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AMINO ACID'S INFLUENCE ON PHYSIOLOGICAL, IMMUNOLOGICAL, AND MICROBIOLOGICAL RESPONSES IN THE BROILER'S INTESTINE

C. BORTOLUZZI[1] and T.J. APPLEGATE[1]

Summary

Even though the intestinal tissues represent a small fraction of the body weight in broiler chickens, its requirement for energy and nutrient is high. The broiler's intestine have a well-coordinated immune system, in association with its commensal microbiota, to avoid the colonization and proliferation of harmful pathogens. However, in commercial situations, due to the modern poultry industry characteristics, there is a high sanitary pressure that may exacerbate the development of diseases, such as coccidiosis and necrotic enteritis. The incidence of these diseases may increase worldwide due to the increasing pressure to limit the use of antibiotic growth promoters in the diets of broilers. For this reason, higher concentrations of some amino acids, known as trophic amino acids, may be beneficial to modulate the intestinal physiology, immunology and microbiology. Trophic amino acids, such as threonine, arginine and glutamine, play a very important role on the intestinal mucosa, and may increase the intestinal turnover rate in cases of tissue damage, helping to heal the damaged tissue after an injury. On the other side, they may help control the over stimulation of the innate immune system (the most expensive in terms of nutrients), and modulate the intestinal microbiome. The objective of this review is to give an insight into the role of trophic amino acids, and report some updated studies of their use in broiler chickens diets.

I. INTRODUCTION

The broiler industry has experienced significant improvements in the past decade. The rapid broiler growth, as well as, environmental and management stressors, and immunological challenges (vaccination and infection) may modify the requirements for essential nutrients. In practical conditions, the requirements for some nutrients may be higher to avoid failures of the immune system or to modulate its response and consequently maintain the performance of the flock. Even though the intestinal tissue represents only about 5% of the body weight, it is responsible for consuming 15 to 30% of the O_2 and proteins in a live organism (Gaskins, 2001) and 20% of the energy (McBride and Kelly, 1990), due to the rapid turnover rate and intense cellular metabolic activity.

An intact intestinal mucosa protects the animal against the uptake of different toxic substances present in the feed, as well as antigens secreted by pathogenic microorganisms and/or the invasion of bacteria. This mucosa also accommodates several immune cells, such as macrophages, polymorphonuclear cells, dendritic cells, and T and B-lymphocytes (Nagler-Anderson, 2001). When the activation of the immune system occurs, the priority of the organism is the proliferation of defense cells, expression of receptors to recognize non-self substances, cytokines, and antibody production; this higher metabolic activity may be directly related to the impairment in the feed conversion ratio.

The intestinal microbiota is responsible for the first line of defense in an animal, regulating cellular permeability, expression of genes in goblet cells and secretion of antimicrobial peptides (Laparra and Sanz, 2010). A well-established intestinal microbiome brings benefits to the host, due to production of vitamins, immune modulation, and inhibition of pathogens; microbial imbalance, on the other hand, may contribute for the development of

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metabolic and immunologic diseases (Jeurissen et al., 2002), and may compete for nutrients with the host (Yang et al., 2009). Therefore, diseases, such as, coccidiosis and necrotic enteritis, besides damaging the epithelial cells, leads to proliferation of undesirable microorganisms, and nutrient competition with the host.

Nutrition aimed at the regeneration of the injured intestinal mucosa can have a significant impact on the health and protection against pathogenic microorganisms, through affecting the microbiota, digestive physiology, immune system and inflammation. An increase in dietary amino acids density has been studied, to reduce the atrophy of the intestinal mucosa, stimulate the local immune system (Wu, 1998), and maintain the balance of the microbiota.

The objective of this review is to give an insight into the role of arginine (Arg), threonine (Thr), and glutamine (Gln) on the physiological, immunological and microbiological parameters of the broiler's intestine, and their beneficial effects for coccidiosis/necrotic enteritis-affected birds.

II. IMPACT OF COCCIDIOSIS AND NECROTIC ENTERITIS ON BROILER'S INTESTINAL PHYSIOLOGY

Two main factors are responsible for maintaining the intestinal mucosa integrity. The first of them is the enterocytes layer, which constitutes a strong barrier. A constant renewal process is observed in the intestine of the chicken as proliferating cells in the mucosal crypts differentiate, predominantly to enterocytes, and migrate to the upper part of the villus, where they are lost through desquamation (Uni et al., 2006). The second of them, is the mucous layers, produced by goblet cells that covers the enterocytes. The mucin-type glycoprotein is able to aggregate different bacterial species, and in some cases, it prevents the attachment of pathogens to the intestinal epithelium. Both enterocytes and goblet cells originate from stems cells located in the crypt region (Uni, 2006).

Additionally, the development of the innate and adaptive intestinal immune system exhibits different spatio-temporal development in broiler chickens (Zhang et al., 2015). The expression of MUC2 gene, a component of innate immune system, rapidly increases from the embryonic day 14 to post hatch day 1 in the ileum and cecum of broiler chickens. The IgA expression, on the other hand, shows slower development from embryonic day 14 to post hatch day 5 and significantly increases up to day 21 and 14 in the ileum and cecum, respectively (Zhang et al., 2015). In infections situations, such as coccidiosis or necrotic enteritis, the mechanisms by which the development of the immune response occurs may change, and nutrients, such as, amino acids may become limiting factors.

Coccidiosis is one of the most important parasitic disease in commercial poultry production systems (Allen and Fetterer, 2002). The parasites invade the intestinal epithelial cell and stimulate the mucus production by goblet cells, as a response to eliminate the coccidia, and it is a predisposing factor for the development of necrotic enteritis (Dahila et al., 2006; Collier et al., 2008). Besides creating an anaerobic environment, essential for the growth of *Clostridium perfringens*, over production of mucus provides a substrate for the proliferation of this bacterium; in addition, the lesions caused by *Eimeria* may increase the availability of substrates from the diet, leading to bacterial misbalance (Pedroso et al., 2012).

Jejunal inflammation in coccidiosis-infected broilers is shown by villus damage, characterized by decreased villus:crypt ratio, crypt dilatation and goblet cell depletion, besides the increase in the expression of inflammatory genes (iNOS, IL-1β, IL-8 and MyD88; Tan et al., 2014). On the other hand, the co-infection of *Eimeria* with *C. perfringens* induces a higher level of inflammation when compared to the birds infected with *Eimeria* or *C. perfringes* alone (Collier et al., 2008), supporting the hypothesis that the host inflammatory

response to eliminate coccidia provides growth advantage for *C. perfringens*. Even though the immunopathology of necrotic enteritis (NE) in chickens is still unclear (Oh and Lillehoj, 2016), several studies have tried to explain the main changes that happen in the intestinal immune cells following NE infection (Park et al., 2008; Collier et al., 2008; Kim et al., 2014).

The activation of the immune system with moderate levels of inflammation is essential for the survival of the birds following an infection, with subsequent tissue repair (Tan et al., 2014). In a recent study, Kim et al. (2014) showed that 1,049 genes were differently expressed in intraepithelial lymphocytes of chickens, in which 601 were increased and 448 were decreased. From these altered genes, all five primary biological functions identified were related to the immune response. This kind of study generates a huge amount of data that has to be well interpreted; however, once we know which genes are activated, different nutritional strategies may be developed with the objective to modulate the immune system, lessen the severity of infection (and/or speed coping/clearance) with the goal of higher survival rates in poultry flocks.

III. AMINO ACIDS AND INTESTINAL HEALTH

The most cited function of the GI tract is related to its importance for the digestion and absorption of nutrients. However, the nutritionists must understand the intestinal immune system and its strict relationship with the microbial community as a separated organ with peculiar nutrient requirements. The nutrient profile used in feed formulations for broiler chickens is based on economically important productive functions, such as: weight gain, feed intake, feed conversion ratio and carcass yield, but not for immunity or disease resistance (Kidd, 2004).

In the last few years, an increasing number of publications have shown the effects of higher concentrations of amino acids on the development and immunity of the GI tract in broiler chickens under normal and challenged situations (Tan et al., 2014a; Tan et al., 2014b; Gottardo et al., 2016; Chen et al., 2016; Rochell et al., 2016a). The effects of coccidiosis on amino acid digestibility has also been evaluated (Adedokun et al., 2016; Rochell et al., 2016b). Rochell et al. (2016) observed that increasing the infecting dose of *E. acervulina*, the ileal digestibility of amino acids decreased linearly, with the exception of tryptophan and glycine. In addition, Adedokun et al. (2016) showed that the apparent ileal digestibility of most of the amino acids, including arginine, threonine and glutamine, were affected by coccidiosis only at 21 d but not at 42 d of age. Dry matter, nitrogen and energy digestibility and metabolizable energy values were lower in coccidiosis challenge birds, and were not restored by amino acid supplementation, while feed efficiency of challenge broilers was positively affected by higher dietary amino acid concentration, specifically: lysine, methionine, threonine, isoleucine, tryptophan, and valine.

The improved feed efficiency without effects on the digestibility measures either in unchallenged or challenged broiler chickens fed higher concentration of amino acids may be due to better development of the intestinal mucosa, and by the availability of amino acids that are related to absorption and utilization of nutrients (Gottardo et al., 2016). Broiler chickens fed higher amino acid (Arg, Thr and Gln) concentrations and challenged with coccidiosis and *Escherichia coli* had significantly more PCNA-positive cells per villus in the jejunum one week after challenge. However, at 2 and 3 weeks post-infection, this number was lower, showing that the amino acids were beneficial in stimulating new cell proliferation right after the challenge (Gottardo et al., 2016).

Amino acids are responsible for the induction of important enzymes for the mitotic process, such as ornithine-decarboxylase for the synthesis of polyamines (Wu et al., 2009). Amino acids can also regulate the expression of genes involved in any other process. For

instance in mammals, studies have shown that dietary supplementation of Arg and Gln increased the expression of antioxidant genes and reduced that of pro-inflammatory genes in the small intestine and adipose tissue (Fu et a., 2005; Wang et al., 2008; Jobgen et al., 2009).

a. Threonine

Threonine (Thr) is an essential amino acid for broiler chickens, and the third most limiting amino acid in corn and soybean meal based diets after methionine and lysine (Lopez et al., 2001). Poultry species do not synthesize threonine *de novo*, which makes it a nutritionally essential amino acid. Thr participates in the synthesis of protein, and its catabolism generates many products important for metabolism, such as glycine, acetyl-CoA, and pyruvate (Kidd and Kerr, 1996).

Broiler chickens have a high Thr requirement for maintenance, when compared to other amino acids, due to its high turnover and high abundance in intestinal secretions (Fernandez et al., 1994). Thr is the major component of intestinal mucin in animals, in which the intestine is responsible for the use of approximately 60% of the dietary Thr, for the synthesis of mucin (Myrie, 2001). Mucin is not digested by the normal mechanism within the GIT, but it is fermented by microorganisms, or secreted in the excreta, which makes it almost unavailable for the animal. Therefore, factors that induce mucin secretion may increase its requirement, and decrease its availability for growth, and compromise the integrity of the intestinal barrier (Stoll, 2006). Faure et al. (2007) reported that sepsis increased the utilization of threonine in rats, and if it is not supplied by the diet, it is covered by muscle protein mobilization.

Studies have indicated that the components of the immune system are sensitive to dietary threonine (Wang et al., 2006; Zhang et al., 2014; Chen et al., 2016; Zhang et al., 2016). In addition to the mucin production, Thr is also a major component of immunoglobulins (Ig), in special IgA secreted by the intestinal mucosa, which contributes to more than 2/3 of all immunoglobulins in the body, and it is essential for maintaining intestinal homeostasis (Brisbin et al., 2008; Slack et al., 2012).

While Zhang et al. (2014) was evaluating the dietary Thr requirement for ducks from 15 to 35 days of age, they observed that serum natural IgY increased linearly when dietary Thr increased, even though Thr had no effect on villus height, crypt depth, goblet cells, and MUC2 gene expression. However, this study did not involve challenge, which may change the requirements. On the other hand, Thr supplementation changed the microbial balance in the intestine, by reducing *Escherichia coli* and *Salmonella* colonies and increasing *Lactobacillus*; in addition, Thr modulated the immune system, by increasing secretory IgA, and downregulating the expression of inflammatory genes, such as INF-γ and IL-1 β (Chen el al., 2016). The effect of decreasing the expression of IL-1β was also observed in coccidiosis-infected broilers fed higher Thr level (1.8 vs. 5.3 g/kg; Wils-Plotz et al., 2013).

A well-established microbiota is able to interact with the intestinal immune system of the animal, and strength its innate defenses. Nutrient composition and flow affects the composition of the microbiome (Pan and Yu, 2014), and the function that the microorganisms are going to perform on the host. Besides the understanding of interactions among members of the gut microbiome, poultry producers are searching for different dietary interventions to enhance bird growth and reduce the risk of enteric infections (Pan and Yu, 2014), in which amino acid manipulation may be one option. As shown by Star et al. (2012), higher dietary concentration of threonine improved production performance of necrotic enteritis-infected chickens, but not the intestinal damage, suggesting that other mechanisms are involved in this process.

b. Arginine

The dietary requirement of Arginine (Arg) for broiler chickens is variable, depending on the strain, grow rate, and due to the nonfunctional cycle of urea in birds (Sung et al., 1991). The genetic material of birds do not encode for the enzyme, carbamoyl phosphate synthetase, that catalyzes the first step of ammonia detoxification and leads to Arg production. As this biochemical cycle is not functional, birds are dependent on the supply of this amino acid in the diet (Fernandes and Murakami, 2010).

In addition to its function as a protein constituent, Arg is related to the synthesis of creatine and polyamines, as a substrate of nitric oxide (NO), and the secretion of insulin-like growth factors (IGF; Fernandes and Murakami, 2010). Polyamines are important for the development of the intestine in newborns (Loser et al., 1999), which may explain the positive effects on performance and small intestine morphology of one-week-old broiler chickens (Murakami et al., 2012). Polyamines are able to stimulate proliferation, migration and apoptosis of intestinal cells (Ruemmele et a., 1999). Therefore, Arg, as a precursor of polyamines, may be considered as a trophic substance, increasing the mitotic process in the crypt-villus region, which may increase the number of cells and the size of the villus (Uni et al., 1998).

The effects of Arg on the immune system of broiler chickens have also been investigated, in normal (Murakami et al., 2014) or in immune-stimulated conditions (Tan et al., 2014a; Tan et al., 2014b). In a coccidiosis challenge study, Tan et al. (2014a) showed that the jejunal inflammation was evidenced by villus damage, crypt dilation and goblet cell depletion. In this study, coccidiosis downregulated the expression of MUC-2 and IgA, but upregulated β-Defensine-8, and inflammatory genes (iNOS, IL-1β, IL-8, TLR4) mRNA expression. Meanwhile, Arg linearly diminished the expression of TLR4, suggesting that the anti-inflammatory effect of Arg is via suppression of TLR4 pathway, which was verified when the inflammation was stimulated by lipopolysaccharide (Tan et al., 2014b).

c. Glutamine

Glutamine (Gln) has been used due to its important function as an energy source in the process of mucosal regeneration after an injury. Gln is considered an important source of energy for enterocytes, being an essential metabolic component for the proliferation of these cells. Gln may reduce the intestinal atrophy, besides its participation on the formation of glutathione, an important system of defense against free radicals (Hunter et al., 1994; Takahashi et al., 1997 e 2006; Kidd, 2004). Gln may be considered an essential amino acid under inflammatory conditions, disease challenge, or surgery (Newsholme, 2001). Yi et al. (2001) observed that 1% of Gln had beneficial effects on the small intestine morphology at 3 and 14 d of age in broiler chickens.

Synthetic Gln in the diet of animals has been studied due to its effects on the intestinal structure and function (Wu et al., 1995; Wang et al., 2008). Gln supplementation to the diet was not able to decrease *Salmonella* shedding in broiler chickens, even though it improved BW gain (Fasina et al., 2010), increased villus height in the duodenum and jejunum, and enhanced antibody (IgA) production (Bartell and Batal, 2007). Coccidiosis vaccinated chickens showed improved feed conversion ratio, deeper crypts in the jejunum and longer villus in the ileum when supplemented with Gln (Luquetti et al., 2016). In weaned piglets, dietary Gln supplementation restored the function of the small intestine, by increasing the expression of genes that are necessary for cell growth and removal of oxidants, while reducing the expression of genes that promote oxidative stress and immune activation (Wang et al., 2008). Even though these results show beneficial effects of Gln on the intestine, there is

a lack of study looking at the effects of this amino acid on chickens' intestinal immune system and microbial balance at a molecular level.

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MICROBIOTA STUDIES IN POULTRY: THE BLACK HOLE OF COMPLEXITY!

R.J. MOORE[1], D. STANLEY[2] and R.J. HUGHES[3]

Summary

Gut health is an important issue when considering how to get the best productivity from a flock. The microbial populations that inhabit the gastrointestinal tract (GIT) of birds play an important role in the establishment and maintenance of a healthy gut. For a number of years we have been studying the composition of the gut microbiota looking for correlations between its structure and the growth performance of broiler birds (Stanley et al., 2012a, 2013a, 2016). Our goal has been to understand how the microbiota influences bird health and productivity and then go on to develop ways to manipulate the interactions to maximise performance and health and produce flocks with more even performance (Moore et al., 2011; Stanley et al., 2012b). In-feed antimicrobial growth promoters have been one way that the host-microbiota interaction has been manipulated. However, in the future, with the need to provide alternatives to the use of antibiotics within production animals, the manipulation of the host-microbiota is likely to rely more heavily on products such as prebiotics and probiotics. Here we review some of the high level findings and conclusions we have drawn from our extensive microbiota studies.

I. COMPLEXITY OF THE GASTROINTESTINAL MICROBIOTA

The explorations of the gut microbiota that we have undertaken have revealed a hitherto unexpected level of complexity in the composition of the microbiota. It is only with the application of advanced methods of analysis that this complexity has been fully revealed. Traditional methods for analysis of the gut microbiota had relied on enumeration of various different classes of bacteria, by culturing on agar plates, using a variety of different media compositions. These methods detected only a limited range of common bacteria. The culturing methods have been largely superseded by molecular methods; first by gel based methods but more recently DNA sequence based methods. It is these powerful new methods, facilitated by the rapid advances in next generation DNA sequencing (NGS) technology, that we have applied to poultry samples to investigate the gut microbiota to an unprecedented level of detail.

When molecular methods of microbiota analysis were first applied to gut samples it became clear that the diversity of bacterial types present within the gut was much broader than that commonly found by culturing methods (Stanley et al., 2012a). Whilst culturing had indicated that there might be a few hundred different types of bacteria the molecular methods now show that there are actually several thousand different types of bacteria in a typical GIT. The apparent discrepancies between evaluations of the GIT microbiota by culturing and molecular methods results from the fact that many of the microbes resident in the gut cannot currently be cultured. There are two principal reasons proposed to explain why the culturing approach misses much of the complexity that is present within the microbiota; (i) we just don't have the correct media to grow some bacteria on and (ii) some bacteria may only grow within a consortium of other bacteria and cannot be cultured in isolation. A consequence of

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the discovery of greater complexity within the gut microbiota is that there are potentially many more types of bacteria that could potentially be harnessed for use as probiotics and competitive exclusion agents. Currently the spectrum of bacteria that have been tested for utility in such products is fairly narrow. If some of the culturing challenges can be addressed we may be able to access many more potential products that could be used to improve bird performance. Even within the well-studied *Lactobacillus* genus the molecular analysis techniques indicate that there are many uncharacterised species of *Lactobacillus* present that that could be a source of new products. Certain genera of bacteria, such as *Lactobacillus*, have been generally regarded as being beneficial to the host. However, the detailed microbiota characterization empowered by culture free methods has repeatedly shown that some members of this genus are often correlated with poor growth performance in broiler birds. Conversely, some bacterial types, such as the clostridia, that have traditionally been considered to be "bad", have some representatives that are actually strongly correlated with high level bird performance (Stanley et al., 2014). It is clear that probiotic potential of a bacterial isolate cannot be accurately deduced simply by knowing what species it is; probiotic activity is a characteristic of a specific isolate.

II. VARIABILITY OF THE GASTROINTESTINAL TRACT MICROBIOTA

In addition to the overall complexity of the microbiota we have also found that there is a second level of complexity which is of even more importance in considerations of how to harness microbiota manipulation for productivity improvements. The emerging story, that has been something of a surprise, is the high levels of variation in microbiota composition between flocks and even between birds in a single flock (Stanley et al., 2013b). Even though birds within a flock generally come from the same hatchery, the same parent stock, are housed together, have access to the same feed and water and environmental conditions and are coprophagic, we still find that they can have quite different microbiota. We have speculated that these differences indicate the importance of the initial microbial colonisation in the earliest hours following hatch. The hatching of chicks takes place in the absence of adult birds and normal brooding and nesting behaviour typical of wild birds. Therefore, newly emerging chicks have no access to "normal" bird microbiota, apart from bacteria residing on or in egg shells if not thoroughly cleaned and fumigated prior to setting. Colonisation is presumably driven by exposure to random sources of bacteria such as those that might be present on shells, in the transport boxes, transmitted from human handlers, present in the first feed they are given and in the initial receiving environment. The stochastic nature of this bacterial acquisition may mean that small variations in the composition of the initial bacterial inoculants may have permanent effects on life-long microbiota composition.

The degree of variation in microbiota composition in bird-to-bird comparisons is certainly much greater between flocks than within a flock. Comparisons within a flock generally show variation in the abundance of different species, genera, and families of bacteria whereas between flocks there can, at times, be very significant differences in phylum abundance. Such large differences in composition are likely to have profound effects on how the host-microbiota meta-organism responds to diets, treatments and medications.

III. PROPERTIES OF A "GOOD" MICROBIOTA

This significant level of variability represents the "black hole of complexity" in the title of this presentation. The finding of great variability between trials was, initially, a great impediment to our goal of identifying specific bacterial strains within the microbiota that were consistently correlated with good performance and hence may have potential for development as probiotics. If the GIT microbiota across trials is very different then there is

no prospect of identifying individual bacterial isolates present across all trials. Despite these complications it has been possible to draw some general conclusions regarding the composition of a favourable microbiota (Stanley et al., 2016). High performance birds generally have more complex microbiotas compared to that seen in low performance birds. The bacterial types within the high performance birds tend to be more evenly distributed such that the microbial populations not dominated by one or a few types of bacteria.

IV. APPLYING MICROBIOTA KNOWLEDGE TO UNDERSTAND GUT HEALTH PRODUCTS

Within the published work that has investigated the efficacy of various prebiotic, probiotic and symbiotic products, including the wide array of phytogenics that have been assessed, there are often large differences in observed efficacy reported for a product from study to study (Blajman et al., 2014). We postulate that the apparently different responses of birds to these products may be caused by large differences in the microbiotas of different flocks (Stanley et al., 2016). It would seem reasonable to expect such products which rely, at least in some part, on their ability to interact with and modify the gut microbiota may have differential effects within radically different microbiotas. For example, consider the case of probiotics. To be truly considered a probiotic a particular organism should have beneficial effects on the host when delivered as a live product. Such a live product potentially interacts with all the other live organisms within the microbiota. Within one particular microbiota setting a probiotic may be able to live synergistically within the microbiota whereas a different microbiota may competitively exclude the probiotic strain or perhaps even actively kill it. This gives us a basis to understand why the reproducibility of many results with prebiotic and probiotic type products can be doubtful.

V. DEVELOPMENT OF A NEW GENERATION OF GUT HEALTH PRODUCTS

More effective products to promote gut health can be developed if the significant level of microbiota variability is taken into account. Much of the variability in GIT microbiota is established during the earliest phase of microbial colonisation of the gut. Therefore, we hypothesise that products aimed at the initial establishment of the microbiota, using microbial/probiotic inoculants in the hatchery, are likely to be effective in producing flocks with a more uniform microbiota. That in turn is likely to result in a flock with more even performance. New approaches need to be taken to the selection and assessment criteria that are applied when developing products that are likely to function by interacting with the mature microbiota. We need to be especially mindful of the need for products that can cope in different gut microbial environments and indeed should build in such assessments to the product development process. Current probiotic products available to the industry need to be used multiple times, sometimes on a daily basis, and even throughout life. This indicates that they have little ability to establish themselves with the GIT microbiota. There may be opportunities to develop a new generation of probiotics that are better able to colonise, establish and grow within the GIT microbiota. The particular need is to identify probiotic bacteria that display these properties in the face of the variable native microbiota that any probiotic product will inevitably encounter. There is likely to be a need to undertake more detailed *in vivo* selection and assessment processes to identify new probiotics. The *in vitro* tests, such as acid and bile resistance, adherence to cultured cells, aggregation ability, etc., that have traditionally been used as preliminary screening mechanisms for probiotic identification are not well suited to addressing the obvious challenge of adaptation to the changing and variable environment that we now know occurs in the GIT.

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MODIFICATION OF THE CHICKEN INTESTINAL EPITHELIAL PHYSICAL BARRIER IN POULTRY BY DIETARY FACTORS

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Summary

The intestinal epithelial physical barrier is the most critical element of maintaining an intact intestinal barrier and made up of a layer of columnar epithelial cells and intercellular junctional complexes including tight junctions, adherens junctions and desmosomes. Tight junctions (TJ), which are formed by proteins including claudins, occludin, junctional adhesion molecule and zonula occludens (ZO), are primarily responsible for the permeability of the paracellular pathway. The function of the intestinal barrier function in poultry is evaluated by measuring intestinal permeability. Few studies have shown the developmental profile of intestinal barrier function and tight junction proteins in the intestinal epithelium of chicks in embryonic phase or/and the early post-hatch period. Several feed additives, including nutrients (i.e. Zn), probiotics, prebiotics, functional polysaccharide, enzymes and epidermal growth factor, were shown to regulate intestinal barrier function by modifying expression and localization of TJ proteins.

I. INTRODUCTION

The animal intestine has the roles of absorbing nutrients and also acting as a barrier to prevent pathogens and toxins from entering into the body and potentially causing disease. Injured intestinal barrier is characterized by increased intestinal permeability, which allows luminal antigenic agents (e.g., bacteria, toxins, and feed-associated antigens) to "leak" across the epithelium to sub-epithelial tissues, to result in inflammation, malabsorption, diarrhea, and potentially systemic disease. In the post-AGP (antibiotic growth promoters) era, nutritional solutions to maintain the integrity of the intestinal barrier is of great importance to get proper functioning of the epithelial cells and to prevent the entry of pathogenic bacteria.

II. THE INTESTINAL EPITHELIAL PHYSICAL BARRIER

The intestinal epithelium forms the largest and most important barrier between internal and external environments of animals. The intestinal epithelial barrier is made up of a layer of columnar epithelial cells that forms the first line of defense between the intestinal lumen and inner milieu. The intestinal epithelial cells are mainly absorptive enterocytes (over 80%) but also include entero-endocrine, goblet, and Paneth cells. The epithelium allows the absorption of nutrients while providing a physical barrier to the permeation of pro-inflammatory molecules, such as pathogens, toxins, and antigens, from the luminal environment into the mucosal tissues and circulatory system. The epithelial selective permeability includes two pathways: the transcellular and the paracellular pathway. The transcellular pathway is

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involved in the absorption and transport of nutrients, including sugars, amino acids, peptides, fatty acids, minerals, and vitamins. As the cell membrane is impermeable, this process is predominantly mediated by specific transporters or channels located on the apical and basolateral membranes. The paracellular pathway is associated with transport in the intercellular space between the adjacent epithelial cells. These epithelial cells are tightly bound together by intercellular junctional complexes that regulate the paracellular permeability and are crucial for the integrity of the epithelial barrier.These junctions allow the passage of fluids, electrolytes, and small macromolecules, but inhibit passage of larger molecules.

The junctional complexes consist of the tight junctions, gap junctions, adherens junctions, and desmosomes. Tight junctions are the most apical and are primarily responsible for controlling permeability of the paracellular pathway (Turner, 2006). Adherens junctions are located beneath the tight junctions and are involved in cell-cell adhesion and intracellular signaling. Both tight junctions and adherens junctions (together known as the apical junctional complex) are associated to the actin cytoskeleton (Anderson, 2001). Desmosomes and gap junctions are involved in cell-cell adhesion and intracellular communication (Garrod and Chidgey, 2008), respectively. The cytoskeleton is an intricate structure of protein filaments that extends throughout the cytosol that is essential for maintaining the structure of all eukaryotic cells. Disruption of the cytoskeleton is linked to the loss of intestinal barrier integrity.

Tight junctions are formed by protein dimers that span the space between adjacent cell membranes. There are over 50 proteins with well recognized roles in tight junction formation. These proteins comprise four integral transmembrane proteins (e.g. occludin, claudins, junctional adhesion molecules (JAM) and tricellulin), and cytosolic scaffold proteins, such as zonula occludens (ZO) proteins. The extracellular domains of the transmembrane proteins form the selective barrier by hemophilic and heterophilic interactions with the adjacent cells. The intracellular domains of these transmembrane proteins interact with ZO proteins (Gonzalez-Mariscal, 2003), which in turn anchor the transmembrane proteins to the perijunctional actomyosin ring (Berkes et al., 2003). The interaction of TJ proteins with the actin cytoskeleton is vital to the maintenance of TJ structure and function. In addition, the interaction of the TJ complex with the actomyosin ring permits the cytoskeletal regulation of TJ barrier integrity. The function of occludin is not yet fully understood, but numerous studies using animals and cell cultures indicate that it is required for TJ assembly and barrier integrity in the intestinal epithelia. Occludin has been linked to the regulation of intermembrane diffusion and paracellular diffusion of small molecules. The claudin proteins are considered to be the structural backbone of TJ. Claudins consist of at least 24 members in humans and mice, and each isoform shows a unique expression pattern in tissues and cell lines. In contrast to their structural similarities, claudins perform different functions and can be roughly divided into two types: those involved in barrier formation (decreasing paracellular permeability) and those in channel pores (increasing paracellular permeability). In the intestines, claudin-1, -3, -4, -5, -8, -9, -11, and -14 can be categorized as barrier-forming claudins, while claudin-2, -7, -12, and -15 are pore-forming claudins (Suzuki, 2013). Several plaque proteins have been identified, including the zonula occludens (ZO) proteins, ZO-1, ZO-2, and ZO-3. Plaque proteins potentially play a central role in TJ

regulation, because they can cause reorganization of the cytoskeleton. Claudin-1、claudin-2 、claudin-3、claudin-5, claudin-16, ZO-1, ZO -2 and occludin are reported in poultry (Kawasaki et al.,1998; Simard et al.,2005; Osselaere et al.,2013).

TJ are not static barriers but highly dynamic structures that are constantly being remodeled due to interactions with external stimuli, such as food residues and pathogenic and commensal bacteria. Regulation of the assembly, disassembly, and maintenance of TJ structure is influenced by various physiological and pathological stimuli. Signaling pathways involved in TJ regulation, and interactions between transmembrane proteins and the actomyosin ring are controlled by several signaling proteins, including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), myosin light chain kinase (MLCK), and the Rho family of small GTPases (Ulluwishewa et al.,2011).

III. ASSESSMENT OF THE EPITHELIAL PHYSICAL BARRIER FUNCTION IN POULTRY

a) Intestinal permeability

Intestinal permeability is defined as the non-mediated diffusion of large (i.e., molecular weight >150 Da), normally restricted molecules from the intestinal lumen to the blood. The primary means of determining intestinal permeability in humans or animals is by measuring the passage of high molecular weight probes across the gastrointestinal tract barrier. In humans, this involves ingestion of a solution containing nontoxic, non-metabolizable substances (such as sucrose, lactulose, sucralose) and assessing their excretion in the urine (Bjarmason et al.,1995; Meddings and Gibbons, 1998). The appearance of probes in the urine indicates loss of barrier function in the gastrointestinal tract. However, that method is unsuitable for poultry because of the mixture of urine and feces. In animal models including poultry, intestinal permeability is usually determined by infusing fluorescent probes, such as fluoroisothiocyanate (FITC)-dextrans, into the intestinal area of interest and measuring plasma concentrations over time (Lambert et al.,1985). The probe horseradish peroxidase was also reported (Cameron et al.,2005).The Ex vivo Ussing chamber is the most sensitive to test the intestinal permeability by measuring transepithelial electrical resistance (TER), as it reflects the opening of the tight junctions between epithelial cells and the paracellular permeability of the intestinal mucosa

b) Bacterial translocation

The disruption in barrier functions was associated with viral and bacterial translocation across the epithelial monolayers. Bacterial translocation is defined as the passage of viable bacteria from the intestinal tract through the epithelial mucosa into extra-intestinal organs. Impaired mucosal surfaces can increase vulnerability of the intestinal epithelium with an augmented risk of bacterial and viral penetration, or bacterial overgrowth in the in the intestine (Magnotti and Deitch, 2005).

c) Plasma LPS concentrations

LPS is a highly pathogenic component of the walls of gram negative bacteria and is found in the intestinal tract in high concentration. Its presence in the portal blood of animal models indicates passage from the intestinal lumen to the circulation. Increased LPS concentration in the systemic circulation likely indicates severe intestinal barrier dysfunction (Hall et al., 2001).

IV. DEVELOPMENT OF THE EPITHELIAL PHYSICAL BARRIER

Kawasaki et al. (1998) determined the developmental expression of occludin in the gastrointestinal tract of 3- to 21-day-old chick embryos and reported that occludin mRNA was first detected by RT–PCR in the chick embryo on day 3 of incubation, by northern blot analysis on day 4, and by western blot analysis on day 5, suggesting that synthesis of occludin begins in the chick embryo at a very early stage of development. The immune-histochemical assay revealed that occludin began to be weakly expressed only along the apical surface of the gastrointestinal epithelium of the 4-day-old chick embryo. As the embryo developed, the immunoreactivity gradually became stronger and formed more complex networks near the apical surface, which indicated that the developmental expression of occludin in the gastrointestinal tract is closely correlated with the morphological as well as functional development of the tight junction. Roberts et al. (2005) reported that the small intestinal epithelial barrier function of broiler chicks hasn't developed well at hatching, and the jejunal TER increased more than 3-folds and the ileal TER increased one-fold during d2 to d11 of age. Jejunal occludin expression increased linearly with age, but did not reach a plateau by d11, even though no effects of age on ileal occludin or on zonula occludens-2 expression were observed. Their work shows that the epithelial barrier function of the ileum is not fully developed in broiler chicks until later than d 14 of age for the ileum. More research are required to develop nutritional solutions beneficial for the small intestinal barrier function development and gut health.

V. MODIFICATION OF THE EPITHELIAL PHYSICAL BARRIER BY DIETARY FACTORS IN POULTRY

a) Zinc

The importance of Zn to intestinal development and function has been demonstrated in many studies, dietary Zn supplementation reduced gut lesion scores and the intestinal permeability and increased expression of ZO-1 and occludin in mammals. Zn deprivation induced a decrease of TER and altered tight and adherens junctions (Finamore et al., 2008 ; Zhang and Guo, 2009). Zhang *et al.* (2012) reported that Zn (as $ZnSO_4$) up-regulated occludin and claudin-1 mRNA expression in the ileum and tended to reduce plasma endotoxin levels in the chickens challenged with *Salmonella Typhimurium*, and indicated that regulation of occludin and claudin-1 expression by Zn could be involved in ameliorating the increased intestinal permeability induced by *Salmonella Typhimurium* challenge. Hu *et al.* (2013) showed that

supplemental ZnO or ZnSO4 did not affect ileal and colonic barrier function and intestinal microflora in broiler chickens, however supplemental 60 mg of Zn/kg as ZnO-MMT (zinc oxide-montmorillonite hybrid) increased colonic TER values, and reduced colonic probe mannitol permeability as well as ileal or colonic inulin permeability of the chickens.

b) Probiotics and Prebiotics

In the study by Rajput et al. (2013), compared to treatments with *Saccharomyces boulardii* and *Bacillus subtilis* B10, the tight junctions of jejunum and ileum of broilers were comparatively loose in the control group, and both *Saccharomyces boulardii* and *Bacillus subtilis* B10 improved the epithelial tight junctions through increasing occludin, claudin-2, and claudin-3 mRNA expression levels in the intestine of the broilers. CAO et al. (2014) reported that *L. fermentum* 1.2029 was able to ameliorate the severity of necrotic enteritis lesions and inflammation and improved the epithelial barrier through increasing claudin-1 and occludin levels in the necrotic enteritis -infected chickens.

Heat stress not only negatively affected the intestinal microbiota balance, also decreased the jejunal TER and increased the jejunal paracellular permeability of FITC-dextrans, and which was correlated the down-regulated jejunal protein levels of occludin and ZO-1 in the broilers. Supplemental probiotic mixture containing *Bacillus licheniformis*, *Bacillus subtilis* and *Lactobacillus plantarum* increased the jejunal protein level of occludin in the broilers (Song et al., 2014). That indicated that dietary addition of the probiotic mixture was effective in partially ameliorating intestinal barrier dysfunction induced by heat stress in broilers.

Song et al. (2013) also reported that supplemental cello-oligosaccharide, a functional oligosaccharide obtained from plant cellulose, increased the jejunal villus height and villus height to crypt depth ratio, as well as decreased jejunal paracellular permeability of FITC-dextran in the broiler chickens.

c) Functional polysaccharides

β-1,3/1,6-glucan from *Saccharomyces cerevisiae* has beneficial effects on both the innate and acquired immune systems, and clearance of pathogens such as *Salmonella, Escherichia coli* and coccidiosis in broiler chickens. The work of Shao et al.(2014) showed that dietary β-1,3/1,6-glucan supplementation could attenuate the intestinal mucosal barrier impairment in the broiler chickens challenged with *Salmonella Typhimurium*, and that could be related to the increased mRNA expression of claudin-1 and occludin, and the increased goblet cell numbers and sIgA level in the jejunum of the broiler chickens. Parson et al.(2014) reported that in chickens, dietary supplementation with soluble non-starch polysaccharide (NSP) plantain NSP reduced invasion *by S.Typhimurium*, as reflected by viable bacterial counts in splenic tissue, and plantain NSP inhibited adhesion of *S.Typhimurium* to a porcine epithelial cell-line and to primary chick caecal crypts *in vitro*.

d) Enzymes

Clostridium perfringens challenge increased the intestinal lesion score and also resulted in passive transcellular permeability and higher plasma endotoxin in the chickens, and dietary addition of xylanase or enzyme complex containing xylanase, glucanase and mannanase could alleviate the alteration caused by *C. perfringens* infection (Liu et al., 2012), indicating that dietary enzyme supplementation could benefit for gut barrier integrity of the *C. perfringens*-challenged chickens.

Lysozyme as a natural antimicrobial protein occurs in a number of animal secretions and is considered an important component of the innate immune system. The addition of exogenous lysozyme significantly reduced the concentration of *Clostridium perfringens* in the ileum and the intestinal lesion scores, and inhibited the overgrowth of *E. coli* and *Lactobacillus* in the ileum and intestinal bacteria translocation to the spleen of chickens challenged with *Clostridium perfringens*, suggesting that exogenous lysozyme could be used to improve the intestinal barrier function of chickens (Liu et al., 2010).

e) Epidermal growth factor (EGF)

EGF is a small amino acid peptide with a broad range of bioactivities on the intestinal epithelium, including the stimulation of cellular proliferation, differentiation, and intestinal maturation. In chickens, EGF reduced jejunal *C. jejuni* colonization and alleviated the dissemination of *C. jejuni* to the liver and spleen. In the *in vitro* study, the pretreatment with EGF abolished the *C. jejuni*-induced intestinal epithelial abnormalities, such as disruption of tight junctional claudin-4, increasing of transepithelial permeability and the translocation of noninvasive *Escherichia coli* C25 (Lamb-Rosteski et al., 2008).

f) Others

Other dietary factors such as threonine (Hamard, et al.,2010), glutamine (Li and Neu, 2009), and flavonoids were also reported to regulate intestinal epithelial barrier in animals or cell lines *in vitro*, but few reports in poultry were found. More nutritional solutions to improve intestinal barrier function and the underlying molecular mechanisms are needed to be investigated.

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EFFECTS OF REALISTIC CONCENTRATIONS OF MYCOTOXINS ON THE FUNCTION AND RESPONSE OF THE CHICKEN'S INTESTINE

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Summary

The gastrointestinal tract (GIT) plays a vital role in ensuring the health and welfare of an animal. Being responsible for multiple crucial functions including gut barrier, nutrient digestion and absorption, gut immunity, and microbial activity, the GIT can consume 20% of all incoming energy of an animal (Cant et al., 1996). A great portion of the consumed energy is attributed to the rapid protein turnover rate of intestinal cells, which can reach as high as 50 to 77% per day in poultry. The physiological and metabolic functionality of the GIT determines the nutrient supply to all other tissues and also ensures both passive barriers and active immunological processes for pathogen clearance. Today's poultry production relies heavily on feed efficiency, thus the health of the GIT must be guaranteed in order to optimize the utilization of dietary nutrients and therefore maximize the performance and welfare of the animal.

I. INTRODUCTION

We often think of mycotoxin's effect on the animal at dosages that would affect performance and cause visual lesions (e.g. oral and dermal lesions from T-2 toxin or fatty liver from aflatoxin). However, recent literature has implicated physiological and immunological effects at lower and more common levels of contamination (even below the EU and US limits) that may ultimately affect GIT functionality. Often neglected, the GIT is the first organ coming into contact with mycotoxins of dietary origin, and can be affected by mycotoxins with greater potency as compared to any other organs. As many of the mycotoxins and their metabolites inhibit protein synthesis, tissues with high levels of protein synthesis and turnover, such as those within the GIT, can be particularly susceptible to their toxic effects. On the other hand, the GIT is repeatedly exposed to mycotoxins at concentrations likely higher than other organ systems. Mycotoxin absorption (and on occasion conversion to either active or inactive metabolites) varies considerably, but will ultimately determine systemic exposure and tissue distribution. Focusing on predominate mycotoxins poultry are exposed to, aflatoxin (AF) is readily absorbed in the proximal GIT (greater than 80%), while ochratoxin (OTA) is moderately absorbed (40%), and deoxynivalenol (DON) and fumonisin (FUM) are minimally absorbed (5 to 20 and 1%, respectively; Grenier and Applegate, 2013). Consequently, the entire gut is exposed to the remaining mycotoxins at high concentrations, which may result in impairment of intestinal functions and also favored growth and colonization of digestive pathogens. In addition, notably, enterohepatic cycling of DON, FUM, and OTA can occur and increase the time and concentration exposure along the intestine. Therefore, the GIT is likely at high risk of mycotoxin toxicity, yet our understanding on the gut health aspect of mycotoxicoses is not thorough. Based on a comprehensive review by Grenier and Applegate (2013), only 83 studies in total were published (as of 2013) on the topic of mycotoxins impact on intestinal processes; this included all mycotoxins on all animal species as well as in vitro studies. Nevertheless, recent literature and results from our work began to implicate that the accumulative effects of low and routine concentrations of major mycotoxins, primarily DON, FUM, and AF, can lead to

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effects on tight junction functionality, active nutrient absorption, and pro-inflammatory cytokine markers. These responses particularly to DON and FUM explain research from several labs wherein coccidial lesion severity is increased and recovery is prolonged, and in other studies where necrotic enteritis lesions have developed.

II. MYCOTOXIN OCCURRENCE

Mycotoxin contamination in animal feed and feed ingredients has been a worldwide concern for decades, and due to the great adaptability of these fungi species, it is becoming an increasingly prevalent and serious risk to the livestock industry globally. The much-cited FAO (Food and Agriculture Organization of the United Nations) estimates suggested that up to 20% of the world's food production is lost due to mycotoxin contamination (FAO, 1997). Conversely, according to a long-term worldwide mycotoxin survey from 2004 to 2011 (Streit et al., 2013), 72% of feed and feed ingredient samples contained at least one mycotoxin out of the 17,316 samples tested. In this survey, approximately 55% of all samples tested were DON positive, with an average concentration of 967 µg/kg. The occurrence of FUM was similar to that of DON, with a positive rate of 54% out of 9,682 samples and an average concentration of 1,689 µg/kg. A total of 27% samples were tested AF positive, with an average concentration of 58 µg/kg.

