds (layers and meat chickens) adapt to new non-cage systems is an area of interest. Potential research into alternative systems, such as the Rondeel™ system, adapted to Australian conditions, might offer an alternative strategy as a compromise.

Priority in ensuring that the definition of outdoor stocking density has relevant scientific evidence behind its implementation is important, especially if new animal welfare standards are to be developed for new alternative production systems. Ensuring the certification schemes are relevant, appropriate and easy to understand is a major consideration and part of this comes from education of the public regarding the poultry

industry, including the positives, negatives, risks and challenges associated with non-cage and free range systems.

Currently, the environmental footprint (including water use) of the poultry industry is relatively small in comparison with some other livestock production systems (Mekonnen & Hoekstra, 2010). However, a move toward increasing numbers of free range poultry systems may change this balance. Understanding issues associated with sustainability of the industry in the face of increased numbers of free range farms, their ability to provide for consumer demand, changing resource requirements, in relation to the unique and variable climatic zones in Australia, is an area where more research is required. Changes in technology should also be considered where possible. For example, these may include new lighting techniques that support lower energy consumption and better animal welfare.

VII. CONCLUSION

These drivers for change; food safety, biosecurity, bird health, retailer and consumer demand, global influences and sustainability are all in some way associated with animal welfare. Animal welfare has emerged as an important contributor to change in livestock agricultural practices worldwide, and there are risks associated with all current poultry production systems. For example, the priority given to behavioural freedom, bird health or sustainability, will determine the type of free range system required by industry and supported by consumers and retailers. Ultimately strong scientific evidence for reasoning behind changes or associated standards in any poultry production system is essential.

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FREE RANGE FARM DEMOGRAPHICS AND PRACTICES IN AUSTRALIA – PRELIMINARY DATA

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Free range poultry egg and meat production is a rapidly growing sector in Australia. Establishing free range production enterprises that meet retailer and consumer demands remains a challenge for the poultry industry. Furthermore, Australia is characterised by a wide array of climatic and topographic regions, some of which may expose free range birds to conditions that may affect their health status and productivity.

In order to evaluate the impact of free range production systems on the needs and challenges of farmers and to identify research priorities, a survey was conducted and distributed via e-mail, flyers, and online platforms. Within three months, 84 farmers responded to the survey, resulting in 56 completed questionnaires and 28 partially completed questionnaires. Of the complete responses, 30 egg farmers (EF) and 26 broiler farmers (BF) each answered 79 questions regarding their farm, range, feed, rearing, production and health status, as well as the environmental impact and their adaption to the free range system.

Layer farmers and BF, representing small and large enterprises, participated from all states. Flock size, stocking density and range design varied widely amongst producers. Broiler farmers were more uniform in their housing standards. Briefly, all BF used litter material in the barn, 65% of those used wood shavings; 96% sowed pastures/ grains, planted trees/ shrubs or both in the range; 96% used permanent ranges/sheds; 96% fed birds in the barn used predominantly automated feeding and drinking systems. On the other hand, LF exhibited a greater housing diversity; 74% used litter material in the barn, 30% of those wood shavings, 67% sowed and/or planted the range; 30% used permanent ranges/sheds, 43% rotated the range usage, 53% housed their chickens in mobile caravans and 50% fed birds on the range, using predominantly pan feeders.

Broiler farmers reported that the major factors causing the mortality were heat stress (64%), predation (55%), impaction (18%) and various diseases (36%) while LF attributed their losses to heat stress (37%), predation (42%), cannibalism (37%), impaction (21%), malnutrition (5%) and various diseases (21%). The survey results showed that 12% of BF and 23% of LF were unsatisfied with their ability to treat or prevent diseases. Farmers chose the free range system for bird welfare reasons (64%), consumer demand (60%) and to produce a better quality product (53%). Producers identified that research should be conducted in welfare (52%), pasture management (54%), nutrition (44%), bird health (44%), housing (40%), and economics (29%).

In summary, this survey presents a wide diversity of the needs and challenges for the free range layer and broiler production sectors in Australia. It has also identified a number of priority areas for research to improve free range production systems.

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CHALLENGES TO FREE RANGE POULTRY IN LOW RAINFALL REGIONS

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Free range poultry production in Australia has increased in market share (40 % layers and 15 % meat chickens in 2013) and continues to grow. New poultry farms are being established to meet increased demand, but many are located within low rainfall marginal regions of southern Australia. Free range certification systems in Australia stipulate there must be palatable vegetation available to poultry on the range at all times. The challenge is to provide palatable vegetation in areas that receive as little as 250mm average annual rainfall with shallow infertile soils and hot dry summers.

The majority of studies on the forages used in free range poultry are based on overseas research with species suited to high rainfall temperate climates (eg. Breitsameter et al. 2013). The limited research from Australia (Glatz and Ru 2004) examined the medium rainfall (445mm average annual rainfall) sheep-cereal zone in South Australia. More recently, Singh et al. (2013) conducted studies to quantify the amount of grass consumed by poultry. The main pasture species was Kikuyu (*Pennisetum clandestinum*). Kikuyu is a perennial tropical grass species that originated from eastern Africa suited to high rainfall or irrigation. New poultry production enterprises in southern Australia, both conventional and free range are being developed in drier marginal agricultural areas. Perennials such as Kikuyu will not persist in drier marginal areas without the use of regular irrigation throughout the summer months. Irrigation will not be sustainable during the high evaporative losses of summer in such areas. In summer, limited water allocations will be used primarily for the watering and cooling of poultry. Forage species suited to drier conditions need to be considered for the marginal poultry production areas of southern Australia. Saltbushes are a possible candidate.

Saltbushes (200 species world-wide) have a wide geographic distribution in Australia and are highly adapted to low rainfall regions. Two species (*Atriplex nummularia* and *A. rhagodiodes*) have the potential to be utilized on the range for free range poultry as a feed source, shelter and shade in low rainfall areas. Research on saltbush and poultry production is scarce (Cilliers et al. 1999 and Furtado et al. 2011). Both studies used slow growing meat chicken breeds fed a diet that included *A. nummularia*. In Australia the predominant meat chicken breeds are Ross and Cobb, bred specifically for fast growth and high feed conversion efficiency. We do not know what impact saltbush may have on modern breeds of free range poultry (meat chickens and layers), particularly in terms of production, nutrition, anti-nutritive factors and welfare.

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