Among all crops, corn is generally the most vulnerable to mycotoxin contamination. Both the percentage and the average contamination level of positive samples were considerably higher in corn than they were in other samples. In finished feeds, FUM was the most commonly detected mycotoxin globally, with 59% (2006) to 78% (2007) of the samples testing positive, while DON was the second most prevalent, detected in 44 to 68% (2005 and 2008, respectively) out of 4,585 samples. While FB contamination remained quite stable throughout the duration of this survey, an increasing trend was observed regarding the prevalence of DON. Notably, 38% contained multiple mycotoxins out of the 17,316 samples tested, posing a challenge for animals because simultaneous exposure to multiple mycotoxins may potentially lead to synergistic interactions.

Table 1 - Mycotoxin survey results (Deoxynivalenol, DON; Fumonisin, FUM) in Australia in feed ingredients from 2013 to 2016. (Results from Biomin survey, unpublished).

	2013	2014	2015	2016
DON				
# of samples	126	58	223	59
Avg. positive (µg/kg)	361	363	94	97
Max. positive	2979	3870	646	740
% positive	25	28	21	24
% positive above threshold[1]	19	7	3	2
FUM				
# of samples	126	57	223	59
Avg. positive (µg/kg)	1059	69	335	385
Max. positive	14301	537	8656	2658
% positive	17	47	41	31
% positive above threshold[2]	4	2	3	7

[1]DON threshold: 200-900 (sow, boar); 150-200 (piglet); 250-1000 (grower, finisher); 200-800 (breeder); 300-1000 (broiler, layer); [2]FUM threshold: 750-1000 (sow, piglet); 1000-1500 (finisher); 1500-2000 (breeder); 2000-3000 (broiler, layer)

In Australia, primary concern of mycotoxin contamination is on DON and FUM. Based on a 4-year (2013-2016) survey by Biomin, 21 to 28% of feed ingredient samples

tested were DON positive, while occurrence for FUM was 17 to 47% (Table 1). The average positive concentrations have decreased over the 4-year period for both DON (2361 to 97 µg/kg from 2013 to 2016, respectively) and FUM (1059 to 385 µg/kg from 2013 to 2016, respectively); however, the percent positive above threshold level for FUM has increased from 2 - 4 to 7% in 2016. Contamination of AF is of less concern in Australia due to the dry weather condition; the contamination levels of which are usually too low and are considered negligible to affect animal production (2013 Biomin Survey resulted in 4% AF positive samples with average concentration of 11 µg/kg in Oceania).

III. MYCOTOXIN ON GUT BARRIER FUNCTION

The gut barrier is the first barrier against ingested contaminants, which protects the luminal end of the intercellular space and regulates water and molecular transport through this paracellular and transcellular barrier (Anderson and Van Itallie, 1995). As the primary component of intestinal barrier, the tight junctions (TJ) act as a fence that blocks the free paracellular diffusion of protein and lipids between the apical and basolateral membranes. Three integral proteins are components of TJ: claudin, occludin, and the junctional adhesion molecule (JAM). Alteration in TJ expression and activity can lead to impairment of the tight junction network, and consequently increase permeability and allow higher translocation of luminal antigens (Chen, 2016).

The majority of previous research on this topic focused on the effects of DON. Using intestinal segments from broiler chicks and laying hens in Ussing chambers, Awad and co-workers have consistently observed a reduced trans-epithelial electrical resistance (TEER), an indication of altered paracellular permeability, when exposed to DON ex vivo; similar effects were also noted in intestinal tissues from birds fed DON (Awad et al., 2004, 2005, 2007, 2008, 2009). Similarly, in Caco-2 cells, DON decreased claudin expression and impaired intestinal barrier (McLaughlin et al., 2004). As pigs are known to be rather susceptible to DON, a recent study from our lab explored the effects of DON in porcine IPEC-J2 cells. When the cells were treated with graded concentrations of purified DON at 4000 ng/ml (which is equivalent to 4 mg/kg), DON significantly reduced trans-epithelial electrical resistance (TEER) at 24 h; at 48 h, 2000 ng/ml DON also exerted significant effect. Correspondingly, the paracellular tracer flux (as determined by FITC-dextran flux) was significantly increased by 4000 ng/ml DON, indicating impaired gut barrier function. In the same study, the mRNA expression of claudin1, 3, and 4 were increased by 1000 to 4000 ng/ml starting at 24 h, while its protein expression decreased with increasing DON. Results of the time-response curve showed that reduction of protein expression began decreasing at 2 h, while their respective gene expression started to increase from 4 to 6 h after treatment, suggesting that the increased claudin mRNA expression is a compensation effect to the disrupted protein synthesis and/or degredation. A subsequent experiment using ubiquitin inhibitor revealed that DON inhibited the claudin protein expression by increasing the ubiquitin-facilitated protein degradation. The question remains as to if protein synthesis pathways are also affected, how DON affects barrier integrity, and what signaling pathways are involved. Interestingly, when DOM-1, an inactivated metabolite of DON, was used as the treatment, gene expression and protein expression of tight junctions were not affected, suggesting that degrading DON to DOM1 in animal feed may be an effective approach to minimize the adverse effects of DON on intestinal barrier (Zhao et al., unpublished data).

Fumonisins (FUM) have strong structural similarity to sphinganine, the backbone precursor of sphingolipids, and thus are known to lead to disruption of sphingolipid metabolism, resulting in impaired membrane formation. Fumonisin B_1 (FB), the most toxic form of FUM, has been reported to lead to reduced TEER (Bouhet et al., 2004), in addition to

reduced tight junction protein expression in the pig ileum (Bracarense et al., 2012). Although the body of literature is still relatively small, especially for mycotoxins other than DON, current evidences suggest that gut barrier integrity can indeed be disrupted by major mycotoxins through interfering with protein synthesis, protein degradation, or membrane formation. Clearly, more in vivo studies are required in different animal species to better understand the effect of each mycotoxin as cell culture models may not perfectly mimic the complex in vivo conditions (e.g. entero-hepatic recycling). Also, conclusions from mRNA expression data should be drawn carefully, as our recent study showed that increased tight junction mRNA expression upon DON exposure did not equate to increased protein expression, but rather, a compensatory effect to the disrupted protein.

a. Mycotoxins and nutrient digestion and absorption

Mycotoxin-induced disturbance of digestive enzyme and nutrient transporters may lead to intestinal disorders, resulting in alteration of nutrient digestibility and absorption, and subsequently the growth of animals. Previous researchers have noted changes in pancreatic digestive enzyme activities and nutrient digestibility upon AF exposure, yet it is difficult to reach a consensus based on the available data. Han et al. (2008) observed increased digestive enzyme activities (protease, amylase, chymotrypsin, and trypsin) yet decreased apparent digestibility of crude protein in 42 d ducks fed 0.02 and 0.04 mg/kg AFB_1. Conversely, laying hens fed up to 2.5 mg/kg AFB_1 for 14 d did not show altered apparent digestibility of dry matter and N, but apparent digestible energy was significantly reduced in those hens compared to control (Applegate et al., 2009). The discrepancies present in the literature can be due to the differences in experimental animals (species, genetic lines, and age), source and concentration of AF, exposure time, nutritional composition of the diets, sampling site, etc. On the other hand, accurate estimation of protein and amino acid digestibility requires the correction for endogenous losses. Results from our most recent study indicated that diet AFB_1 contamination at 1.5 mg/kg has the potential of increasing endogenous N loss in broilers by 22%; similarly, amino acid losses from the AFB_1-treated birds were all higher than those of control birds by approximately 20 to 30%. Exposure to AFB_1 also significantly reduced standardized ileal N, amino acid, and energy digestibility (Chen et al., 2016). During aflatoxicosis, the increased endogenous N flow may come largely from sloughed mucus layer and/or increased secretions from the pancreas (proenzymes from pancreatic cells) and small intestine. The intestinal epithelium cells have a very high protein turnover rate; thus it is essential that adequate substances are provided for the protein synthesis, while a reduced N and amino acid digestibility and reduced ileal digestible energy may lead to an insufficient nutrient and energy supply to the intestinal cells, thus affecting normal activities and functions of these cells. Therefore, the increased maintenance cost along with the decreased ability of animals to utilize dietary nutrients are indeed factors that lead to the array of metabolic disturbances during aflatoxicosis (Chen et al., 2016).

Information regarding the effects of DON and FUM on nutrient digestion is relatively limited; nevertheless, several previous studies have looked at how they modulate the active nutrient absorptive processes. Reduced small intestinal villi height is mostly found after FB and DON challenge (Awad et al., 2006, 2011; Yunus et al., 2012; Girgis et al., 2010), which indicated intestinal epithelial damage, and may lead to decreased absorption of dietary nutrients. Consistently, utilizing the Ussing Chamber method in cell culture models, multiple studies have revealed that FB and DON could interfere with glucose absorption (Maresca et al., 2001, 2002; Lessard et al., 2009). Maresca et al. (2002) demonstrated that the inhibition of nutrient uptake by DON is through a specific modulation on intestinal transporters rather than nonspecific cell damage. Compared to DON and FUM, studies on AF effect on nutrient

absorption is still scarce. while In broiler chicks and laying hens, AFB_1 had no effect on jejunal villi histology (Applegate et al., 2009; Chen et al., 2016), while decreased villus height and villus/crypt ratio in ducks was observed (Wan et al., 2013). Notably, a very consistent trend of increased mRNA expression of jejunal peptide and amino acid transporters were observed in broiler chicks upon AFB_1 challenge, both on the brush border side ($b^{0,+}$AT, EAAT3, PepT1, rBAT) and the basolateral side (yLAT1 and 2). This might be a compensatory effect of transporter gene expression where a higher mRNA production is needed to increase translation process in order to restore impaired protein, and/or a higher absorption rate is necessary to compensate for decreased amino acid digestibility. In addition, this may also suggest an increased requirement for amino acids absorption and subsequent protein synthesis during aflatoxicosis (Chen et al., 2016).

In addition to an individual mycotoxin contamination, contamination of feedstuffs with multiple mycotoxins occurs very often and evaluation of multi-contamination effects in livestock animals is necessary. In laying hens, feeding a diet contaminated with both AF and OTA resulted in a more pronounced effect on reducing dietary metabolizable energy than when either toxin was fed alone, which occurred through a significant increase in the maintenance energy requirement (Verma et al., 2007). Feeding hens a diet naturally contaminated with multiple *Fusarium* mycotoxins also led to further decreased nutrient digestibility and metabolizable energy (Danicke et al., 2002). In broiler chicks that were challenged with both DON and FB, a synergistic effect of DON and FB were observed on reducing N digestibility, especially during a coccidiosis challenge (Grenier et al., 2016). Evidently, multi-contamination poses a bigger challenge for animals because simultaneous exposure may potentially lead to synergistic interactions. Thus there is a need to further assess their interactions in animals to better understand whether a particular combination may lead to antagonistic, additive, or synergistic effects. However, caution must be taken when conducting such studies, especially when evaluating nutrient digestion and absorption, that the results must not be confounded by the effects of mold(s) on the nutrient and energy content of the feed ingredient (Murugesan et al., 2015).

b. Mycotoxin and gut immunity

Besides nutrient digestion and absorption, the GIT also plays essential roles in immune responses as up to 70% of the immune defenses are located in the GIT (Grenier and Applegate, 2013). The major players in the intestinal immune responses include the GALT (gut-associated lymphoid tissue), Peyer's patches (aggregated lymphoid nodule located in ileum), mesenteric lymph nodes, and cecal tonsils, which are responsible for producing immunocompetent cells upon infection. Additionally, localized responses along the GIT are facilitated by the mucus, intraepithelial immune cells, and epithelial cells (Grenier and Applegate, 2013).

Although the immunosuppressive property of AF is well accepted, the modulation of GIT immune system has been less studied. Considering the potent toxicity of AFB_1 and its inhibitory effect on protein synthesis and activity, it is expected that feeding AF-contaminated feeds can interfere with gut immune responses similar with or more intense than those seen with other mycotoxins, and thus awaits further research. Indeed, a very recent study by Jiang et al. (2015) first revealed that AF exposure (0.6 mg/kg) can decrease the intestinal IgA cell numbers and negatively affect the mRNA expression of IgA, pIgR, IgM, and IgG in 21 day-old broilers. In the meantime, studies using other mycotoxins have clearly revealed modulation on the immune balance and intestinal immune responses during parasitic, bacterial, and viral infections. Altered cytokine expression within the intestine upon exposure to DON in vivo, ex vivo, or in vitro has been documented, where a consistent trend

of upregulated pro-inflammatory cytokines (IL--1β, Il-6, IL-8, and TNF-α) as well as Th1, Th2, and T-regulatory cytokines can be observed (Grenier and Applegate, 2013). Similar responses have also been noted in pigs exposed to FUM (Bracarense et al., 2012). Thus the question remains as to whether mycotoxicoses may also influence the animal's ability to mount an effective and timely immunological response to infections. Antonissen et al. (2014) examined the effect of DON on the development of necrotic enteritis (NE), a disease perpetuated from initial intestinal damage and secondary exposure to the gram positive bacterium *Clostridium perfringens*, in broiler chicks, and found that occasional occurrence levels of DON (3 to 4 mg/kg) increased the percentage of birds with NE from 20 to 47%. Their results clearly demonstrated that DON predisposed the broilers to and exacerbate the severity of NE by reducing the intestinal epithelial tight junction integrity, thus increasing plasma protein/amino acid leakage into the intestinal lumen which provides the necessary nutrients for *Clostridium perfringens* proliferation.

Another predisposing factor to necrotic enteritis is mucosal damage caused by coccidiosis, a disease caused by protozoal parasites of the genus *Eimeria,* and is one of the most costly diseases in today's broiler production. Girgis et al. (2010) reported that broiler breeder pullets fed diets containing *Fusarium* mycotoxins (at a concentration that was lower than that could negatively affect performance) showed impaired intestinal recovery and delayed immune response from enteric coccidial lesions. Similarly, broilers exposed to OTA had higher lesion and oocyst in the intestine upon coccidial challenge by *Eimeria acervulina* (Koynarski et al., 2007a, b) and higher number of *Salmonella typhimurium* in duodenum and cecum, with the presence of acute enteritis (Fukata et al., 1996) compared to control birds. In our most recent study by Grenier et al. (2016), the interactive effect of subclinical doses of DON and FB in broilers with or without a coccidial challenge was investigated. When broilers were fed a co-contaminated diet with 1.5 mg/kg DON and 20 mg/kg FB while challenged with *Eimeria spp*, intestinal lesions and oocysts numbers in the jejunum of challenged birds were more severe in birds fed mycotoxins than birds fed a control diet. Ingestion of DON and FB also affected the partitioning of performance reduction (between the fraction due to change in maintenance vs. change in feed efficiency). Meanwhile, the upregulation of cytokines (IL-1β, IL-6, IL-8, and IL-10) following coccidial challenge was further increased in the gut of birds fed diets contaminated with DON and FB. The authors concluded that at subclinical doses, DON and FB may have little impact in unchallenged birds, but clearly can result in amplified severity of coccidiosis in challenged birds by disturbing the animal's metabolic and immunologic functionality. This is attributed to their mechanisms of toxicity, where DON inhibits protein synthesis via binding to ribosomes while FB disrupts the sphingolipid metabolisms through inhibition of the ceramide synthase; thus both toxins act on active and dynamic cellular processes. Interestingly, the type of interaction of DON and FB in this study was highly dependent on the endpoint evaluated, where synergistic effects were observed on nutrient digestibility, while additive effects were found on multiple pathogenicity markers (intestinal lesion and oocyst number) as well as markers for gut inflammation (IL-8, IL-10, and SOCS1). Antagonistic effects were also observed on 3 endpoints assessed. Therefore, while endpoints were crucial when investigating the interactions of mycotoxins, other factors may also influence the outcome, which may include animal species, exposure age, and concentrations used. Based on the results from this study, even mycotoxins from the same fungal origin and similar mode of action may result in antagonistic effects. Further experiments using different mixture of mycotoxins are therefore needed to better understand the effects of mycotoxin interaction. Nevertheless, exposure to low concentrations of mycotoxins can clearly result in metabolic and immunological disturbances in chickens and consequently lead to increased severity and outbreak of pathologies, such as in coccidiosis.

IV. SUMMARY

Although the body of literature on mycotoxins' effects on the GIT functionality is still relatively small, current evidence clearly suggest a direct and/or indirect impact of several major mycotoxins on gut barrier integrity, nutrient digestion and absorption, and gut immunity. The main mechanisms by which DON, FUM, and AF affect growth through the gut may include 1) interfering with protein synthesis and degradation, leading to impaired tight junction protein complex, and therefore impaired gut barrier; 2) increasing endogenous nutrient loss and interfering with digestive enzyme synthesis and/or activities, leading to reduced nutrient digestibility; 3) damaging intestinal villi and disrupting the normal activity of nutrient transporters, resulting in reduced nutrient absorption; 4) altering cytokine expression and providing nutrients for luminal pathogen proliferation (through impaired gut barrier which increases plasma nutrient leakage), resulting in an amplification of the severity of infection; 5) increasing the maintenance requirement of the gut while sparing nutrients for immune function needs, and thus decreasing nutrient availability.

Notably, the concentrations used in many of these aforementioned studies are considered low or sub-clinical and from survey data, more occasional dosages rather than routine. For instance, in several studies, DON at concentrations $\leq$ 4 mg/kg showed a significant negative effects on barrier integrity and nutrient digestibility, and increased the birds' susceptibility to multiple infections in poultry species. However, poultry were traditionally considered quite tolerant to DON; the EU maximum guidance level of DON for poultry feed is 5 mg/kg. Therefore, these mycotoxins at even lower concentrations that may not lead to significant growth impairment may still pose a risk to GIT functions and may predispose the animals to other infections, leading to greater production loss. Clearly, this gut health aspect of mycotoxicoses is not yet fully understood and deserves further research. In particular, more in vivo studies are required to elucidate how mycotoxins modulate barrier functions and nutrient digestive and absorptive processes. Also, as it is a common practice to include multiple feedstuffs in practical diets, the risk of simultaneous exposure to multiple mycotoxins increases in the field compared to research settings. Future research should elucidate feeds that are co-contaminated to account for mycotoxin interactions.

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MOLECULAR APPROACHES TO IMPROVING FEED EFFICIENCY: ROLE OF THE MITOCHONDRIA

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Summary

The modern broiler has been transformed into an elite feed converter, with some producers currently reporting that 42 day old birds gain 1 kg of wet weight for every 1.35 kg dry weight consumed. This performance is largely (~90%) a consequence of intense selection for improved genetics. In an effort to gain a better understanding of individual variation in chicken feed efficiency our group has been exploring the biology of the mitochondrion at multiple levels of organisation. The mitochondrion is the organelle where much biochemical energy transformation occurs in the cell. Using Cobb-Vantress industrial birds as our primary experimental resource we have explored the tissue content, structure and function of the mitochondrion and its relationship to growth, development, efficiency and genetic background. We have drawn on a number of molecular technologies to inform these questions: particularly, Single Nucleotide Polymorphism (SNP) genotyping, quantitative Polymerase Chain Reaction (PCR), genome-wide transcriptome screening and mass-spectrometry based proteomic analyses. While much remains to be understood, recent highlights include 1) variation in muscle mitochondrial content that is associated with performance phenotypes 2) altered muscle mitochondrial gene and protein expression in birds differing in feed efficiency 3) variation in isolated mitochondrial function in birds differing in feed efficiency and 4) evidence for an unexpected role for the mitochondrially-localised progesterone receptor in altering bird muscle metabolism.

I. INTRODUCTION

Animal production is the process by which chemical energy stored in feed is converted into value-added products for human consumption, such as meat and eggs. At the level of an individual animal or bird, production thus encompasses the biological processes of behaviour, feeding, digestive physiology including the role of symbiotic gut microbiota and cellular metabolism. The last of these is the focus of this article. In contrast to the biomedical literature, efficiency in agricultural science specifically refers to lean tissue deposition. While fat accumulation can clearly be seen as the product of an 'efficient' metabolism - the feed energy has been acquired and stored rather than liberated – because excess fat is trimmed off most animal carcasses it is considered wasteful. As is true for all biological systems, the cost of commercially valuable lean tissue deposition in chickens is ultimately paid for by a high energy intermediate molecule called Adenosine Triphosphate (ATP). With this in mind, the biochemistry of ATP production and use has to be considered central to our understanding of the feed efficiency. This has implications for the characteristics of feed efficient phenotypes.

The average mammalian cell contains ~1 billion molecules of ATP which are fully recycled every 2 minutes (Hoagland et al., 2001). Humans turnover the equivalent of their entire body weight in ATP on a daily basis (Tornroth-Horsefield and Neutze 2008), although at any one time the total is about 250g. This staggering flux of energy is in large part

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attributable to the 'engine-room' of the cell, the mitochondrion. In fact, in the presence of oxygen the vast majority of ATP is synthesised by the mitochondria, transforming multiple sources of chemical feed energy into a common, usable currency. In brief - and working backwards for convenience – energetically depleted ADP is phosphorylated to energy rich ATP by an enormous mitochondrial molecular motor called the ATP synthase. The synthase motor is rotated by a 'downhill' flow of protons (H^+), termed the proton motive force, that has been set up across the innermost of two mitochondrial membranes. This can be compared, by way of analogy, to the energy transformation that occurs when a cyclist peddles a bicycle, with one form of energy coupled to another by the chain on the bike. Because protons bear a charge as well as determine pH, the flow is essentially an attempt to renormalise an electrochemical potential. It is believed 4 protons are required to flow through the ATP synthase to make a single molecule of ATP.

The direct connection of this electrochemical potential to the activity of the ATP synthase complex is called 'chemiosmotic coupling' and bequeathed to its principal advocate (Mitchell 1961) a hard-won Nobel Prize. Mitchell's ideas were originally ridiculed by the conservative scientific community who favoured a traditional biochemical explanation (i.e. the making and breaking of chemical bonds in a sequence of reactions exploiting high energy intermediates). By way of contrast, Mitchell's electrochemical proposal is biophysical in nature. Unlike his contemporaries he concentrated on thermodynamic principles rather than searching for intermediates and developed his theory largely in the absence of any supporting data. The psychological stress of rejection and isolation taken together with Mitchell's mild mannered personality culminated in depression and gastric ulcers. He withdrew from the scientific community, resigned from his academic position and resorted to running experiments out of a home laboratory funded by personal wealth. It would be several decades before Mitchell's view of the bioenergetic world eventually triumphed over that of his peers, but it is now safe to say the basic tenets of his theory have proven to be fundamentally correct.

The 'uphill' supply of protons in chemiosmosis comes from reducing agents, primarily in the form of NADH. These protons travel through a mitochondrial redox system set up in parallel as four protein complexes (NADH dehydrogenase, succinate dehydrogenase, cytochrome b and cytochrome oxidase) termed the Electron Transport Chain (ETC) that are embedded in the innermost mitochondrial membrane. The combination of events involving the capacity of the ETC to drive the motor of the ATP synthase is called Oxidative Phosphorylation or OxPhos. In turn, the NADH proton donor is a major product of the mitochondrial Citric Acid Cycle whose bioenergetic input is Acetyl CoA, a metabolite often considered the metabolic cross-roads. Acetyl CoA is universally produced by the digestion and catabolism of all protein, carbohydrate (by glycolysis) and lipid (by beta-oxidation) consumed in the diet or derived from body reserves. Thus, cellular respiration can be viewed as the process by which chemical energy in food produces Acetyl CoA produces ATP. The mitochondrion is a central player in all of the biochemical transformation post Acetyl CoA, and many of the events prior to it, such as the beta-oxidation of fat.

With a few rare (and functionally enlightening) exceptions, every cell contains a single, continuous branching network of mitochondria. Indeed, the overall metabolic potential of every cell is governed by the size and structure of its mitochondria, setting what is known as its aerobic capacity. Aerobic capacity is high in endurance athletes and low in sprinters, and declines during ageing. When analysed in standard histological cross-section these connected tubes appear to be discrete oval organelles, but this is illusory. For this reason we shall refer to the amount of mitochondria in a cell as its mitochondrial content, not number. In terms of basic structure, the mitochondrion is an organelle with a centre called the matrix bound by two semi-permeable membranes, the inner (IMM) and the outer (OMM).

The matrix contains the majority of the ~1000 mitochondrial proteins, such as those engaged in the Citric Acid Cycle and β-oxidation. On the other hand, a much smaller number of specialised transporter proteins are embedded in the two membranes.

As mentioned earlier, the OxPhos activity (4 ETC complexes plus the ATP synthase) plus a number of other protein complexes are embedded in the less permeable IMM. Various mitochondrial import (e.g. pyruvate translocase) and export (e.g. Voltage Dependent Anion Channel, VDAC) proteins are embedded in the OMM. The OMM proteins help provide the mitochondrion with access to fuel for combustion, but also allow delivery of freshly minted ATP and metabolic waste to the cytoplasm for cellular consumption and disposal, respectively. Further, the mitochondrion maintains close physical connections with other organelles, particularly the endoplasmic reticulum and peroxisome with which it cooperates. Finally the mitochondrial matrix contains its own genome. This is a vestige of its ancestry as a free-living bacterium that in modern eukaryotes acts symbiotically within the host cell. In humans, the mitochondrial genome encodes 37 genes - 2 rRNAs, 22 tRNAs and 13 protein-coding genes. The regulation of mitochondrial function requires that messenger RNA expression from the mitochondrial genome is coordinated with that of the much larger nuclear genome.

In our collective research, we have mainly focussed on mitochondria located in the skeletal musculature. This is on the grounds that, in addition to its commercial value, muscle 1) contributes a substantial amount to bird mass and resting metabolism 2) diversity in muscle fibre composition sets up the clear possibility for substantial individual to individual variation and 3) the tissue is highly plastic and responsive to environmental cues. This combination of high mass, high responsiveness and high potential for variability is a unique feature of the skeletal musculature. It also opens up the possibility for manipulation by environmental cues. Except for the experimental data section on mitochondrial content (section IV), we made repeated use of an unusual feed efficiency resource – two groups of birds (n = 8 in each group) from within a single genetic line with one group expressing a ~1.5 fold higher FE (HFE) than the other group (LFE) between 42-49 days. However, before discussing muscle mitochondria, it is important to consider mitochondria and avian function.

II. MITOCHONDRIA AND DIFFERENT AVIAN SPECIES

Contrasting the mitochondrial phenotypes of species that are extreme metabolic performers is a useful approach for understanding what is achievable with mitochondrial adaptation. For example, to take the big picture viewpoint: the highly athletic hummingbird has a pectoralis mitochondrial content of ~35% (Mathieu-Costello et al., 1992). In the late 1970s the much more sedentary broilers were found to contain a pectoralis mitochondrial content nearly 10-fold lower, 4% (Kiessling 1977). The current value is unknown. If one accepts that the mitochondrion is the engine-room of the cell, then the mitochondrial content determines engine size. By this reasoning, the broiler possesses a particularly small engine. The small-engine phenotype is partly a consequence of the ancestral species, the red jungle fowl, bearing a very unusual metabolism dominated by low mitochondrial content sprint type IIB muscle fibres even in the wild, free-ranging state. Interestingly, a low muscle mitochondrial content appears common to all ground-dwelling bird species in the Phasianidae taxonomic group cf. turkeys, pheasants, grouse and quail. They use short burst flights reliant on anaerobic ATP production to escape predators, as reviewed by (Askew and Marsh 2002).

In broilers, subsequent selection for growth and domestication may well have lowered muscle aerobic capacity further, as a tendency towards low mitochondrial content type IIB muscle phenotypes is observed in numerous growth and efficiency selected production species (Jackson et al., 1997, Deveaux et al., 2001, Lefaucheur et al., 2004, Bouley et al.,

2005, Lehnert et al., 2007, Lefaucheur et al., 2011). Why is this? Physiological spare capacity arguments (Diamond and Hammond 1992) suggest that reducing mitochondrial content lowers the energetic cost associated with organelle maintenance (Hudson et al., 2008, Hudson 2009). To take a vehicular analogy, Toyota Corollas consume less fuel than high performance drag racers - even at common speeds. This economic design argument explains in part why 'drag-racing' hummingbirds must feed almost constantly while on the wing, and enter a specialised metabolic hibernation to fend off the threat of starvation every night. A starker contrast to the sedentary, highly feed efficient broiler is hard to imagine.

III. MITOCHONDRIA IN METABOLICALLY ADAPTED TISSUES

It is also valuable to examine mitochondrial performance in tissues with highly adapted forms of metabolism. A fine example comes from comparing brown versus white fat. The former is a tissue adapted for profligate heat production and is energetically wasteful / inefficient. The latter is adapted to store energy and is relatively inert / efficient. These two opposite functions are largely mitochondrial in origin. Firstly, unlike white fat, brown fat has a very high mitochondrial content, giving it its brown colour. Secondly, the ATP synthase complex is actually replaced by a specialised membrane pore protein called Uncoupling Protein 1 (UCP1). This serves to wastefully dissipate the proton motive force without producing ATP – a process called, the 'futile cycle.'

This mitochondrial adaptation is analogous to taking the chain off a bicycle so that the kinetic energy in the cyclist's muscles is no longer coupled to the wheels turning. In both the cyclist and the brown fat cell, the original source of energy is ultimately liberated as heat. Mitochondrial uncoupling is also a consequence of other factors sometimes collectively called proton leak, which can apply in tissues like skeletal muscle. These factors include membrane lipid composition, and possibly the impact of gene orthologs of UCP1, such as UCP2 and UCP3 both of which are expressed in muscle. Uncoupling has implications for feed conversion efficiency as it provides a mechanism for determining the proportion of feed energy (in the form of metabolised Acetyl CoA) that is available for lean growth (in the form of ATP). Increasing uncoupling wastes more feed energy on heat production, leaving a smaller proportion of feed energy available for ATP production that could service production. This would be expected to reduce feed conversion efficiency at the level of the whole bird. Finally, red blood cells, particularly in mammals, and to a lesser extent in birds, are devoid of mitochondria. This reflects their specialised role as carriers of oxygen rather than consumers of it.

As mentioned above, ATP foots the bioenergetic bill in all living systems. Notably expensive (ATP demanding) cellular processes include the maintenance of trans-membrane ion gradients, particularly the Na^+K^+ plasma membrane transporter which, on its own, typically consumes 20 out of every 100 ATP molecules produced by any given cell. This explains why organisms considered to be energetically frugal, such as tailed amphibians and lungfish (both of whom persist in oxygen-poor environments) are built of individually very large cells (Szarski 1983). For surface area to volume reasons they possess less total plasma membrane across which the Na^+ and K^+ gradients must be maintained. This lessens their ATP demand on a per unit tissue basis. Along these lines, the type IIB fibres that exclusively constitute the breast muscle of broilers have the largest cross-section of all the possible sprint and endurance muscle fibre types, thereby presumably achieving the same energy saving goal. Putting all this to one side, whether energetically profligate like a hummingbird or frugal like a broiler, all living systems must ensure ATP demand is met by adequate supply. Failure to do so quickly leads to either pathology (in the short term) or death if left uncorrected.

IV. BROILER MUSCLE MITOCHONDRIAL CONTENT

Despite the fact that muscle mitochondrial content is a promising source of individual variation that may impact any number of bioenergetic phenotypes, no production species had been screened for individual variation until our recent work in Cobb-Vantress broilers (Reverter et al., submitted). There are a number of ways of quantifying mitochondrial content, with electron microscopy based analysis on serial sections the most direct. Another approach is to count the number of copies of mtDNA in a tissue sample as a proxy. This method is convenient as it means an unbiased DNA extraction (which is simple and cheap) contains the compositional information. We have designed a duplex high-throughput qPCR assay to quantify the number of mtDNA copies per nucleus in broilers. This correction for nDNA yields a per cell (or per unit tissue mass) expression.

Using this method we detected substantial variation in the order of 5-fold across both breast and thigh muscle in 80 birds, despite the animals being inbred members of a single genetic line. Further, we found that any bird with a particularly low breast mitochondrial content also tended to possess a low thigh content, consistent with systemic regulation of mitochondrial content across the birds musculature. In terms of commercial phenotypes, birds with particularly low breast mitochondrial content were more muscular (higher breast muscle yield) and also possessed higher abdominal fat. Unfortunately, we did not have feed efficiency (FE) phenotypes for this particular resource, so the relationship of bird FE to muscle mitochondrial content remains conjectural and a major focus of future work. During the composition of this article it has been established that low mitochondrial content in cattle liver is associated with higher feed efficiency (Kong et al., 2016). This is a striking finding that supports the physiological spare capacity arguments previously applied to metabolic efficiency in humans and production animals (Hudson et al., 2008, Hudson 2009).

V. MUSCLE MITOCHONDRIAL STRUCTURE

In comparing species with very different aerobic capacities (i.e. mitochondrial contents) it has been found that the OMM is not folded, and the extent of IMM folding is largely constant, except for in highly oxidative endotherms like hummingbirds. The increased folding in organisms like hummingbirds provides a mechanism for connecting more of the matrix to the IMM (shortening diffusion distance) and also for embedding more IMM proteins per volume of matrix. In principal, this could allow higher rates of ATP production without having to pay the cost of maintaining more matrix (although the maintenance costs of the additional IMM would still need to be met). IMM folding can be visualised using Transmission Electron Microscopy (TEM). We have never morphometrically analysed mitochondria by TEM in broilers so we do not know for sure whether IMM (or even OMM) architecture has been modified by domestication or subsequent genetic selection. This suggests there is scope for more basic research in this area.

Using the independent FE resource described above (HFE and LFE), (Iqbal et al., 2004) used a Western blotting antibody-based strategy probing for a set of IMM proteins in breast muscle. They discovered that ANT1, cytochrome b, cytochrome c, core II, core I and COXII all were lower ($P < 0.05$) in the HFE birds. No differences were detected for IMM proteins NAD3, NAD4, NAD5, NAD6, NAD7, 70S and alpha-ATPase. The protein abundances were expressed per unit muscle tissue, not per unit mitochondria. Given we might expect increased IMM folding to house more IMM proteins *across the board*, this combination of data tends to indicate the HFE birds have a subtle change in IMM composition favouring a reduction in certain IMM proteins. To complicate matters, a recent mass-spectrometry based proteomic study on the same tissue samples (Kong et al., 2016), suggested a trend towards increases, not decreases, in a number of ETC proteins. One

potential confound is that differential oxidation of proteins (either pre- or post-extraction) could alter protein migration on a gel. This could lead to artefacts in mass-spectrometry based detection but would have no effect on antibody-based approaches. Having said this, the apparent conflict remains unresolved at this time.

VI. MUSCLE MITOCHONDRIAL FUNCTION AND ACTIVITY

The definitive way to measure mitochondrial function is to measure respiratory performance as unintrusively as possible. Traditionally, cells or tissues are homogenised then isolated mitochondrial preparations assayed spectrophotometrically under various conditions. Using such as approach on the HFE and LFE birds, (Iqbal et al., 2004) found HFE birds had significantly higher activity in all four ETC complex activities, with a particularly dramatic increase in complex IV activity. These data were expressed per unit mitochondrial protein implying a change in mitochondrial function independent of any change in content. Further, the HFE birds showed greater coupling efficiency between the IMM membrane potential and ATP synthesis. Collectively, these data imply that proton pumping across the IMM through the ETC could be higher in the HFE birds and that the electrochemical gradient produced is more efficiency coupled to ATP synthesis. Taken together this would be consistent with an increased potential for ATP production on a per unit mitochondrial basis. Recently, a new high-throughput technology called the Seahorse flux analyser has become available that allows mitochondrial and non-mitochondrial sources of ATP production to be assayed using intact cells with minimal disruption, but this approach has not been applied to broiler muscle cells in primary culture.

To gain more molecular detail into how mitochondrial metabolism and other aspects of muscle structure may differ with feed efficiency, we have used genome-wide transcriptome (mRNA) screening and mass-spectrometry based proteomics on total breast muscle homogenates. We were encouraged to find that both technologies identified a tendency for the HFE birds to express less slow muscle contractile isoforms and associated machinery (Kong et al., 2016, Bottje et al., revised manuscript submitted). This suggests a muscle fibre composition shift towards the whiter, type IIB phenotype in line with prior expectation. However, surprisingly both approaches (mRNA and protein) indicated a subtle, but coordinate increase in the mitoproteome (including but not limited to ETC proteins) in HFE birds. This suggests either an increased mitochondrial content and / or activity in the HFE birds. This conclusion appears somewhat contrary to the lower IMM proteins in HFE birds as based on antibody detection (Iqbal et al., 2004) and entirely contrary to the physiological spare capacity argument i.e. small engine animals are more feed efficient. We are currently working on reconciling these issues. At this point in time, we should emphasise that VDAC, resident in the OMM, was among the most upregulated mitochondrial proteins in HFE birds based on the proteomic data. VDAC shuttles ADP in and ATP out of the mitochondria, a clear driver of overall energetic flux and homeostasis. A promising analytical approach for the future is to try to better disentangle mitochondrial content versus structure at a molecular level by treating matrix, IMM and OMM mitochondrial proteins independently – rather than as a group - in the genome-wide screening analyses.

Furthermore, we used two different approaches to predict upstream causal regulation from downstream patterns of differential expression, whether mRNA or protein. Gratifyingly, both identified perturbations in progesterone signalling as a major driver in the muscle of HFE birds (Kong et al., 2016, Bottje et al., revised manuscript submitted). We also showed in a quail muscle cell line that the progesterone receptor localises to the mitochondrion. This functional link between progesterone signalling and avian mitochondria further implicates the mitochondrion as a key player in the two broiler FE groups. The progesterone signalling

prediction is particularly interesting given progesterone is added to combination mix hormone growth promotants that collectively increase FE by 20% in cattle, but very little is known about its particular role in bird muscle biology.

VII. GENETIC SELECTION ON GENES ENCODING MITOCHONDRIAL PROTEINS

Drawing on a genetic resource of ~200 birds in each of 4 Cobb lines we have used an in house haplotype detection algorithm to find segments of DNA apparently under selection in one or more of the lines (Hudson et al., submitted). We specifically explored the 507 mitochondrial proteins (out of a total 1045) for which we could find gene matches in the broiler 50K SNP data. Among other findings, we detected dramatically different allele frequencies for 2 SNP in the gene region encoding *AGK* which encodes the mitochondrial acylglycerol kinase (unpublished data). For one of these SNP the effect was particularly striking. The two lines possessing an elevated FE had the AA allele in the highest frequency, whereas the two lines possessing the lower FE had BB as the highest frequency allele. *AGK* has recently been identified as a possible functional candidate within a feed efficiency Quantitative Trait Loci in Cobb broilers (Reyer et al., 2015) using a bird population independent from those we analysed.

VIII. METABOLIC FLUX CONTROL

According to (Conley 2016) there are 3 control points in muscle ATP flux as regards meeting the enhanced demand during intense exercise: electron transport chain activity, coupling efficiency and ATP synthesis. These can be exploited to increase ATP production 50-fold compared to rest. Some of these control points can apparently be modified by diet, at least in mammals. For example, dietary nitrate through beetroot juice consumption elevates mitochondrial ATP synthesis per oxygen uptake by increasing coupling efficiency (Jones 2014). Secondly, the mitochondrially-targeted antioxidant SS-31 was able to not only improve coupling efficiency but also increase capacity for ETC flux (Siegel et al., 2013). Both dietary treatments were rapid, apparently working within 1 hour of administration. Further to this, a number of dietary compounds have been identified that can upregulate mitochondrial content in mammalian tissues, including high fat diets in general (Jain et al., 2014), ketone esters (Srivastava et al., 2012) and curcumin (Wang et al., 2015) to name a few.

At the molecular level there are several known flux control points. These include citrate synthase, the rate-limiting enzyme in the Citric Acid Cycle, and regulation of the pyruvate dehydrogenase complex to control the flux of Acetyl CoA derived from carbohydrate. Outside of the mitochondrion, in the cytosol, resides AMP kinase whose job is to monitor overall ATP supply versus demand. If mitochondrial ATP supply is failing to meet cellular demand, AMP kinase sets in motion a chain a set of events to accelerate mitochondrial ATP production. Further to this, our gene expression data in multiple species and circumstances has pointed to *PDK4, CKMT1A* and *UCP3* as particularly responsive to - or responsible for - metabolic perturbation. These genes encode the following mitochondrial proteins 1) pyruvate dehydrogenase kinase 4, a fuel decision making enzyme, which inhibits the production of Acetyl CoA from carbohydrate, thereby forcing the supply to come from beta-oxidation of fat 2) mitochondrial creatine kinase which transfers high energy phosphate from ATP to creatine in order to replenish cytosolic Phosphocreatine and 3) Uncoupling Protein 3 which helps export excess fatty acids out of the mitochondria and into the cytosol during times of dietary fuel excess.

IX. CONCLUSIONS AND FUTURE WORK

Revisiting this body of work reinforces a need to better structurally characterise broiler mitochondria in terms of both content and morphology. A basic TEM analysis quantifying the folding of the IMM and OMM with regard to the mitochondrial matrix (mitochondrial structure) would help interpretation the mountain of molecular data we are currently analysing. It would also help check the status of modern broiler muscle aerobic capacity (mitochondrial content) versus those birds analysed by Kiessling back in the late 1970s. Inclusion of wild red jungle fowl would clarify a number of outstanding questions relating to the consequences of domestication and subsequent selection on growth and efficiency. This 'big picture' contrast of the ancestral bird would surely help place the physiology of the modern domestic animal in better context. The relationship between variation in mitochondrial content and bird feed efficiency still needs to be established empirically. Finally, mitochondrial content and function is highly responsive to exercise, temperature and diet as well as genetics. In mammals *PPARGC1A* is the master regulator coordinating the cellular response to these influences, but the gene and protein are not so well characterised in birds. There appears to be plenty of room to explore the basic biology of the broiler mitochondrion as well as opportunity to inform current industry practices. Possible paths to industry impact include informing existing genomic predictions for animal breeding purposes or alternately developing novel feedstuffs containing bioactives (such as the beetroot nitrate example) relevant to mitochondrial function.

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STARCH DIGESTIVE DYNAMICS IN BROILER CHICKENS

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Summary

Starch digestibility coefficients are static values that cannot account for the kinetic progressions of energy utilisation and growth performance. The site and rate of starch digestion can influence bird performance in addition to the extent of starch digestion. The utilisation of sorghum starch in broiler chickens is inferior to maize despite the fact that they are ostensibly similar feed grains. Concentrations of kafirin and certain phenolic compounds inherent in sorghum are almost certainly contributing factors to the poor starch/energy utilisation in birds offered sorghum-based diets. Starch and energy utilisation in broiler chickens are influenced by whole grain feeding, exogenous phytases and additional strategies. However, deliberations of starch digestive dynamics should not exclude protein; because, as discussed, starch and protein digestive dynamics should be considered in tandem.

I. INTRODUCTION

The starch component of feed grains is the major energy source in broiler diets and ileal starch digestibility in maize-based diets, if not wheat and sorghum, are of a very high order (Truong et al. 2016a). However, sites and rates of starch digestion are equally important so that starch digestive dynamics merit consideration. Starch digestive dynamics may be influenced by numerous factors including grain type (eg sorghum), whole grain feeding, exogenous phytase and reducing agents. These feed additives may influence not only static digestibility coefficients but positively manipulate rates and sites of starch digestion to generate a better balance of available nutrients for poultry. Importantly, any consideration of starch digestive dynamics should not be isolated from protein digestive dynamics and both kinetic factors are discussed in this review. Finally, RVA starch pasting profiles of grain sorghum may be indicative of its feed grain quality for chicken-meat production.

II. FACTORS INFLUENCING (SORGHUM) STARCH UTILISATION

Starch utilisation will vary among feed grains and it is generally recognised that the utilisation of the starch/energy component of sorghum is sub-standard under Australian conditions (Black et al., 2005). Certainly, on the basis of *in vitro* (Giuberti et al., 2012) and *in vivo* (Truong et al., 2016a) data the digestibility of sorghum starch in poultry is inferior to that of maize. Truong et al. (2016c) offered broilers nutritionally equivalent diets based on six different grain sorghums from 7 to 28 days post-hatch. Starch digestibilty coefficients averaged 0.684 in the proximal jejunum , 0.753 in the distal jejunum, 0.838 in the proximal ileum, and 0.871 in the distal ileum (range: 0.851 to 0.888). This incomplete digestion of starch (0.871 corresponds to 12.9% resistant starch) is both noteworthy and entirely consistent with the balance of our previous findings; only rarely have we observed ileal starch digestibilities of more than 0.900 in sorghum-based diets. This quantity of resistant starch at the ileo-caecal junction could trigger the 'ileal brake', which delays gastric emptying and intestinal transit time (Martinez et al., 1995) and compromises growth performance. In comparison, a mean ileal starch digestibilty coefficient of 0.950 (range 0.873 to 0.993) was

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found in broilers offered maize-based diets in a review of eleven studies (Truong et al., 2016a), which clearly reflects the relatively inadequate starch/energy utilisation in broilers offered sorghum-based diets.

The incomplete digestion of sorghum starch could be due to intrinsic or extrinsic factors; however, the amylose:amylopectin ratios of our sorghum samples exhibited remarkably little variation so the focus has been on the contributory 'starch extrinsic' properties of sorghum. Kafirin is the dominant protein fraction in grain sorghum and it is present as protein bodies that, together with starch granules, are embedded in the glutelin protein matrix of the endosperm (Selle et al., 2010). It is generally accepted that kafirin interferes with sorghum starch utilisation via biophysical and/or biochemical starch-protein interactions (Taylor and Emmambux, 2010), although the precise mechanisms have yet to be clarified. Nevertheless, this assertion appears to be valid as there was a negative linear relationship (r = -0.655; P = 0.015) between dietary kafirin levels and ME:GE ratios (effectively, efficiency of energy utilisation) in broilers offered diets based on nine sorghum varieties [LVP 3, LVP 5, FW, Tiger, Block I, HP, Liberty #2, MP, JM] in five feeding studies (Selle et al., 2016; Truong et al., 2015b, 2015c, 2016c, 2016d) as shown in Figure 1. Consequently, the real possibility that kafirin proportions of sorghum protein have increased in recent decades as an inadvertent outcome of breeding programs (Selle, 2011) becomes a concerning, fundamental issue.

Sorghum contains the highest phenolic compound concentrations amongst the feed grains and while Australian sorghums do not contain condensed tannin (Khoddami et al., 2015) it does appear that 'non-tannin' phenolics (eg flavan-4-ols, ferulic acid) have deleterious effects on starch utilisation. From the same database as above, there was a negative linear relationship (r = -0.569; P = 0.042) between total phenolic compound concentrations and ME:GE ratios as shown in Figure 2. Consequently, our contention that the established anti-nutritive properties of phenolic compounds are not solely the province of condensed tannin but are partially shared by the balance of polyphenols and phenolic acids found in grain sorghum. White sorghums axiomatically contain less polyphenols than red varieties and red polyphenolic pigments can bind with starch granules in grain sorghum (Freeman and Watson, 1971). White sorghums may also contain less phenolic acids and, anecdotally, are considered to be superior to red varieties by both pig and poultry nutritionists in Australia but white sorghums probably represent less than 5% of the national harvest. White sorghums are considered to be not as viable agronomically as red varieties, an issue that ideally should be addressed.

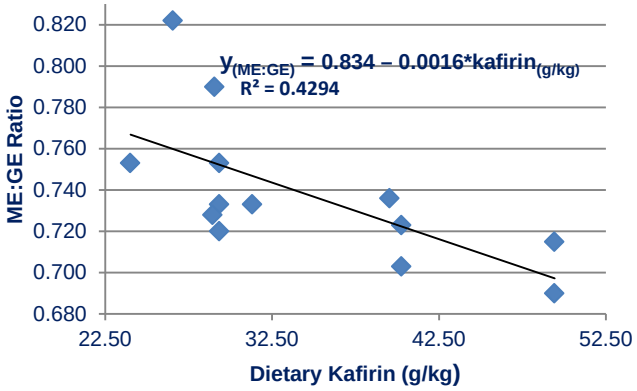

Figure 1 - Linear relationship (r = -0.655; P = 0.015) between dietary kafirin levels and ME:GE ratios in broilers offered diets based on 9 sorghum varieties.

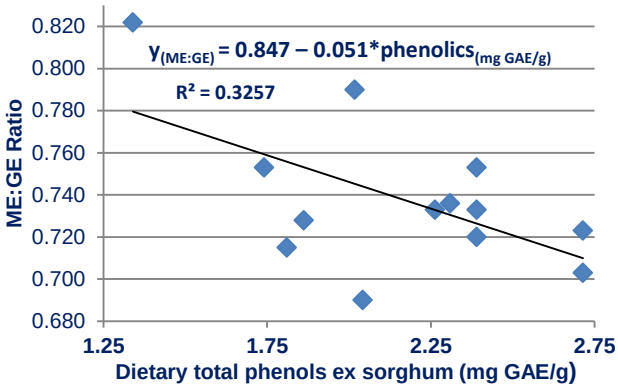

Figure 2 - Linear relationship (r = -0.569; P = 0.042) between dietary phenolic compound levels and ME:GE ratios in broilers offered diets based on 9 sorghum varieties.

Grain size and pelleting temperature have little influence on starch utilisation in sorghum grain (Selle et al., 2016); however, there is evidence that starch digestibility is influenced by physical disruption and hydrothermal and processes (Liu et al., 2015). Under whole grain feeding regimes, some of the grain components are not subject to hammer-milling and steam-pelleting and are more slowly digested as a consequence. In one study (Truong et al., 2016b) whole wheat was added either pre- or post-pelleting where the second approach generated more robust responses in relative gizzard weights and energy utilisation. Gizzard weights were significantly correlated with distal ileal starch digestibility coefficients and nutrient utilisation parameters and it was evident that a heavier and more functional gizzard was the primary driver of performance responses. It follows that the greater physical disruption of starch granules and endosperm protein matrices by the grinding action of heavier, more muscular gizzards facilitates substrate access for amylase in the small intestine. There is also the likelihood that heavier gizzards will prompt increased episodes of reverse gastro-duodenal peristalsis and the reflux of pancreatic amylase into the gizzard may amplify the extent of starch digestion. This study confirmed the advantages of whole grain feeding, which appeared to be driven by greater extents of starch digestion allied to heavier relative gizzard weights.

Exogenous phytase is almost invariably included in broiler diets and may positively influence starch digestion. Pursuant to the digestion of starch, intestinal uptakes of glucose are mainly in association with sodium via Na^+-dependent transporters (SGLT-1) that are driven by Na^+-K^+-ATP-ase or the activity of the 'sodium pump'. Phytase has been found to increase the re-absorption or retrieval of Na along the small intestine in three separate investigations to profound extents (Truong et al., 2014; 2015a; 2016d). It is possible that phytate depresses Na pump activity by causing a depletion of Na in small intestinal enterocytes through the hyper-secretion of sodium bicarbonate into the duodenum; therefore, it follows that phytase addition would improve the Na status of enterocytes, sodium pump activity and the co-absorption of nutrients, including glucose, with sodium. Our contention is that the negative impact of phytate, and the reciprocal positive effect of phytase, is more on the absorption of glucose than the digestion of starch.

A series of feeding studies have investigated inclusions of the reducing agent sodium metabisulphite in sorghum-based diets (Liu et al., 2014; Selle et al., 2014, 2016b; Truong et al., 2016d). With one exception (Truong et al., 2015b), sodium metabisulphite has generated improvements in energy utilisation (AME, ME:GE ratios, AMEn) quite often to tangible extents. Sulphite reducing agents, including sodium metabisulphite, have the capacity both to reduce disulphide cross-linkages and to depolymerise starch via oxidative-reductive reactions. However, it appears that the reduction of disulphide cross-linkages in the periphery of kafirin protein bodies and an attenuation of starch-protein interactions may be the important mode of action. If so, the benefits of sodium metabisulphite may not extend equally to other feed grains.

III. STARCH AND PROTEIN DIGESTION DYNAMICS

Both sorghum and phytase studies have indicated that condensed starch:protein disappearance rate ratios are likely to advantage broiler performance. In two maize-based diets (Truong et al., 2015a; 2016d), phytase supplementation narrowed starch:protein disappearance rate ratios where starch:protein disappearance rate ratios were correlated to weight gain and ME:GE ratios in sorghum-based diets (Truong et al., 2015c). As shown in Figure 3, there was a negative, linear relationship ($r = -0.766$; $P < 0.005$) between proximal ileal starch:protein disappearance rate ratios with 40-day weight gains where "protein" equals the sum of 16 amino acids. This demonstrates the interactive impacts of glucose and amino

acid intestinal uptakes on broiler performance. It is evident from this relationship that increases in protein disappearance rates advantage weight gain and ME:GE; whereas, increases in starch disappearance rates are disadvantageous. This response appeared to stem from phytase increasing the digestion and disappearance of both protein and starch but, importantly, the impact of phytase on protein is more pronounced.

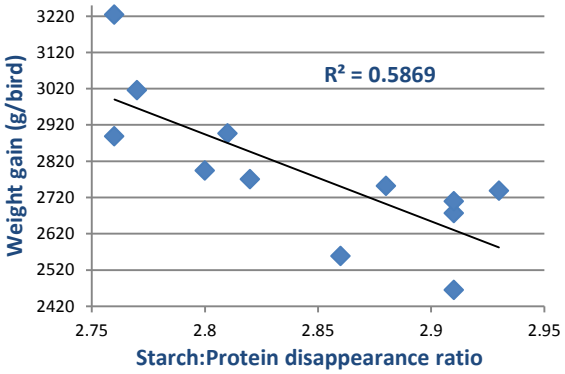

Figure 3 - Linear relationship (r = -0.766; P = 0.003) between starch:protein disappearance rate ratios in the proximal ileum and weight gain at 40 days post-hatch. Adapted from Truong et al., (2015a).

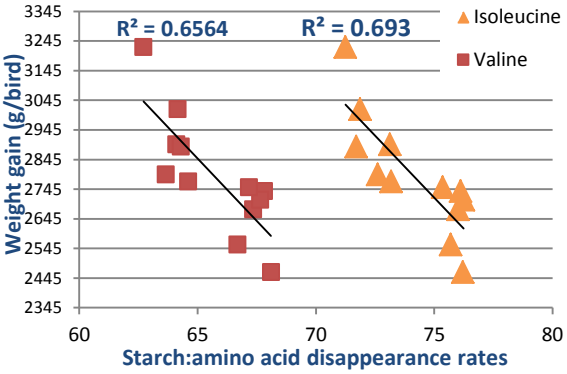

Figure 4 - Linear relationship between starch: isoleucine (r = -0.832; P < 0.001) and starch:valine (r = -0.810; P < 0.001) disappearance rate ratios and weight gains at 40 days post-hatch. Adapted from Truong et al., (2015a).

Furthermore, starch disappearance rates in relation to disappearance rates of amino acids showed that two branched-chain amino acids, isoleucine (r = -0.832; P < 0.001) and valine (r = -0.810, P < 0.001), were the most significantly correlated of the sixteen amino acids (Figure 4). Taken together, the multiple linear regression equation for the relationship (r = 0.833; P < 0.005) is as follows:

y (40-day weight gain) = 8984 - 76.6*starch:isoleucine - 7.8*starch:valine

It appears evident that branched-chain amino acids, especially isoleucine and valine, may play a pivotal role in the dynamics of starch and protein digestion. Amongst the essential amino acids, branched-chain amino acids are vulnerable to catabolism in the gut mucosa (Chen et al., 2009). The antagonism between branched-chain amino acids is established and may stem from competition for intestinal uptakes and/or systemic catabolism. Phytase has been shown to generate profound increases in the proximal jejunal digestibilities of amino acids by in the order of 50% (Truong et al., 2015a) and this 'proximal shift' in the sites of amino acid absorption may reduce their catabolism in the gut mucosa. If so, this would increase the portal flux of amino acids and enhance their post-enteral availability for protein deposition. The effect of phytase on starch and protein digestive dynamics in general terms, and perhaps especially the impact of phytase on branch-chained amino acids, and in respect of glucose versus amino acid catabolism in the gut mucosa to generate energy certainly appears to merit further attention.

IV. RVA STARCH PASTING PROFILES

In conclusion, it appears that RVA starch pasting profiles may hold potential for predicting the quality of sorghum as a feed grain for chicken-meat production (Truong et al., 2016e). In a meta-analysis of five broiler bioassays it was found that peak, holding, breakdown and final RVA viscosities were positively correlated with ME:GE ratios to significant extents as were peak and breakdown RVA viscosities with AMEn. Instructively, concentrations of kafirin

and total phenolic compounds were negatively correlated with peak RVA viscosities to significant extents across thirteen sorghums. That RVA starch pasting profiles appear to be predictive appears linked to kafirin and total phenolic compound concentrations in sorghum as it seems both factors depress RVA starch viscosities *in vitro* and, in turn, depress energy utilisation in birds offered sorghum-based diets. Both kafirin and phenolic compounds are effectively unique to grain sorghum and for this reason RVAs starch pasting profiles may not be as indicative for other feed grains.

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PRE- AND POST-PELLET WHOLE GRAIN ADDITIONS TO POULTRY DIETS INVESTIGATED VIA NUTRITIONAL GEOMETRY

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Summary

Diets containing 600 g/kg wheat were offered to 360 male Ross 308 broiler chicks as ten blends of ground wheat and whole wheat added either pre- or post-pelleting from 7 to 28 days post-hatch. A combination of 172 g/kg ground wheat, 256 g/kg pre- and 172 g/kg post-pellet whole wheat generated the optimal FCR of 1.469 as determined by a response surface design. A quadratic relationship indicated that a relative gizzard weight of 16.88 g/kg was associated with an FCR of 1.479 and there was a positive correlation ($r = 0.438$; $P < 0.001$) between relative gizzard and pancreas weights.

I. INTRODUCTION

Whole grain (WG) may be incorporated into poultry diets either before (pre-pellet WG) or following (post-pellet WG) the steam-pelleting process and are offered as either intact pellets or as a whole grain-pelleted concentrate blend. The second approach is more commonly adopted in Australia, but there is little comparative data to indicate their relative value. FCR and energy utilisation responses to whole grain feeding have been attributed to heavier and more functional gizzards which are more commonly observed with post-pellet WG addition (Liu et al., 2014). However, some studies suggest there may be additional mechanisms. Wu et al. (2004) reported both pre- and post-pellet whole grain additions generated significant improvements in FCR and AME but pre-pellet WG, did not increase gizzard weights in contrast to post-pellet WG. In addition, 25% pre-pellet WG did not influence relative gizzard weights (8.50 versus 7.94 g/kg) in comparison to post-pellet WG inclusions (15.50 versus 7.94 g/kg) in Abdollahi et al. (2016). Nevertheless, pre-pellet WG additions generated improvements in FCR and AME at 21 days post-hatch. One possible explanation advanced by Truong et al. (2015), is that starch digestion rates of whole grains are inherently slower than processed wheat. Thus, pre-pellet whole grain may be generating positive responses from changes in starch digestive dynamics within the avian gut without influencing gizzard functionality. Therefore, in this study, nutritional geometry was used to compare ground grain with both pre-pellet and post-pellet WG additions in an 'equilateral triangle' response surface design to evaluate the performance of various blends on parameters of growth performance, gizzard and pancreas characteristics.

II. MATERIALS AND METHODS

The equilateral triangle design is shown in Figure 1. The three apical treatments of the response surface design comprised (1A) a standard diet containing 600 g/kg ground wheat, (2B) the same diet containing 600 g/kg pre-pellet whole wheat, and (3C) the same diet containing 300 g/kg ground wheat and 300 g/kg post-pellet whole wheat. The balance of seven treatment blends was based on blends of the apical treatments (Table 1). The ten dietary treatments were offered to a total of 360 male Ross 308 chicks from 7 to 28 days post-hatch. Where appropriate, wheat was ground through a 3.2 mm hammer-mill screen prior to

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steam-pelleting through a 4.00 mm die at an 80°C conditioning temperature. Growth performance, gizzard and pancreas characteristics were determined by procedures described in Truong et al. (2016). Data was analysed using IBM® SPSS® Statistics (IBM Corporation. Somers, NY) and response surfaces were generated with R 3.0.3 software, as outlined in Liu et al. (2016). A probability level of less than 5% was considered statistically significant. The study complied with guidelines approved by the Animal Ethics Committee of The University of Sydney.

Table 1 - Schedule of ten dietary treatments

Treatment	Diet 1 (%)	Diet 2 (%)	Diet 3 (%)
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

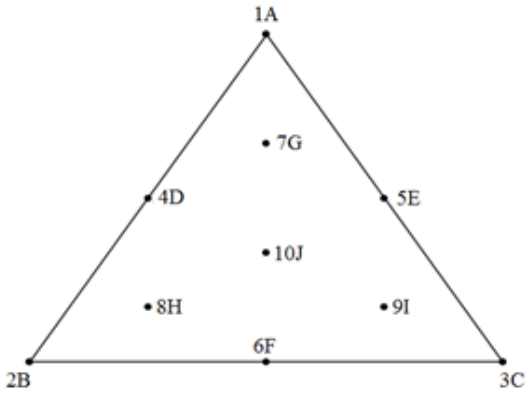

Figure 1 - Diagramatic representation of dietary treatments.

III. RESULTS

Effect of dietary wheat incorporations on growth performance, gizzard and pancreas characteristics from 7 to 28 days post-hatch are shown in Table 2. WG feeding numerically depressed feed intakes and significantly depressed ($P = 0.05$) weight gains in 7 ex 9 dietary treatments containing WG. Significant differences were observed in FCR ($P < 0.015$) as treatment 3C was 6.86% inferior (1.525 versus 1.452; $P < 0.001$) in comparison to treatment 10J. The relationship between grain form and FCR ($r^2 = 0.999$; $P < 0.001$; lack of fit $P > 0.120$) may be predicted using R 3.0.3 software from the following equation (Figure 1c) where D_1, D_2 and D_3 represent the proportions of diet 1A, 2B and 3C, respectively:

$$FCR = 0.01491D_1 + 0.01512D_2 + 0.01550D_3 - 2.638\times10^{-5}D_2D_3$$

Therefore, a blend of 42.7% Diet 2 and 57.3% Diet 3 will generate the optimal FCR of 1.469, which corresponds to 172 g/kg ground grain, 256 g/kg pre-pellet whole grain, 172 g/kg post-pellet whole grain in a diet containing 600 g/kg wheat. Significant differences were observed for relative gizzard weight and contents ($P < 0.005$); however, there were no

significant differences for relative pancreas weight and gizzard pH (P > 0.05). There was a significant quadratic relationship (P < 0.043; r = 0.331) between relative gizzard weight and FCR such that a 16.88 g/kg gizzard weight will generate a 1.479 FCR and heavier gizzards would compromise FCR. Both relative gizzard weights (r = 0.438; P < 0.001) and their contents (r = 0.298; P < 0.025) were positively correlated with relative pancreas weights.

Table 2 - Effect of dietary wheat incorporations on growth performance, gizzard and pancreas characteristics from 7 to 28 days post-hatch.

Treatment	Weight gain (g/bird)	Feed intake (g/bird)	FCR (g/g)	Relative gizzard weight (g/kg)	Relative pancreas weight (g/kg)	Relative gizzard contents (g/kg)	Gizzard pH
1A	1544b	2326	1.508bc	16.00a	2.26	4.91a	3.03
2B	1415a	2152	1.525bc	18.50cd	2.54	7.45c	2.74
3C	1414a	2202	1.559c	21.62e	2.57	6.49bc	3.11
4D	1494ab	2209	1.479ab	16.83ab	2.51	5.24ab	3.09
5E	1472ab	2218	1.507b	18.12bc	2.37	5.54ab	3.16
6F	1434a	2168	1.478ab	18.82cd	2.42	6.15abc	3.05
7G	1458a	2165	1.518bc	18.70cd	2.51	5.29ab	3.19
8H	1448a	2155	1.490ab	18.71cd	2.49	7.70c	3.18
9I	1418a	2165	1.484ab	19.96d	2.60	6.46abc	3.13
10J	1459a	2118	1.452a	18.26bc	2.45	7.40c	2.96
SEM	28.398	48.089	0.0183	0.5517	0.1095	0.5557	0.2025
Significance (P =)	0.050	0.194	0.011	< 0.001	0.562	0.003	0.657
LSD (P < 0.05)	80.7	-	0.0517	1.567	-	1.579	-

abcde Means within columns without common suffixes are significantly different at the 5% level of probability

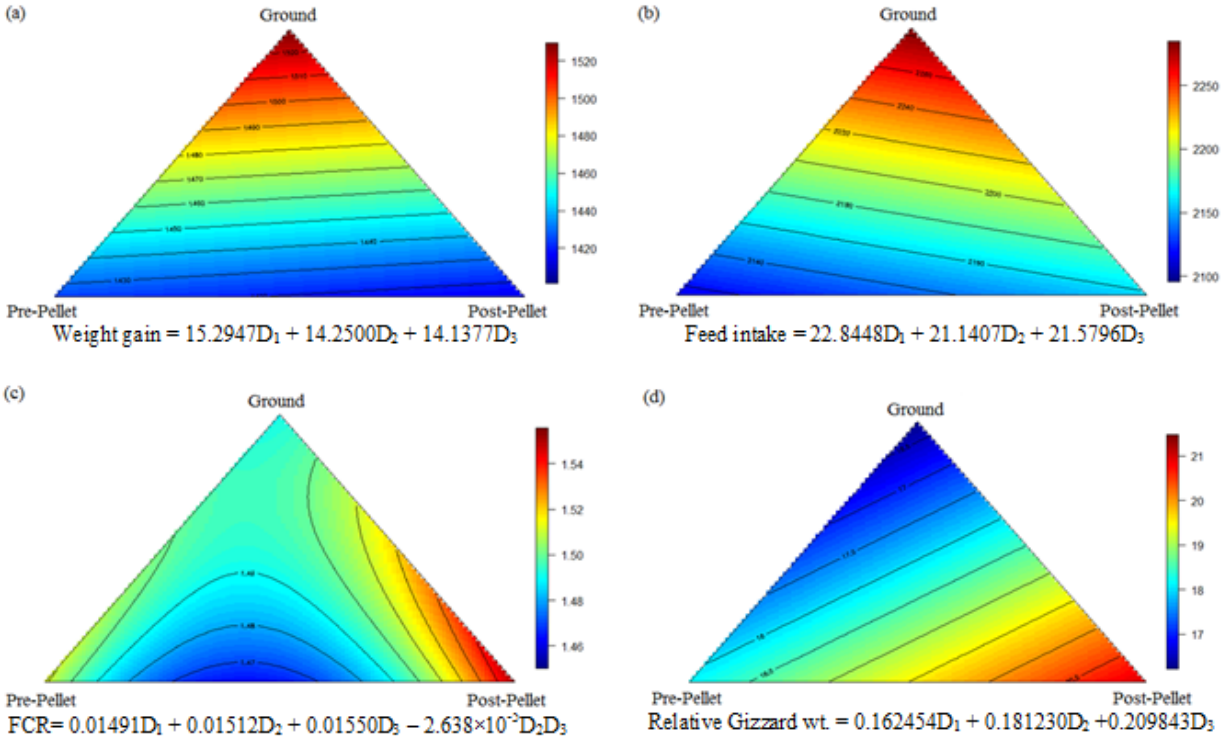

Figure 2 - Response surface plots of three basal diets and; weight gain (a), feed intake (b), feed conversion ratio (c), and relative empty gizzard weight (d).

IV. DISCUSSION

One of the prime reasons for the transition from mash to pelleted diets was to avoid selection, sorting, rejection and feed wastage (Abdollahi et al., 2013). Conversely, offering birds a pelleted concentrate-WG blend provides the possibility of feed wastage from "feed-flicking", a phenomenon that is observed in the field. In this study we observed feed-flicking in diets 5E, 9I and especially in 3C, probably due to the relatively greater post-pellet WG component. Consequently, feed intake of treatment 3C were exaggerated which would have negatively influenced FCR. This may have contributed to the outcome that a blend of ground grain, pre- and post-pellet WG was associated with superior FCR. Nevertheless, as mentioned, pre-pellet whole grain can improve FCR and energy utilisation with only modest increases in relative gizzard weights. One possible explanation for responses to WG feeding is that the wheat component remains intact as it is not hammer-milled. This probably results in slower starch digestion rates which may advantage bird performance (Truong et al., 2015). In the present study, gizzard weights and their contents were positively correlated with pancreas weights. Thus, the implication is that more functional gizzards influence the pancreas, as peptide end products of pepsin digestion can stimulate pancreatic activity via the release of enteric hormones including CCK and gastrin (Krehbiel and Matthews, 2003). Presumably, this would increase the secretion of an array of endogenous enzymes into the duodenum to enhance nutrient digestion.

It is evident from the current study that a blend of pre- and post-pellet WG generated superior feed efficiency. Clearly, post-pellet WG is more susceptible to feed-flicking. Therefore, pre-pellet WG addition should not be overlooked for chicken meat production because it will attenuate problems associated with feed wastage. While tandem inclusions of pre- and post-pellet WG would be a novel approach, this study suggests that it may have merit.

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STRATEGIES FOR ALLEVIATING COCCIDIOSIS WITH A MICROENCAPSULATED BLEND OF ORGANIC ACIDS + ESSENTIAL OILS OR WITH MICROENCAPSULATED BLEND OF ESSENTIAL OILS FOR BROILERS

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Summary

Coccidiosis, a parasitic disease, continues to siphon the financial benefits in commercial poultry farming due to its adverse effects on animal performance. Two experiments were conducted to 1) assess the efficacy of in-feed supplementation of a microencapsulated blend of organic acids + essential oils (MOA + MEO; 300 g/t) and of a microencapsulated blend of essential oils (MEO; 500 g/t) in alleviating the impact of coccidiosis and 2) to investigate the potential of an anti-coccidial program based on a combination of in-feed MEO (300g/t) with an ionophore (salinomycin – SAL; 66 ppm) for broiler chickens. In the first trial, the chickens were challenged with a mixture of *E. acervulina, E. maxima* and *E. tenella* by oral gavage and in the second trial, by contaminated mixed feeds. Zootechnical performance, lesion scores for each intestinal segment corresponding to each *Eimeria* strains (Johnson and Reid, 1970) and total oocysts counts in feces were analyzed for both experiments. In experiment 1, birds supplemented with MOA + MEO and MEO had lower ($P < 0.05$) coccidiosis lesion scores for each intestinal segment and lower ($P < 0.05$) oocyst counts compared to the challenged control birds. Average weight gain of birds with MEO supplementation was higher ($P < 0.05$) than the challenged control and similar ($P > 0.05$) to MOA + MEO birds. Microencapsulated essential oils improved ($P < 0.05$) feed conversion by 18 points compared to the challenged control but was similar ($P > 0.05$) to the MOA + MEO group from the time of the inoculation to end of rearing. In experiment 2, average lesions scores were similar ($P > 0.05$) between birds supplemented with SAL, MEO + SAL, MEO but also lower ($P < 0.05$) than the challenged control. Birds receiving MEO + SAL obtained the lowest total oocysts counts followed by birds with SAL although similar but SAL group was also similar to birds with MEO and with trial product Rx compose of plant extracts, essential oils and organic acids. At end of rearing, birds with MEO + SAL improved ($P < 0.001$) feed conversion and body weight gain compared to all other treatments. However, SAL ranked second followed by MEO and Rx, which were similar for feed conversion and body weight gain as well as statistically improved compared to challenge control ($P < 0.001$). In conclusion, both studies demonstrated the efficacy of MOA + MEO, MEO and MEO + SAL in alleviating the negative impact of coccidiosis on intestinal health and broiler performance. The anti-coccidial properties of these products may form basis for their use as part of an effective anti-coccidial strategy in commercial broiler farms.

I. INTRODUCTION

Coccidiosis continues to be recognized as one of the most expensive enteric disease of the poultry industry worldwide, and was estimated to an annual cost of USD$1.8 billion (Zhang et al., 2013) in terms of prevention, treatments, and loss in productivity. Moreover, the industry is changing: new anti-coccidials are scarce; resistance to coccidiostats is taking over (Chapman 1997); vaccination has its load of management concerns for producer; and performance boosters, like *3-nitro*, are being banned. With the global chicken industry

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moving toward using fewer drugs and complied to please the 21st century consumer, alternatives that will provide a certain control over this parasite alone or with other medication are in-demand. The objective of experiment 1 was to evaluate the anticoccidial efficacy of an in-feed microencapsulated blend of organic acids + essential oils and a microencapsulated blend of essential oils. Experiment 2 was conducted to investigate the efficacy of a microencapsulated blend of essential oils as a performance enhancer when in combination with an ionophore.

II. MATERIALS AND METHODS

Two experiments were conducted in the same research center, the Southern Poultry Research (SPR) in the United-States. For both trials, the chickens were challenged with a mixture of *E. acervulina*, *E. maxima* and *E. tenella* (challenge model proprietary to SPR) administered either orally by gavage or mixed with feed. The experimental design was done according to randomized complete block design for the two trials. Table 1 details the parameters for each experiment.

In experiment 1, a total of 320 off-sex male (byproduct from broiler female line) chicks were distributed in each battery cages. Cages were randomly assigned to one of the following treatments in 8 replicates: 1) an unchallenged control (UNC), 2) a challenged control (CC), 3) CC supplemented with microencapsulated blend of essential oils at 500 g/t of feed (MEO) or 4) CC supplemented with microencapsulated blend of organic acids + essential oils at 300 g/t of feed (MOA + MEO). Birds and non-consumed feed were weighed at day 0, 14, 20 and 28.

In experiment 2, a total of 2400 birds (mixed-sex Cobb x Hubbard) were allocated to one of the 6 treatments in 8 replicates: 1) unchallenged control (UNC); 2) challenged control (CC); 3) CC with trial product Rx (Rx) compose of plant extracts, essential oils and organic acids at 700 g/t; 4) CC with a microencapsulated blend of essential oils (MEO) at 500 g/t; 5) CC with MEO at 300 g/t and salinomycin 66 ppm (MEO + SAL); and 6) CC with salinomycin 66 ppm (SAL). Birds were reared in floor pens on a 10 cm built-up dirt litter separated by curtain sidewalls and reaching a density of 0.07 m^2/bird to mimic commercial conditions. Birds and unconsumed feed were weighted at days 14, 23 and 35.

Table 1 - Detailed parameters, days and action taken for each experiment.

Parameters	Experiment 1		Experiment 2	
	Days	Action	Days	Action
Rearing period	28	Cages	35	Floor pens
Supplementation	D0-28	Additives through out	D14-35	Additives from D14
Inoculation	D14	Individual gavage	D17	Mixed with feed
Lesions scores	D20	4 birds necropsied[1]	D23	5 birds necropsied[1]
Feces collection[2]	D19	Mixed feces	D23 & 35	Mixed feces

[1]Humanely euthanized and scored according to Johnson & Reid (1970) method.
[2]Feces were collected and quantified by fecal flotation for coccidia oocysts per gram of litter (OPG).

Birds received the routine vaccination at the hatchery and were provided with feed and water *ad libitum*. Cages or pens were checked twice daily, unusual observations and mortality in addition to most probable cause of death were recorded. Temperature, humidity, ventilation and lighting were controlled according to age and rearing environment in order to maximize birds' comfort during time of trial.

Data for performance parameters, mortality, intestinal lesions score and oocysts count per gram of fecal material were compiled and analyzed by ANOVA (using Random

Complete Block Design) and compared by Tukey's test or Fisher's LSD test when ANOVA P values were significant ($P < 0.05$).

III. RESULTS AND DISCUSSION

In experiment 1, the challenge model applied was successful, supported by the superior results in performance, OPG counts and lesion scores of the unchallenged treatment (Table 2). Comparing the treatments challenged with coccidiosis, birds supplemented with MOA + MEO and MEO had lower ($P < 0.05$) coccidiosis lesions score for each intestinal segment corresponding to an *Eimeria* species and also had lower ($P < 0.05$) oocysts counts than the challenged birds. The average body weight gain of the MEO and MOA + MEO groups were higher ($P < 0.05$) than the challenged control group. Birds receiving MEO had the best ($P < 0.05$) feed conversion followed by MOA + MEO when compared to the challenged control at 14-28 days. These results suggest that the lower lesion score and reduction in excretion of oocysts is able to give an edge to birds and translate in enhanced performance for birds supplemented with MEO and MOA + MEO. Although feed conversion was numerically better but not significant, treatments with MEO had better weight gain than CC with no differences with the MOA+MEO treatment. The microencapsulated essential oils blend in this study had the greatest protection effect for coccidiosis, as similarly observed by Remmal et al. (2013) and Giannenas et al. (2003) in poultry. The industry is in great need of an effective anticoccidial, which should demonstrate a broad-spectrum of activity as well as enhance performances in broilers as pointed out by McDougald (1982).

Table 2 - Performance at 28 days, total oocysts counts and lesions score for experiment 1.

Treatments	BWG[1] (kg)	FC	OPG	Lesions score[2]		
				duodenum	ileum	ceca
UNC	0.631^{a}	1.665^{c}	0^{c}	0^{c}	0^{c}	0^{c}
CC	0.355^{c}	2.502^{a}	164477^{a}	3^{a}	2.2^{a}	2.1^{a}
MEO	0.430^{b}	2.231^{ab}	63533^{c}	2.6^{b}	1.7^{b}	1.7^{b}
MOA + MEO	0.406^{b}	2.318^{b}	43014^{c}	2.5^{b}	1.7^{b}	1.1^{b}

[1] BWG: body weight gain; FC: feed conversion; OPG: oocyst per gram of feces; MEO microencapsulated essential oils; MOA: microencapsulated organic acids.
[2] Each intestinal segment is related to the lesion score of one of the *Eimeria* species: duodenum (*E. acervulina*), ileum (*E. maxima*) and ceca (*E. tenella*).
[abc] Means within a column with different superscripts differ ($P < 0.05$; Tukey test).

In experiment 2, the challenge model applied was also successful and the unchallenged birds presented superior results in performance, OPG counts and lesion scores. Comparing the treatments challenged with coccidiosis, birds receiving SAL, MEO + SAL and MEO had similar average lesions score ($P = 0.37$), however lower ($P < 0.05$) than the challenge control. In addition, MEO and Rx treatment groups were similar. Total oocysts counts at D23 revealed that birds supplemented with MEO + SAL provided the lowest ($P < 0.001$) counts followed by SAL group, which were statistically similar. Total oocysts counts for MEO, Rx and SAL birds group were also similar. At day 35, birds with MEO + SAL presented best result for feed conversion ($P < 0.001$) and body weight gain ($P < 0.001$). Birds receiving SAL ranked second with 4 points on feed conversion difference with MEO + SAL, 5 points with MEO and 6 points with Rx ($P < 0.001$). MEO and Rx birds were similar for feed conversion and body weight gain. However both were better ($P < 0.001$) than challenge control for feed conversion only, not for body weight gain. These results suggest that MEO at 500g/t or MEO at 300g/t combined to SAL can be part of a strategy as an aid to coccidiosis challenged by reducing lesions scores and oocysts counts as well as improving the

performance of chickens challenged with coccidiosis. Product Rx and MEO offer also a promising avenue to improve control of coccidiosis. In accordance with these findings, Bozkurt et al. (2014) observed that a mixture of essential oils was able to reduce the severity of lesions when compared to other feed additives in chickens, but was not tested with the SAL.

Table 3 - Performance at 35 days, total oocysts counts and lesions score for experiment 2.

Treatments	BWG[1] (kg)	FC	OPG	Av. lesions score
UNC	1.818^{a}	1.660^{a}	812^{d}	0^{d}
CC	1.664^{cd}	1.847^{e}	23425^{a}	3^{a}
Product Rx	1.647^{d}	1.800^{d}	14171^{b}	2.5^{b}
MEO	1.665^{cd}	1.785^{d}	16834^{b}	2.2^{bc}
MEO + SAL	1.747^{ab}	1.700^{b}	6298^{cd}	1.9^{c}
SAL	1.729^{bc}	1.741^{c}	11407^{bc}	1.9^{c}

[1] BWG: body weight gain; FC: feed conversion; OPG: oocyst per gram of feces; MEO microencapsulated essential oils; MOA: microencapsulated organic acids.
[abcde] Means within a column with different superscripts differ ($P < 0.05$; LSD test).

In conclusion, the in-feed tested microencapsulated blend of organic acids + essential oils at 300g/t and the microencapsulated essential oils at 500g/t can be used as a strategy to diminish the negative impacts of coccidiosis in broiler chickens. In addition, the tested microencapsulated essential oils at 300g/t combined with an ionophore (salinomycin; 66ppm) can be used to prevent performance losses due to coccidiosis challenges.

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REDUCING UNDIGESTED PROTEIN AVAILABLE AT THE INTESTINAL WALL

X.U. ARBE[1] and M.S. BEKKER

An experiment was conducted to evaluate the effects of supplementing an inherently thermo stable protease (CIBENZA® DP100) in broiler chickens fed diets based on single raw materials, soya bean meal and rice bran to evaluate the reduction of undigested protein and amino acids of each raw material. The semi-purified diets are shown in Table 1. A total of 210 Ross 308 male broilers of 21 days old were assigned to 5 treatments with 6 pens per treatment and 7 chickens per pen. Data were analyzed as factorial in randomized complete block design using the General Linear Models procedure of SAS® and the significant differences between treatment groups were detected by Duncan's multiple range test. The differences among the treatment were considered significant at P-value≤ 0.05. Test diets included: T1 (-ve control): a N free diet; T2: a 96.2% soya bean meal diet; T3: T2 with protease at 0.05% of the diet; T4: a 96.2% rice bran diet; T5: T4 with protease at 0.05% of the diet. Before 21 days of age there was a 5 day adjustment period to the diets. At 21 days, excreta collection was done during a 96-hour period and dried for metabolic energy calculations. After finishing the body weight and total feed intake of birds in each cage was recorded. The birds were sacrificed for collecting ileal content and freeze dried for digestibility analysis. The ileal protein digestibility was calculated on a dry matter basis. T2 and T4 showed lower AME and AMEn than the treatments supplemented with protease. As seen in Table 2 there was an increase of the amino acid digestibility in the diets supplemented with protease (T3 and T4). Digestibility improved significantly ($P < 0.05$) in several essential amino acids (Met, Cys, Trp, Arg, Val) and non-essentials (Phe, His, Asp, Gly). The improvement of digestibility in the rice bran was higher than in soya bean meal. The addition of an exogenous protease improved the digestibility of amino acids resulting in a reduction of the undigested protein available as nutrients for the growth of pathogenic bacteria.

The use of reduced crude protein diets is becoming popular to increase bird health but must come at the expense of performance. Judicious use of exogenous protease to maximize digestion of the protein fraction in these diets has multiple benefits. Accelerated dynamics of amino acid digestion in the gut leads to reduced protein escape to the hind gut. This in turn reduces intestinal fermentation and colonization by pathogenic bacteria. Reduction in these pathogenic bacteria allows for increased expression of growth in intestinal tissue and more efficient nutrient absorption.

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Table 1 - Constituents of the semi purified diets.

	SBM Diet	Rice Bran Diet	Protein Free Diet
SBM	96.2		
Rice bran		96.2	
Corn starch			54.39
Dextrose			32.4
Cellulose			4
Soybean oil			4
Dicalcium phosphate	2.3	2.3	3.17
Calcium carbonate	0.8	0.8	1.04
Vitamin-mineral premix	0.2	0.2	0.2
Sodium chloride	0.2	0.2	0.4
Choline Chloride			0.1
Chromic oxide	0.3	0.3	0.3
Total	100	100	100

Table 2 - Digestibility of amino acids in a semi-purified diet.

	Rice Bran			Soy Bean Meal		
Amino Acid	T4 No Protease % Digestible	T5 Protease 500g/t % Digestible	Difference % Improvement	T2 No Protease % Digestible	T3 Protease 500g/t % Digestible	Difference % Improvement
Met	32.21	44.01	11.8	60.23	67.99	7.76
Cys	35.52	44.04	8.52	39.8	49.74	9.93
Lys	24.38	29.98	5.6	63.21	69.63	6.41
Thr	31.68	35.11	3.43	54.39	60.62	6.23
Trp	36.94	50.55	13.61	59.51	66.31	6.8
Val	36.23	42.01	5.78	56.87	63.89	7.02
Arg	55.01	63.55	8.54	66.35	68.85	2.5

INTESTINAL MICROBIOTA OF CHICKENS RAISED IN WIRE CAGES AND DEEP LITTER HOUSING ARE NOT DIFFERENT

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Contribution of the gastrointestinal microbiota to animal health, overall wellbeing and productivity is well appreciated. The chicken gastrointestinal microbiome has been studied for more than four decades to understand its characteristics and function. Its microbial community composition is dependent mainly on diet (Apajalahti et al., 2001), and to a lesser extent host systemic response, gastrointestinal secretions and type of litter material being used (Torok et al., 2009). However, composition of the ileal and caecal microbiome can significantly differ (Mohd Shaufi et al., 2015). Here, we extend these findings by characterising the effects of the housing systems (deep litter vs cages) on the ileal and caecal microbiota of broiler chickens using high-throughput culture independent DNA sequencing (Illumina).

Ninety six day-old male chicks were randomly divided into six cardboard pens of size 95.0cm x 95.0cm x 65.0cm (LxWxH) covered with wood shavings and six cages of size 90.0cm x 70.0cm x 50.0cm (LxWxH) kept in an environment-controlled room with eight birds per replicate. Birds were raised for 35 days on a wheat and soybean based diet. Ileal and caecal contents of 12 randomly selected birds from three cages and three pens were collected at day 35, then flash frozen in liquid nitrogen and stored at -80 ^{0}C. Genomic DNA was extracted and the intestinal microbial profiling was performed by amplicon sequencing of the V6 to V8 variable regions of the 16S rRNA gene. QIIME software was used to process sequencing data. The 16S rRNA sequences were clustered into operational taxonomic units (OTUs) at 97% DNA sequence similarity and identified by using Basic Local Alignment Search Tool (BLAST). Data was analysed with vegan and other general packages in R.

Permutational multivariate analysis of variance of microbial community data indicated that there was no significant difference in microbial community structure both in the ileum ($P > 0.05$) and caecum ($P > 0.05$) associated with housing. The dominant OTUs in the ileum were *Lactobacillus* and the dominant OTUs in caecum were *Faecalibacterium* both in the cages and deep litter pens. However, the relative abundance of two of the OTUs representing *Lactobacillus* were significantly lower ($P < 0.05$) in the ileum of the birds raised in cages than in the birds kept in deep litter pens. Relative abundance of one OTU representing *Ruminococcus* was significantly increased in the caecum of birds in cages compared to those in the deep litter pens. Microbial diversity (Shannon index) was similar ($P > 0.05$) in both housing systems (rarefaction with sequencing depth of 4000 reads per sample in both sites).

In conclusion, intestinal microbiota of chickens raised in cages and deep litter housing are similar.

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EFFECTS OF PROBIOTIC *BACILLUS AMYLOLIQUEFACIENS* H57 ON PERFORMANCE AND INTESTINAL MICROBIOTA OF CHICKENS

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Probiotics (direct fed microbials) are a potential alternative to antibiotic growth promoters for improving animal production and preventing enteric pathogen infections. We are studying the effects of the novel, spore forming strain, *Bacillus amyloliquefaciens* H57 (H57) on productivity and the intestinal microbiota of poultry.

A sorghum and soybean based mash diet with or without H57 spores (~10^7cfu/g), was fed to day old male, broiler chicks for 21 days. Chick growth and feed consumption was measured weekly and the ileal and caecal contents of 24 birds (2 from each replicate cohort of 15 chicks) were collected on day 21. Culture independent microbial profiling of the digesta samples was undertaken by sequencing V6 to V8 variable regions of the 16S rRNA gene with Illumina sequencing. Data was analysed with QIIME (Caporaso et al., 2010) and vegan (Oksanen et al., 2016) and other general packages in R.

Permutational multivariate analysis of variance of operational taxonomic units from control and H57 treated birds indicated that H57 significantly modified the microbial community structure both in the ileum ($P < 0.005$) and caecum ($P < 0.005$). Microbiota diversity (Shannon index) was significantly reduced ($P < 0.05$) in the ileum by dietary H57 addition (control 4.35 vs H57 group 3.92) while diversity was not affected in the caecum (rarefaction with sequencing depth of 25,000 reads per sample in both sites) with a shift in the relative abundance of multiple bacterial genera in both sites. The most prominent change was an increase in the relative abundance of *Bacteroides* in the caecum from 0.0002% in control birds to 17.4% in the H57 treated birds; becoming the most dominant taxon. Quantification of H57 in digesta samples by real time qPCR indicated that H57 did not multiply in the intestine.

Together with the change in microbial community structure, H57 significantly improved ($P < 0.005$) growth rate. The average daily weight gain of H57 fed chicks was improved by some 6.9% (34.8 g/day/control bird vs 37.2g/day/H57 bird) resulting in a higher ($P < 0.01$) body weight at day 21(845g vs 896g). Similarly, H57 improved ($P < 0.05$) the feed conversion ratio by about 6% during the study without effect on feed intake.

In conclusion, dietary supplementation of the probiotic *Bacillus amyloliquefaciens* H57 to chickens modified the intestinal microbiota and improved growth and feed efficiency.

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EVALUATION OF ANTI-INFLAMMATORY CAPACITIES OF *BACILLUS*-BASED PROBIOTICS USING AN *IN VITRO* INTESTINAL CELL MODEL

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With the evolution of genetic potential of modern broilers, transforming the large feed intake into protein deposition is a real challenge for the digestive tract. The mucosa, being the first line of defence, plays a key role in maintaining gut health and its physiological functions and is thus crucial to support animal performance. Intestinal inflammatory response is the consequence of imbalance of the homeostasis in relation with various digestive stress conditions (Peterson and Artis, 2012). Anti-inflammatory properties of probiotics have been demonstrated but are devoted to specific strains (Hardy et al., 2013). The objective of this study was to investigate the anti-inflammatory properties of the new *Bacillus subtilis* probiotic strain 29784 in comparison with two other commercially available *B. subtilis* strains, (Bs A and Bs B) at the gut level.

An *in vitro* model based on human intestinal epithelial cells (Caco-2 cells) was used to evaluate the ability of these three *B. subtilis* strains to prevent inflammation in stimulated and non-stimulated conditions. Vegetative cells of each *B. subtilis* strains, or a positive control, epigallocatechin gallate (EGCG) known for its anti-inflammatory properties (Kim et al., 2006), were overnight applied to a 14-day differentiated Caco-2 cells monolayer established in a transwell system. Caco-2 cells were then exposed, or not, to the inflammatory mediator IL-1β during 6 hours. Trans Epithelial Resistance (TER) and IL-8 production were then monitored as indicators of intestinal permeability and inflammation, respectively (Maresca et al., 2008).

As expected, TER was improved by the positive control EGCG in both standard and stimulated conditions. Whereas Bs A decreased TER, Bs B had no effect and *B. subtilis* 29784 increased it. IL-1β induced inflammation as shown by an increase in IL-8 production. All strains tested were able to significantly reduce IL-8 level. However, *B. subtilis* 29784 was the only strain able to fully reduce the inflammatory response, as shown by the equal level of IL-8 secreted by the IL-1β -stimulated cells treated with *B. subtilis 29784*, as well as EGCG, and the non-stimulated cells.

Our results clearly show that different *B. subtilis* strains can have different levels of efficacy in modulation of inflammatory response and intestinal permeability. *B. subtilis* 29784 thus appears an efficient probiotic solution enhancing intestinal barrier and reducing inflammatory status.

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AN OVERVIEW OF THE POULTRY CRC'S ACHIEVEMENTS

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Summary

The Poultry CRC commenced operation on 1 July 2003 and will end on 30 June 2017. It has been a highly successful CRC, providing solutions to numerous key challenges facing the Australian poultry industry in the areas of research, education, communication, and outreach activities. In brief, it has tremendously enhanced the Australian poultry industry's capacity to travel down the path of sustainable growth well into the future. The following is a succinct summary of its achievements.

a) Frontier science and state-of-the-art innovations

The Poultry CRC's investment in over 176 research projects has dealt with many pressing issues for the industry. As just one example demonstrates, the Poultry CRC has established over 30 rapid tests at The University of Melbourne's Asia-Pacific Centre for Animal Health, providing industry with a one-stop shop for swift diagnosis and enhanced disease surveillance. Turning to innovations, the Poultry CRC has achieved many breakthroughs, including the discovery of netB, a novel bacterial toxin, which overturned a 30 year dogma that alpha toxin is the causative agent of necrotic enteritis in chickens. At its peak, the Poultry CRC held 57 patents, marking out its leading position in many areas of poultry science around the world.

b) Future leaders and workforce

The Poultry CRC has supported 16 interns, 25 postdoctoral fellows, 77 postgraduate students, and 33 honours students, as well as hundreds of students who have undertaken our three poultry specific undergraduate courses. These new industry entrants have significantly reduced the skills shortage in the Australian poultry industry that existed when the CRC started, and will be a source of industry leadership and essential skills for many years to come. For example, of our 16 interns, 10 continue to work in industry and 2 in academia. In addition, 22 of our graduates are currently working in the industry and 18 in academia or research. With an additional 30 students still working to complete their degrees, there will be even more graduates entering the poultry industry or poultry related research. Furthermore, our Avian Health Online course conducted through The University of Melbourne has enabled 39 poultry health professionals to upgrade their skills at either a diploma level or a master's level.

c) National network with global leverage

The Poultry CRC has constructed a highly effective collaborative network of participant research organisations informed by end-users. The collective expertise of the network generates innovative and unique research initiatives. It solves the problem of critical mass, where no single research institute in Australia has the expertise and facilities to solve the complex scientific and industrial problems the poultry industry will face in the future.

I. RESEARCH CHALLENGES

The Cooperative Research Centres (CRC) Programme was established in 1990 with the aim of encouraging private sector investment in collaborative research to deliver frontier science

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and practical solutions to industry problems. It also has a strong education component with a focus on producing graduates with skills relevant to industry needs.

The Poultry CRC successfully secured two terms of funding, with a total operational life of 14 years from 1 July 2003 to 30 June 2017. The aim of the first term CRC was "*to enhance the competitiveness of the Australian egg and chicken meat industries and supporting industries through the application of strategic programs delivering cost-effective and socially responsible production of safe, quality poultry products for domestic consumption and for emerging export markets*". The objective of the second term CRC was "*to help Australia achieve sustainable, ethical poultry production as our population grows."*

The Poultry CRC is an industry-driven CRC and as such its research is primarily applied. The nature of applied research is that it creates incremental gains, rather than the quantum leaps that blue sky research produces from time to time. To address highly complex industry challenges, the Poultry CRC put together an integrated program of research, development and education. This involved the majority of end-users and researcher providers in Australia with significant international participation, covering all key facilities and expertise related to the research focus of the CRC. The mature and genuine collaborative network formed as a result has developed a holistic view of the poultry industry's needs and implemented many effective solutions to them.

a) CRC 1st Term - Australian Poultry CRC

The Australian Poultry CRC commenced operation on 1 July 2003 with five core participants, Australian Egg Corporation Limited, Bioproperties Pty Ltd, Rural Industries R&D Corporation, The University of Melbourne and University of New England. Most key egg and chicken meat producers were also members of the Australian Poultry CRC. The focus of the Australian Poultry CRC was to increase productivity of the Australian poultry industries through the delivery of solutions in nutrition, vaccines and disease diagnostics, environment and welfare, and education and utilisation.

A total of 106 research projects, 27 postgraduate students, 14 honours students, and 12 postdoctoral scientists were supported during the six years. The research projects delivered some lasting solutions to industry. The examples include:

- The use of structural materials in feed to enhance gut health, leading to a paradigm shift in feed processing and presentation;
- Setting up the delivery of a comprehensive set of rapid diagnostics tests for poultry diseases at The University of Melbourne's Asia-Pacific Centre for Animal Health, cutting the time required for disease diagnosis from days to hours and creating a one-stop shop for industry diagnostic service;
- Commercialisation of Vaxsafe PM®, offering a unique live attenuated vaccine for the control of Fowl Cholera due to infection with virulent homologous or heterologous strains of *Pasteurella multocida*; and
- Production of a comprehensive set of teaching materials for poultry health, welfare, nutrition and environment, leading a solid foundation for delivering vocational education and training for the industry.

b) CRC 2nd Term – Poultry CRC

With overwhelming support from the industry and research partners, we were successful in obtaining a second term of funding for the Poultry CRC, which commenced operation in early 2010. Poultry CRC has three programs, i.e., Health and Welfare, Nutrition and Environment, and Food Safety and Egg Quality.

A total of 71 research projects, 50 postgraduate students, 19 honours students, and 13 postdoctoral scientists were supported in the Poultry CRC. It focussed on addressing practical problems facing the poultry industries, such as production and welfare of free range birds, addressing wet litter problems, identification of the spotty liver pathogen, the use of canola seeds and meals in both layer and broiler diets, minimising avian flu risks, mitigating waste streams, addressing skills shortage and extension. In addition, the Poultry CRC continued the work done previously on a number of vaccines, including:

- development of an ILTV vaccine as well as live and killed versions of a necrotic enteritis vaccine;
- investigation of a potential Campylobacter vaccine;
- extensive evaluation of Salmonella vaccines; and
- the delivery of an MG vaccine and a haemorrhagic enteritis vaccine for turkeys (both for Minor Use).

II. INTERNSHIPS, VET SECTOR, AND SCHOOLS PROGRAM

As the Poultry CRC's education program was summarised earlier, this section only covers our internship, vocational education and training (VET), and schools programs. In the year 2000, when the idea of submitting a CRC proposal for the poultry industry was first canvassed, one of the common issues identified was the difficulty for the poultry industry to attract veterinarians. In order to help industry with this issue, an industry internship program was introduced shortly after the commencement of the Australian Poultry CRC. Despite establishing the framework, it took five years' of stellar effort by some of our dedicated industry veterinarians to attract the first interns. As of 2016, the Poultry CRC has attracted 16 interns with 10 of them continuing to work in the industry with an additional 2 working in academia.

Poultry CRC also sponsored the Avian Health Online course at The University of Melbourne. This course enabled 39 poultry health professionals to upgrade their skills to either masters or diploma level. In conjunction with the TAFE NSW New England Institute in Tamworth, the Poultry CRC developed extensive resources for the delivery of VET courses across the country. Employees of many of our industry partners took up training at Certificate III level to further their skills and employment in the poultry industry. In addition, the Teachers' Resource Kit we produced has been used by nearly a thousand schools throughout Australia.

Furthermore, we have collaborated with the NSW Department of Education to produce "Keeping Poultry in Schools" video series, which have been made available to all schools in Australia via YouTube and other channels. Our "Chicken Embryo Development" animation, which was funded from part of a grant we received from the WPSA for outreach activities, is a standout educational tool with over 2 million views on YouTube.

III. UTILISATION AND EXTENSION

The Poultry CRC's approach to research and education is to produce products that are of use to the Australian poultry industry. It was realised from the start that increased utilisation of our research and education outcomes could only come from effective two-way communication between researchers and industry. This is because real solutions are much more likely to arise if researchers start with a clear understanding of the problems the poultry industry faces. Therefore, the Poultry CRC has had a heavy emphasis on end-user involvement in identifying pressing issues, evaluating projects and selecting students. This approach has helped the CRC focus on transferrable projects and intellectual property capable

of commercialisation. Thus, in the Poultry CRC, utilisation and extension are viewed as two overarching areas, embedded in as many research and education projects as possible.

The Poultry CRC knows that communication underpins its public and industry interface. It places great importance on events like the Australian Poultry Science Symposium, Poultry Information Exchange (PIX), the AECL Industry Forum, and its own Ideas Exchange as these are great forums that facilitate lasting interactions between researchers and industry. Of course, our electronic newsletters (eChook), mailouts (Chook Run; final reports; factsheets), and face-to-face meetings are also important features of the Poultry CRC's communication strategy.

IV. CONCLUSION

The Poultry CRC has laid a solid foundation for the Australian poultry industry in science and technology, diagnostic capability, and workforce. This foundation will act as a stable platform from which the next generation industry leaders and scientists the Poultry CRC has produced will drive the industry forward in a sustainable manner. In addition, a transition body, Poultry Hub Australia based at the University of New England, will maintain and expand the collaborative network established by the Poultry CRC to serve our poultry industry's research and educational needs into the future.

VITAMIN D IN LAYING HENS: HOW HIGH IS HIGH ENOUGH?

M.E. PERSIA[1], T. WANG[2] and K.A. LIVINGSTON[3]

Summary

Human vitamin D status has been in question for the past 15 years as a resurgence of vitamin D deficient childhood rickets has reoccurred after being eradicated in the 1930s (Holick, 2006; Welch, 2000). In 2009, Lite suggested that up to three-quarters of U.S. teens and adults are deficient in vitamin D due, in part, to lack of sun exposure, and such deficiency is associated with osteomalacia (weak muscles and bones), reduced immune functions and increased inflammation (Lite, 2009; NIH, 2009). Other more recent reports have this number much lower, but vitamin D deficiency is still a major concern even in the developed world (Cashman *et al.*, 2016). This recent trend might be related to increased awareness of the relationship between sun exposure and skin cancer. The American Cancer Society reports that skin cancer is the most common form of cancer with about 5.4 million basal and squamous cell skin cancers diagnosed each year (ACS, 20016). The current recommendation is limit ultraviolet (UV) exposure by protecting skin with clothing, wearing wide-brimmed hats, using sun screen, wearing sunglasses and/or seeking shade (ACS, 2016). These recommendations seem to have been effective as the incidence of melanoma of the skin has continued to increase, but the rate of increase has fallen by decade starting in 1975 (NCI-SEER, 2016). Although these recommendations have been effective at reducing incidence of melanoma, they may have had certain unintended consequences on vitamin D status, contributing to the vitamin D deficiency noted above.

I. INTRODUCTION

In 2010, the Institute of Medicine in the United States reviewed current literature and changed the estimated average requirement of 400 IU/day to a recommended dietary Allowance (RDA) of 600-800 IU/day in the United States (IOM, 2010). This was completed under the assumption that due to skin cancer concerns, sun exposure was minimal. To this end, few natural foods contain significant concentrations of vitamin D to meet the new recommended dietary allowances, which necessitates fortification of the diet or specific foods with vitamin D to satisfy the current and future recommendations. Consumers in the United States are familiar with the fortification of animal products with vitamin D as milk has contained supplemental vitamin D since the beginning of the 1930's (Harman and Steenbock, 1935). The fortification of milk with vitamin D is generally recognized as the key factor in the elimination of rickets in the United States. Although fortification of milk with vitamin D is an important dietary source it supplies less than 50% of the current RDA. Therefore it is important to find alternative foods to increase the daily intake of vitamin D in the diet to maintain high circulating concentrations of this vital nutrient. In 2011, USDA surveyed table egg vitamin D content and updated the value to 40 IU/egg, reflecting the increased amount of vitamin D fed to laying hens for bone and shell quality (J Exler, KY Patterson, and JM Holden, USDA, Beltsville Human Nutrition Research Center, Beltsville, MD). Although this represents a tremendous increase in egg vitamin D content, raw data from the report indicated commercial content of vitamin D above 400 IU/egg showing the variation of egg vitamin D

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content due to dietary supplementation and the efficient transfer of lipid and lipid soluble molecules from the hen to the egg.

At has been previously shown that dietary vitamin D is transferred to the egg yolk in laying hens (Mattila et al., 1999). The authors used a six week feeding period, to understand the relationship among dietary vitamin D content and egg yolk vitamin D content, noting a strong correlation between the two (r = 0.995) over dietary vitamin D concentrations of 1,064, 2,496, and 8,640 IU/kg feed. A follow up study showed that supplementation of laying-hen diets with 12,000 IU/kg of vitamin D resulted in eggs that contained more than 5 times the "normal" amount of vitamin D (Mattila et al., 2003). Although this report demonstrated transfer of vitamin D it was limited in scope (only a single feeding dose was evaluated and the longer-term study suggested that after prolonged feeding transfer of vitamin D to the egg might be down regulated). Additional experiments have validated these results on vitamin D transfer and added exploration of combinations of vitamin D and vitamin D metabolites as sources of vitamin D activity with additive effects (Browning and Cowienson, 2014). These high vitamin D eggs have been used as a food source and have been shown to increase human vitamin D status (Hayes *et al.*, 2016). The previous reports do make a strong argument for the effectiveness of vitamin D feeding in laying hens and demonstrate a safe response of feeding up to approximately 12,000 IU/kg feed, but questions remain as to when vitamin D supplementation does become toxic to the laying hen. Morrisey *et al*. (1977) reported that 40,000 IU/kg feed was the maximal concentration of supplemental vitamin D in laying hen diets that did not cause negative hen health effects but the next highest level of evaluation was 400,000 IU/kg feed resulted in hen renal tubular calcification.

II. HYPOTHESIS AND PROJECT OBJECTIVE

Feeding increasing concentrations of supplemental vitamin D to laying hens will increase egg vitamin D concentrations. The objective of this proposed study was to identify the threshold of the supplemental dietary vitamin D that corresponds to the peak vitamin D content of the egg. Although 40,000 IU/kg has been demonstrated to be safe when fed to laying hens, supplementation of laying hen diets with 400,000 IU/kg has been shown to cause negative health and performance effects. This study was designed to evaluate several doses below the safe dose of 40,000 and one above the safe dose, but still well below the known toxic dose of 400,000 IU/kg.

III. MATERIALS AND METHODS

Treatment design is outlined in Table 1. The experimental diets will be fed to three consecutive pens of three hens (Hy-Line W-36) each allowing eight replicate groups and 72 total hens for each treatment. Vitamin D_3 was selected as the form of dietary supplemental Vitamin D due to cost, transfer to egg and toxicity comparisons to other vitamin D metabolites.

Table 1 - Treatment design

Treatment	Vitamin D_3	Calculated IU/kg feed
1	Control (C)	2,200
2	C + 7,500 IU/kg diet	9,700
3	C + 15,000 IU/kg diet	17,200
4	C + 22,500 IU/kg diet	24,700
5	C + 100,000 IU/kg diet	102,200

The experimental diets were fed to W-36 laying hens starting at the onset of the egg laying cycle and continued for the duration of the cycle, approximately 18 to 54 weeks. Hen management followed UEP guidelines (UEP, 2002) and diets were formulated to breeder recommendations. The experimental diets were mixed every two weeks to minimize the exposure of supplemental vitamin D to degradation due to feed storage. Feed was delivered daily and feed residual determined weekly. Feed intake and conversion (g eggs/g feed intake) was calculated. Hen mortality will be recorded daily.

Eggs were collected daily and production performance recorded. Eggs were saved from two consecutive days per week during the first four weeks (19-23); two consecutive days every two weeks for the next 10 weeks 23-33; and two consecutive days every four weeks for the remaining duration of the experiment (32-58). The saved eggs were weighed to determine egg size and mass produced. Sixteen eggs per experimental treatment (two from each replicate group) were be broken and separated to determine shell, yolk and albumin weights. The egg yolks were pooled to determine total lipid, phospholipid, fatty acid composition and total unsaponifiable matters. Egg yolks were pooled to result in four replicate samples for Vitamin D determination by HPLC (Byrdwell, 2009; Jakobsen et al 2004). The shells were used to determine shell thickness and Haugh units were measured and yolk color will be scored on a color fan. The remaining eggs were used to determine yolk physical and functional properties, including viscosity of raw yolk, and emulsification parameters.

At the conclusion of the experiment, all hens were visually inspected for keel bone integrity and soft tissue calcification to determine effects of long-term feeding of supplemental vitamin D on skeletal and soft tissue integrity. The right tibia was collected from three birds per replicate group to determine fat-free tibia bone ash as an indicator of general skeletal health and Ca and P status.

Statistics on the performance data and egg weights, egg component weights, Haugh units and yolk color scores were analyzed using ANOVA with repeated measurements. Performance data were analyzed with new diet mixing every two weeks and egg data were analyzed when eggs were collected at either 1, 2 or 4 week intervals. There were no interactions over time so main effects are presented. If significance was detected ($P \leq 0.05$), Tukey's honestly significant test was employed to separate treatment means. Egg quality characteristics and vitamin D content and transfer rates were analyzed using ANOVA.

IV. RESULTS AND DISCUSSION

Observation of the performance data from 19 to 58 weeks suggests no difference among the various concentrations of supplemental vitamin D. Egg production over the 40 week period was between 91.4 and 93.1% hen housed, without any significant differences among the control fed birds and vitamin D supplemented birds, although there were some inconsistent differences among the vitamin D supplemented birds. Feed intake again showed no significant differences among the control feeds and the vitamin D supplemented feeds, with over all feed intake ranging from 91.8 to 99.2g per hen per day. There were no differences in daily egg mass or overall feed efficiency (g egg/kg feed intake). These data are in agreement with previous data that reported no differences in performance among control fed birds and birds supplemented with 5,000 to 15,000 IU/kg vitamin D (Browning and Cowieson, 2014; Mattila *et al*., 2004). Egg weights were not significantly different among the treatments, but some egg component weights were significantly different (Table 3). As with total egg weight, albumen weights were not different among treatments, but egg yolk weight was significantly increased from the birds receiving 24,700 IU/kg in comparison to both the control (2,200 IU/kg) and the 102,200 IU/kg treatments. There were significant differences among the shell

weights of the 24,700 and 102,200 IU/kg supplemented hens, but none of the experimental treatments were different from the eggs of the control fed birds. These results are in general agreement with Browning and Cowienson (2014) where up to 10,000 IU/kg vitamin D did not significantly alter egg weights, shell breaking strength, or yolk weights. Haugh units, a measurement of albumen protein quality was not significantly changed by vitamin D treatment, but yolk color was significantly increased with the 102,200 IU/kg vitamin D treatment (Table 3). Yolk color is the first response measured where the highest concentrations of vitamin D fed might have altered the response, but the question remains, is this modest, but significant difference in yolk color indicative of negative performance?

Table 2 - Performance of laying hens fed 2,200, 9,700, 17,200, 24,700, and 102,200 IU D_3/kg diet of dietary cholecalciferol.[1]

Dietary cholecalciferol (IU D_3/kg)	Hen-housed egg production (%)	Feed intake (g/h/d)	Egg mass (g/h/d)	Feed efficiency (g egg/kg feed)
2,200	92.6^{ab}	98.6^{ab}	50.7	528
9,700	93.1^{a}	98.3^{ab}	51.3	534
17,200	91.4^{b}	97.8^{b}	50.3	531
24,700	92.0^{ab}	99.2^{a}	51.6	537
102,200	92.3^{ab}	98.6^{ab}	51.2	538
Pooled SEM	0.48	0.37	0.53	6.8

[1] Data are means of 8 groups of 9 Hy-Line W36 laying hens from 19 to 58 wk of age. Adapted from Persia *et al.*, 2013.
[a–b] Means within columns with no common superscript differ significantly ($P \leq 0.05$)

As with Haugh units, there were no significant differences among egg yolk moisture content, unsaponifiable matter, viscosity, and emulsification properties due to hen dietary vitamin D treatment (Table 4). The emulsion and emulsification data are supported in the literature as Mattila and co-workers (2003) did not show any differences in these measurements with whole eggs. At the end of the 40 week experiment, hen tibia bones were collected for fat-free ash determination. As with performance there we no effects of supplemental dietary vitamin D on fat-free tibia ash and all bones appeared normal and healthy.

Table 3 - Egg characteristics from laying hens fed 2,200, 9,700, 17,200, 24,700, and 102,200 IU D_3/kg diet of dietary cholecalciferol.[1]

Dietary Cholecalciferol (IU D_3/kg)	Egg Weight (g)	Albumen Weight (g)	Yolk Weight (g)	Shell Weight (g)	Haugh Unit	Yolk Color[2]
2,200	56.2	34.0	13.9^{bc}	6.83^{ab}	89.4	5.34^{b}
9,700	56.2	34.1	14.0^{abc}	6.78^{b}	88.8	5.35^{b}
17,200	56.3	34.0	14.1^{ab}	6.86^{ab}	88.4	5.27^{b}
24,700	57.1	34.5	14.2^{a}	6.91^{a}	89.0	5.33^{b}
102,200	56.3	34.5	13.8^{c}	6.78^{b}	89.7	5.51^{a}
Pooled SEM	0.31	0.23	0.09	0.044	0.58	0.050

[1] Data are means of eggs collected from 8 groups of 9 Hy-Line W36 laying hens over 19 to 58 wk of age. Adapted from Persia *et al.*(2013).
[2] Roche yolk color score fan (DSM Nutritional Products, LLC, Parsippany, NJ).
[a–c] Means within columns with no common superscript differ significantly ($P \leq 0.05$).

Table 4 - Moisture content, unsaponifiable matters, viscosity, and emulsification properties of egg yolks from hens fed various concentrations of dietary vitamin D.[1,2]

Dietary Cholecalciferol (IU D_3/kg)	Moisture (%)	Unsaponifiable matter (% of yolk)	Viscosity (Pa s)	Emulsification capacity (g oil/g yolk)	Emulsion stability (%)
2,200	50.0±0.1	1.1±0.1	1.3±0.1	57.8±2.2	50.0±0.0
9,700	49.9±0.4	1.2±0.3	1.3±0.2	58.9±3.7	51.8±5.6
17,200	50.1±0.3	1.0±0.1	1.3±0.2	60.3±2.4	44.7±4.1
24,700	50.3±0.5	1.1±0.1	1.2±0.2	60.3±4.9	49.3±1.3
102,200	49.8±0.3	1.1±0.1	1.3±0.2	60.1±2.6	51.0±5.8

[1] Values are means ± standard deviations. Adapted from Yao *et al.*, 2013.
[2] The means of each parameter had no significant difference among the diets at p =0.05 (n=4).

The performance and egg quality data presented might suggest that up to 102,200 IU/kg of vitamin D is safe and efficient to feed to laying hens, but it is also important to consider general vitamin D metabolism and regulation before drawing conclusions. The feeding of vitamin D to laying hens for 40 weeks resulted in a linear increase in egg yolk vitamin D content from the 9,700 to the 24,700 IU/kg feed fed birds (Figure 1), validating previous data and expanding the range of response to almost 25,000 IU/kg (Browning and Cowienson, 2014; Mattila *et al*., 2003; Mattila *et al*., 1999). But looking at the transfer of vitamin D from the diet to the egg yolk for the 102,200 IU/kg treatment it is clear that more vitamin D is being transferred to the yolk at these high concentrations of dietary vitamin D than in any of the other treatments. This point is made abundantly clear when considering the vitamin D transfer rates of the five dietary treatments (Figure 2). The lowest rate was noted in the control eggs were approximately 5% transfer occurred, the 9,700 to 24,700 IU/kg treatments resulted in a significant increase in transfer rate, but at a controlled rate of 8 to 9%. Once the 102,200 IU/kg diets were fed, transfer rate was uncontrolled at approximately 25 to 30% transfer of vitamin D from the diet to the egg yolk. This break down of vitamin D metabolism does suggest at least the beginning stages of toxicity although performance over a 40 week experiment was not negatively altered.

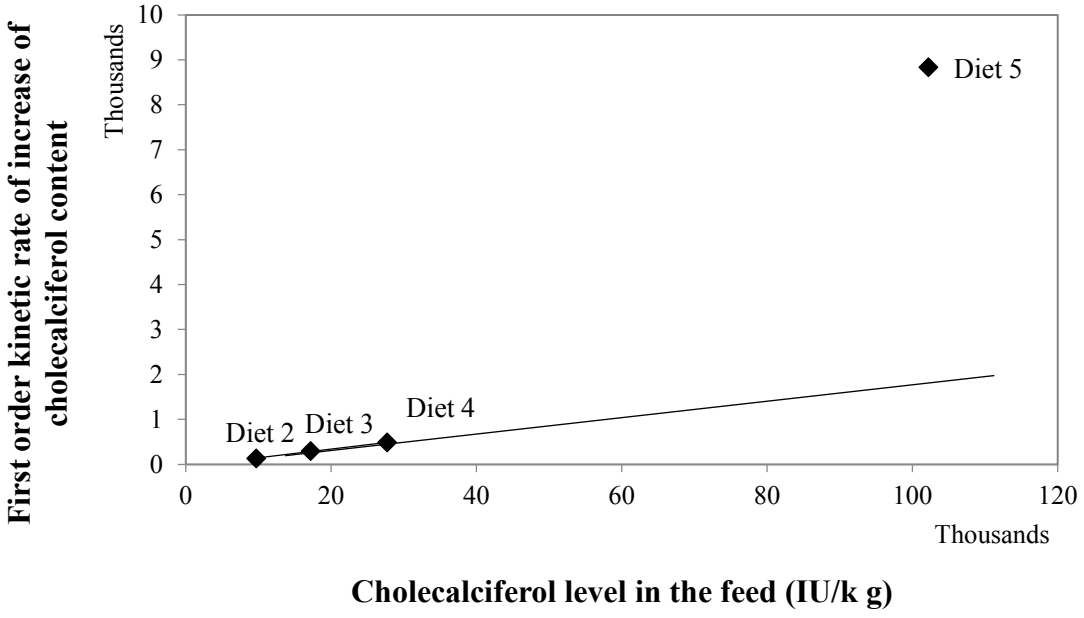

Figure 1 - The relationship of cholecalciferol content in yolk and dietary cholecalciferol level in the feed of diet 2 to 5. The linear equation applies to diet 2 to 4, not to diet 5. Adapted from Yao *et al.*, 2013.

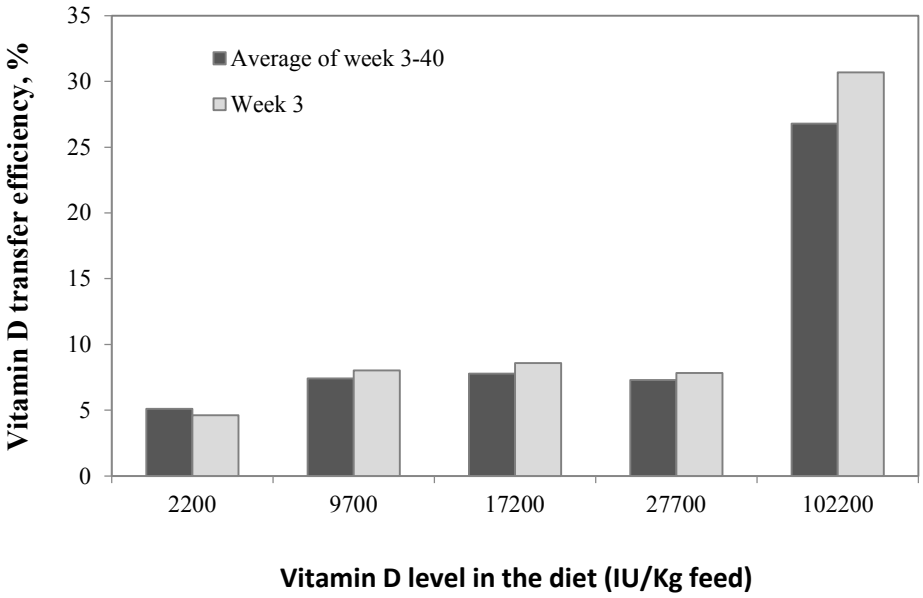

Figure 2 - Effect of dietary cholecalciferol level on egg cholecalciferol transfer efficiency. The control (2,200 IU/kg of vitamin D) is lower than 9,700, 17,200 and 27,700 IU/kg vitamin D which are all lower than 102,200 IU/vitamin D ($P \leq 0.05$). Adapted from *Yao et al.*, 2013.

These data add to our estimate of vitamin D toxicity data and help to better define those rates in laying hens. Currently the highest concentration of vitamin D that has been fed without noticeable toxicity effects has been 40,000 IU/kg feed (Morrisey *et al.*,1977), and the remaining research reports and data presented here all agree that concentrations under 40,000 IU/kg are not toxic to laying hens. There are few data points collected above this threshold, but Morrisey and co-workers (1977) did evaluate 400,000 IU/kg with vitamin D toxicity noted including calcification of soft tissue. The current data would suggest that 102,200 is beginning to become toxic due to the failure of the model to control transfer of dietary vitamin D into the yolk of the egg. Therefore at this time we can conclude that 40,000 IU vitamin D/kg feed or less is safe and tolerable for laying hens, but dietary concentrations above 100,000 are becoming toxic to the hens. The data presented in this document are based on either short or long term feeding in adult animals and do not reflect that application of high vitamin D feeding over the pullet rearing phase limiting interoperation. It would be interesting to better understand the long term growth, performance and health effects of higher vitamin D feeding over the entire life cycle of the laying hen, not just the adult egg production phase.

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VITAMIN D ENRICHMENT OF EGGS FOR HUMAN HEALTH

L.C. BROWNING[1,2]

Summary

Epidemiological surveys indicate a recognised chronic insufficiency of vitamin D within the human population. Vitamin D is essential for good skeletal health, healthy skin and a sound immune system and chronic insufficiencies may cause many diseases including osteoporosis, cancer and diabetes. There are few naturally occurring foods which contain significant dietary levels of vitamin D, however the modern commercial laying hen has been shown to be unique in its ability to efficiently transfer vitamin D metabolites from its diet into egg yolk. Consequently egg yolk represents one of the few foods which is rich in both vitamin D_3 and 25-hydroxyvitamin D_3. An experiment demonstrated that a single egg from a laying hen supplemented with increased levels of vitamin D_3 and 25-hydroxyvitamin D_3 has the potential to contribute significantly to the recommended daily intake of vitamin D for both adults and children without detrimental effect on production parameters.

I. INTRODUCTION

Vitamin D is an essential nutrient in vertebrate nutrition for the maintenance of good health and wellbeing however epidemiological studies in most countries indicate a widespread vitamin D insufficiency in the population which potentially may have a detrimental effect on health.

Vitamin D is primarily sourced from sunlight (UVB 290-320nm) acting on skin, hence it has historically been called the sunshine vitamin. Within the skin 7-dehydrocholestrol is converted to pre-vitamin D_3, which is rapidly converted to cholecalciferol (vitamin D_3) by the presence of body heat. Vitamin D_3 is further converted to 25-hydroxyvitamin D_3 (25(OH)D_3) in the liver and 25(OH)D_3 represents the storage form of vitamin D_3. 25-hydroxyvitamin D_3 is transported in the serum on specific proteins to the kidneys where it is further hydrolysed to 1, 25 dihydroxyvitamin D_3 (1,25(OH)2D_3) which is the true active metabolite of vitamin D in the body. 1,25 dihydroxyvitaminD_3 is often called a hormone. The term vitamin D designates a group of the closely aligned compounds vitamin D_3, and 25(OH)D_3, which are fat soluble and possess anti-rachitic activity.

II. ROLE OF VITAMIN D IN BONE HEALTH

The major function of vitamin D is to maintain serum calcium concentrations within the physiologically homeostatic range and in order to maintain such tight homeostatic control vitamin D regulates the transfer of calcium and phosphorus across the gastro-intestinal wall and the subsequent mineralization of bone tissue (Borle 1974, Fraser 1975). In children a deficiency of vitamin D will cause bone tissue not to properly mineralize and this disease known as rickets was very widespread in the late 19th and early 20th centuries in industrialized countries. In an older human adults, osteoporosis, a disease characterized by low bone mass and micro-architectural deterioration of bone tissue leading to reduced bone strength and a consequent increase in fracture risk (Holick 2007).

Osteoporosis is estimated to affect 200 million women worldwide (Kanis et al. 2008).

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It is widespread with approximately 33% of women 60 to 70 years of age and 66% of women 80 years of age or older have osteoporosis ((Larsen et al. 2004). Furthermore approximately 47% of women and 22% of men 50 years of age or older will sustain an osteoporotic fracture in their remaining lifetime (Holick 2007).

III. THE ROLE OF VITAMIN D IN CANCER

Strong biological and mechanistic bases indicate that vitamin D plays a role in the prevention of colon, prostrate and breast cancers. Both prospective and retrospective epidemiological studies indicate serum levels less than 50 nmol/L of $25(OH)D_3$ to be associated with 30 to 50% increased risk of incident colon, prostrate and breast cancer and high associated mortalities from such cancers (Gorham et al. 2005, Giovannucci et al. 2006, Garland et al. 2006).

Vitamin D is also believed to play a role in maintaining the immune system (Brown et al. 1999, DeLuca and Zierold 1998) and helping to maintain healthy skin (DeLuca and Zierold 1998) and muscle strength (Brown et al. 1999). Additional evidence indicates that vitamin D insufficiency might be associated with diseases such as multiple sclerosis (MS) such that living below 35 degrees latitude for the first 10 years of life reduces the risk of multiple sclerosis by approximately 50% (Ponsonby et al. 2002). Furthermore women who ingested more than 400 IU of vitamin D per day had a 42% reduced risk of developing multiple sclerosis (Munger et al. 2004) . Similar observations have been made for rheumatoid arthritis (Merlino et al. 2004) and osteoarthritis (McAlindon et al. 1996). Recent research has shown that vitamin D status has a role in preventing lung cancer development (Chen et al. 2015).

IV. THE ROLE OF VIAMIN D IN DIABETES

Several studies suggest vitamin D supplementation early in life may reduce the development of type 1 diabetes. For 10,366 children in Finland who were given 2000 IU of vitamin D_3 per day for the first year of life and were followed for 31 years, the risk of type 1 diabetes was reduced by approximately 80% (Hyppönen et al. 2001).
Another study showed that a combined intake of 1200mg of calcium and 800 IU of vitamin D lowered the risk of type 2 diabetes by 33% as compared to a daily intake of less than 600 mg of calcium and less than 400 IU of vitamin D_3 (Pittas et al. 2006).

V. THE VITAMIN D STATUS IN AUSTRALIA

Though the importance of the physiological role vitamin D is widely accepted there is also an increased recognition that the vitamin D nutritional status of the human population is lower than optimum (Chapuy et al. 1997). The Working Group of the Australian and New Zealand Bone and Mineral Society; Endocrine Society of Australia; Osteoporosis Australia 2005 has defined vitamin D nutritional status in the population as follows: (NHMRC 2006)

Not deficient vitamin D	=	serum $25(OH)D_3$ greater than 50 nmol/L.
Mild deficiency vitamin D	=	serum $25(OH)D_3$ levels between 25 - 50 nmol/L.
Moderate deficiency vitamin D	=	serum $25(OH)D_3$ between 12.5 – 25 nmol/L.
Severe deficiency vitamin D	=	serum $25(OH)D_3$ less than 12.5 nmol/L.

In Australia, a survey was undertaken within three regions located at different latitudes, Southeast Queensland region, Geelong region and Tasmania. They found a vitamin D insufficiency or mild deficiency (≤ 50nmol/L) in 40.5% of women in Southeast Queensland, 37.4% in the Geelong region and 67.3% in Tasmania. It was concluded that

vitamin D insufficiency is common within Australia irrespective of the latitude (van der Mei et al. 2007). Van der Mei et al. suggested a need to pursue other means to achieve vitamin D adequacy in Australia. Details of this study are found in Figure 1.

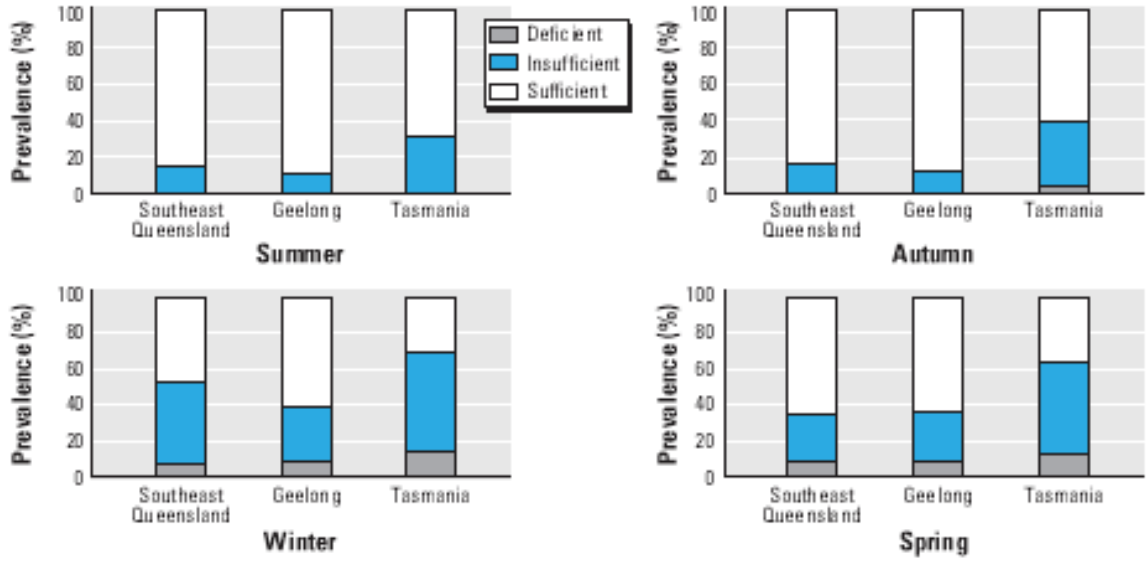

Figure 1 - Prevalence of vitamin D deficiency (≤25 nmol/L), vitamin D insufficiency (26-50 nmol/L), and vitamin D sufficiency (>50 nmol/L) for women <60 years of age in southeast Queensland (latitude 27°S), Geelong (latitiude 38°S), and Tasmania (41-43°S) by season.

The population groups of greater risk in Australia and New Zealand are the older persons, particularly those in institutionalμ care who may have a severe deficiency of 22-67% and a mild deficiency of 45-84%, and dark skinned peoples and veiled women may have a mild deficiency of vitamin D in up to 80% of cases (NHMRC 2006). People who always wear protective clothing, always use sunscreen and those who have intestinal, hepatic, renal or cardiopulmonary disease may be at increased risk (Fuller and Casparian 2001). It has been found that 57% of hospitalized patients have Vitamin D deficiency (Thomas et al. 1998).

VI. RECOMMENDED VITAMIN D ALLOWANCES

The Australian National Health and Medical Research Council (NHMRC) has published its recommendations for vitamin D intake based on life stage and gender (NHMRC 2006):

Age	AI Adequate Intake
0-12 months	200 IU/day
1-18 years	200 IU/day
19-50 years	200 IU/day
51-70 years	400 IU/day
>70years	600 IU/day

The NHMRC has recommended upper levels of intake of Vitamin D based on studies looking at the effect of vitamin D on serum calcium in humans. There is some animal evidence of oral Vitamin D causing non- calcified atherosclerosis of large arteries (Taura et al. 1979, Toda et al. 1985, Valdivielso et al. 2009) and therefore NHMRC remain cautious in their recommendations for high doses of vitamin D.

Age	Upper Limit of Intake (UL)
0-12months	1000 IU/day
1year to 18years	3,200 IU/day
Adults, including Lactation and Pregnancy	3,200 IU/day

The daily recommended dietary allowance (RDA) for vitamin D by the USA Food and Nutrition Board 2010 is 600 IU from 1-70 years of age, and 800 IU from 70 years of age. Egg yolks are a rich in vitamin D (Parrish and Richter 1979) because of an efficient transfer by the domestic fowl of vitamin D from the feed into the yolk and unlike other animal-based foods the egg yolk contains both D_3 and 25(OH)D_3. In fact eggs contain higher levels of 25(OH)D_3 than any other animal-based food such as fish and meat (Mattila 1995). The fact that eggs contain 25(OH)D_3 is significant because in human nutrition 25(OH)D_3 has five times the relative biological vitamin D activity as compared to D_3 (NHMRC 2006). 25-hydroxyvitamin D_3, is the principal metabolite of vitamin D found in circulating blood plasma in all vertebrate species and represents the major storage form of vitamin D with a half- life of 15 days (Horst and Littledike 1982). 25-hydroxyvitamin D_3 is available for dietary supplementation of livestock feed in the form of a commercial vitamin D supplement.

VII. EXPERIMENT 1

An experiment was conducted to investigate the effect of supplementing the diet of laying hens with additional D_3 and 25(OH)D_3 on the vitamin D content of the egg yolk, overall performance and the change of vitamin D content of egg yolk with time (Browning and Cowieson 2014). A total of 162 Isa-Brown laying hens of 58 weeks of age were divided into nine treatment groups of 18 birds with six replicates of three 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. Prior to the commencement of the trial all birds were fed for four weeks on the control diet with 2,500 IU D_3/kg (acclimatisation period). During the subsequent trial period of 9 weeks, the birds were fed a mash diet *ad libitum* with various levels of vitamin D_3 and 25(OH)D_3 as shown in Table 1. The photoperiod regime was 16 hours of light and 8 hours of dark. Water was supplied *ad libitum.*

In practical feeding of livestock and human supplementation, vitamin D quantities are usually expressed in terms of International Units (IU) where 1.0 µg vitamin D3 equals 40 IU. In human nutrition 25(OH)D_3 has five times the level of vitamin D potency as compared to vitamin D_3, that is 1.0 µg of 25(OH)D_3 equals 200 IU of vitamin D (NHMRC 2006).

Table 1 - Outline of dietary treatments.

Diet	Treatment	Added D_3* IU/kg	Added 25(OH)D_3**µg/kg
1	Normal D_3	2,500	0
2	Normal D_3 + 34.5 µg 25(OH)D_3	2,500	34.5
3	Normal D_3 + 69.0 µg 25(OH)D_3	2,500	69.0
4	Medium D_3	5,000	0
5	Medium D_3 + 34.5 µg 25(OH)D_3	5,000	34.5
6	Medium D_3 + 69.0 µg 25(OH)D_3	5,000	69.0
7	High D_3	10,000	0
8	High D_3 + 34.5 µg 25(OH)D_3	10,000	34.5
9	High D_3 + 69.0 µg 25(OH)D_3	10,000	69.0

*DSM Nutrition l Products Pty Ltd Rovimix® D_3 500
**DSM Nutritional Products Rovimix®HY·D®

At the end of the 9 week trial a total of five eggs per replicate (30 eggs per treatment) were collected and their yolks separated, weighed, blended and freeze dried prior to analysis for D_3 and 25(OH)D_3 content. The egg yolks were analysed at the government owned Australian Measurement Institute The average weight of the egg yolks per replicate was used to calculate the D_3 and 25(OH)D_3 content of egg yolk per replicate (6 replicates per treatment).

VIII. RESULTS

Table 2 - Effect of various levels of D_3 and 25(OH)D_3 supplementation on D_3, 25(OH)D_3 and total vitamin D content of egg yolk (Browning and Cowieson, 2014).

D3 (IU /kg)	25(OH)D3 (µg /kg)	D3 /100g egg yolk (IU)	25(OH)D3 /100g egg yolk (IU)	Total vitamin D† /100g egg yolk (IU)	Total vitamin D1 /egg yolk (IU)
2500	0	6.48^{cd} (±1.462)[2]	1.61^{f} (±0.757)	580^{e} (±201.7)	96^{f} (±33.1)
2500	34.5	5.96^{cd} (±1.444)	3.30^{d} (±1.448)	898^{d} (±315.2)	152^{e} (±50.2)
2500	69.0	4.90^{d} (±0.532)	4.49^{c} (±1.242)	1094^{cd} (±246.2)	185^{de} (±41.4)
5000	0	10.51^{c} (±2.270)	2.06^{ef} (±0.752)	832^{de} (±186.1)	146^{e} (±39.6)
5000	34.5	7.43^{cd} (±1.673)	4.51^{c} (±1.011)	1199^{c} (±261.5)	213^{cd} (±50.1)
5000	69.0	8.07^{cd} (±3.022)	5.81^{b} (±0.656)	1484^{b} (±172.1)	256^{bc} (±27.5)
10000	0	26.17^{ab} (±8.280)	3.01^{de} (±0.392)	1649^{b} (±325.1)	270^{b} (±56.4)
10000	34.5	23.60^{b} (±5.208)	3.71^{cd} (±0.481)	1686^{b} (±173.0)	285^{b} (±25.6)
10000	69.0	30.85^{a} (±5.384)	8.05^{a} (±1.117)	2845^{a} (±251.3)	478^{a} (±39.40)
Pooled SEM		*1.648*	*0.381*	*99.3*	*16.98*
Model P		*<0.01*	*<0.01*	*<0.01*	*<0.01*
		Main Effects			
2500		5.78^{c}	3.13^{c}	858^{c}	144^{c}
5000		8.67^{b}	4.12^{b}	1172^{b}	205^{b}
10000		26.87^{a}	4.93^{a}	2060^{a}	344^{a}
P		*<0.01*	*<0.01*	*<0.01*	*<0.01*
	0	14.39^{a}	2.23^{c}	1021^{c}	171^{c}
	34.5	12.33^{a}	3.84^{b}	1261^{b}	217^{b}
	69.0	14.61^{a}	6.12^{a}	1808^{a}	307^{a}
	P	*N.S.*	*<0.01*	*<0.01*	*<0.01*
		Interaction Terms			
D^3*25(OH)D^3		*N.S.*	*N.S.*	*P<0.01*	*P<0.01*

Means in columns with no common superscript differ significantly. †Total vitamin D (IU) calculated by addition of D_3 where 1.0 µg of D_3 equals 40 IU vitamin D and 1.0 µg of 25(OH)D_3 equals 200 IU vitamin D.

The total vitamin D level, expressed in terms of international units, is shown in Table 2. The addition of 25(OH)D_3 in the diet of laying hens not only significantly increased the 25(OH)D_3 content of egg yolk ($P < 0.01$) but also significantly increased the D_3 content of egg yolk ($P < 0.01$). Consequently there was a significant positive interaction ($P < 0.05$) between D_3 and 25(OH)D_3 when 25(OH)D_3 was included in the diet. 25-hydroxyvitamin D_3 appeared to increase the deposition of D_3 into egg yolk.

The highest concentration of 478 IU of vitamin D in the egg was achieved at the highest level of D_3 in combination with the highest level of 25(OH)D_3. There was no mortality in any treatment group during the trial and there was no significant difference between any of the nine treatments in respect to egg weight, egg mass, feed intake or feed conversion efficiency.

A second objective of this experiment was to determine the rate of change in total vitamin D content of yolk following additional vitamin D supplementation. Eggs were collected at 0, 3, 6 and 9 weeks and both the D_3 and 25(OH)D_3 content of their egg yolks were determined at each interval by the National Measurement Institute. The results are shown in Figure 2. The greatest change in vitamin D content of egg yolk was achieved in the first three weeks, after which there appeared to be a plateauing of total vitamin D content.

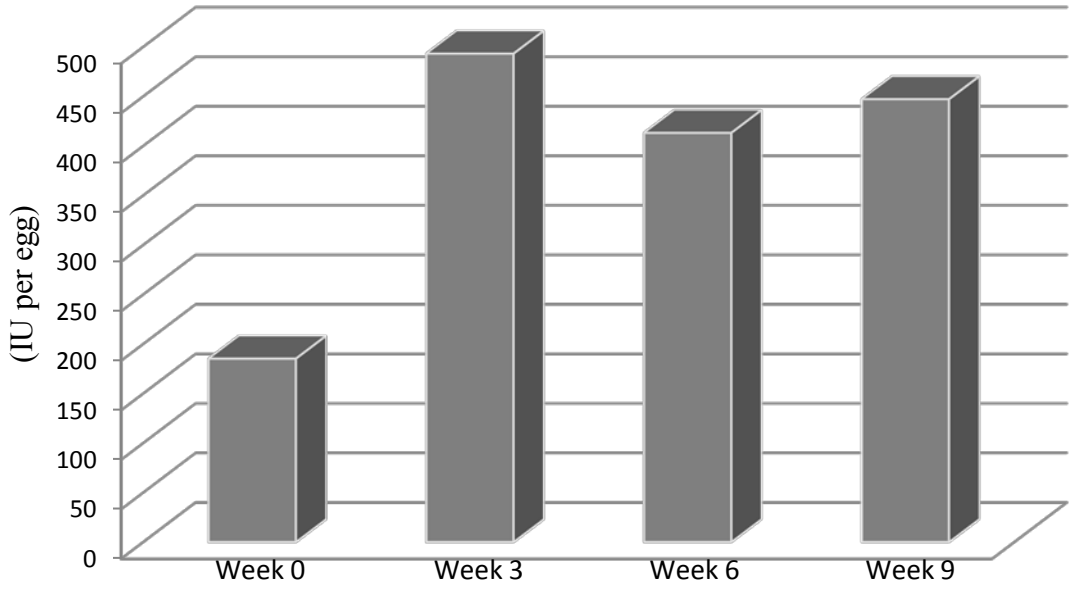

Figure 2 - Change in vitamin D content of yolk with time (Browning and Cowieson, 2014).

IX. EXPERIMENT 2

A second field experiment was conducted by Browning LC in 2014 to investigate the effect of cooking on the D_3 and 25(OH)D_3 content of egg yolk (unpublished data). Eggs were collected from ISA-Brown laying hens on a commercial laying farm. The birds were fed a diet supplemented with both D_3 and 25(OH)D_3. The cooking process was immersion in boiling water for 10 minutes. There were 15 samples of yolk analysed for both fresh and cooked yolks, and each sample was a homogenous blend of 10 egg yolks, that is 300 eggs were used in the 30 samples. The D_3 and 25(OH)D_3 content of the egg yolks for both fresh and cooked yolks were analysed by the Australian Government National Measurement Institute.

X. RESULTS

Table 3 – Effect of cooking on D_3, 25(OH)D_3 and total vitamin D content of egg yolk. (unpublished data)

	Average 25(OH)D_3 content per egg yolk (IU)	Average D_3 content per egg yolk (IU)	Average total vitamin D content per egg yolk (IU)
Fresh Yolk (n=15)	202 ± 12.5*	105 ± 15.0	307 ± 20.5
Cooked Yolk (n=15)	183 ± 12.5	87 ± 15.0	271 ± 20.5
P value (Probability)	NS	NS	NS

*Standard Error Mean

The effect on the vitamin D content of eggs placed in boiling water for 10 minutes is shown in Table 3. There was an approximate loss of 12% in total vitamin D content of egg yolk which was comprised of a 10% loss in D_3 and a 17% loss in 25(OH)D_3. It may be hypothesized that cooking for only five minutes in boiling in water (a more realistic scenario) may have reduced the loss of vitamin D metabolites. The reduction in the total vitamin D content of egg yolk with cooking is in line with previous research by Mattila et al. (1999) who found a 10% reduction in the vitamin D_3 content of egg yolk with cooking.

XI. DISCUSSION

The supplementation with higher levels of D_3 in combination with 25(OH)D_3 markedly increased the total vitamin D content of the egg. Furthermore, it was found that cooking reduced the total vitamin D content of egg yolk by approximately 10 to12%. In these studies the feeding of 5,000 IU D_3 in combination with 69 µg 25(OH)D_3 would produce an egg with approximately 250 IU of vitamin D which after cooking would produce an egg with about 220 IU of total vitamin D.

The current NMMRC recommendation for an 'Adequate Intake' of Vitamin D for children and adults up to 50 years of age is 200 IU of vitamin D. The feeding of 5,000 IU D_3 with 69 µg 25(OH)D_3 could produce a single enriched vitamin D egg which after cooking would meet the Adequate Intake for vitamin D as determined by the NHMRC. For those persons older than 50 years and 70 years, the consumption of at least one enriched vitamin D egg per day, would significantly contribute to the recommended 'Adequate Intake' of 400 IU and 600 IU respectively of vitamin D as recommended by the NHMRC.

XII. CONCLUSION

This research has shown the supplementation of the diet of the laying hen, with conservative, practical and inexpensive levels of vitamin D_3 and 25-hydroxyvitamin D_3 will significantly increase the vitamin D_3 and 25-hydroxyvitamin D_3 content of the egg. The daily consumption of one cooked egg, from a hen supplemented with 5,000 IU of vitamin D3 and 69 µg 25hydroxyvitamin D_3, would contribute significantly to the NHMRC daily recommended vitamin D intake. It is hypothesized that an improved vitamin D status within the general population would improve overall health by helping to reduce bone diseases, cancers and diseases associated with reduced immune function.

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ENRICHED VALUE ADDED EGGS FOR IMPROVED HUMAN HEALTH OUTCOMES

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Summary

Eggs are an easy and convenient vehicle to address nutritional deficiencies within the community. Layer hen eggs were enriched with iodine, vitamin D, lutein and a combination of all three nutrients to levels that enabled specific nutritional claims to be made for all treatments. Allowable health claims were restricted to the iodine and vitamin D enriched eggs in accordance with the Australia New Zealand Food Standards Code. Egg enrichment was achieved by feeding hens specific formulated diets for a period of six weeks. None of the experimental diets impacted negatively on egg production, feed intake, egg weight or egg quality. Hens receiving diets enriched with lutein had significantly higher yolk colour scores as compared to eggs from the other dietary treatments investigated. Sensory analysis of boiled and scrambled enriched and control eggs was done to assess consumer preference. Sensory analysis demonstrated that egg enrichment had no detrimental impact on consumer acceptability, and in fact for the attributes of appearance and aroma, egg enrichment increased consumer acceptability. For both hard boiled and scrambled eggs, there were no significant differences in flavour, texture, aftertaste or overall liking of enriched eggs as compared to control eggs. The outcomes of this work could put a competitive edge on value added "functional" eggs within the market.

I. INTRODUCTION

Functional foods are those that provide a demonstrated physiological benefit or reduce the risks of chronic disease above and beyond basic nutrition. They are a rapidly advancing area of the food industry due to consumers becoming increasingly concerned about overall health, as well as avoiding debilitating illnesses. An aging population and increased disposal income are assisting growth in the functional foods market. A review entitled "*Supercharged Eggs: Path to Market"* (SARDI internal report, Functional Foods Focus Program) was undertaken regarding the nutritional status of the Australian population to provide rationale for functional egg development. The most common nutritional deficiencies identified in Australia included calcium, iodine, iron, zinc, vitamin D and omega-3 fatty acids. In addition to considering the above nutrients, carotenoids (specifically lutein) were also examined as potential targets for egg enrichment due to their reported role in eye health. Based on the literature review, the following novel functional eggs were identified: 1) eggs enriched with lutein, which protect eyes from harmful high-energy blue light and oxidative damage and could be targeted at elderly Australians; 2) eggs enriched with iodine for optimising iodine intake of childbearing aged women, in particular pregnant and lactating women with increased iodine requirements; 3) eggs enriched with vitamin D to reduce prevalence of vitamin D deficiency, especially in the winter months, and among institutionalised individuals with limited mobility who have limited sun exposure, and the elderly who have reduced ability to synthesise vitamin D from sunlight; 4) and eggs enriched with all of the above nutrients, to improve intakes of all nutrients and, therefore, potentially provide multiple health benefits.

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II. MATERIALS AND METHODS

All experiments were conducted in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (NHMRC, 2004). Hyline Brown layer hens were fed either a control commercial diet or one of four experimental diets (SARDI proprietary formulation) enriched with iodine, vitamin D, lutein or a combination of all three nutrients for six weeks during late lay (commencing at 65 weeks of age). Hens (n=216) were housed in layer cages (n=3 birds/cage) at SARDI Pig and Poultry Production Institute, Roseworthy Campus and received the control diet (n=16 cages) or one of four experimental diets (n=14 cages/diet). During the feeding trials, egg production, feed intake, hen weight gain over the trial and feed conversion ratio (FCR; g feed/g egg produced) were measured to assess bird performance. For egg quality, eggs for compositional and sensory analysis were collected over a week during the sixth week of feeding the experimental diets. Egg quality measures taken were: egg weight; albumen quality (Haugh Units); yolk colour (Roche colour fan); shell thickness; and shell weight as a percentage of whole egg weight. Egg quality measures were done eight weeks after egg collection and eggs were stored at 12^{o}C. Chemical analysis was done on eggs stored at 12^{o}C for four weeks before whole egg contents were pooled (n=3 eggs/cage), frozen, freeze dried and sent to National Measurement Institute (Sydney, Australia) for analysis of iodine, vitamin D3, 25(OH)D3 and lutein. Sensory analysis (consumer acceptance) was done on both boiled and scrambled eggs from the layer trial at SARDI Food Safety and Innovation Sensory Laboratory. A total of 12 sensory sessions were held over 3 days, with a total of 120 consumers participating in the tastings (n=60 consumers/cooking method). Consumers were presented with one sample at a time and asked to score their liking of each sensory attribute (appearance, aroma, flavour, mouthfeel/texture, aftertaste), as well as their overall liking. Sensory and egg compositional data were statistically analysed using R software (v3.1.3, R Core Development Team, 2015). Egg production and quality data were analysed by Analysis of Variance (ANOVA) using a generalised linear model (GLM), with differences between treatments determined using Duncan's multiple range test (SAS for window v9.1). Nutritional and health claims for enriched eggs were evaluated against Standard 1.2.7 within the Australia New Zealand Food Standards Code (Food Standards Australia New Zealand, 2016).

III. RESULTS

There were no significant ($P > 0.05$) differences in egg production (hen day egg production %) or hen performance (hen start weight, end weight, weight gain or feed conversion ratio) associated with feeding the experimental diets. Overall mean hen day egg production was 83.7%, while mean feed intake and feed conversion ratio were 132.58 gm and 2.23, respectively. Yolk colour was the only significant difference in egg quality measures (Table 1). Egg yolk from hens receiving diets supplemented with lutein had significantly higher yolk colour scores as compared to egg yolks from birds receiving the control, iodine or vitamin D supplemented diets.

Compositional analysis of eggs from the trial are shown in Table 2. Birds fed diets enriched with iodine had significantly higher levels of iodine (iodine and combination treatment groups) in the egg as compared to other treatments. Likewise, eggs obtained from hens supplemented with lutein (lutein and combination treatment groups) had significantly higher levels of lutein as compared to other treatments. 25(OH)D3 was significantly higher in enriched eggs from hens receiving the combination diet, as compared to all other treatments.

Significant differences in consumer sensory preference (appearance and aroma) were detected among hard boiled enriched egg treatments (Table 3). The combination enriched eggs had significantly higher liking scores on average as compared to the control, iodine and

Table 1 - Egg production.

Diet	Egg weight (gm)	Shell thickness (micron)	Yolk colour (Roche)	Shell weight % of egg weight	Haugh Units
Control (n=24)	61.02 ± 4.61	352.50 ± 28.93	10.25 ± 1.11[b]	9.13 ± 0.71	47.54 ± 12.17
Iodine (n=21)	61.79 ± 4.15	352.86 ± 38.62	10.76 ± 0.62[b]	9.23 ± 1.02	42.86 ± 13.12
Vitamin D (n=21)	59.73 ± 4.70	341.43 ± 33.06	10.05 ± 1.86[b]	9.00 ± 0.84	48.86 ± 16.88
Lutein (n=21)	60.18 ± 5.77	338.10 ± 36.83	12.14 ± 0.96[a]	8.82 ± 0.87	48.19 ± 14.61
Combination (n=21)	61.79 ± 4.15	346.19 ± 29.06	12.14 ± 1.11[a]	9.17 ± 0.79	49.67 ± 10.54
P value	0.0817	0.4751	0.0001	0.3308	0.6824

Data are expressed as mean ± standard deviation.
Values within a row that do not share a common letter are significantly different.

Table 2 - Compositional analysis of enriched and control eggs.

Analysis	Hen Diet					
µg/egg[#]	Control	Iodine	Vitamin D	Lutein	Combination	*P* value
Iodine	20.04±3.09[a]	275.18±40.33[b]	22.78±3.19[a]	23.00±3.34[a]	273.07±22.21[b]	< 0.0001
Vit D3	0.50±0.16	0.39±0.11	0.44±0.38	0.38±0.11	0.40±0.25	0.7037
25(OH)D3	0.28±0.10[a]	0.27±0.08[a]	0.41±0.09[ab]	0.29±0.05[a]	0.44±0.09[b]	0.0027
Lutein	29.49±4.68[a]	49.12±8.65[a]	53.09±12.77[a]	1342.23±85.64[b]	1361.29±264.87[b]	< 0.0001

Data are expressed as mean (µg/egg) ± standard deviation.
Values within a row that do not share a common letter are significantly different.

Table 3 - Consumer acceptability scores of hard boiled enriched and control eggs.

Attribute	Treatment					
	Control	Iodine	Lutein	Vitamin D	Combination	*P* value
Appearance	4.32 ± 1.71[a]	5.23 ± 1.81[bc]	5.75 ± 1.87[cd]	4.72 ± 1.73[ab]	6.20 ± 1.68[d]	<0.001
Aroma	5.27 ± 1.42[a]	5.45 ± 1.31[ab]	5.63 ± 1.45[ab]	5.50 ± 1.43[ab]	6.05 ± 1.17[b]	0.026
Flavour	5.73 ± 1.54	6.30 ± 1.53	6.15 ± 1.52	6.43 ± 1.44	6.08 ± 1.48	0.116
Texture	5.73 ± 1.74	5.73 ± 1.77	5.98 ± 1.62	5.95 ± 1.96	6.03 ± 1.41	0.794
Aftertaste	5.55 ± 1.63	5.87 ± 1.61	5.85 ± 1.62	6.22 ± 1.35	5.70 ± 1.51	0.190
Overall liking	5.48 ± 1.64	5.87 ± 1.70	6.02 ± 1.62	6.15 ± 1.54	6.02 ± 1.48	0.187

Mean consumer acceptability score ± standard deviation.
Values within a row that do not share a common letter are significantly different (P<0.05).

vitamin D enriched eggs for appearance. The lutein enriched eggs also scored significantly higher liking for appearance on average as compared to the control and vitamin D enriched eggs, and the iodine enriched eggs scored higher for liking in appearance than the control eggs. There were also statistically significant differences in aroma with the combination enriched eggs having significantly higher liking scores on average than the control eggs.

For the scrambled eggs, a significant difference in appearance was observed among treatments ($P = 0.043$), however, no significant pairwise differences were observed. The combination enriched eggs scored the highest for appearance, while the vitamin D enriched eggs scored the lowest. No significant differences were observed for any of the other sensory attributes investigated for scrambled eggs.

IV. DISCUSSION

The average cost of egg production in Australia is around 95 cents per dozen or $1.45/kg (Poultry Hub 2015). The main factors influencing egg production are: cost of feed ingredients; cost of rearing pullets from day-old to point of lay, or purchasing pullets at point of lay; level of mechanisation on the farm; and hen mortality. Supplementing layer hen diets with vitamin D, iodine and/or lutein did not impact negatively on egg production or performance of layer hens. The only factor that would be influenced is the price of additional feed ingredients. Based on the enrichment levels targeted in this project, additional costs per tonne feed would range from less than 1% for vitamin D enrichment to 18% for a combination iodine, vitamin D and lutein enrichment. Consumer surveys have indicated that > 60% of US consumers are prepared to purchase enriched eggs and > 70 % would pay US$0.50 more per dozen (Singh et al., 2012).

Egg enrichment had no detrimental impact on consumer sensory acceptability of the product and in fact for the attributes of appearance and aroma, egg enrichment increased consumer acceptability. Consumers significantly preferred the appearance of hard boiled eggs enriched for iodine, lutein and a combination of iodine, lutein and vitamin D as compared to control eggs. Consumers also significantly preferred the aroma of hard boiled eggs enriched with a combination of iodine, lutein and vitamin D as compared to control eggs. For both hard boiled and scrambled eggs, there were no significant differences in flavour, texture, aftertaste or overall liking of enriched eggs as compared to control eggs.

Nutrient content claims could be made for all the enriched eggs, although allowable health claims were restricted to the iodine and vitamin D enriched eggs. A nutrient content claim is a claim regarding the presence or absence of a nutrient or biologically active substance. A general level health claim is a health claim that does not refer to a serious disease or biomarker of a serious disease. Currently, the Australia New Zealand Food Standards Code does not allow for any health claims about lutein that link its presence in eggs with a health effect, unless the claim is substantiated by a systematic review.

The gross value of Australian and South Australian egg production has experienced consistent growth in recent years. While the gross value of egg production in Australia increased by 8.7% from 2012-13 to 2013-14, South Australia alone experienced a growth of 31.4% during this same period (Australian Bureau of Statistics, 2015). The estimated value of egg production at farm gate prices in 2013-14 was AUD$625.5 million for Australia and AUD$33.4 million for South Australia; an increase of 10.9% and 48.4% since 2012-13, respectively. Outcomes of the project could put a competitive edge on value added eggs within the market, with valid functional claims, while helping to address some nutritional deficiencies within the community.

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QUANTIFICATION OF MITOCHONDRIAL COUNT AND PROTOPORPHYRIN IX ASSOCIATED GENE EXPRESSION IN RELATION TO DIFFERENT STAGES OF EGGSHELL FORMATION AND NICARBAZIN CHALLENGE IN THE SHELL GLAND OF LAYING HENS

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Summary

Mitochondrial count per cell and genes involved in the synthesis of protoporphyrin IX (PP IX) were quantified from the shell gland tissue of laying hens at different time-points (TP) and under nicarbazin (N) challenge (TP+N) in relation to eggshell formation. PP IX was measured in 1 g of tissue and of eggshell. TP+N challenge had no significant effect on mitochondria per cell or expression of PP IX synthesis related genes between the control and N groups except for *ALAS1*. The mitochondria per cell were significantly lower in the N group at the 15 hr time-point. In both the control and N groups, the expression levels of all genes except *CPOX* were significantly affected by different time-points. PP IX per gram of tissue was significantly lower in the N group compared with the control group. PP IX in the eggshell decreased linearly over time in the N group, but remained constant in the control group. Egg weight and shell thickness were similar in the control and N groups. In conclusion, mitochondria per cell did not vary significantly with different stages of eggshell formation, nor with the expression levels of genes associated with PP IX synthesis and/or deposition. Feeding nicarbazin caused down regulation of *ALAS1* that resulted in reduced production of PP IX in the shell gland tissue and in eggshells.

I. INTRODUCTION

In commercial laying hens, regular egg formation can dramatically change the energy metabolism of key organs such as the oviduct, liver and adipose tissue. At the cellular level, alterations of nutrient and energy requirements coincide with changes in the mitochondria, the main site of high-yielding ATP-generating reactions in cells (Lentz et al., 2010). The mitochondrial genome is multi copy per cell and their number varies depending on energy demands of a cell, the organ, the age, and sex of the organism, and patho-physiological condition (Fuke et al., 2011; Phillips et al., 2014). Chicken mtDNA has been relatively less studied compared to other species. In the epithelial cells of the shell gland, mitochondria are of particular interest, owing to the high demand for energy used in the biogenesis of the eggshell. Nicarbazin produces reversible pharmacological effects by causing complete discolouration of brown egg pigment, which is dosage dependent (McClary, 1955; McLoughlin et al., 1957). Thus, this model can be easily reproduced in studies to investigate effects of nicarbazin on eggshell formation in laying hens. Understanding the molecular basis for the effect of nicarbazin may also provide an understanding of the mechanisms by which shell colour decreases in response to other factors which have been less well-defined.

II. MATERIALS AND METHODS

Oviposition time was recorded by video camera, eggs were collected and shell colour (L*) was measured with a spectrophotometer using the L*a*b* colour space system. The L* measurements represent the intensity of brown eggshell colour; the higher the value, the lighter the colour of the eggshell. The post-oviposition times (time-points) were 5, 15 and

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23.5 hr for the four groups of hens selected. The hens were selected and divided in such a way that the mean values of the variables measured were not significantly different among the groups. Hens were killed with CO_2 at specific time-points and shell gland tissue was collected and stored in RNALater at -20°C until further processing. Primers were either sourced from published literature or designed in NCBI database (Table 1).

a) Total DNA and RNA extraction

Total DNA and RNA were extracted with TRIsure (Bioline, Australia) protocol. The quantity of total DNA and RNA in 2 µL of each sample was determined by optical density readings in a NANODROP-8000 spectrophotometer. In order to achieve absolute quantification of mtDNA, a recombinant plasmid vector was constructed by cloning 137 bp fragments of each of the mtDNA and gDNA, using TOPO® TA Cloning® Kit for sequencing (LifeTechnolgies, Australia) as per the manufacturer's protocol. The PureLink® Quick Plasmid Miniprep Kit (ThermoFisher Scientific, Australia) was used for the extraction of recombinant plasmid DNA. The extracted total RNA was purified using an RNeasy Mini Kit as per the manufacturer's instructions.

b) Quantitative PCR

For absolute quantification of mtDNA, qPCR was performed on DNA samples in duplicate. With each PCR run, recombinant plasmid DNA (10^{-2}~10^{-7} dilutions) was included in the same gene disc. The master-mix was prepared as per the protocol of the SensiFAST™ SYBR® No-ROX Kit (Bioline, Australia). No template control (NTC) was also included to detect possible contamination. Thermocycling conditions for a 2-step PCR were: polymerase activation at 95°C for 3 minutes, denaturation at 95°C for 5 seconds and annealing at 60°C or 63°C for 30 seconds. The PCR products were examined on a 1.5 % agarose gel to estimate the size of the amplicons for specificity. PCR amplification efficiencies and correlation coefficients (R^2) were determined with the amplifications of a series of six 10-fold dilutions. The qPCR data for the genes were processed further when the PCR amplification efficiency was in a range of 94 % to 105 %, and linear correlation coefficient $R^2 > 0.980$ were considered of high standard.

For the gene expression studies, samples were run in triplicate along with the NTC and no reverse transcriptase (-RT) control. Master-mix was prepared as per manufacturer's protocol using the SensiFAST SYBR® Lo-ROX One-Step RT-PCR Kit (Bioline, Australia). The PCR products were examined on a 1.5 % agarose gel as described earlier.

c) Measurement of protoporphyrin IX in tissue and eggshell

A 0.25 g whole piece of tissue was weighed into a 10 mL tube containing 4 mL of 3N HCl. The tissue was homogenized and stored in a refrigerator for 3 hours. The tissue tubes were centrifuged at 800 ×*g* for 30 minutes at 4°C and the absorbance of the supernatant was read in a spectrophotometer at PP IX specific absorbance wavelength of 412 nm. Standard dilutions (0 to 6.87 nM) prepared from protoporphyrin IX di-sodium salt were read at the same wavelength in order to construct a standard curve for the calculations of the amount of PP IX per gram of tissue. For measuring the amount of PP IX in the eggshells, the method described by Samiullah and Roberts (2013) was followed.

Table 1 - Base sequence of the primers used in this study.

Gene names	Abbrev. used	Primer sequence (5-3)	Amplicon length (bp)	Annealing temperature (°C)	Accession No.	Reference
NADH dehydrogenase subunit 4	ND4[a]	F:CGCAGGCTCCATACTACTCG R:TTAGGGCACCTCATAGGGCT	137	60	NC_001323.1	this study
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*[b]	F: GGTCACCAAGAAGGTGGAGA R: GACAGTGCCCTTGAAGTGTC	137	63	NC_006088.3	this study
SLC25A38 solute carrier family 25, member 38	*SLC25A38*	F: AGACACGGTATGAGAGTGGA R: ATCCCAGAGAAAGGTGCGTC	139	63	XM_418818.3	this study
5-aminolevulinic acid synthase	*ALAS1*	F: GGTGGACAGGAAAGGTAAAGA R: ACTGGTCATACTGGAAGGTG	197	60	NM_001018012.1	Li et al., 2013
ATP binding cassette subfamily C member 6	*ABCB6*	F: CTCAACTGGTTCGGCACCTA R: TTCACTGCATCCTTCACCTCC	107	60	XM_015290086.1	this study
Coproporphyrinogen oxidase	*CPOX*	F: GAGAGGACGGTATGTGGAGT R: TTTGGGATTGCGGAGAAC	187	60	XM_004938236.1	Li et al., 2013
ATP binding cassette subfamily G member 2	*ABCG2*/ BRCP	F: CCTCCTTGTAAACCTCCCTT R: GTAATCTTCACCAGAGCACCTT	208	65	XM_421638.4	Li et al., 2013
Ferrochelatase	*FECH*	F: TGCTTTGCCGATCACAT R: CACGGTTCACCACAGACAT	112	63	U68033.1	Li et al., 2013
Feline leukemia virus subgroup C cellular receptor 1	*FLVCR*	F: ACAACAGACTACAGTCCTCGTGC R: ATTGTGCGTTTCTAAGCCATCT	239	60	ENSGALG00000 009807	Zheng et al., 2014
Hydroxymethylbilane synthase	*HMBS*	F: GGCTGGGAGAATCGCATAGG R: TCCTGCAGGGCAGATACCAT	131	60	XM_417846.2	Yin et al., 2011
Hypoxanthine Phosphoribosyltransferase 1	*HPRT1*	F: ACTGGCTGCTTCTTGTG R: GGTTGGGTTGTGCTGTT	245	60	NM_204848.1	Yang et al., 2013

[a]Gene was used to amplify mtDNA; [b]Gene was used to amplify gDNA.

d) Statistical analysis

Mitochondria were enumerated by the quantification of their DNA copies in a cell and genomic DNA copies were used to represent cell numbers in the samples (Miller et al., 2003). The cloned plasmid DNA with ND4 and GAPDH genes inserts were converted into plasmid DNA copies/µL in six different dilutions for analysis. Plasmid copy number was calculated based on the concentration of plasmid DNA and its molecular weight. The cloned plasmid DNA amplification cycle (Cq) values were then used to construct a standard curve to calculate the mtDNA and gDNA copies per diploid cell. The mtDNA copies were divided by the 2 × gDNA copies to get the absolute copy number of mtDNA/diploid cell.
For the gene expression studies, raw Cq values were imported into qbase+ version 3.0 (Biogazelle, Belgium) and analysed against the two reference genes. The normalized relative quantities (NRQ) values were exported and analysed in SPSS using GLM. All other parameters data were analysed in SPSS using GLM module unless stated.

III. RESULTS

The mitochondria per cell in the shell gland tissue was not significantly different ($P>0.05$) between the control and nicarbazin challenge groups, and there was no significant effect of different time-points (Figure 1A). However, there was a statistically significant interaction ($P<0.05$) between the time-points and nicarbazin challenge (Figure 1B).

The mitochondria per cell were significantly lower at the 15 hr time-point in nicarbazin challenge group compared with the control group at the same time-point. The mean expression levels of all seven genes studied were not significantly different in the control and nicarbazin challenge hens except for *ALAS1* (Table 2). All the genes except for *CPOX* were significantly affected by time-points, both in the control and nicarbazin challenge groups. Different genes expressed differently at different time-points. The expression levels of *SLC25A38* and *FLVCR* was significantly higher at 15 hrs both in the control and nicarbazin challenge groups. The expression level of *ALAS1* was significantly higher at 15 hrs compared with the 5 and 23.5 hrs in the control group. However, in the nicarbazin challenge groups, *ALAS1* expression level showed no significant difference between 5 and 15 hrs. The expression level of *ABCG2* was significantly higher at 5 hrs compared with both the 15 and 23.5 hr groups in the control and in the nicarbazin challenge groups. The expression level of *FECH* was the same in the control and nicarbazin challenge groups, occurring at 23.5 hrs.

There was a significantly lower amount of PP IX per gram of shell gland tissue in the nicarbazin challenge compared with the control groups.

In the control groups, the amount of PP IX significantly increased at the 23.5 hr post-oviposition time whereas, in the nicarbazin challenge groups, the amount of PP IX declined (Figure 1C,D). Egg weight and shell thickness were not significantly different between the control and nicarbazin challenge groups.

Within each treatment, superscript letters (^{a,b,c}) across a row denote significantly different results. TP is time-point, while N denotes nicarbazin challenge.

Table 2 - Mean expression levels of candidate target genes.

Gene	Control			Nicarbazin			P value		
	Time-point (hr)			Time-point (hr)					
	5	15	23.5	5	15	23.5	TP	N	TP*N
SLC25A38	0.751 ±0.06^{b}	1.586 ±0.06^{a}	0.791 ±0.08^{b}	0.757 ±0.05^{b}	1.824 ±0.10^{a}	0.842 ±0.10^{b}	<0.0001	0.1567	0.3481
ALAS1	1.344 ±0.11^{b}	1.667 ±0.07^{a}	0.600 ±0.03^{c}	1.212 ±0.09^{a}	1.276 ±0.06^{a}	0.509 ±0.12^{b}	<0.0001	0.0104	0.2148
ABCB6	1.327 ±0.11^{a}	1.003 ±0.11^{ab}	0.866 ±0.08^{b}	1.322 ±0.12^{a}	1.033 ±0.07^{a}	0.732 ±0.11^{b}	0.0002	0.6805	0.7290
CPOX	1.080 ±0.06	1.016 ±0.05	0.958 ±0.08	1.059 ±0.06	1.042 ±0.01	0.899 ±0.04	0.0700	0.7199	0.7765
ABCG2	1.220 ±0.09^{a}	0.945 ±0.04^{b}	0.952 ±0.11^{b}	1.468 ±0.15^{a}	0.803 ±0.06^{b}	0.868 ±0.12^{b}	0.0002	0.9335	0.1560
FECH	0.749 ±0.04^{b}	0.668 ±0.03^{b}	1.792 ±0.06^{a}	0.820 ±0.03^{b}	0.770 ±0.03^{b}	1.817 ±0.08^{a}	<0.0001	0.1467	0.7750
FLVCR	0.806 ±0.03^{c}	1.396 ±0.09^{a}	1.007 ±0.07^{b}	0.777 ±0.04^{b}	1.125 ±0.08^{a}	1.099 ±0.17^{a}	0.0002	0.3850	0.1792

Values are mean of normalized relative quantities (NRQ) ± standard error. Relative quantities for individual gene are scaled to the average across all unknown samples per target gene.

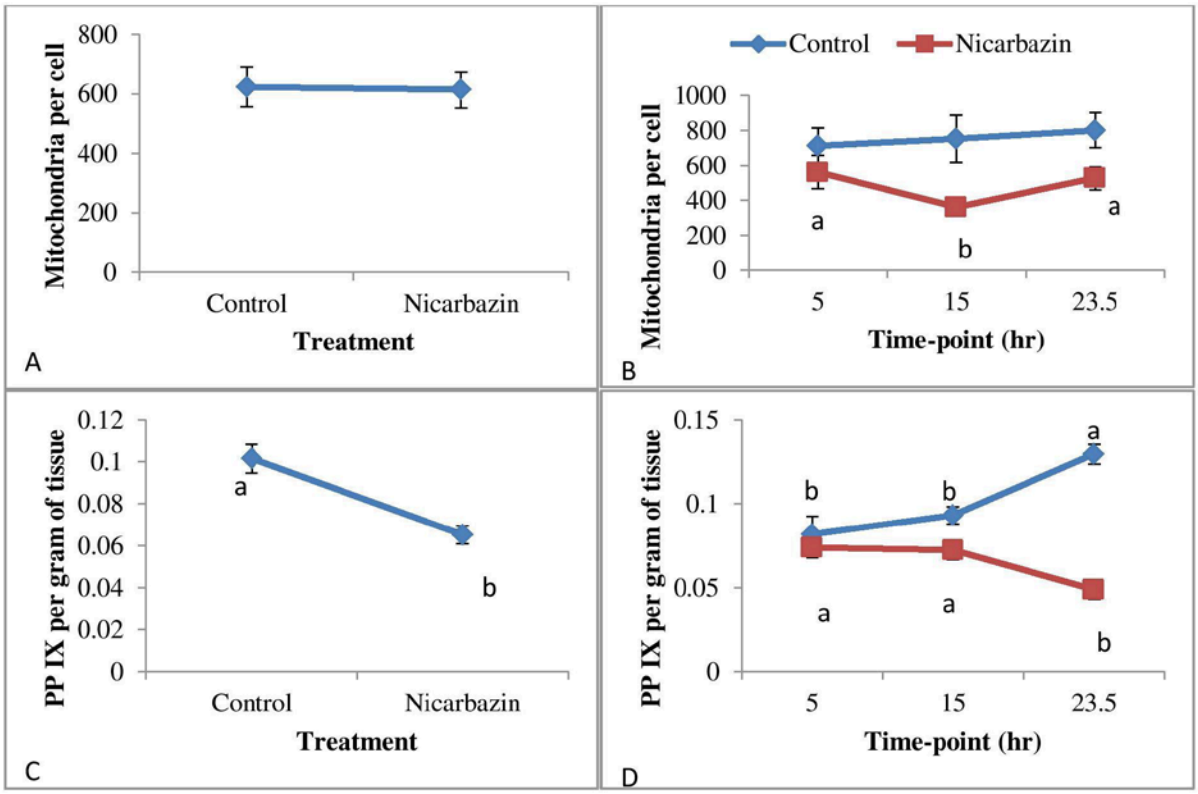

Figure 1 - Mitochondria per cell and PP IX per gram of shell gland tissue affected by time-points of eggshell formation and nicarbazin challenge. A). Mitochondria per cell in the shell gland tissue between control and nicarbazin challenged laying hens. B). Mitochondria affected by different time-points and nicarbazin challenge. C). PP IX (in nM) per gram of shell gland tissue between the control and nicarbazin challenged hens. D). PP IX affected by three different time-points and nicarbazin challenge.

IV. DISCUSSION

Comparing the gene expression data between control and nicarbazin challenge groups, *ALAS1* was the only gene affected by nicarbazin. Nicarbazin inhibited *ALAS1* expression and thus a lower amount of PP IX was produced in the shell gland. A significantly higher level of PP IX in tissue in the control group, compared with the nicarbazin group, further indicates that nicarbazin can disrupt the mechanism involved in PP IX synthesis. The current model suggests that nicarbazin disrupts PP IX synthesis by affecting the expression levels of the *ALAS1* gene. The linear decrease in PP IX per gram of shell indicated that PP IX production declined with feeding of the drug on consecutive days.

The non-significant difference in egg weight and shell thickness between the control and nicarbazin challenge groups indicates that nicarbazin does not affect genes involved in eggshell formation including the transport of calcium and phosphorus across the shell gland cells. However, these variables were not measured directly, and the nicarbazin was fed only for a short period of time.

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PREDICTING FOOD INTAKE IN LAYING HENS AND ITS IMPLICATIONS FOR IMPROVING ECONOMIC EFFICIENCY

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Summary

Predicting food intake is the key to being able to optimise the feeding of farm animals. To predict food intake in any animal it is necessary to know what the animal is attempting to achieve, i.e. it is necessary to describe its genetic potential. In the case of a laying hen this requires an understanding of systems such as the attainment of sexual maturity and the physiological control of egg production. The potential performance, that is, the number of eggs produced over the production cycle of a single laying hen, is dependent on the age at which it becomes sexually mature, on its internal ovulatory cycle length and egg weight, and the rates at which these change over time, all of which are genetically determined and may be satisfactorily modelled. Using stochasticity, the potential laying performance of a flock may then be simulated from these individual responses. It is then possible to predict the amount of energy and of each nutrient that is required to meet the maintenance and potential laying performance of each hen so that the voluntary intake of a feed of any given composition can be predicted and the consequences on performance can be determined. The constraining effect of high temperature on food intake and performance must be incorporated into such a model. It may not be economically justifiable to meet the requirements of the most demanding hens in a population: the economic optimum intake of nutrients would depend on the relative cost of feeding these birds and the revenue derived from the sale of the eggs they produce. The optimisation process takes account of all these factors in maximising or minimising the objective function, which would be expected to differ between laying hens and broiler breeders, for example.

I. INTRODUCTION

By describing the potential performance of a flock of commercial laying hens it is possible to calculate the daily nutrient intakes required to meet the potential performance of each individual in terms of numbers and weights of eggs produced over the production cycle. This would improve the possibility of optimizing the composition of the feed offered to the flock. In order to achieve these goals, a comprehensive understanding is required of the factors influencing the attainment of sexual maturity in these birds, of the ovulatory cycle and how this changes during the laying cycle, of the changes that occur in egg and body component weights over time and, ultimately, of the physiological and environmental factors that may prevent each bird from consuming sufficient of a given feed to meet its potential performance each day. Without a comprehensive simulation model that incorporates all these concepts it is unlikely that the consequences of offering feeds of different quality to flocks of laying hens kept in different environments on the rate of egg production and the weight and composition of the eggs produced could be accurately predicted. Nor is there a better way of optimizing the feeding of these birds than being able to predict these consequences before the feed is offered to the flock.

Much of the information required to develop such a model has been published and many of the relevant publications will be referred to here. But there are still some concepts that have not been researched, where further information would be valuable. Thus, by developing a simulation model for predicting responses in laying hens the relevant

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information from the literature has been synthesized into a workable theory for each system, the various systems have been integrated and gaps in our knowledge of these systems have been identified.

II. PREDICTING THE AGE AT SEXUAL MATURITY

The age and body weight of a bird on the day it lays its first egg has a very strong influence on future egg weight and the number of eggs laid, which are important considerations for both the layer and broiler breeder industries. These characteristics can be modified by lighting and/or the nutritional control of growth: in full-fed, egg-type hens, a ten-day delay in sexual maturity that has been achieved through a lighting programme results in an increase of 1.3 g in mean egg weight and a reduction of seven eggs over 52 weeks lay, but the total egg output will be similar (Lewis and Morris, 2006). Clearly, to predict the laying performance of a hen, her age at sexual maturity must first be defined, and this can be predicted.

Important considerations in predicting sexual maturity in hens are that gonadal development will take place whatever lighting programme is used, that lighting modifies the age at sexual maturity, that changing photoperiods have a greater influence than do constant photoperiods (Lewis and Morris, 2006), and that the response of a broiler breeder to light differs from that of a commercial laying hen because broiler breeders, unlike commercial laying hens, exhibit photorefractoriness (Lewis *et al*., 2003). The attainment of sexual maturity is therefore under both genetic and environmental influences, with broiler breeders still exhibiting photorefractoriness, while this has been eliminated in laying pullets by selection.

In full-fed commercial pullets, lighting is the most important environmental factor influencing age at first egg (AFE) (Lewis *et al*., 2002). When pullets are reared under constant daylengths the length of the photoperiod used can influence AFE (Lewis *et al*., 1998), and when one or two changes are made to the daylength during rearing, the length of each photoperiod also has an influence (Lewis and Perry, 1994; Lewis *et al*., 1996). While the initial and final photoperiods are the principal components of a lighting programme influencing AFE in full-fed pullets, the effects of a given change in photoperiod are not the same at all ages. Also, the advance in AFE for birds started on 8-h photoperiods and given a single increment in photoperiod at a defined age is proportional to the size of the increment up to about 13 h (Lewis *et al*., 1998), but not for longer final photoperiods (Lewis *et al*., 1996).

Lewis *et al*. (2002) proposed a model to predict AFE of full-fed pullets when changes were made to the photoperiod during rearing. The four components of the empirical model, each of which is calculated separately, deal with: (i) the genetic differences in AFE in birds maintained on constant photoperiods from hatching; (ii) the change in AFE as a function of age at transfer to the final photoperiod; (iii) the acquisition of sensitivity to increases in photoperiod in the young pullet; and (iv) the onset of spontaneous rapid gonadal development, that is, the proportion of birds maturing under the influence of the initial photoperiod, without responding to a late change in photoperiod. Lewis and Morris (2008) then modified their original model to accommodate subsequent evidence related to the effect of follicle stimulating hormone (FSH) on the process of sexual maturity. They found that when two opposing changes in photoperiod are given within an interval of <30 days, rate of sexual maturation is determined by the change in circulating FSH concentration achieved during the time of the second photoperiod. If a decrease in photoperiod is given within a week of an increase, a period in which circulating FSH concentration rises very little, AFE will not be significantly different from constant short-day controls.

This empirical model for commercial laying pullets enables the prediction of AFE for individuals making up a laying flock. Using appropriate means and standard errors for each of the parameters in the models it is possible to allocate randomly an AFE to each bird in the simulated flock, which contributes to its potential rate of laying, as will be described in the next section.

III. MODELLING POTENTIAL EGG OUTPUT

Describing the potential rate of lay of a laying hen is complex because of the number of interacting factors involved, and the fact that the potential varies over time within each individual. The mathematical model of Etches and Schoch (1984), based on the theory of Fraps (1955), demonstrated that two functions, representing two independent but interacting systems of the hen's asynchronous ovulatory cycle, were able to predict realistic ovulation times and intra-sequence ovulation intervals. Johnston and Gous (2003) extended this model by defining a set of continuous functions, representing the changes required to the values of the different parameters, such that the prediction of any sequence length is possible.

Mean rate of lay in a flock of hens at a particular age is determined by the individual patterns of sequential laying at that time. Within a population of birds, individuals of the same age show considerable variation about a mean sequence length, which may be due to variation in the length of the open period for luteinizing hormone release, or variation in follicular dynamics. This variation may be accounted for using mean values and standard errors for each of the parameters in the model (Johnston and Gous, 2003). Such a population of birds would generate a range of ovulation times, the distribution of which is unimodal and positively skewed in young hens, becoming bimodal with age. Reproductive senescence in hens manifests as an increase in the intra-sequence ovulation and oviposition intervals with time, as well as an increase in the number of pause days.

Different approaches have been used to model the decline in rate of lay over time. Most of these have been empirical in nature (Gavora *et al*., 1971; McNally, 1971; McMillan *et al*., 1986; Foster *et al*., 1987; Yang *et al*., 1989; Koops and Grossman, 1992; Fialho and Ledur, 1997), all of which are severely limiting when making use of a mechanistic approach for describing the decay in the rate of laying of an individual hen over time. Emmans and Fisher (1986) suggested that the hen's internal cycle length increased with time from first egg, resulting in a linear decline in the rates of ovulation and oviposition with age. They suggested that, at the start of the laying period, some hens had the capacity to lay at a rate greater than one egg in 24 h, but that laying performance of these birds was constrained by the external cycle length. Eventually, the internal cycle length would become longer than the external cycle length, when ovulation rate would begin to decline. However, there is evidence to show that sequence length tends to rise initially (Lewis and Perry, 1991; Johnston, 2004), with most hens exhibiting a single characteristically long (prime) sequence about the time of peak egg production, which then declines at different rates between individuals (Robinson *et al*., 1990), so the model of Emmans and Fisher (1986) is unsatisfactory in describing the change in ovulation rate over time.

In order to reproduce these changes in sequence length over time, the internal cycle length initially needs to be long (usually more than 24 h), before decreasing with advancing time from first egg to close to, or below 24 h, and subsequently increasing. Internal cycle lengths longer than the external cycle length will cause the time of lay to be later each day, whereas those shorter than the external cycle length will enable the hen to lay long sequences with oviposition occurring at a similar time each day (Morris, 1978). Internal cycle lengths are under genetic control and can be manipulated (Foster, 1981), thus the constraining effect of the external cycle length on potential rate of lay may be reduced either by reducing the

internal cycle length or making use of ahemeral cycles greater in length than 24 h (Morris, 1978). External cycle lengths longer or shorter than 24 h can be accommodated when such an approach is used. When the ovulation curves of individuals in the flock are integrated, the characteristic laying curve is faithfully reproduced. The slope of the initial rise in flock egg production to peak rate of lay is influenced by the distribution of ages at sexual maturity and by the lengths of the individual prime sequences. The incidence of internal laying at onset of maturity plays a role in modifying rate of lay but not ovulation rate. The persistency of lay after peak will be determined by the rate at which sequence lengths of individual hens shorten over time, as well as by the number of pause days. Hence the prediction of sequence length is a logical step in predicting the performance of a flock of laying hens over an entire laying cycle.

The reproductive rates of flocks of commercial laying hens and broiler breeders may be simulated by making use of the Monte Carlo simulation method, which requires the choice of appropriate values for the means and standard errors of the parameters in the various equations used to simulate ovulation rate, the rate of decay in internal cycle length and the incidence of pause days (Johnston and Gous, 2006, 2007a,b,c). The potential performance of each hen in the population is simulated in this way, thereby producing information necessary for predicting the nutrients required by each hen on each day of lay. For more precision in determining these nutrient requirements, the weight of the egg and the proportions of yolk and albumen in the egg need to be known, and these can be modelled as described below.

IV. MODELLING EGG WEIGHT AND COMPOSITION

When modelling the nutrient requirements of a hen over a production cycle, based on the daily outputs of each nutrient, egg weight needs to be predicted as the sum of the three components, since each has a unique chemical composition, and these proportional changes will therefore influence the nutrient requirements of the hen. Egg weight increases as hens age, but the eggs contain proportionally more yolk and less albumen and shell. However, at a given age, larger eggs contain proportionally more albumen (Johnston and Gous, 2007b). Yolk weight is dependent mainly on the genotype, but within a strain because it is related to hen age it may be calculated using an appropriate (exponential) function. Allometric functions may then be used to predict albumen weight from yolk weight and shell weight from the weight of the egg contents. The methods described by Johnston and Gous (2007b) for this purpose appear also to work satisfactorily for broiler breeders (Gous and Nonis, 2010) as long as appropriate functions are used to describe the relationships between age and yolk weight, albumen and yolk weight, and shell and egg content weight. These relationships differ not only between laying hens and broiler breeders, but also between strains.

The position of an egg in the sequence also influences the proportions of the weight of the egg and its components. The weight of consecutive eggs within a sequence gradually decreases (Belyavin *et al*., 1987; Miyoshi *et al*., 1997) and similar patterns have been observed with yolk weights (Bastian and Zarrow, 1955; Zakaria *et al*., 1984; Zakaria, 1999) with the heaviest yolks occurring more frequently in the first two places of a sequence (Gilbert, 1972). Egg shell weight tends to be heaviest in the terminal egg of the clutch (Miyoshi *et al*., 1997) presumably because the lag in oviposition time is longest for this last egg, which means a longer period of time is devoted to shell deposition.

When nutrient intake is constrained this will have consequences on both rate of lay and egg weight: Morris and Gous (1988) showed that these are equally reduced when the feed is marginally deficient in an amino acid, but that as the deficiency becomes more severe, rate of lay is reduced to a far greater extent than is egg weight. So there is very little scope for the laying hen nutritionist to manipulate egg size without also affecting rate of lay. The

practice of altering the amino acid (usually methionine) content of the feed as a means of altering egg size and not rate of lay, which is commonly applied when economic conditions appear to warrant this (Leeson and Summers, 2005) appears attractive because the coefficient of variation for rate of lay is very high (around 25%) whereas that for egg weight is only between 6 and 8%. So it is more difficult to show statistically significant differences in rate of lay between treatments than in egg weight, and consequently the conclusion that a decrease in amino acid supply reduces egg weight but not rate of lay is incorrect.

Because an amino acid deficiency influences rate of lay more than egg weight, this relative change in the outputs (number and weight) needs to be accounted for when determining the revenue derived from the sale of eggs.

V. PREDICTING BODY WEIGHT AND COMPOSITION OF A HEN

A large proportion of the daily intake of energy and amino acids by a laying hen is used for maintenance, so the prediction of the bird's maintenance requirement, when determining her optimum daily intake of energy and amino acids, is of considerable importance. In most factorial models these maintenance requirements are based on body weight, but because body lipid does not need to be maintained (Emmans and Fisher, 1986), a more accurate basis for calculating these requirements would be the body protein content of the bird. Emmans and Fisher (1986) and Fisher (1998) have raised this issue in the past, and the concept has been successfully incorporated into some broiler (EFG Software, 1995) and pig (Ferguson *et al*., 1997) growth models. But little useful information is available on the carcass protein content of layers or broiler breeders during lay or the extent to which this varies over time, to enable such calculations to be made of the maintenance requirements of these birds.

In the early period of lay there appears to be an increase in the mean weight of body protein in a flock of hens, a large part of this being the growth of the ovary and oviduct during the period when the pullet reaches sexual maturity (Bowmaker and Gous, 1989). Differences in age at sexual maturity between birds in the flock will also contribute to the variation in the apparent increase in body protein weight during this period, with early maturing birds no longer growing, while those not yet sexually mature continue to grow until they have laid their first egg.

In laying hens it is well established that body protein content is maximal at sexual maturity and that little further protein growth occurs during lay (Fisher and Gous, 2008). It has been demonstrated in mammals that protein growth does not occur when the animal is in a lactating state, equivalent to the egg production state in hens. Sows, for example, show very little protein growth, if any, during gestation (Shields and Mahan, 1983; King, 1987), while they may lose considerable amounts of body protein during lactation (Whittemore and Yang, 1989) unless adequately fed (Coop and Kyriazakis, 1999).

As the weight of body protein remains relatively stable throughout the laying period, and as any growth in body protein may be regarded as taking place among non-laying hens only, it should not be necessary to assume that protein growth is obligatory when determining nutrient requirements of laying hens. Also, because changes in body lipid content are the consequence of the way in which the hen has been fed, it is unnecessary to make provision for any obligatory gain in body lipid during lay. Maintenance requirements may thus be considered to be constant over the laying period for those birds that continue to lay in closed cycles, and these should be based on the body protein content at the age of first egg.

VI. PREDICTING FOOD INTAKE

To be of any real value, models that attempt to optimize the feeding of laying hens must be capable of predicting voluntary food intake. In most models, where food intake is an input, it

is naive to believe that feeding programmes can be successfully optimized given that the composition of the food offered has such an influence on voluntary food intake. Food intake must therefore be an output from, and not an input to, a model. A reproducing animal needs to be supplied with nutrients in order to meet the requirements for maintenance of the body and for reproduction. The theory of food intake and growth proposed by Emmans (1981, 1989) is based on the premise that birds attempt to grow at their genetic potential, which implies that they attempt to eat as much of a given feed as would be necessary to grow at that rate. The same principle can be applied to laying hens (Emmans and Fisher, 1986). To calculate the daily energy and nutrient requirements of a laying hen, her protein weight (for maintenance) and potential protein and lipid output (in eggs) needs to be known. By comparing the requirements for these functions with the content of nutrients in the feed the 'desired' feed intake can be determined: this is the amount of feed that would be needed to meet the requirement for the first limiting nutrient in the feed (Emmans, 1981). The bird may not be capable of consuming this amount of feed, its intake possibly being constrained by either the bulkiness of the feed or the inability to lose sufficient heat to the environment. In this case feed intake will be less than desired and performance would be compromised.

This theory has been shown to predict food intake and hence growth and carcass composition with considerable accuracy (Ferguson and Gous, 1997, 2002; Ferguson *et al*., 1997; Wellock *et al*., 2004). Burnham *et al.* (1992) and Gous *et al*. (1987), among many others, have shown that broilers and laying hens increase food intake as the limiting nutrient (usually an amino acid) in the feed is reduced, attempting thereby to obtain sufficient of the limiting nutrient. The common misconception that 'birds eat to satisfy their energy requirements' is clearly naive and of no value in predicting voluntary food intake.

In determining nutrient requirements, rules must be applied to account, for example, for the size of an amino acid pool for potential albumen formation (which must be filled before ovulation can proceed), and for the rates at which lipid can be deposited in, or withdrawn from, body reserves as a means of accounting for differences in energy balance. If it is assumed that birds and animals have an inherent ratio of body lipid to protein, which they attempt to maintain at all times (Emmans, 1981, 1989), where possible, the bird will make use of excess lipid reserves as an energy source. This has an impact on the voluntary food intake of hens, with energy being stored on non-laying days and being utilized on laying days, which would tend to buffer the changes in food intake required on these days. Presumably there is a minimum amount of body lipid that needs to be maintained (Gous *et al*., 1990) that will be unavailable as an energy source.

Voluntary food intake must be constrained at high environmental temperatures to ensure that the body temperature of the hen remains constant. Similarly, at very low temperatures energy intake would need to increase to account for the additional heat required for thermogenesis. These constraints can be accommodated by defining the maximum and minimum amounts of sensible and evaporative heat that may be lost at any given environmental temperature. Ideally, the environmental heat demand should be used to define these limits, this being the interaction between temperature, relative humidity and wind speed. The effect on reproductive performance of the constraint on food intake can then be successfully modelled, emulating the results of Marsden & Morris (1987).

To summarise, the critical features of a model to predict food intake in hens would be predictions of the body protein weight of the bird and her potential egg output on each day, from which nutrient requirements for maintenance and output may be calculated; a description of the nutrient content of the feed on offer; and a description of the environmental heat demand under which the bird is housed. Although the principle of predicting food intake is the same for growing and reproducing birds, the description of potential growth and of egg output differs markedly between the two.

VII. IMPROVING ECONOMIC EFFICIENCY

Until recently, mechanistic models developed for poultry have dealt with the simulation of responses in a single bird. Such responses are usually linear to the point where the genetic potential is reached (Fisher *et al*., 1973). Poultry nutritionists are interested in responses to nutrients in economically important outputs such as body weight (or protein) gain, breast meat yield, egg output, numbers of chicks produced per hen, etc. Because such responses are usually measured using groups of birds, they are invariably curvilinear, being the result of integrating the responses of individuals making up that population (Fisher *et al*., 1973). Populations of birds therefore cannot have 'requirements' for nutrients: what nutritionists seek are the optimum economic dietary contents of each nutrient, and for this they need to know how populations respond to increasing dietary contents of the essential nutrients. Descriptions of such responses, whilst taking account of marginal costs and revenues, are therefore invaluable in determining how to maximize or minimize the objective function chosen for any given commercial operation. In the commercial laying hen model described here the theory is applied to an individual and then a population is simulated using appropriate means and standard errors for the variables concerned. The responses obtained are acceptable representations of reality, and are thus ideal for determining the optimum method of feeding these simulated flocks.

Optimizing the feed and feeding programme for a flock of laying hens can be achieved with three components, namely, a feed formulation program, an egg production model and an optimization routine. The flow of information for such a procedure bears similarities to the continuous quality improvement model of Deming (1986), which consists of four repetitive steps (Plan, Do, Check, Act). This continuous feedback loop is designed to assist managers to identify and then reduce or eliminate sources of variation. In the case of the nutritionist, the optimizer defines nutritional constraints for practical layer or breeder feeds, which are passed to the feed formulation program where the least-cost feed that meets these constraints is determined. The characteristics of this formulated feed are then passed, as input, to the laying hen model. The performance expected from this feed when given to a defined flock of hens in a given environment is predicted by the model, and this predicted performance is then passed to the optimizer to complete the cycle. The next cycle starts with the optimizer modifying the feed specifications, moving, according to some in-built rules, to an optimum point. A single feed could be fed throughout the laying period, or different feeds might be more beneficial as the flock ages. The objective function to be maximized or minimized can be defined in terms of any output from the simulation model, but realistically would be an economic index of some sort. An example for a laying flock would be to maximize margin over feed cost, based on the value of the eggs being sold and the cost of feeding. In the case of broiler breeders, because of the high value of the hatched chick, the objective would be to maximize the number of hatching eggs per hen.

VIII. CONCLUSIONS

The major limitation in determining the optimum economic amino acid and energy supply for a flock of laying hens has been the inability to predict how much of a given food the flock would consume. Thus, even though it is possible to determine the optimum intakes of these nutrients as their marginal costs and the marginal revenue for eggs change, it has not been possible to convert these into concentrations in the feed with any degree of certainty. The model described here now offers the possibility of being able to describe the potential reproductive performance of the hens making up a laying flock, from which it would be possible to predict food intake, and as a result the optimization of feeds for the laying flock is now possible. Predicting food intake is only possible once the potential laying performance of

each hen can be predicted, which is in itself dependent on a large number of interacting systems, all of which can now be simulated, although not perfectly.

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FLOCK UNIFORMITY - IS IT IMPORTANT AND HOW IS IT ASSESSED?

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Summary

Reduced flock uniformity in live weight can result in significant economic losses to chicken meat processors. There is a lack of publicly-available information on commercial flock uniformity in Australia, which raises the question "What is a reasonable benchmark for commercial flock uniformity expressed as the coefficient of variation (CV) in average live weight at a given age"? Proxy estimates of CV for live weight derived from three large scale chicken growth experiments were 6.6% for males (3,157 g/bird) and 6.1% for females (2,714 g/bird) at 5 weeks of age, 7.3% for males (3,620 g/bird) and 8.8% for females (3,044 g/bird) at 6 weeks of age, and 6.7% for males (4,286 g/bird) at 7 weeks of age. CVs were sensitive to breed, sex, diet formulation and source of feed, with each of these factors influencing uniformity in live weight. Overall, an optimistic estimate of achievable uniformity in live weight of meat chickens under experimental conditions is about 6%.

I. INTRODUCTION

Flock uniformity is a key performance indicator and economic driver in commercial practice. Madsen and Pedersen (2010) pointed out that in the USA wholesale purchasers of chicken meat insist on supply of carcasses within a narrow weight range, and that failure to meet these specifications can incur severe economic losses to the processor. In Australia, losses could exceed AUD$127M per annum assuming that 5% of throughput totalling 1,159,602 tonnes chicken meat per annum is downgraded by 40% due to out-of-range weight specifications. From these brief comments it is easy to see that flock uniformity in live weight is a very important matter. The question is just what is a benchmark figure for flock uniformity in commercial practice?

Flock uniformity can be expressed as the coefficient of variation in live weight, with increased CV values synonymous with decreased uniformity, or in other words, a wider spread in live weights above and below the flock average. Here CV is calculated as statistical variance in live weight expressed as a percentage of the mean value of the flock. Facts and figures are hard to ascertain, but it is clear that flock uniformity can decrease if close attention is not paid to nutrition, vaccination, husbandry, health and hygiene. For example, Ciftci and Ercan (2003) reported CV for live weight increasing from 8.7 to over 10% when males were fed less homogeneous feed. Madsen and Pedersen (2010) reported CV for live weight increasing from 6.7% to 9.5% when DL-methionine supplementation was reduced from 1.2 g/kg to 0.8 g/kg, and CV for breast meat yield rising from 8.7% to 11.7 % with this reduction of 0.4 g/kg DL-methionine supplementation.

Scarcity of publicly-available information on commercial flock uniformity in Australia prompted us to derive estimates from large scale growth studies conducted on the Roseworthy Campus of the University of Adelaide as a proxy for commercial benchmarks in the absence of further information. This report covers three experiments in which live weights of individual birds were measured at the end of each growth study, and at hatch and then weekly for five weeks in one of the studies.

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II. MATERIALS AND METHODS

Each of the three experiments took place in a controlled-temperature chicken shed (36 x 12 metres) on the Roseworthy Campus, University of Adelaide. The shed was thoroughly cleaned and fumigated by commercial contractors between experiments, and fresh sawdust and shavings litter was used. Breeder recommendations for air temperature and ventilation were followed. The shed was divided into 48 floor pens, each measuring 2.6 x 2.6 metres. A solid state logic controller fully integrated the operation of fans for cross-ventilation and internal circulation, heating by gas-fired brooders (one at each end of the shed), and cooling by water evaporation through activation of a pump for re-circulation of water through a bank of inlet pads mounted on the wall opposite to the exhaust fans. Daily records were kept on air temperatures inside and outside the shed, thermostat settings for heaters, cooler and fans, and light dimmers. Commercial feed from two tube feeders per pen and water via a nipple drinker line were available at all times. Dimmed lights were controlled by a programmable digital time clock set to breeder recommendations.

In Experiment 1, 1,200 male and 1,200 female Cobb 500 chickens, derived from breeder hens given commercial diets with 1,000 and 2,000 ppm betaine, were given a commercial starter, grower and finisher diets suited to this breed. Male and female progeny were reared separately. Birds were weighed individually on day of hatch, and at weekly intervals for five weeks. In Experiment 2, 1,920 male and 1,920 female Ross 308 chickens were fed commercial diets sourced from four different feed mills but with the same nutrient specifications. Male and female progeny were reared separately. These birds were weighed individually at six weeks of age. Experiment 3 involved 1,920 male birds from three genetic breeds fed either a diet formulated to Cobb 500 or a diet formulated to Ross 308 recommendations. Birds were weighed individually at 48 days of age. In each experiment, live weight and CV were recorded for each pen of birds for analysis of variance by generalised linear models.

III. RESULTS

Live weight (g/bird) and live weight uniformity expressed as the coefficient of variation from three separate experiments are summarised in Tables 1-3.

Table 1 - Effects of age on live weight (g/bird) and live weight uniformity expressed as the coefficient of variation (CV) of Cobb 500 chickens (Experiment 1).

Age (in weeks)	Live weight	CV
0	43	8.5
1	213	7.6
2	585	8.1
3	1,245	7.5
4	2,132	7.1
5	3,151	6.6

In Experiment 1 (Table 1), breeder diet and sex had no significant effects ($P > 0.05$) on live weight or flock uniformity of progeny at any age. Females were 2,714 g/bird with CV 6.1% and males were 3,157 g/bird with CV 6.6% at five weeks of age.

Table 2 - Live weight (g/bird) at six weeks of age and live weight uniformity (CV) of Ross 308 chickens fed commercial diets from four different feed mills (Experiment 2).

Feed mill	Live weight		CV [1]	
	Female	Male	Female	Male
A	2,989	3,597	7.77 c	6.99 c
B	3,084	3,616	9.77 ab	7.49 c
C	3,079	3,654	7.92 bc	7.02 c
D	3,024	3,614	9.85 a	7.66 c

[1] CV means with a common letter are not significantly different (P > 0.05).

In Experiment 2 (Table 2), the interaction between feed mill and sex was not significant (P > 0.05) for CV. Female chickens fed diets from mills B and D were significantly less uniform (P < 0.05) than females fed diets from mills A and C, and male chickens irrespective of mill. There was no effect of mill (P > 0.05) on uniformity of male chickens. For live weight, the interaction between feed mill and sex was not significant (P > 0.05), nor did the source of feed have any effect (P > 0.05) on live weight of either sex, however males were significantly heavier (P < 0.0001) than females (3,620 vs 3,044 g/bird).

Table 3 - Effects of breed and diet on live weight (g/bird) and live weight uniformity (CV) of male chickens at seven weeks of age (Experiment 3).

Breed	Diet	Live weight	CV
1	Cobb	4,143 c	7.36 a
1	Ross	4,267 bc	6.45 ab
2	Cobb	4,240 c	6.37 ab
2	Ross	4,223 c	7.15 a
3	Cobb	4,399 ab	6.60 ab
3	Ross	4,445 a	6.00 b

Means with a common letter are not significantly different (P > 0.05).

In Experiment 3 (Table 3), the main effects of breed and diet were not significant (P > 0.05), but the interaction was virtually significant (P = 0.0501), indicating inconsistent differences due to diet within breed (Table 3). Overall, CVs for Cobb and Ross diets were 6.8% and 6.5%, respectively. The CVs for breeds 1, 2 and 3 were 6.9, 6.8 and 6.3%, respectively. Breed had a highly significant effect (P < 0.001) on both live weight. Live weights for breeds 1, 2 and 3 were 3,424, 3,441 and 3,562 g/bird, respectively, with breed 3 being significantly heavier than other breeds. Diet and the interaction between breed and diet on live weight were not significant (P > 0.05).

III. DISCUSSION

Coefficients of variation in market weight of meat chickens under experimental conditions ranged between 6 and 10% depending on sex, breed and diet. From the results presented above it is evident that a coefficient of variation of 6% from large scale growth experiments was the best outcome achieved. CVs were sensitive to differences in breed, sex, diet formulation and source of feed, with each of these factors influencing uniformity in live weight with potential to reduce flock uniformity. Can commercial flocks do better than this? Possibly, yes, as birds in experimental flocks are routinely subjected to additional handling and are exposed to regular disturbance when research staff allocate feed manually to individual feed hoppers in pens. On the other hand, periods of feed deprivation during regular thinning of flocks for processing, and routine handling of cohorts of birds to assess flock average weight, could be detrimental to uniformity on commercial farms. If not already

known by processors, it would be highly desirable to determine industry benchmarks for homogeneity in live weight and carcass traits by direct measurement of a large number of individual birds rather than by mass weighing of a few hundred to estimate the flock average.

Finally, renewed research efforts to assess live weight of chickens by computer-assisted image analysis (De Wey et al. 2003) and automated weighing stations (Chedad et al. 2003) is warranted given rapid advances in these technologies in the last 10-15 years.

IV. CONCLUSIONS

Overall, flock uniformity, expressed as the coefficient of variation in market weight of meat chickens under experimental conditions, ranged between 6 and 10% depending on sex, breed and diet.

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INVESTIGATION OF VARIATION IN FEED EFFICIENCY AND EGG QUALITY IN LAYING HENS

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Summary

The objective of this study was to characterise the individual feed conversion ratio (FCR) of a cohort of laying hens and investigate the relationship between FCR and egg quality. The study was divided into two phases (preliminary and primary). The preliminary phase focused on measuring the FCR of 140 Isa Brown laying hens (birds = 28 weeks old), over a study period of seven weeks. From the preliminary phase, the 15% most efficient (FCR < 1.7; n = 20) and 15% least efficient (FCR > 2.1; n = 20) hens were identified, and designated as high feed efficiency (HFE) and low feed efficiency (LFE) groups respectively and subsequently monitored for feed intake, egg production and egg quality assessment during the primary phase for a duration of six weeks (birds = 55 weeks old). Feed intake, egg production and egg quality were measured weekly over the six-week primary period. Primary FCR results showed significant differences between groups, with HFE maintaining lower FCR than LFE birds. Internal egg quality testing revealed significant differences between groups. The HFE group had greater albumen weight and height and a greater Haugh Unit score and albumen: yolk ratio when compared with the LFE group. The LFE group had a heavier yolk weight when compared with the HFE group. No significant differences were found between groups in egg weight, height, width and shell weight or thickness, nor were there any differences in yolk width or height. This study demonstrates considerable variation exist in the feed efficiency of layer hens, individual feed efficiency is stable over a productive cycle, with an indication that egg quality between HFE and LFE birds do differ significantly, particularly in albumen quality.

I. INTRODUCTION

Laying hens have an impressive egg laying capacity, producing approximately 21 kg of eggs over a laying cycle of 72 weeks. Despite such success, there is evidence to suggest considerable variation in the efficiency with which laying hens convert feed to eggs. Feed constitutes the major cost in the egg industry, accounting for approximately 70% of the total cost of production (Willems et al., 2013), It is therefore important to understand the variation in efficiency with which laying hens convert feed to eggs. Moreover, there have been few studies exploring the link between feed efficiency and egg quality in hens. The aim of this study is to investigate the presence and extent of variation in feed efficiency in laying hens and the consequences of any variation on a range of egg characteristics and egg quality parameters.

II. MATERIALS AND METHODS

The study was divided into an initial (preliminary) screening period and a subsequent comparative (primary) period. In the initial screening period, 140 Isa Brown (28 weeks old) were randomly selected and housed individually in 25 × 50 × 50 cm cages for 8 weeks to facilitate individual weekly feed intake and daily egg production. Birds were offered ad libitum water and access to a commercial layer diet comprised primarily of wheat and

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soybean meal. During the initial screening period individual egg production and feed consumption was measured to allow weekly determination of feed conversion ratio (FCR).

At the end of the preliminary phase, FCR mean values and lay % were calculated for each individual bird and were used to select birds for the primary phase. Birds with < 85% lay were excluded from the primary phase study. From the preliminary phase, the 15% most efficient (FCR < 1.7; n = 20) and 15% least efficient (FCR > 2.1; n = 20) hens were identified, designated as high feed efficiency (HFE) and low feed efficiency (LFE) groups respectively and subsequently monitored for FCR and egg quality assessment during the primary phase (6 weeks, hens 55 weeks old). Egg quality testing and assessment was conducted once a week for 6 weeks (n = 10 per group) testing for internal and external quality, including egg, albumen and yolk weight, height, and width. Weight was measured using a TSS digital scales, with height and width measured pole to pole using a digital calliper. Albumen height was measured using an albumen height gauge. Egg shell thickness was measured using a digital caliper. Haugh unit values were calculated using the formula: $100 \times \log (h - 1.7 \times w^{0.37} + 7.6)$ where h = height of the albumen in mm, w = egg weight in g (Sekeroglu & Altuntas, 2008). The top and bottom 15% of birds categorised by average FCR were designated HFE and LFE respectively. Data were checked for normality using the univariate procedure of SAS. Data were analysed using the generalised linear model procedure of SAS (SAS Institute) with feed efficiency group as the main effect. All data are presented as least square means ± standard error of the mean (SEM). Means were separated using the Tukey-Kramer method. The probability value which denotes statistical significance was $P < 0.05$.

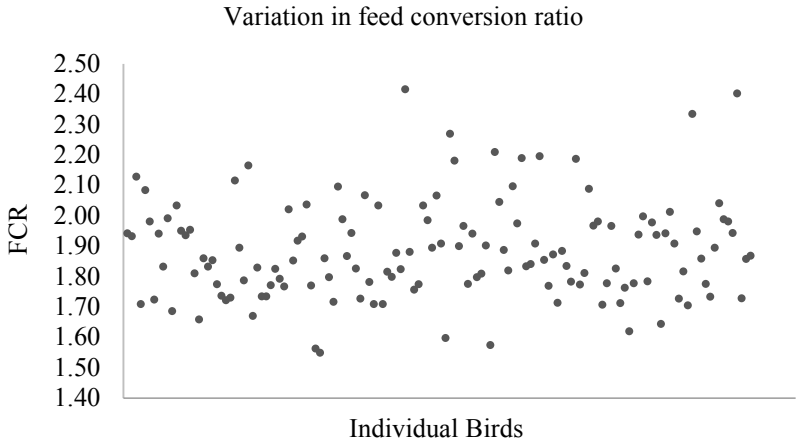

Figure 1 - Variation in feed efficiency; Average feed conversion ratio of 140 birds observed during the preliminary screening stage (8 weeks). The top and bottom 15% of birds based on average FCR were then selected for the subsequent primary phase to assess FCR and egg quality.

III. RESULTS

Results of FCR between the two groups revealed LFE birds to have a significantly ($P < 0.001$) higher FCR during the primary phase than HFE birds (Table 1). There were no significant differences found between groups in external egg and egg shell quality measurements. Internal quality of eggs between groups revealed significant differences, particularly in albumen quality (Table 1). Birds in the HFE group had significantly heavier albumen weight ($P < 0.001$) and greater albumen height ($P < 0.041$) than LFE birds. No significant difference was found in albumen width between groups. HFE birds had lower yolk

weight than LFE birds (Table 1). There were no significant differences in yolk width and height. Measurements of albumen:yolk weight ratio and Haugh unit score (Figure 1 & 2) revealed significant differences between groups, with HFE birds having a greater Haugh unit score and albumen:yolk ratio than the LFE group.

Table 1 - Feed conversion ratio and egg quality HFE and LFE groups.

	Measurement	HFE	LFE	SEM	P value
	FCR	2.0	2.30	0.055	0.001
Egg	Width (mm)	44.8	44.6	0.208	0.49
	Weight (g)	67.4	65.38	0.870	0.12
	Height (mm)	58.7	57.45	0.057	0.447
Egg Shell (dry)	Total weight (g)	6.40	6.40	0.066	0.97
	Top (mm)	0.32	0.33	0.006	0.21
	Side (mm)	0.35	0.35	0.008	0.78
	Bottom (mm)	0.32	0.33	0.005	0.17
Albumen	Width (mm)	67.79	70.97	1.36	0.12
	Weight (g)	41.07	37.19	0.97	0.011
	Height (mm)	7.78	6.24	0.33	0.041
Yolk	Width (mm)	37.74	38.62	0.432	0.16
	Weight (g)	17.37	18.93	0.302	0.002
	Height (mm)	17.22	16.91	0.191	0.27

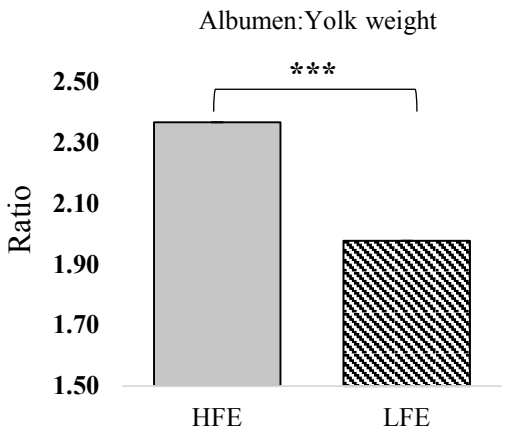

Figure 1 - The effect of feed efficiency group on albumen: yolk ratio (mean ± SEM; *n* = 10); HFE – High feed efficiency group, LFE – Low feed efficiency group.

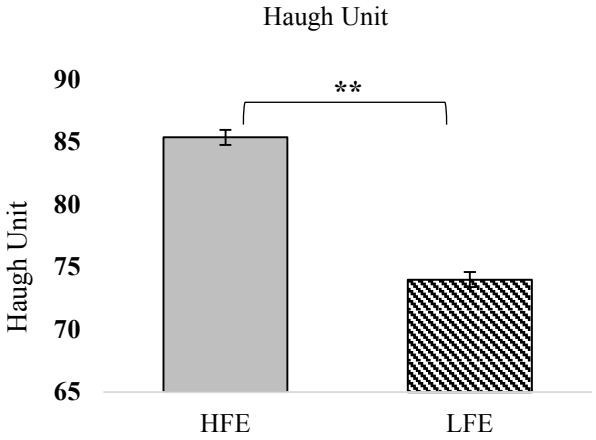

Figure 2 - The effect of feed efficiency group on Haugh Unit (mean ± SEM *n* = 10); HFE – High feed efficiency group, LFE – Low feed efficiency group.

IV. DISCUSSION AND CONCLUSION

The aim of this study was to investigate the variation and persistency of individual feed conversion ratios (FCR) of laying hens and associations with egg quality. Birds designated as HFE in the preliminary phase (eight week duration, birds 28 weeks of age) continued to have lower FCR in the primary phase (six weeks duration, birds 55 weeks of age). The primary phase revealed a significant difference in FCR between groups, with LFE birds having a higher FCR than LFE birds. This observation suggests that individual FCR may be

established early in the production cycle and maintained over time. Birds designated as HFE produced eggs, which had greater albumen quality, in particular, weight, height, albumen: yolk weight ratio and Haugh Unit compared with LFE birds. Yolk weight in LFE birds was found to be significantly heavier than HFE birds. While speculative, the higher yolk weight in designated inefficient birds could be attributed to the higher energy intake and requirement of the birds and their ability to effectively utilise dietary lipids and consequently depositing higher levels of cholesterol within the yolk (Hussein et al., 1993). Furthermore, the observed relationship between FCR and internal egg quality may be attributed to differences in metabolic efficiency and thus extent of nutrient utilisation, in particular dietary protein and fat, with HFE birds having contrasting metabolic rates and nutrient utilisation compared to LFE birds. Despite HFE designated birds demonstrating heavier albumen weight compared to LFE birds, egg weight and thickness between groups revealed no difference. Similar findings in the disassociation of albumen weight and egg weight have been previously reported (Şekeroğlu and Altuntaş, 2008). The findings of this study suggest that FCR has no effect on external egg qualities between efficient and inefficient birds. The overall results of this study suggest that feed efficiency differs substantially among individual laying hens. Furthermore, birds designated as having high feed efficiency had improved egg quality parameters related to albumen, while birds designated as low feed efficiency had heavier yolks. Furthermore, to the authors' knowledge, studies looking at the relationship between FCR and egg quality are few. Further research on this relationship are merited, by exploring the role of protein and lipid utilisation in birds of varying feed efficiency to determine the post-absorptive fate of amino and fatty acids relevant to egg albumen and yolk quality.

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MEAT AND BONE MEAL: A NECESSITY IN LAYER DIETS?

D.C. CRESWELL[1]

Summary

The Australian layer feed industry generally utilises meat and bone meal (MBM) in layer diets at inclusions of 4-6% to meet the bird's requirement of 0.40% available Phosphorus (aP) or greater, as dictated in layer breeder manuals. However, when a requirement figure of 0.30% (a more accurate reflection of the aP requirement) is used, in addition with an elevated inclusion of high efficacy phytase, MBM is both uneconomic and unnecessary to provide the P requirement. This paper demonstrates this standpoint through a formulation exercise, using lower levels of aP together with phytase to result in a lower cost diet.

I. INTRODUCTION

Do we need to use MBM in layer diets? The answer is quite simple. Yes, if it is necessary and economic to do so. In this paper I will demonstrate that MBM is no longer economic or necessary to use in layer diets, providing we understand phosphorus requirements for poultry and use high inclusions of an efficacious phytase.

Australian layer diets commonly use of MBM as a source of supplemental phosphorus, usually at 4-6%. Nevertheless, the era of high efficacy phytases has dramatically changed the approach to formulation to achieve phosphorus adequacy in layer diets.

How should we approach layer formulation in order to provide adequate phosphorus? Firstly, we need to understand the requirement for aP. Recent research suggests it is not more than 220 mg/day (Angel R, 2011., Francesch M et al., 2005., Karcher DM et al., 2006., Keshavarz et al., 2003., Lei QB et al., 2011., Liu YG, 2012., Snow JL et al., 2005., Tan ZK et al., 2011). If this figure is translated to a 110 gram feed intake, we have a dietary level of 0.20% aP. Allowing for a reasonable safety factor, we can round this figure to 0.25% aP. In the following formulation exercise, I have used a generous aP requirement of 0.30% aP.It is noteworthy that there is no research to support the aP requirement recommendation of breeders, which is 0.40% or higher (Hy Line Management Guide Brown Commercial Layers 2016). This manual recommends 460 mg/day from 17-35 weeks, 420 mg/day from 35-55 weeks and 380 mg/day from 56-74 weeks. Secondly, we need to define the levels of available P in our ingredients. These are suggested in Table 1 for feedstuffs sourced locally. Finally, we need to select an appropriate phytase. In the formulation exercise I will use a 600 FTU phytase inclusion which releases 1.95 kg aP/t feed, or 0.195% (Table 2).

Several published trials report the ability of phytase enzymes to provide supplemental phosphorus in a diet in which all inorganic P has been removed. These trials follow a similar design; a positive control diet is formulated with 0.40% available P and containing about 16 kg/t DCP. A negative control diet is also included in which all the DCP has been removed. A third treatment, in which phytase has been added to the negative control is also included. In all cases phytase has restored all egg production parameters to the level of the PC treatment. One such trial is shown in Table 3.

[1] Creswell Nutrition, Sydney, Australia; dcreswell@bigpond.com

Table 1 - Phosphorus content of Australian feed ingredients.

Ingredient	Total P,%	Available P, %
Wheat	0.30	0.12
Sorghum	0.27	0.075
Barley	0.40	0.16
Corn	0.30	0.065
Millrun	1.00	0.20
Soybean meal	0.69	0.24
Canola Meal	1.00	0.20
MBM	4.3	3.00
DCP 18	18	15.3
MCP 21	21	21

Table 2 - Nutrient matrices for phytase[1].

Nutrient	Matrix Values	Nutrient	Nutrient release
ME, Mj/kg	2367	ME, Mj/t	284
Calcium, %	1790	Calcium, kg/t	2.15
Available P, %	1625	Available P, kg/t	1.95
Sodium, %	375	Sodium, kg/t	0.45
SID, %		SID, g/t	
Lysine	190	Lysine	230
Methionine	42	Methionine	50
MC	417	MC	500
Tryptophan	208	Tryptophan	250
Threonine	367	Threonine	440
Arginine	142	Arginine	170
Isoleucine	279	Isoleucine	335
Valine	250	Valine	300

[1] Quantum Blue (ABVista, United Kingdom)

Table 3 - Effect of phytase on egg production parameters[1].

Item	PC	NC	NC + phytase
Treatment	NPP 0.26% with 7.5 kg DCP	DCP removed	DCP removed + phytase
Final weight, kg	1.87[a]	1.68[b]	1.93[a]
Egg production, %	84.4[a]	77.6[b]	84.8[a]
Feed intake, g/d	117.8[a]	113.8[b]	116.5[ab]
Egg weight, g	63.1	62.5	62.3
FCR, g/g	2.18	2.28	2.18
Mortality, %	2.78[b]	13.89[a]	2.78[b]

[1] From Liu and Zhang, 2013 [ab] $P<0.05$

II. FORMULATION EXERCISE

A formulation exercise was conducted for a 110 gram layer diet using different aP levels, without and with phytase. Prices are in Australian dollars (AUD), and represent prices within NSW in May/June 2016. Costs of MBM and soybean meal are given as equal at $650/t. Results of the formulation exercise are shown in Table 4.

Table 4 - Layer diets formulated with different available P levels and without and with phytase.

Ingredient	Cost, $/t	1	2	3	4
Wheat 11 (enzyme)	270	673.57	683.71	700.71	0
Sorghum 9	240	0	0	0	613.33
Soybean Meal Argentina	650	0	18	43	86
Expeller Canola Meal	420	141	150	150	150
MBM 50	650	97	27	0	0
Millrun	255	0	28	7.8	52
Canola Oil	1200	4.2	0	0	0
Limestone	120	76	85	90	90
Salt	240	1.9	1.6	1.9	2.4
Sodium bicarbonate	540	1	1	1	1
L Lysine HCL	1824	1.5	2.1	2.1	1.6
DL Methionine	5500	1.6	1.1	1.0	1.6
L Threonine	2442	0.4	0.4	0.4	0.28
L Valine	11300	0	0.2	0.2	0
L Isoleucine	15000	0.06	0	0	0
Choline chloride 60	1450	0.6	0.6	0.6	0.5
Vitamins/trace minerals	5000	1	1	1	1
Xylanase	20000	0.1	0.1	0.1	0.1
Phytase 5000	18000	0	0.12	0.12	0.12
Red pigment	85000	0.04	0.04	0.04	0.04
Yellow pigment	82000	0.03	0.03	0.03	0.03
Total, kg		1000	1000	1000	1000
Cost, $/t		346.48	324.48	322.34	319.65
Shadow price of MBM, $/t				586.8	582.5
Nutrient minimums					
ME, MJ/kg				11.72	11.72
SID, %					
Lysine				0.762	0.762
Methionine				0.350	0.350
M+C				0.686	0.686
Tryptophan				0.161	0.161
Threonine				0.571	0.571
Arginine				0.786	0.786
Isoleucine				0.594	0.594
Valine				0.701	0.701
Calcium, %				3.80	3.80
Available P, %				**0.40**	**0.30**
Sodium, %				0.19	0.19
Choline, ppm				1250	1250

III. RESULTS AND DISCUSSION

Diet 1: Uses 0.4% available P and no phytase. This would be the formulation approach used over 15 years ago, before phytase was widely used in layer diets. Note the very high inclusion of MBM at 97 Kg/t, and a diet cost of $346.48/t. MBM has completely replaced soybean meal in this diet.

Diet 2: Continues to use 0.4 % available P but phytase was offered to the formulation. Here we see large reductions in both MBM inclusion (to 27 kg/t) and in feed cost (to $324.48/t).

Diet 3: Uses the more correct requirement of 0.30% aP and phytase. Cost of diet falls further to $322.35/t, and more importantly, MBM is no longer economic. Shadow price of MBM is $586.8/t

Diet 4: The previous 3 diets were based on wheat. Sorghum is however also commonly used in layer diets, and is lower in available P than wheat (Table 1). Therefore, it is important to run a formulation with sorghum replacing wheat. This diet also excludes MBM, which holds a shadow price of $582.5/t.

Therefore, this formulation exercise demonstrates an aP requirement of 0.3%, together with high inclusions of an efficacious phytase minimizes feed cost, and renders MBM uneconomic to use.

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INFLUENCE OF CLASS OF BIRDS ON THE APPARENT ILEAL DIGESTIBILITY OF NUTRIENTS AND ENERGY UTILISATION

M.R. ABDOLLAHI[1], A. MTEI[1], N. SCHREURS[1], V. RAVINDRAN[1] and G. CHANNARAYAPATNA[2]

Summary

The present experiment investigated the influence of class of birds on the coefficient of apparent ileal digestibility (CAID) of nutrients and energy utilisation in diets with different dietary fibre concentrations. A 2 × 3 factorial arrangement of treatments was used with two dietary fibre contents (Low and High) and three classes of birds (broilers, pullets and layers). Increasing dietary fibre concentration had no effect on the CAID of dry matter (DM), starch, and nitrogen-corrected apparent metabolisable energy (AMEn) in broilers and pullets, but increased these parameters in layers, resulting in a significant diet type x class of bird interaction ($P < 0.01$). At both fibre concentrations, broilers had higher ($P < 0.05$) nitrogen digestibility compared to pullets and layers. In low fibre diets, pullets and layers showed the highest and lowest fat digestibility, respectively, with broilers being intermediate; however, the differences in CAID of fat evened out in high fibre diets, resulting in a diet type x class of bird interaction ($P < 0.001$). A significant ($P < 0.05$) interaction between diet type and class of birds was also observed for CAID of neutral detergent fibre (NDF) and gross energy (GE). Increasing dietary fibre concentration did not affect the digestibility of NDF and GE in broilers, but markedly increased it in pullets and layers. Overall, the current results reject the hypothesis that layers would utilise nutrients better than broilers and pullets, and suggest that feed texture might have more pronounced impact on digestive tract development and function in layers than broilers and pullets.

I. INTRODUCTION

The utilisation of nutrients and energy by poultry can vary depending on the class of birds, but studies comparing nutrient digestibility for different type of birds are scant. Limited published data are available on the effect of bird type on the digestibility of protein, amino acids (AA) (Huang et al., 2006; 2007) and energy utilisation (Mollah et al., 1983). Huang et al. (2006) determined the coefficient of apparent ileal digestibility (CAID) of protein and AA in 7 feed ingredients using broilers, layers and roosters and reported that the class of birds can influence protein and AA digestibility in some feed ingredients. These researchers found that the AA digestibility for maize, wheat and sorghum were higher in broilers than in layers and roosters.

Moreover, research examining the utilisation of nutrients and energy in pullets compared to broilers and layers (Kimiaeitalab et al., 2016) is scant, and the possible interaction between dietary fibre content and class of birds has not received much attention. It was hypothesised that an interaction might exist in digestibility responses of the different type of birds to diets with different dietary fibre contents, with layers showing better digestion efficiency for all nutrients especially in high fibre diets. The objective of the present study was to elucidate the influence of diet type (low and high fibre) and class of birds (broilers, pullets and layers) on ileal nutrient digestibility and energy utilisation.

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II. MATERIALS AND METHODS

Two diets with different fibre contents (Low and High; Table 1) were formulated and mixed at the same time using the same batch of ingredients for the 3 assays (with broilers, pullets and layers). Both assay diets were based on maize and soybean meal and did not contain any synthetic amino acid sources. The assay diet with high fibre also contained palm kernel meal, canola meal and oat hulls as high fibre ingredients. Titanium dioxide was included in the diets (5g/kg) as an inert marker for the ileal digestibility measurements. From the two diets, 6 experimental treatments were developed using three different classes of birds (broilers, pullets and layers). The 3 assays, with broilers, pullets and layers were conducted concurrently. The diets for the three assays were mixed at the same time.

Broiler Assay: Day-old male broilers (Ross 308) were obtained from a local hatchery, raised in floor pens and fed commercial broiler starter (d 1 to 21) and finisher (d 22 to 35) diets. On d 35, a total of 96 broilers with the uniform body weight (average 2.60 kg) were selected and assigned to 24 cages, so that the average initial weight per cage were similar. Two adjacent cages were treated as a replicate (four birds per cage, eight birds per replicate). Each of the two broiler treatments were randomly assigned to six replicates and offered the assay diets on d 35.

Pullet Assay: A total of 96 pullets (Hy-Line Brown Commercial, 10-week-old) of uniform body weight (average 1.05 kg) were obtained from a local layer farm and allocated to 24 cages. Two adjacent cages were treated as a replicate (four birds per cage, eight birds per replicate) and the two assay diets were randomly assigned to six replicates containing eight birds each.

Layer Assay: A total of 96 layers (Hy-Line Brown, 59–week-old) of uniform body weight (average 1.95 kg) were obtained from a local layer farm and allocated to 24 cages. Two adjacent cages were treated as a replicate (four birds per cage, eight birds per replicate) and the two assay diets were randomly assigned to six replicates containing eight birds each.

In all three assays, birds were fasted for 12 h before the introduction of assay diets. The assay diets, in mash form, were offered ad libitum for 7 days prior to the collection of ileal digesta. Water was freely available throughout the trial period.

Feed and total excreta output of each replicate were quantitatively measured over the last four days of the assay for the determination of nitrogen-corrected apparent metabolisable energy (AMEn). After 7 days on assay diets, all birds per replicate were euthanised by intravenous of sodium pentobarbitone, and digesta were collected from the lower half of the ileum (Ravindran et al., 2005), for the determination of CAID of dry matter (DM), nitrogen (N), starch, fat, neutral detergent fibre (NDF) and gross energy (GE).

III. RESULTS AND DISCUSSION

The influence of diet type and class of birds on the CAID of nutrients and AMEn is shown in Table 1. Increasing dietary fibre concentration had no effect on the CAID of DM, starch, and AMEn in broilers and pullets, but resulted in pronounced increases in layers, causing a fibre x class of bird interaction ($P < 0.01$). Despite the fact that the three classes of birds showed positive N digestibility responses to the high fibre diet, differences in the magnitude of responses resulted in a significant ($P < 0.01$) diet type x class of bird interaction. Feeding high fibre diets was associated with N digestibility increases by 4.6, 6.5 and 16.5% in broilers, pullets and layers, respectively.

In low fibre diets, pullets and layers showed the highest and lowest fat digestibility, respectively, with broilers being intermediate; however, the differences in the CAID of fat evened out in high fibre diets, resulting in a diet type x class of bird interaction ($P < 0.001$). It is likely that the better gut development, especially gizzard function, may have contributed to

better digestive process. In addition, most of the dietary fat content originated from intact fat contained within maize in low fibre diets, and from the supplemental soybean oil in high fibre diets. This could be another reason for the enhanced fat digestibility in high fibre diets compared to low fibre diets in which lipids encapsulated within cell walls of maize grain.

A significant interaction between diet type and class of bird was observed for the CAID of NDF ($P < 0.05$) and gross energy ($P < 0.001$). Whilst increasing dietary fibre concentration did not affect NDF and GE digestibility in broilers, it increased it in pullets and layers.

The primary purpose of this study was to investigate (i) the general belief that laying hens utilise dietary nutrients and energy more efficiently than broilers, and (ii) whether dietary fibre concentration and bird type would interact on the nutrient digestibility and energy utilisation. While acknowledging the significant interaction between diet type and class of birds for all parameters in the present study, the current findings did not support the hypothesis, and the general belief, that digestion efficacy is higher in layers than broilers. Generally speaking, broilers showed higher nutrient digestibility and energy utilisation, either statistically or numerically, than layers with the differences being more pronounced in low fibre diets. It is also worth noting that all the measured parameters, except AMEn, were suppressed in low fibre diets, possibly due to poor gut development.

Despite the effects of dietary fibre concentration in different class of birds shared a similar general trend, major differences were noted in the magnitude of responses. In general, severe reductions in nutrient digestibility and AMEn were observed in layers fed low fibre diets, but these were mostly restored in high fibre diets. Several studies have shown that broilers require a minimal amount of fibre in their diet to optimise digestive tract functionality and nutrient digestibility (Hetland et al., 2003; Amerah et al., 2009). The present findings that nutrient digestibility and energy utilisation are more influenced by dietary fibre content in layers than broilers and pullets appear to suggest that the digestive tract development and function in layers is more sensitive to feed structure than broilers and pullets.

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Table 1 - Influence of diet type and class of bird on the coefficient of apparent ileal digestibility (CAID)[1] of dry matter (DM), nitrogen (N), starch, fat, neutral detergent fibre (NDF), gross energy (GE), and N-corrected apparent metabolisable energy (AMEn, MJ/kg DM)

Item		CAID						Energy
Diet type	Class of bird	DM	N	Starch	Fat	NDF	GE	AMEn
Low fibre	Broilers	0.685a	0.798b	0.950b	0.696c	0.275a	0.736a	12.93bc
	Pullets	0.604c	0.754c	0.981ab	0.749b	-0.058b	0.661c	13.35a
	Layers	0.495d	0.673d	0.771c	0.569d	-0.051b	0.550d	11.72e
High fibre	Broilers	0.666ab	0.835a	0.978ab	0.943a	0.340a	0.738a	12.66c
	Pullets	0.628bc	0.803b	0.994a	0.952a	0.234a	0.712ab	13.04ab
	Layers	0.627bc	0.784b	0.963ab	0.924a	0.270a	0.688bc	12.16d
Pooled SEM		0.0194	0.0099	0.0139	0.0139	0.0442	0.0167	0.119
Main effects								
Diet type								
	Low fibre	0.595	0.742	0.901	0.671	0.055	0.649	12.67
	High fibre	0.640	0.807	0.978	0.940	0.281	0.712	12.62
Class of bird								
	Broilers	0.675	0.817	0.964	0.820	0.307	0.737	12.79
	Pullets	0.616	0.779	0.988	0.850	0.088	0.686	13.20
	Layers	0.561	0.728	0.867	0.746	0.110	0.619	11.94
Probabilities, P ≤								
Diet type		0.007	0.001	0.001	0.001	0.001	0.001	0.652
Class of bird		0.001	0.001	0.001	0.001	0.001	0.001	0.001
Diet type x Class of bird		0.002	0.002	0.001	0.001	0.013	0.001	0.005

Means in a column not sharing a common letter (a,b,c,d,e) are significantly different (P < 0.05).

[1] Each value represents the mean of six replicates (eight birds per replicate), measured after 7 days on assay diets.

[2] Each value represents the mean of six replicates (eight birds per replicate), measured over the last four days of the assay.

EXPLORING STAKEHOLDER VIEWS TOWARD POULTRY WELFARE USING ONLINE FORUMS

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Evidence suggests that there is variation in support for poultry farming practices amongst stakeholder groups. An exploration of this variation can help understand the nature of these differences, and identify strategies to work towards convergence.

Online focus groups were used to explore attitudes and beliefs relevant to poultry welfare in two studies: furnished cages for layers (Study 1), and intensification of meat chicken farming (Study 2). Participants completed pre- and post-forum surveys gauging their perceptions and knowledge about poultry farming practices, and took part in written exchanges with each other through online forums.

In study 1, 23 participants (general public, n = 19; animal advocacy group, n = 3; industry, n = 1) discussed furnished cages to house laying hens across three asynchronous online forums (lasting 14 days each, participants posting at their convenience) and one synchronous forum (instant messaging for 1.5 h). Results suggest that synchronous forums appear better to canvass participant levels of support or opposition towards a particular topic and explore the beliefs underlying these decisions, as posts were more frequent and spontaneous. Asynchronous forums appear better to inform participants on a topic because they offer a vehicle to discuss and share information, and therefore possibly change opinions. The changes in the responses between the pre- and post-forum surveys indicate that participants did learn about the topic of furnished cages. More people correctly identified furnished cages in the post- compared to pre-forum survey, and they were more likely to rate the welfare of hens in furnished cages higher after forum participation. Although forum participation may not change long-held opinions about animal welfare in general, knowledge about a specific practice may increase as participants become more informed.

In study 2, 25 participants (general public, n = 8; animal advocacy group, n = 11, meat chicken industry, n = 3; research or veterinary practice, n = 3) discussed meat chicken intensification and welfare across six online forums, all synchronous forums. Main reasons for intensification support were perceptions of improved bird health, and perceptions that it is a cost-effective, sustainable production system. Reasons for opposition included perceptions that a large number of birds are kept in close proximity and have limited ability to perform natural behaviours. Misunderstandings about current practices were clarified in forums which contained industry representation. Participants agreed on the need for enforceable standards and industry transparency. Objective knowledge of intensification increased after forum participation, but support for intensification did not change over time, counter to assertions that lack of knowledge results in lack of support for some practices.

Engaging stakeholders can provide valuable information regarding perception and knowledge of farming practices on animal welfare. Online surveys and forums were an effective way of gathering information about perceptions and reasons for approval or disapproval of specific poultry management practices, as well as gauging objective knowledge of those practices. Online forums may therefore be a useful new tool to engage stakeholders in a practical and low-cost manner, with anonymous input, although participant recruitment methods require optimisation, especially to stimulate industry engagement.

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ON-RANGE CHOICE FEEDING OF BLACK SOLDIER FLY LARVAE DOES NOT INFLUENCE RANGE USAGE OF FREE-RANGE LAYING HENS

I. RUHNKE[1], C. NORMANT[1,2], C. LEE[3], G.N. HINCH[1], R. SWICK[1] and D CAMPBELL[1,3]

In Australia, on-range feeding of free-range laying hens can be frequently observed and is especially common in mobile sheds. While the biosecurity risk of on-range feeding cannot be overemphasised, the impact of on-range feeding on range usage remains unknown. The present study investigated the effect of on-range insect feeding on range usage of free range laying hens.

A total of 120 ISA brown laying hens (20 hens/pen) at 43 weeks of age were housed with access to indoor feeders, drinkers, perches, nesting boxes and an outdoor range with one bio-secure feeding station. Indoors, all hens received *ad lib* a typical Australian wheat-soy based layer diet formulated to breed nutrient specification. Pop holes to the range were open daily from 9 am – 7 pm. The outdoor feeders were empty for 3 control pens while the 3 treatment pens had their feeders filled with dried Black Soldier Fly (*Hermetia illucens)* larvae (BSF). BSF larvae were offered to the hens of the treatment group *ad lib* for the duration of 6 weeks (feeding period). Ranging activity was recorded during the first and last 7 consecutive days of this feeding period. Individual ranging data were arranged 2 x 2 factorial (time point x treatment) and analysed using the General Linear Model. All statistical analyses were processed in SPSS statistics 24.

All hens used the range every single day. There were no significant differences between the control and treatment group at any time point. However, the average number of visits/day and the total number of visits significantly decreased over time ($P < 0.001$). Results are displayed in Table 1. In conclusion, additional on-range feeding with Black Soldier Fly larvae did not affect the range usage of free-range laying hens. Further observations of the behavioural time budgets between hens with and without on-range feed are warranted.

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Table 1 - Range usage of control hens compared to hens with on-range choice feeding.

Parameters	Treatment	Time[1]		P - value		
		Week 1	Week 6	Treatment	Time	Treatment *Time
Average time on range/day (h)	Control	5.82± 0.12^{a}	5.12± 0.15^{b}	0.675	0.000**	0.299
	Choice fed	6.05± 0.13^{a}	5.03± 0.20^{b}			
Total time on range (h)	Control	40.8± 0.83^{a}	35.9± 1.07^{b}	0.675	0.000**	0.299
	Choice fed	42.4± 0.94^{a}	35.2± 1.40^{b}			
Average visits on range/day	Control	25.0± 1.13^{a}	18.0± 0.80^{b}	0.056	0.000**	0.027*
	Choice fed	21.1± 0.87^{a}	18.3± 0.93^{b}			
Total visits	Control	175.1± 7.92^{a}	125.9± 5.61^{b}	0.056	0.000**	0.027*
	Choice fed	147.7± 6.07^{a}	127.9± 6.47^{b}			
Average min. time outside/visit (h)	Control	0.016± 0.00	0.032± 0.00	0.123	0.589	0.978
	Choice fed	0.062± 0.04	0.079± 0.05			
Average max. time outside/visit (h)	Control	0.975± 0.03	1.13± 0.08	0.058	0.369	0.164
	Choice fed	1.19± 0.06	1.16± 0.08			
Minimum time outside (h)	Control	0.004± 0.001	0.006± 0.001	0.061	0.587	0.032*
	Choice fed	0.006± 0.001	0.005± 0.001			
Maximum time outside (h)	Control	1.46± 0.06^{b}	1.72± 0.14^{b}	0.011*	0.303	0.451
	Choice fed	1.94± 0.15^{a}	1.98± 0.19^{a}			

[1]Mean values ± standard error of the mean of 60 hens.

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EFFECT OF RANGE ACCESS AND ZINC BACITRACIN ON ILEAL GENE EXPRESSION IN BROILERS

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Summary

Using a whole genome approach we studied the response of broilers, at a transcriptional level, when given access to outdoor range as compared to conventionally housed birds and fed an antibiotic growth promoter free diet (AGP-) vs. a diet containing Zinc Bacitracin (AGP+). Gene expression was analyzed in tissue obtained from the distal ileum and using GeneChip® Chicken Genome Array. Amino acid synthesis and immune regulatory pathways were seen to be up-regulated, while membrane and trans-membrane integrity related pathways were down-regulated in birds which had access to range as compared with conventionally housed birds. For birds that were fed AGP- diets, steroid synthesis, inflammatory response, lipid metabolism and membrane related transport pathways were up-regulated, while pathways related to disruption of bacterial cell-wall via peptoglycan biosynthesis in conjunction with sugar metabolism were down-regulated. Understanding the underlying mechanisms that regulate pathways related to range access and non-inclusion of AGP in diets can help design alternative strategies and products for rearing birds in welfare oriented systems.

I. INTRODUCTION

Free range broiler production in Australia is characterized by two main factors, access to range and non-inclusion of antibiotic growth promoter (AGP) in diets. Knowledge of affected metabolic pathways and biological functions can provide insight into the complex interplay of production systems and physiology. This can lead to development of intervention strategies that can be used to target the production gap between conventional and free range broilers (Durali *et al.,* 2012) both in terms of nutritional solutions for effects of range access and alternatives to antibiotic growth promoters. Access to range leads to added activity and exposure to many influences resulting in differential expression of genes as compared to confined housing systems. Housing systems can lead to differential gene expression influencing muscle fiber accretion (Yin *et al.* 2014), fat deposition (Li *et al.,* 2015) and immune response (van Loon *et al,* 2004) among others. AGPs are known to be associated with chicken growth and performance (Sevane *et al.* 2014), cell growth (Li *et al.* 2012), energy metabolism (Li *et al.,* 2012), while reinforcing the immune status of animals (Lee *et al.,* 2012; Alizadeh *et al.,* 2016; Kim *et al.*, 2016).

II. MATERIALS AND METHODS

A total of 1440 Cobb 500 as-hatched broilers were allocated to one of four treatments with twelve replicates in a 2x2 full factorial arrangement, the factors being free-range or conventional production, with or without in-feed antibiotic (zinc bacitracin (15%) (30 mg/kg feed)). Day old chicks received numbered wing tags on the day of placement and were randomly allocated to 48 pens (30 birds per pen) with *ad libitum* access to feed and water.

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Feed was formulated according to Cobb 500 nutrition specifications and produced in the Poultry Research Foundations' feed mill. Range access was made available to birds from day 21 onwards.

On day 42, six male birds in each pen were selected, euthanized and ileal tissue collected and stored in Invitrogen™ RNA*later*™ Stabilization Solution (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. Samples were stored at –20°C until analysed. Tissues were homogenised with a Qiagen Tissue Ruptor and RNA was isolated with a Qiagen RNeasy mini kit. RNA quantity and quality were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and an Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Labelled cRNA (n = 6 per treatment) for all samples was prepared using the Ambion (Paisley, UK) WT Expression Kit following the manufacturers' instructions. Labelled cRNA was hybridised to a GeneChip® Chicken Genome Array (Affymetrix) for 16 h at 45°C, followed by washing, staining and finally scanning in an AffymetrixGeneChip Scanner 3000 7G at the Nutrigenomics Center, Alltech Inc. Nicholasville, USA.

GeneSpring GX 10.0 (Silicon Genetics, Redwood, CA, USA) was used to qualify and normalise microarray data and to perform statistical and gene expression pattern analyses. To analyse pathway, network and function of genes that were differentially expressed, the dataset was uploaded into DAVID (Database for Annotation, Visualisation and Integrated Discovery) a web-based program (Huang da, 2009). Each identifier was mapped to its corresponding gene object using gene ontology (GO) terms. Genes that showed a fold-change (FC) of >1.2 were considered for KEGG pathway analysis. Top biological functions were determined based on number of molecules involved and significant enrichment score ($P < 0.05$).

III. RESULTS

A total of 914 (555 up-regulated and 418 down-regulated) gene transcripts were found to be differentially expressed ($P < 0.05$) in samples from birds that had access to range as compared to those reared in conventional housing systems. A total of 767 (361 up-regulated and 406 down-regulated) gene transcripts were found to be differentially expressed ($P < 0.05$) in samples from birds fed an AGP- diet as compared toAGP+.

Seventy four up-regulated and 56 down regulated genes with FC ≥ 1.2 were annotated with GO terms when range access was granted and 28 up-regulated and 22 down regulated genes were annotated when AGP was excluded in the diet. These genes were then used for further pathway and functional analysis as shown in Table 1.

IV. DISCUSSION

Of the up-regulated pathways in free range birds, a deep change of pace in amino-acid metabolism is seen related to immune response (histidine), inflamation (arachidonic acid) and lymphocyte proliferation (alanine, aspartate and glutamate metabolism). Arginine biosyntheis is of great importance due to its role in both innate and acquired immunity. Available evidence shows that arginine is required for defense against a number of microorganisms and parasites (Wu and Meininger., 2002) which may be why this pathway is up-regulated in free range production systems. Cytokine receptors, also up-regulated, are soluble extracellular proteins or glycoproteins that are crucial intercellular regulators in innate as well as adaptive inflammatory host defenses, cell growth, differentiation, and restoration of homeostasis (Oppenheim., 2001). FoxOs on the other hand can accelerate glucose production, reduce insulin secretion, decrease fat and muscle masses leading to spare energy (Nakae *et al*., 2008) which can be considered useful for birds in free range systems. Down-regulation of PPAR

signalling and pyrimidine metabolism pathways are indicative of inability to meet the metabolic demands of the bird. Focal adhesion and ECM receptor is a type of adhesive contact between the cell and extracellular matrix through the interaction of the transmembrane proteins connected to the actin cytoskeleton. Down-regulation of these pathways indicates impaired growth and differentiation.

Lack of zinc bacitracin (AGP-) shows up-regulated pathways related to steroid biosynthesis, where key enzymes are considered as important targets for endocrine-disrupting chemicals, affecting growth and development (Sanderson, 2006). Glycerophospholipid metabolism and ether lipid metabolism pathways were related to lipid metabolism and regulating membrane related transport (Spector and Yorek, 1985). Cytokines and inflamatory response was also up-regulated in the absence of zinc bacitracin. Antibiotic synthesis was down-regulated in the AGP- birds. Bacitracin is known to disrupt bacterial cell wall by influencing membrane permeability and peptidoglycan biosynthesis in conjunction with carbohydrate and sugar metabolisms in host (Storm, 1974) which may be the reason for related pathways being down-regulated in the AGP- group as compared to the AGP+ group. Understanding the underlying mechanisms that trigger biological pathways to cope with range access and non-inclusion of AGP in diets can help design targeted strategies and products for improving production in welfare oriented systems.

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Table 1 - Gene expression, number of annotated genes, KEGG pathways enriched by genes with fold change >1.2, affected enzymes, and top biological functions based on number of molecules, P-value and activation score in the ileum of 6-week old free range broilers vs conventionally housed broilers.

Gene expression	Annotated genes with FC ≥1.2 (n)	KEGG Pathways	Genes involved in pathway enrichment	Biological functions
Free range vs. Conventional housing Up-regulated	74	Biosynthesis of amino acids, Histidine metabolism, Arachidonic acid metabolism, Alanine, aspartate and glutamate metabolism, Arginine biosynthesis, Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, FoxO signaling pathway	ASL2 GCTA3 HPGDS HMNT IL22RA2 RAG2	Immunity, Inflammatory host response, Energy metabolism
Free range vs. Conventional housing Down-regulated	56	Pyrimidine metabolism, PPAR(Peroxisome proliferator-activated receptors) signalling pathway, Focal adhesion, ECM (extracellular matrix)-receptor interaction	CMPK2 CPEB2 FABP4 LCT MMP1 TNN	Growth and development, Trans-membrane helix, Integral components of the membrane
AGP- vs AGP+ Up-regulated	28	Steroid biosynthesis, Glycerophospholipid metabolism, Ether lipid metabolism, Cytokine-cytokine receptor interaction	DHCR24 ST6GALNAC3 CCR4 CXCL13L2 IDI1 PLD4	Growth and development, Trans-membrane –transport, Cytokines and inflammatory response
AGP- vs AGP+ Down-regulated	22	Biosynthesis of antibiotics, Glycolysis/Gluconeogenesis, Fructose and mannose metabolism, Starch and sucrose metabolism, Carbon metabolism, Insulin signaling pathway	HK1 MRPL2	Energy metabolism, Membrane permeability

EFFECTS OF CORTICOSTERONE INJECTION AT EMBRYONIC DAY ELEVEN ON BROILER GROWTH AND TONIC IMMOBILITY

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Summary

Elevated corticosterone in ovo can have significant lasting impacts in avian species, including poultry. Stress in broiler breeder hens can lead to elevated corticosterone in the egg, exposing the developing embryo, leading to lifelong changes. This study aimed to mimic these effects by injecting broiler eggs with corticosterone or phosphate buffered solution at embryonic day eleven, into the chorioallontoic membrane. Differences were seen in growth during the final week, with corticosterone injected birds lighter. Behavioural changes were also observed in males, using tonic immobility testing, at day 14 ($P < 0.05$). These changes show that exposure to elevated corticosterone at embryonic day eleven has long-term impacts on chick growth and behaviour. Further research into this area and larger trials using this injection time point would are needed to be conducted to further look at these changes.

I. INTRODUCTION

Hens exposed to an environment of increased stress can have significantly elevated circulating corticosterone levels (de Jong et al., 2002), increasing deposits of corticosterone within the egg (Saino et al., 2005). Embryos within the egg are exposed to this increase in corticosterone during development, and changes to the hypothalamic-pituitary-adrenal (HPA) axis can occur (Ahmed et al., 2014b). These changes can then go onto to impact the lifelong health of the embryo with reductions in growth (Hayward and Wingfield, 2004) immune response (Henriksen et al., 2013) and changes in behaviour (Ahmed et al., 2014a). In humans and other species these changes occur through a process known as developmental programming and have been proven to have significant impacts later in life (Barker et al., 2006). This area is still being researched and understood, and this study aimed to further understand the lifelong impacts of in ovo exposure of elevated corticosterone in chicken embryos on lifelong growth and behaviour.

II. METHODS

a) In Ovo Injections

A total of 144 eggs were collected from Baiada hatchery and incubated at the Roseworthy Campus, University of Adelaide. At embryonic day eleven 96 eggs were injected with corticosterone (1µg in 100µl phosphate buffer solution) and the remaining eggs injected with the phosphate buffered solution (PBS) only. This dose rate was chosen as it was used in previous studies (Ahmed et al., 2014a; Ahmed et al., 2014b) as a high dose and has therefore been demonstrated to have measurable effects throughout the lifetime of the embryo. Also, corticosterone concentrations can vary substantially between eggs and hens (Janczak et al., 2006) and as we had no control over the hens, we wanted to ensure the dose was high enough that the corticosterone group was significantly elevated compared to the control group.

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Solutions were injected into the chorioallantoic (CAM) membrane sing a 1mL insulin syringe and needle after a hole was made using a 23G needle. After the injection, the hole was sealed and the egg continued through incubation as normal.

b) Blood sampling

At 35 days of age, 32 birds were blood sampled via the jugular vein. Samples were centrifuged at 3500rom for five minutes, and the plasma collected and stored at -20^{o}C. Sample were then sent to the School of Animal Biology, University of Western Australia where it underwent Radioimmunoassay testing for corticosterone using a Corticosterone 125I RIA KIT (MP Biomedical, Orangeburg, NY).

c) Animal Husbandry

After hatch, chicks were weighed, feather sexed and placed into treatment pens. Chicks from each treatment (corticosterone or PBS) and sex were placed together. Chicks were monitored daily for signs of ill health and unwell birds were culled. Throughout the trial chicks had *ad libitum* access to water and a commercial broiler diet and were individually weighed weekly for the duration of the trial (6 weeks).

d) Tonic Immobility Test

All birds undertook a tonic immobility test (TI) at 14 days of age. Birds were placed onto their back and restrained for 20 seconds, where after the restraint was removed and birds allowed to flip over onto their front. The time taken for birds to do this was recorded in seconds, with a maximum score of 60 seconds possible. Results from the test were then placed into categories of slow or fast responses, with fast being 0-29 seconds and slow being 30-60 seconds.

e) Statistical tests

Statistical tests were performed using the IBM SPSS program, version 21 with a P value < 0.05 considered significant. A Fischer's exact test was used for the tonic immobility data.

III. RESULTS

a) Average Daily Gain

During the six week grow out trial, birds that were injected with corticosterone and PBS in ovo, maintained similar average daily gain (ADG) throughout the first five weeks of the trial. However, a significant split is seen between the two groups from week five to week six, where by week six corticosterone injected birds had a significantly lower ADG (96.446±3.815) compared to PBS treated birds (121.822±6.474).

b) Plasma Corticosterone

At day 35, plasma corticosterone (ng/mL) was significantly ($P < 0.05$) different between corticosterone and PBS injected birds. Corticosterone treated birds had significantly lower levels (480.66 ± 45.12) compared to PBS birds (648 ± 53.93).

c) Tonic Immobility

At day 14, no significant difference was seen between response times in the tonic immobility test between in ovo injection treatments ($P > 0.05$). There was however a significant difference in speed between males injected with corticosterone and PBS ($P < 0.05$). A higher number of corticosterone injected males were slow to flip over (*n=23)* than were fast (*n=11)*.

IV. DISCUSSION

a) Average Daily Gain

Average Daily Gain (ADG) was unaffected for the majority if the trial, but was significantly impacted by treatment during the final week of growth. Corticosterone treated birds grew less during this final week than PBS birds, with other trials reporting similar reductions in growth after exposure to increased corticosterone in ovo (Ahmed et al., 2014a). Corticosterone can have many lasting impacts on the body, and if elevated can mean decreased IGF-1 (Scanes, 2011), which may lead to a reduction in growth. Unfortunately, IGF-1 levels were not recorded in this trial so it is unclear if there were differences between treatments.

Also, broilers grow rapidly, with a 400% increase in broiler growth in the last 50 years (Zuidhof et al., 2014) and therefore develop much faster than other species. In humans and other species exposed to altered uterine environments, metabolic diseases including diabetes can arise (Hales, 2001), but are often not seen until later in life (Barker et al., 2006). This may explain why these changes in the broilers were not observed until the final week of growth. However, metabolic measures were not recorded in this trial so future work would need to include these measures on birds that have been exposed to elevated corticosterone in ovo to better understand the impacts on metabolism and growth and why they are occurring.

b) Plasma Corticosterone

In this study plasma corticosterone was significantly different between in ovo treatments at day 35. Surprisingly, the birds injected with corticosterone had lower levels than the PBS injected birds which may mean that there is a down-regulation of corticosterone occurring later in the life of the bird. Impacts on growth were still seen from day 35, with corticosterone still supressing growth as in other trials with increased corticosterone exposure in ovo (Hayward and Wingfield 2004). This suggests that there may be an interaction effect of corticosterone but this would need much more study to understand.

c) Tonic Immobility

TI is used to assess the fear response in birds, with the speed of the response indicating the level of fear/stress the bird is experiencing (Wang et al., 2013). At day 14 only males showed a significant difference in speed, with corticosteroid treated males responding slower, while females remain unaffected. Other studies have shown similar results with corticosterone treated birds responding slower to the test (Ahmed et al., 2014a). Males were affected while females were not and this may mean that they are more sensitive to the corticosterone elevation. A similar study where birds were exposed to an early life stress resulted in a dampened response to the stress (Goerlich et al., 2012). Male offspring of these birds also had a dampened stress response, suggesting males may be more sensitive to corticosteroid changes during development. However further research into understanding mechanisms behind these behavioural changes and differences between males and females.

V. CONCLUSION

Injection into the CAM at embryonic day 11 can be used in poultry to mimic the stress levels of feed restricted breeder hens. Using this technique, we found reductions in average daily gain of corticosterone treated birds in the final week of growth, which may be due to changes in growth factors and metabolic disorders but more work in this area is need. Behaviour was also altered, with males impacted at day 14 and responding slower to the test. The reasons for this sex effect is unclear but may be due to increased sensitivity in males to corticosterone but more work to investigate this is needed. Elevated corticosterone in ovo can have significant impacts on broiler growth and behaviour but the reasons for these changes needs to be researched further to better understand the mechanisms behind them.

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EFFECTS OF EARLY ENRICHMENT ON RANGE USE IN FREE-RANGE LAYING HENS

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Free-range laying hen production systems are perceived to be preferable for hens' ethological needs. However, not all hens use the range daily with some hens rarely venturing outdoors (Campbell et al., 2016). Free-range hens are typically reared indoors until point-of-lay, when they are first provided outdoor access. The outdoor environment exposes hens to unpredictable conditions including variable weather and predation, in comparison to the controlled, sheltered indoor environment in which pullets are reared. Thus hens may be reluctant to use the outdoor range. Modifications to housing environments or management practices during rearing can alter hen behaviour as adults within indoor housing systems (Janczak and Riber, 2015) but there are few data on the impacts of early enrichment on range use in free-range systems.

In this study, 300 Hy-line Brown day-old chicks were reared in 2 rooms (n = 150 chicks/room) with unpredictable, variable environmental enrichments provided in 1 room from 4 – 21 days, and standard rearing conditions (non-enriched) in the other room. From 21 days onwards, all birds were provided the same housing conditions. At 12 weeks of age, pullets were transferred to an experimental free-range facility, equally divided into 6 pens (n = 3 enriched rearing treatment, n = 3 non-enriched rearing treatment) and all birds leg-banded with microchips. From 22 – 41 weeks of age, hens were provided daily range access with individual range use tracked via radio-frequency identification technology. From 39 – 41 weeks of age the range was shrunk to 20% of its original size to measure hens' range use responses to environmental change. Additionally, occurrences of natural hen disturbance behaviours (hens suddenly running towards the pop holes) on the range were decoded from video recordings at 23-25 and 35-36 weeks of age.

All data were analysed using General Linear Models in JMP® 12.1.0. Results showed the enriched hens on average, spent more hours on the range daily in the first 3 weeks of range access (22 – 25 weeks of age) ($P = 0.03$) including more individual visits to the range ($P < 0.0001$). Conversely, during weeks 35-38, the non-enriched hens spent more hours on the range daily ($P < 0.0001$) with more visits to the range ($P = 0.002$). After the range was reduced in size all hens spent less time on the range but there was no difference between treatment groups in the magnitude of reduction in daily time ranging ($P = 0.11$). The enriched hens however did increase their number of visits to the range more than the non-enriched-reared hens did ($P = 0.005$), spending less time per individual visit ($P < 0.0001$). There were no differences between treatment groups ($P = 0.11$) in occurrence of natural disturbance behaviours but significantly fewer disturbances on the range occurred at 35 – 36 weeks of age compared to 23 – 25 weeks of age ($P < 0.0001$).

These preliminary results show environmental enrichments provided during early development increased range use when birds were first provided range access but over time this pattern changed. The rearing treatments impacted birds' responses to environmental change but further replication of the enrichment rearing treatment, including trialing different types of enrichment (e.g. manipulable versus structural) are needed.

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EFFECT OF ARABINOXYLANS AND XYLO-OLIGOSACCHARIDES ON NET ENERGY IN BROILERS

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Summary

This study examines the effects of arabinoxylans and arabinoxylo-oligosaccharides (AXOS) on performance and net energy in broilers. These effects were assessed by feeding broiler diets containing either pure arabinoxylans, AXOS produced by exposing arabinoxylans to xylanase *in vitro* or arabinoxylans in combination with xylanase, from d10 to d21. FCR was numerically lower (1.15 vs 1.20) and metaboliseable energy intake was higher (P=0.049) in birds fed the diet with 2% AXOS compared with those fed the diet with 2% Arabinoxylans.

I. INTRODUCTION

Xylans and arabinoxylans are cell wall non-starch polysaccharides that are abundant in monocotyledonous plants, such as cereals. The presence of these pentosans has a direct negative impact on energy availability of a diet in monogastric species (Choct and Annison, 1990). These negative effects can be combatted by supplementing the diet with xylanase, which partially depolymerises the xylans and arabinoxylans, reducing the number of sugars in the molecular chains. These smaller sugars can be utilised more efficiently, which has a direct positive impact on overall energy utilisation of the cereals. Additionally, the arabinoxylo-oligosaccharides (AXOS) produced by depolymerisation of arabinoxylans can be selectively fermented by intestinal bacteria, which instigates positive effects on the composition and activity of gastrointestinal microbiota. This study examines whether it is more advantageous for arabinoxylans to be hydrolysed into AXOS *in situ* via supplemental enzymes, or to feed diets with AXOS that has been prepared *in vitro*. This was assessed by observing the impact on performance and energy utilisation of feeding broiler diets that contain either 2% pure arabinoxylans, 2% AXOS or 2% arabinoxylans in combination with xylanase. Net energy (NE) was assessed to determine the true energy value of the diets, as this method takes into account energy lost as heat and differences in metabolic utilisation of ME of nutrients for maintenance and production requirements (Noblet et al., 2010).

II. MATERIALS & METHODS

Male day-old Ross 308 chicks (n=90) were housed in 15 floor pens, 6 birds per pen. The birds were fed a standard wheat-soybean meal based starter diet from d0-10. They were then allocated to one of three dietary treatments from d10-21; a standard wheat-soybean meal based grower diet supplemented with either an additional 2% arabinoxylan, 2% AXOS or 2% arabinoxylan with 16,000 BXU xylanase (Econase® XT 25, AB Vista Feed Ingredients, Marlborough, UK). The pure arabinoxylan was isolated from a starch milling by-product by ethanol precipitation at the University of New England, and the AXOS was then produced by hydrolysing this arabinoxylan with 16,000 BXU xylanase. All diets were formulated to meet Ross 308 nutrient specifications and feed and water was provided *ad libitum* throughout the trial.

On d15, 2 birds per pen were allocated to one of fifteen closed-circuit calorimeter chambers, 5 replicates per dietary treatment. Birds were acclimatised to the chambers for four

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days prior to collecting data and calculation of heat production (HP). Metabolisable energy (ME) was determined by total collection of excreta and net energy was calculated as ME minus heat increment (Noblet et al., 2010). Individual bird feed intake and body weight gain was measured from d10-21.

III. RESULTS

FCR was lowest numerically in birds fed the diet with 2% AXOS. Feed intake and FCR was numerically highest and body weight gain (BWG) lowest in birds fed the diet with 2% Arabinoxylans.

Nitrogen retention was significantly higher in birds fed the diet with 2% AXOS and 2% arabinoxylans + xylanase compared to those fed the diet with 2% arabinoxylans. Analysis of ME of the diets did not reveal any difference, but birds fed the diet with 2% AXOS had significantly higher ME intake compared to those fed the diet with 2% Arabinoxylans.

Table 1- Effect of diets containing either 2% arabinoxylan, 2% AXOS or 2% arabinoxylan + xylanase on individual bird performance, energy balance and efficiency of energy utilisation in broilers from d10-21.

Diet	2% Arabinoxylans	2% AXOS	2% Arabinoxylans +Xylanase	SEM	*P*-Value
Performance					
Feed Intake (g)	996	967	988	7	0.524
BWG (g)	830	839	851	5	0.720
FCR	1.20	1.15	1.16	0.03	0.309
Energy Value (DM basis)					
ME feed (kJ/g)	13.51	13.48	13.47	0.15	0.995
MEn feed (kJ/g)	12.73	12.64	12.49	0.15	0.825
NE feed (kJ/g)	10.50	10.88	10.63	0.16	0.662
NE:ME	0.77	0.81	0.79	0.01	0.143
Energy Partition (kJ/b/d)					
ME	1547	1631	1631	32	0.532
NE	1163	1358	1289	22	0.056
HI	431	423	449	8	0.410
Energy/nitrogen balance (kJ/kg $BW^{0.70}$/d)					
ME intake	1497^{b}	1682^{a}	1631^{ab}	32	0.049
NE intake	1090	1240	1186	26	0.057
HP	814	817	831	4	0.122
HI	388	402	413	5	0.081
RE	683	865	801	32	0.054
Retained N (g/d/b)	2.50^{b}	3.06^{a}	3.46^{a}	0.12	0.001
Respiratory quotient					
RQ	1.03	1.02	1.02	0.00	0.363

ME Intake= Metabolisable energy intake (kj/kg $BW^{0.7}$/d);
NE intake= Net energy intake (kj/kg $BW^{0.7}$/d);
HP= Heat production (HP/$BW^{0.7}$/d);
FHP= Fasting heat production ($FHP^{0.7}$= 450);
HI=heat increment of feeding calculated as HP-$FHP^{0.7}$ x $BW^{0.7}_{fed}$;
RE = Retained energy (MEI-HP);
RQ=respiratory quotient calculated as the volume of CO_2 expired/the volume of O_2 consumed;
ME= Metabolisable energy measured by total excreta collection for indirect calorimetry (3 d);
NE= Net energy (RE+FHP/per g of DM intake);
NE/b/d = Net energy /bird/day (RE + $FHP^{0.7}$ x $BW^{0.7}_{fed}$)

IV. DISCUSSION

Measurements of NE provide an estimate of efficiency of utilisation, and in this study suggest that the diet containing 2% arabinoxylans required comparatively more digestive and metabolic effort for the birds to utilise, meaning this diet was less efficient at providing energy for maintenance and production. This may be partly because the weight and relative proportion of energetically active organs, such as the gastrointestinal tract and pancreas was greater in birds fed this diet (Wu et al., 2004), which increased the total cost of maintenance. The observed numerical positive effect of xylanase on energy efficiency in this study is likely because it reduced intestinal viscosity and improved nutrient digestibility.

Results from this study suggest that AXOS has a positive effect on broiler performance and energy utilisation. When AXOS is fermented in the caeca it produces readily absorbed short chain fatty acids which can be used as an energy source (Choct et al., 2010); for example butyrate is released which has anti-inflammatory properties, fuels epithelial cells and increases intestinal epithelial integrity (De Maesschalck et al., 2015). AXOS also selectively stimulate beneficial bacteria, namely bifidobacteria, and non-digestible carbohydrates act as the main source of energy during microbial proliferation in the hindgut (Eeckhaut et al., 2008). An interesting observation from this study was that feeding AXOS prepared *in vitro* resulted in numerically higher ME and NE intake and NE/b/d compared with feeding the arabinoxylans in combination with xylanase. This was probably because depolymerisation of NSP *in situ* is not instantaneous, and hence AXOS generation in the gut via the use of enzymes is not as efficient as feeding AXOS directly. The findings from this study suggest that AXOS has potential to be an efficacious prebiotic in broiler diets.

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EFFICIENCY OF XYLANASES FROM FAMILY 10 AND FAMILY 11 IN PRODUCTION OF XYLO-OLIGOSACCHARIDES FROM WHEAT ARABINOXYLANS

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Summary

This study investigated the effect of xylanases from family 10 and family 11 on the production of arabinoxylo-oligosaccharides (AXOS) from wheat arabinoxylan, and identified the impact that pH had on this process. Xylanase from family 10 had greater hydrolytic efficiency and resulted in heightened AXOS production compared with xylanase from family 11 or family 10 and 11 combined. The greatest total conversion of arabinoxylan into AXOS was observed at pH 2.5, with approximately 37% conversion.

I. INTRODUCTION

Arabinoxylans in cereal fibres are unable to be digested in the small intestine of monogastric animals, but beneficially affect the host by stimulating health-promoting bacteria in the distal gut (Sanchez et al., 2008). Arabinoxylo-oligosaccharides (AXOS) and non-substituted xylo-oligosaccharides (XOS) are produced by hydrolytic degradation of arabinoxylans by endo-β1,4-xylanases (De Maesschalck et al., 2015; Broekaert et al., 2011). The structure of XOS vary in degree of polymerization, degree of substitutions, monomeric units and types of linkages. The number of xylose residues in the backbone can vary from 2 to 10; for example xylobiose ($C_{10}H_{18}O_9$), xylotriose ($C_{15}H_{26}O_{13}$), xylotetraose ($C_{20}H_{34}O_{17}$) and xyloglucan (consisting of xylo hepta, octa and nona saccharides). Selective fermentation of XOS has been shown to instigate positive effects on the composition and activity of gastrointestinal microbiota, thus suggesting XOS can be defined as a prebiotic. The prebiotic properties are dependent on the impact that enzymatic treatment has on degrees of polymerization and substitution. Xylanases are classified into enzyme families based on their catalytic modules; within the classification system xylanases are usually grouped into glycoside hydrolase (GH) families 10 and 11, based on amino acid sequence similarity. Family 10 and family 11 xylanases differ from each other with regards to their specificities, in that family 11 xylanases are active exclusively on substrates that contain D-xylose, whereas family 10 xylanases exhibit greater catalytic versatility as they have a more flexible structure (Biely et al., 1997), and can hence hydrolyze highly substituted xylans more efficiently. Family 10 xylanases also display greater activity on short AXOS than family 11, signifying presence of smaller binding sites. The conditions that xylans and xylanases are exposed to also dictate oligosaccharide production. Changes in pH cause the protein structure of enzymes, particularly the tertiary structure as the pH is acidified, to alter. Enzyme pH profile is dependent on the pKas of the catalytic residues, for examples residues that contribute positive charges and hydrogen bonds lower the pKa value (Joshi et al., 2001). The aim of this study was to investigate the effect of xylanases from family 10 and family 11 on production of xylo-oligosaccharides from wheat arabinoxylan, and identify the impact that pH has on this process.

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II. MATERIALS & METHODS

Xylan was isolated from an arabinoxylan-rich wheat milling by-product by the alkaline extraction procedure described by Choct and Annison (1990). The xylan was hydrolyzed by xylanases from glycoside hydrolase family 10 (Econase XT 25, AB Vista Feed Ingredients, Marlborough, UK), family 11 (Econase XT, AB Vista Feed Ingredients, Marlborough, UK) and family 10 and 11 combined in equal quantities. Two grams of xylan were suspended in 25ml of citrate buffer (pH 5.4) containing 0.02% azide. The pH of the solution was adjusted to either 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 or 7 (+/- 0.05) using 0.1M HCl and 0.1M NaOH, measured using a digital pH meter. The total volume of HCl and NaOH added was recorded. 0.44ml of 16,000 BXU/g xylanase (either family 10, family 11 or equal amounts of family 10 and 11 (8,000 BXU/g xylanase of each)) was added to the samples. The temperature of the samples was then adjusted to 42°C in a water bath and was maintained at this temperature for 120 minutes. All the samples were then adjusted to pH 7 (+/- 0.05) with 0.1M NaOH, and the total volume of NaOH added was recorded. The samples were then centrifuged for 10 minutes at 3000 x g and the aqueous phase was collected and filtered through 22µm syringe filters. Standards of 0, 1000, 2500 and 5000µl of xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xyloglucan (X_7, X_8 and X_9) (Megazyme, Wicklow, Ireland, UK) were prepared with QH_20. 500µl of prepared standard or sample was then combined with 500µl of 0.3M NaOH and 500µl of 0.5M 1-Phenyl-3-methyl-5-pyrazolone. The sample was then incubated at 70°C for 30 minutes, cooled to room temperature and then pH was adjusted to pH 7 using 0.3M HCl. 800µl of the resulting solution was washed with 400µl of chloroform and centrifuged for 10 minutes at 3000 x g and the organic phase was discarded. This was repeated 3 times. The resulting aqueous phase was than diluted 1:3 with QH_2O and then filtered through a 22µm syringe filter. Analysis of the standards and samples was carried out by HP1100 HPLC, 5uL injection using Thermo Scientific BDS Hypersil C18 column (150x4mm), maintained at 30°C. Mobile phase was sodium phosphate buffer (40mM, pH 8) and acetonitrile (79:21 v/v) at flowrate 0.8ml/min. Detection was at 254nm.

III. RESULTS

The greatest degradation of arabinoxylans into AXOS was found at pH 2.5, with approximately 37% of the total xylan being converted into AXOS. This was followed by pH 3 and pH 2 in which approximately 29% and 23% respectively of the total xylan were converted into AXOS (Table 1). At pH 2.5 and 3 the AXOS remaining following hydrolysis were predominantly larger oligosaccharides, with approximately 12% and 15% xyloglucans respectively and approximately 8% and no detectable xylobioses at pH 2.5 and 3 respectively. At pH 2 however there was equal distribution of the resulting AXOS, with approximately 7% xylobioses and xylotetraoses and 5% xylotrioses and xyloglucans. The lowest total conversion of xylan into AXOS was found at pH 5 and 5.5.

Interactions between xylanase family and pH were observed for the total and all the individual AXOS analyzed in this study ($P < 0.001$). Xylobiose production was greater in the presence of the family 10 and family 11 xylanases individually compared with the combination of them together at pH 2, but at pH 2.5 it was greater in the presence of family 10 and 11 combined and family 10 compared to family 11. At pH 2 and 2.5 family 10 xylanases produced more xylotrioses than family 11 and family 10 and 11 combined, but at pH 6.5 family 11 xylanases produced more xylotrioses than family 10 and family 10 and 11 combined.

Table 1 - Total proportion of xylan converted into xylo-oligosaccharides by xylanase (g/100g).

pH	Xylobiose	Xylotriose	Xylotetraose	Xyloglucan	Total
2	6.98	4.83	6.77	4.54	23.14
2.5	8.42	10.51	6.26	11.97	37.16
3	0.01	3.54	10.99	14.72	29.26
3.5	0.26	1.23	2.50	2.98	6.97
4	0.52	0.38	0.59	7.70	9.20
4.5	0.40	0.13	3.47	6.67	10.67
5	0.17	0.24	0.93	4.41	5.75
5.5	0.00	0.40	0.50	4.50	5.40
6	0.12	0.83	3.04	3.45	7.44
6.5	0.18	1.85	5.84	2.32	10.18
7	1.79	1.92	3.48	1.33	8.52
SEM	0.87	0.89	0.92	1.20	3.10
Xylanase					
Family 10	2.14	3.95	4.64	5.26	15.99
Family 11	1.82	1.64	4.04	6.03	13.53
Family 10 & 11	1.18	1.47	3.33	6.45	12.42
SEM	0.23	0.65	0.31	0.29	0.86

IV. DISCUSSION

The optimum scenario is an almost complete degradation of xylan into xylobioses and xylotrioses, as hydrolysis of xylan eliminates its anti-nutritional effects and AXOS have been shown to present prebiotic properties, particularly in monogastric species. In this study it was observed that the lower the pH the greater the degradation of the xylan into smaller saccharide units such as xylobiose disaccharides and xylotriose trisaccharides, and the higher the pH the more longer-chained saccharides were left undegraded. The lowest total conversion of xylan into AXOS was found at pH 5 and 5.5, which was surprising as the pH optimum for the specific xylanases used in this study is believed to be approximately 5 to 5.5.

Exposing the xylan to the family 10 xylanase resulted in greater production of total AXOS than family 11 or family 10 and 11 combined. This suggests that family 10 xylanases are able to cleave more linkages closer to the substituent groups and hence hydrolyze substituted xylan to a comparatively greater extent. Xylobiose production was greater in the presence of the family 10 and family 11 xylanases individually compared with the combination of them together at pH 2. This may be because in combination both enzymes were at a concentration of just 8,000 BXU/g compared to 16,000 BXU/g, potentially resulting in just partial degradation as opposed to complete hydrolysis. Also, the two enzymes potentially inhibited each other by partially degrading and altering the structure of the substrate, therefore reducing the availability of the optimum form of the substrate for the opposing xylanase. At pH 2.5, however, xylobiose production was greater when the xylan was in the presence of xylanases from family 10 and 11 combined and family 10 compared to family 11. The positive effect observed when the enzymes were in combination may be because at this pH the enzymes worked cooperatively (Collins et al., 2005), the family 11 xylanase were less efficacious so did not obstruct the family 10 xylanase or that the products resulting from the actions of the family 10 xylanase were further hydrolyzed by the family 10 enzyme (Biely et al., 1993). The comparatively heightened degradation induced by the family 10 xylanases is likely due to the lack of specificity and versatility of family 10 xylanases and because this was the optimum pH for this xylanase family. In conclusion, the findings from this study suggest that xylanases from family 10 have greater hydrolytic efficiency compared

to those from family 11 and family 10 and 11 combined, and that pH of the environment has a significant impact on xylan degradation. This could have a direct impact on the focus of xylanase research and understanding of how xylanase works in the gastrointestinal tract.

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TAKING PHYTASE SUPERDOSING FROM SCIENTIFIC CONCEPT TO COMMERCIAL APPLICATION: A UK EXAMPLE

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Summary

Scientific research is based on carefully designed experiments, performed in carefully controlled conditions with the outcome measured as accurately as possible, using a range of parameters. In commercial practice, the reality is that performance is measured on farms with a variety of confounding factors influencing the few parameters that are ultimately considered. In order to get nutritional concepts accepted by commercial nutritionists, it is often required to do 'commercial testing' to bridge the gap between these two realities. This paper gives a case study of a possible approach, and nicely shows some potential pitfalls in doing this.

I. INTRODUCTION

Phytase superdosing has been shown to increase broiler performance, both in terms of growth rate and in FCR, combining in a typical improvement of 3-4 points weight corrected FCR as compared to the use of a standard dose of phytase (Walk *et al.* 2013; Walk *et al.* 2014). Commercial evaluations can take the form of replicated pen studies, house to house comparisons or farm to farm comparisons, either simultaneously run or compared over time. In this case it was decided to use a combined approach of comparing both across farms and time, as it was not possible to split farms and guarantee accurate collection of data.

II. MATERIALS AND METHODS

Within a UK broiler integration successive crops on 25 farms over a four month period were allocated either to Control or Superdosing in an alternating manner. This resulted, for most farms, in either one or two crops being fed Control diets with the other one being fed Superdose diets. For Control crops all feeds contained both xylanase and phytase at standard levels (Econase XT at 16,000 BXU/kg and Quantum Blue at 500 FTU/kg, AB Vista) and the diets were typical UK vegetable diets based on wheat and soya. For the Superdose crops the only dietary difference was an increase in the dose of the phytase from 500 FTU/kg to 1500 FTU/kg. Some (n=2) crops were excluded from final analysis where the killing programme was adjusted to produce Christmas birds or other identified factors affected the results. In the end, 32 Control crops and 23 Superdose crops were used in the analysis.

The main performance parameters (Average Weight, daily gain, Feed/bird, Weight for age, and FCR) for Control and Superdosed flocks were subjected to an ANOVA using the standard least squares procedure of JMP 13.0 (SAS Institute Inc., Cary, NC). The statistical model included QB dosage, farm, average age and month of clear date as co-variates.

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III. RESULTS

Table 1 - The mean, min, max and standard deviation of the measured production parameters.

	Mean	Min	Max	Standard Deviation
Av Wt (kg)	2.23	2.03	2.57	0.1
Daily Gain (g/d)	62.45	57.42	68.62	2.28
Feed / bird (kg)	3.63	3.3	4.16	0.19
Wt FCR (kg/kg)	1.66	1.54	1.84	0.05
Wt for Age (kg)	2.74	2.52	2.92	0.08

There were a number of significant co-variate effects of farm, average age and month of clear date. Farm had a significant effect on average weight, daily gain, EPEF, weight corrected FCR. Average age had a significant effect on average weight, feed consumption per bird and meat production per metre squared. Average performance measures; weight, daily gain, and weight for age were significantly ($P \leq 0.05$) increased in Superdose flocks. Superdosed flocks also had a tendency for a higher feed per bird ration and greater meat yield per square metre (see Table 2).

Table 2 - Effects of Superdosing on production measures. * indicates ($P \leq 0.05$) ˙ indicates a tendency ($P < 0.10$) towards significance.

	LSM		
Variable	Control	Superdosed	Significance
Average weight (kg)	2.27 ± 0.07	2.31 ± 0.07	*
Daily gain (g/d)	63.79 ± 2.04	64.9 ± 2.05	*
Weight for age (kg)	2.79 ± 0.07	2.83 ± 0.07	*
Feed/bird (kg)	3.54 ± 0.17	3.61 ± 0.17	.

Although most other parameters showed numerical improvements, there were no further statistically significant differences, demonstrating the inherent variability of parameters under commercial conditions. Financial calculations, done by the integration and taking into account the extra cost of the phytase, showed a difference in margin over feed and chick of £0.0098 per chick, which is an economically significant improvement.

IV. DISCUSSION

This retrospective analysis of commercial performance and health data indicates that Superdosing provided significant system benefits including improved average weight, daily gain and weight for age as well as a tendency towards significant improvements in meat yield per square metre and higher feed consumption per bird. Whilst the average results are sufficiently interesting for the commercial nutritionist to base a decision on, the dataset also contains some observations useful for future studies of this kind. Firstly, the range of results between farms was large, yet normal, for commercial conditions (see Table 1). Given the number of flocks/farms involved, this data can be viewed with some confidence however, if a given pair of farms or flocks were to be compared, then clearly any outcome could be possible, even though the conditions on these farms were fairly standardised and management was generally considered to be good. Additionally, there were a number of significant co-variate effects of farm, average age and month of clear date on production and health parameters showing the importance of considering these when making commercial comparisons.

Commercial evaluations require careful thought, planning and analysis to get the best possible conditions for comparing treatments. Where this is done and the results achieved are sufficiently convincing, then a promising scientific concept can be evaluated and translated into a commercial application.

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NEGATIVE IMPACTS OF PHYTATE ARE SUBJECT TO DIGESTIBLE LYSINE:STARCH RATIOS IN POULTRY DIETS

S.Y. LIU[1], P.V. CHRYSTAL[2], A.F. MOSS[1] and P.H. SELLE[1]

Summary

The objective of this study was to investigate the influence of phytate on growth performance in Ross 308 broiler chickens offered different levels of digestible lysine, starch and lipid in twelve maize-soy diets from 7 to 28 days post-hatch. Diets with high lysine:starch ratios generated lower feed intakes ($P < 0.001$) and diets with higher lipid concentrations reduced weight gains ($P < 0.005$). Importantly, there were interactions ($P < 0.01$) between dietary phytate-P concentrations and lysine:starch ratios for weight gain and FCR. Phytate had a more pronounced negative impact on growth performance in broiler chickens offered diets with low digestible lysine and high starch levels.

I. INTRODUCTION

Phytate (*myo*-inositol hexaphosphate; IP_6) is a ubiquitous component of human foods and animal feedstuffs of plant origin. Phytate may form binary protein-phytate aggregates at pH lower than the isoelectric point of proteins, compromise starch digestibility, and impede intestinal uptakes of glucose and amino acids via Na^+-dependent transport systems (Selle *et al.*, 2015). Thus the aim of this study was to determine the impact of dietary phytate levels with different protein (digestible lysine), starch and lipid concentrations on broiler performance.

II. MATERIALS AND METHODS

Twelve iso-energetic diets based on maize and soybean-meal were formulated to contain three levels of digestible lysine:starch ratios (0.025, 0.040 and 0.055), two levels of phytate concentrations and correspondingly high and low lipid concentrations (Table 1). All diets contained similar ideal protein ratios and were cold-pelleted and then crumbled. Each of the twelve dietary treatments was offered to 3 cages (6 birds per cage) or a total of 216 male Ross 308 chicks from 7 to 28 days post-hatch. Initial and final body weights were determined, and 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 FCR calculations. ANOVA and tests of significance were performed using JMP® 9.0.0 and differences were considered significant at $P < 0.05$. This feeding study complied with guidelines of the Animal Ethics Committee of The University of Sydney.

III. RESULTS AND DISCUSSION

Growth performance results are shown in Table 2 where the 1.4% mortality rate was not influenced by dietary treatments ($P > 0.25$) Broiler chickens offered diets with higher lysine:starch ratios had significantly lower feed intakes (1716 g/kg, $P < 0.001$); however, lipid and phytate concentrations did not influence feed intake. Diets with high dietary lipid concentrations had lower weight gains (1416 versus 1484 g/bird, $P < 0.005$). There were significant interactions ($P < 0.01$) between dietary phytate-P concentrations and lysine:starch

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ratios for weight gain and FCR. Phytate had a more pronounced negative impact on growth performance in broiler chickens with the dietary context of low digestible lysine and high starch. As illustrated in Figure 1 the interactions where increasing phytate-P from 2.6 to 3.0 g/kg depressed weight gain by 15.6% (1628 versus 1374 g/bird), 7.55% (1550 versus 1433 g/bird) and 5.45% (1395 versus 1319 g/bird) in diets containing lysine:starch ratios of 0.025, 0.040 and 0.055, respectively. Similarly, increasing dietary phytate levels correspondingly compromised FCR by 21.6 (1.130 versus 1.374 g/g), 16.0% (1.124 versus 1.304 g/g) and 3.04%.

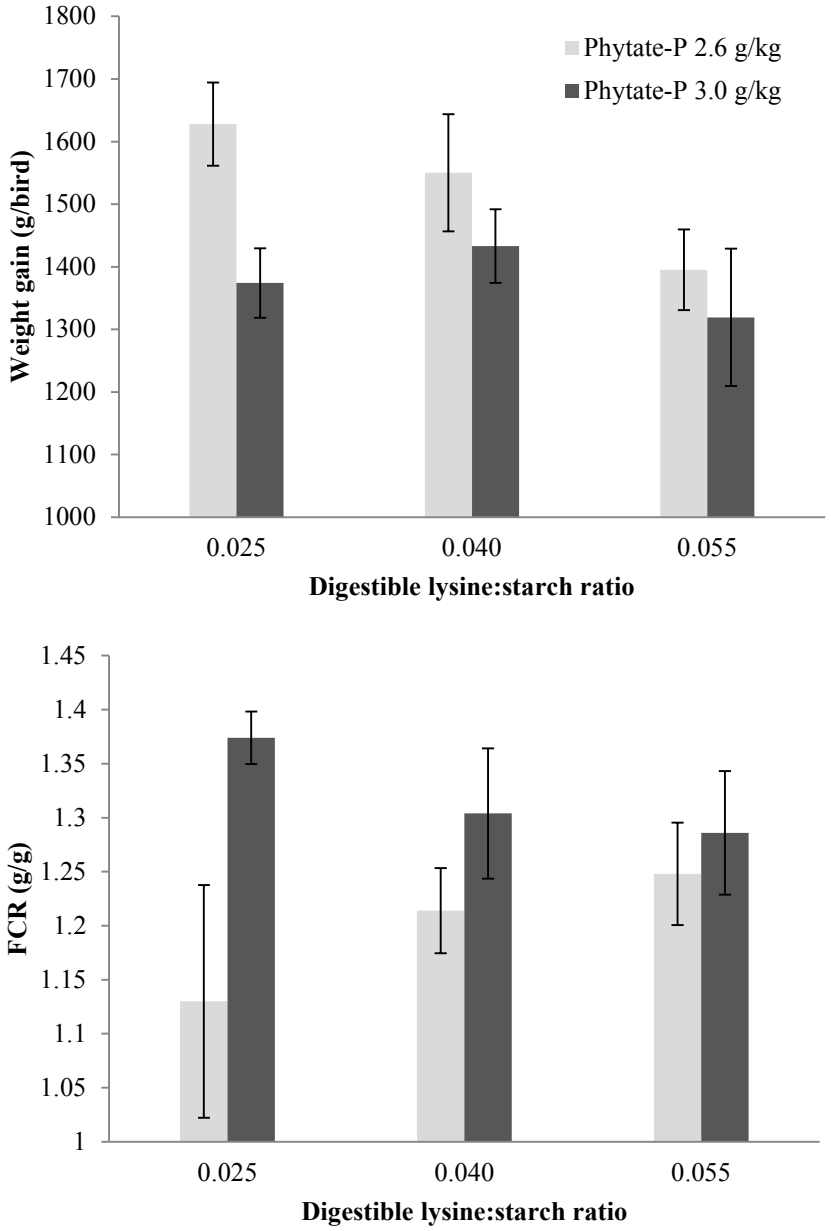

Figure 1 - The interaction between phytate-P concentrations and lysine: starch ratios on weight gain and FCR in broiler chickens from 7-28 days post-hatch.

Broiler chickens offered diets 1 to 6, with typical phytate-P concentrations of 2.6 g/kg, had an average FCR of 1.197 and weight gain of 1524 g/bird from 7-28 days post-hatch, which represents respective improvements of 18.9% and 9.9% relative to 2014 Ross 308 performance objectives. Phytate generated the greatest negative impacts on weight gain and FCR in birds fed diets with the lowest digestible lysine:starch ratio. Phytate has the capacity

to inhibit amylase activity *in vitro*, Sharma *et al.* (1978) and Nitsan *et al.* (1991) suggested that amylase does not attain maximum activity until 8 days post-hatch. While amylase activity may not limit starch digestion in poultry, any compensatory hypersecretion of amylase to counter phytate induced inhibition would increase endogenous amino acid flows and decrease apparent amino acid digestibilities.

The higher 3.0 g/kg phytate-P concentration depressed gain and feed conversion within the context of significant interactions between phytate and digestible lysine:starch ratios. One possibility is that these interactions were driven by the adverse impact of phytate on 'protein' rather than starch. It may be deduced that protein:phytate ratios were positively correlated with weight gain (r = 0.579; P = 0.049) and negatively correlated (r = -0.667; P = 0.018) with FCR. The probabilities of the corresponding correlations with starch:phytate ratios were not significant. Thus, the negative influence of phytate on protein/amino acid utilisation (Selle *et al.*, 2012) appears to be diluted by increasing dietary protein. However, dietary treatments contained variable quantities of synthetic amino acids ranging from 1.86 to 8.76% of total protein (Table 1). Moreover, synthetic amino acid concentrations were positively correlated with weight gain (r = 0.747; P = 0.005) and negatively correlated with FCR (r = -0.664; P = 0.024), which indicates their efficient utilisation.

Remarkably, increasing synthetic lysine:phytate ratios linearly improved weight gain (r = 0.682; P = 0.015) and FCR (r = -0.660; P = 0.019); therefore, broilers responded to unbound synthetic lysine better than protein-bound lysine as diets contained equal quantities of digestible lysine. This suggests that the phytate impacts more negatively on protein-bound lysine as a consequence of protein-phytate interactions. Lysine may be both a critical and unique amino acid as lysine may be conserved in tissue pools (Benevenga and Blemings, 2007), which would improve synthetic lysine utilisation if this applies to poultry. Lysine HCl has been shown to up-regulate intestinal uptakes of lysine and some other amino acids (Torras-Llort *et al.*, 1998) and increase ileal digestibilities of several additional amino acids (Selle *et al.*, 2007). Given the increasing interest in low protein-high synthetic amino acid broiler diets, the outcomes of the present study should be instructive. The performance of broiler chicks offered diet 1 was outstanding and this maize-soy diet contained a low 186 g/kg protein content, 4.9 g/kg lysine HCl and 16.3 g/kg protein derived from synthetic amino acids. This suggests that the performance of poultry on low-protein diets can be highly satisfactory and the task is to identify and rank the dietary factors contributing to this outcome. In this study it appears that a combination of low digestible lysine:starch ratios, low lipid and phytate concentrations were contributing factors to a dietary context in which synthetic amino acids were effectively utilised.

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Table 1 - Diet composition, calculated and analysed nutrient specifications in experimental diets for broiler chickens from 7-28 days post-hatch.

Ingredients (g/kg)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
Oats	0	105	210	66	279	493	0	91	182	0	188	376
Maize	679	361	43	572	296	21	591	423	256	609	349	89
Maize starch	0	82	164	8	4	0	0	0	0	0	0	0
Soybean meal	216	332	447	266	289	312	203	286	370	160	202	244
Canola meal	21	11	0	0	5	11	127	93	60	150	146	142
Soybean oil	13.4	45.4	77.5	27.5	66.8	106.1	24.5	48.9	73.3	21.4	58.3	95.1
Lysine HCl	4.9	3.2	1.5	2.4	2.0	1.5	1.5	1.6	1.7	2.7	2.1	1.4
Methionine	3.6	3.7	3.8	2.7	2.8	3.0	2.1	2.7	3.4	2.3	2.2	2.2
Threonine	2.2	1.5	0.9	1.0	0.9	0.8	0.3	0.6	1.0	0.9	0.7	0.5
Tryptophan	0.2	0.1	0.0	0.3	0.2	0.0	0.2	0.2	0.1	0.4	0.2	0.0
Valine	2.3	1.4	0.5	0.8	0.5	0.2	0.0	0.3	0.6	0.8	0.4	0.0
Arginine	2.3	1.2	0.0	1.5	0.7	0.0	0.9	0.8	0.8	2.1	1.1	0.0
Isoleucine	1.9	0.9	0.0	0.4	0.2	0.0	0.1	0.1	0.2	0.8	0.4	0.1
Salt	1.0	1.6	2.3	1.8	1.9	2.0	2.1	2.1	2.0	1.7	1.9	2.1
Sodium Bicarbonate	3.7	2.8	1.9	2.6	2.3	2.0	2.0	2.1	2.2	2.6	2.2	1.8
Limestone	8.2	7.1	5.9	8.0	6.9	5.9	7.4	7.0	6.6	7.3	6.4	5.5
Dicalcium Phosphate	16.6	17.8	19.0	17.0	18.1	19.2	15.6	16.3	17.0	15.4	16.5	17.6
Choline Cl	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Premix	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Celite™	20	20	20	20	20	20	20	20	20	20	20	20
Calculated												
Metabolisable energy	12.75	12.75	12.75	12.75	12.75	12.75	12.75	12.75	12.75	12.75	12.75	12.75
Protein	186	215	243	192	199	206	195	201	206	190	200	210
Lipid	48	72	95	59	99	138	67	87	107	67	103	138
Starch	460	357	255	420	315	209	400	323	246	412	311	209
Calcium	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Available P	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Phytate P[1]	2.6	2.6	2.6	2.6	2.6	2.6	3.0	3.0	3.0	3.0	3.0	3.0
Digestible lysine	11.5	12.76	14.01	10.5	11	11.5	10	11.75	13.49	10.3	10.9	11.5

[1]Calculation was based on phytate phosphorus concentrations in ingredients reported in Selle *et al.* (2003)

Table 2 - The influence of phytate-P concentrations on growth performance in broiler chickens offered diets contain different lysine: starch ratios and lipid concentrations.

Diet	Lysine: Starch ratio (%/%)	Lipid concentration	Phytate -P (g/kg)	Feed intake (g/bird)	Weight gain (g/bird)	FCR (g/g)	Mortality (%)
1	0.025	Low	2.6	1849	1634	1.134	0.0
2	0.040	Low	2.6	1823	1621	1.126	0.0
3	0.055	Low	2.6	1927	1633	1.180	5.6
4	0.025	High	2.6	1830	1468	1.247	0.0
5	0.040	High	2.6	1762	1443	1.222	0.0
6	0.055	High	2.6	1715	1347	1.274	0.0
7	0.025	Low	3.0	1881	1370	1.373	0.0
8	0.040	Low	3.0	1895	1378	1.375	5.6
9	0.055	Low	3.0	1806	1436	1.258	0.0
10	0.025	High	3.0	1930	1430	1.351	0.0
11	0.040	High	3.0	1756	1387	1.269	5.6
12	0.055	High	3.0	1629	1251	1.302	0.0
	SEM			57.0	36.6	0.0351	2.78
Main effects							
Lysine: Starch ratio	0.025			1862[a]	1501	1.252	1.4
	0.040			1873[a]	1492	1.259	1.4
	0.055			1716[b]	1357	1.267	1.4
Lipid concentration	Low			1830	1484[a]	1.239	1.9
	High			1804	1416[b]	1.279	0.9
Phytate-P concentration	2.6			1818	1524	1.197	0.9
	3.0			1816	1375	1.321	1.9
P-value							
Lysine: Starch ratio				< 0.001	<0.000	0.841	1.000
Lipid concentration (L)				0.436	0.004	0.061	0.569
Phytate-P concentration (P)				0.958	<0.000	<0.000	0.569
Interactions							
L:S × L				0.429	0.099	0.266	0.282
L:S × P				0.479	0.006	0.001	0.282
L × P				0.367	0.282	0.882	0.569
L:S × L × P				0.192	0.166	0.900	0.282

Table 3 - The interactions between phytate-P concentrations and lysine: starch ratios on weight gain and FCR in broiler chickens from 7-28 days post-hatch.

Lysine: Starch ratio (%/%)	Phytate-P concentration (g/kg)	Weight gain (g/bird)	FCR (g/g)
0.025	2.6	1628[a]	1.130[d]
0.025	3.0	1374[cd]	1.374[a]
0.040	2.6	1550[b]	1.214[c]
0.040	3.0	1433[c]	1.304[ab]
0.055	2.6	1395[c]	1.248[bc]
0.055	3.0	1319[d]	1.286[bc]

ILEAL AMINO ACIDS DIGESTIBILITY IN RESPONSE TO INCREASING PHYTASE DOSE OR MCP LEVELS IN BROILERS

Y. DERSJANT-LI[1] and C. KWAKERNAAK[2]

Summary

This study tested the effect of increasing phytase dose on ileal AA digestibility, AMEn and performance in Ross 308 male broilers fed corn/soybean meal based test diets from 5-21 days of age. A negative control diet (NC) was formulated without inorganic P, and supplemented with four different dose levels of phytase, with either a *Buttiauxella* sp phytase up to 1050 FTU/kg feed or an *E.coli* phytase up to 1810 FTU/kg feed. Three PC diets with addition of 0.6, 1.2 and 1.8 g P from MCP/kg feed to NC were used as reference. Increasing phytase dose showed a linear increase in ileal dig AA and AMEn, a greater efficacy of *Buttiauxella* phytase was found compared to *E. coli* phytase based on the slope ratio. *Buttiauxella* sp phytase at 1050 FTU/kg increased ileal AA digestibility, AMEn and reduced FCR ($P < 0.05$) compared to the PC with 1.8 g/kg P from MCP and revealed an extra-phosphoric effect.

I. INTRODUCTION

Commercial poultry feed is formulated mainly with plant based ingredients including cereals and oil seeds. These ingredients provide low available P because 70-80% of P is in the form of phytate, which is poorly utilized by mono-gastric animals. In addition, it is well known that phytate can bind to proteins mainly at the stomach pH and bind to minerals mainly at the intestinal pH and therefore reduce the availability of other nutrients such as amino acids. Phytase has been traditionally used at 500 FTU/kg with the primary objective of improving phytate P digestibility and therefore reduce the excretion of P to the environment. Recent studies demonstrated that phytate degradation rate was positively co-related to amino acids digestibility (Amerah et al, 2014; Liu et al. 2014; Truong et al, 2015). The objective of this study was to determine ileal AA digestibility and AMEn in response to increasing phytase dose in broilers. Performance and tibia ash were also measured.

II. MATERIALS AND METHODS

The trial comprised in total twelve dietary treatments with 6 replicates/ treatment. A negative control (NC) diet was formulated with 2900 kcal/kg AMEn, 21% CP, 0.18% retainable P, 0.25% phytate-P, and 0.65% Ca. NC was supplemented with 4 graded levels of either a new generation *Buttiauxella* sp phytase (expressed in *Trichoderma reesei,* highly active at low pH and wide pH range) or an *E coli* phytase (expressed in *Pichia pastoris*) at targeted dose level of 250, 500, 750, 1000 FTU/kg feed. Three positive control diets (PC) were used to determine the response of the broilers to increasing retainable P levels, with addition of MCP to supply 0.6, 1.2 and 1.8 g p per kg feed to NC. Dietary Ca was increased by 0.8 g/kg in the PC diets to maintain a reasonable Ca/P ratio. In order to assure a similar diet composition, apart from Ca, P and phytase, one basal mixture of feed was produced, containing 0.25% titanium dioxide as an inert dietary marker. This batch was divided to 12 sub-batches, and were used for production of the PC diets (adding MCP, diamol and $CaCO_3$) and the test diets (adding phytase and diamol). No coccidiostat or other enzymes were added to the diets. Diets were analysed for P and Ca content and the analysed values were very similar to formulated

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values. The phytase premix samples and dietary samples were analysed by LUFA, Germany (an independent laboratory) to determine the phytase activity. The analysed activity was higher than targeted activity for *E coli* phytase and therefore the analysed activity was used for both phytase sources being 300, 530, 890 and 1050 FTU for the *Buttiauxella* phytase and 440, 960, 1500 and 1810 FTU for the *E.coli* phytase. One-day-old Ross 308 male broilers were housed in a large floor pen during the 5 day pre-experimental period and were fed a pelleted, nutritional adequate broiler starter diet. At 5 days of age 1152 healthy broilers were allocated to 72 two-tier balance cages (16 broilers per cage) based on a weight class system to achieve similar mean BW per cage at the start of the experiment. The experimental diets were pelleted and fed to the birds at *ad libitum* from 5-21 days of age. Water was freely available during the whole period.

Ileal contents were collected at the last day of the experiment. Ileal chyme samples were freeze-dried, ground and analysed for dry matter, Ti and amino acids (excl. Tryptophan). Excreta were collected per cage during three successive days (18, 19 and 20), for N and AMEn measurement. The data were analysed as a randomised block design by analysis of variance (ANOVA) using GenStat® statistical package for Windows (14th edition). A cage was the experimental unit. Treatment means were compared by LSD. P≤ 0.05 is statistically significant.

III. RESULTS

Increasing phytase dose resulted in linear increase in AMEn and ileal digestible AA, while ADG, FCR and tibia ash responded in a more curvilinear manner. Ileal total amino acid (sum of 17 AA) content increased linearly with increasing phytase dose, however, the slope was greater with *Buttiauxella* phytase than *E. coli* phytase (see Figure 1), indicating a greater efficacy. Increasing inorganic P level from 0.6 to 1.8g/kg form MCP numerically increased ileal digestible total AA, however, *Buttiauxella* phytase at 1050 FTU/kg significant increased ileal digestible total AA compared to all three PC diets and the *E. coli* phytase at the similar dose. Similar dose response was seen with AMEn (Figure 2).

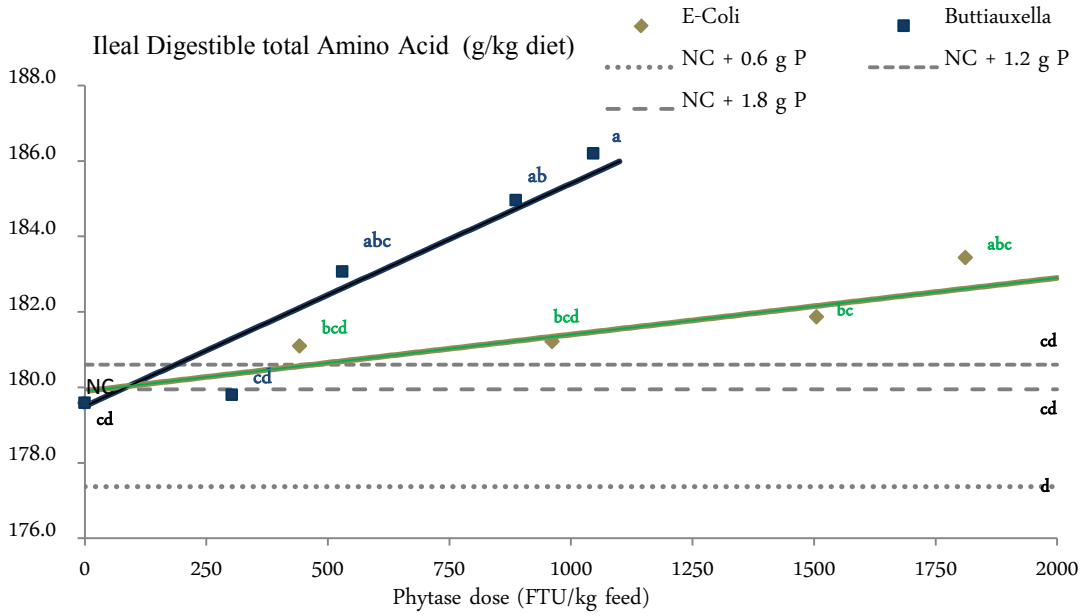

Figure 1 - Ileal digestible total amino acids (17 amino acids) in response to increasing phytase dose and inorganic P levels (a,b,c: significant difference at P<0.05).

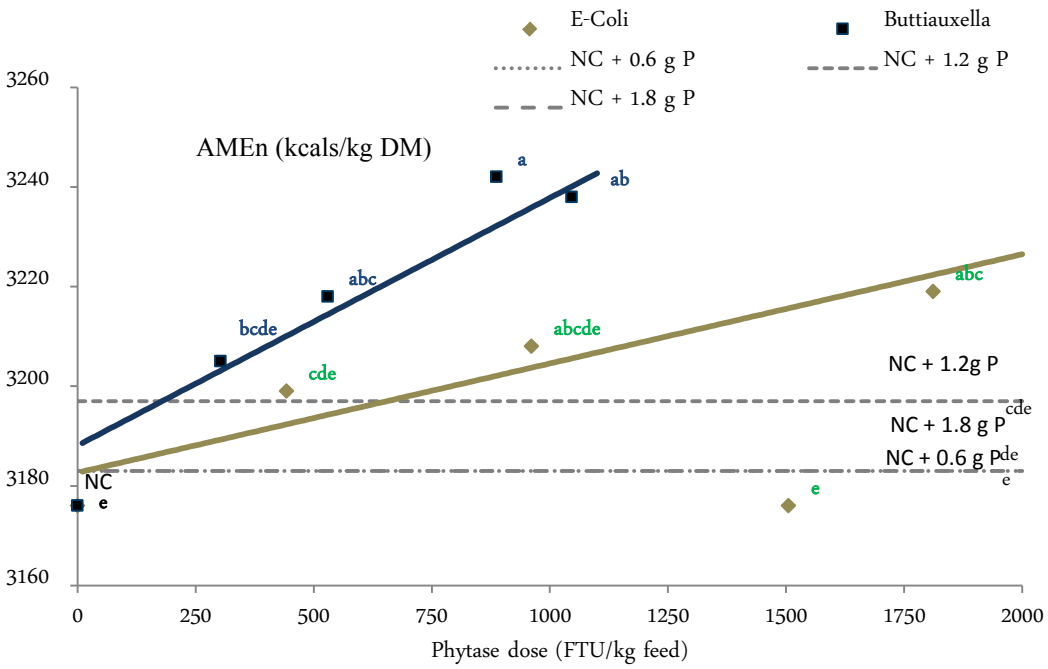

Figure 2 - AMEn in response to increasing phytase dose and inorganic P levels (a,b,c: significant difference at P<0.05).

The digestible AA and AMEn results were corresponding to the significantly lower FCR with *Buttiauxella* phytase at 1050 FTU/kg, compared to NC, PC and the E. coli phytase at 1500 FTU (Figure 3).

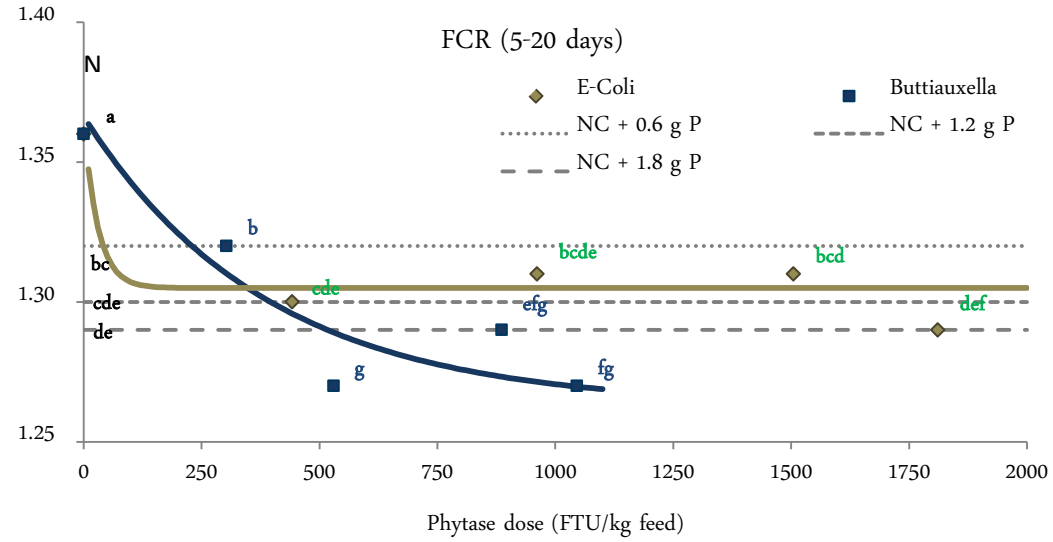

Figure 3 - FCR in response to increasing phytase dose and inorganic P levels (a,b,c: significant difference at P<0.05).

Body weight gain (BWG, data not shown) and tibia ash data (Figure 4) showed that *Buttiauxella* phytase at 1050 FTU/kg was equivalent to inorganic P added at 1.8g/kg from MCP, while *E. coli* phytase at 1500 FTU/kg maintained BWG and tibia ash at a level comparable to PC with 1.2g/kg P from MCP.

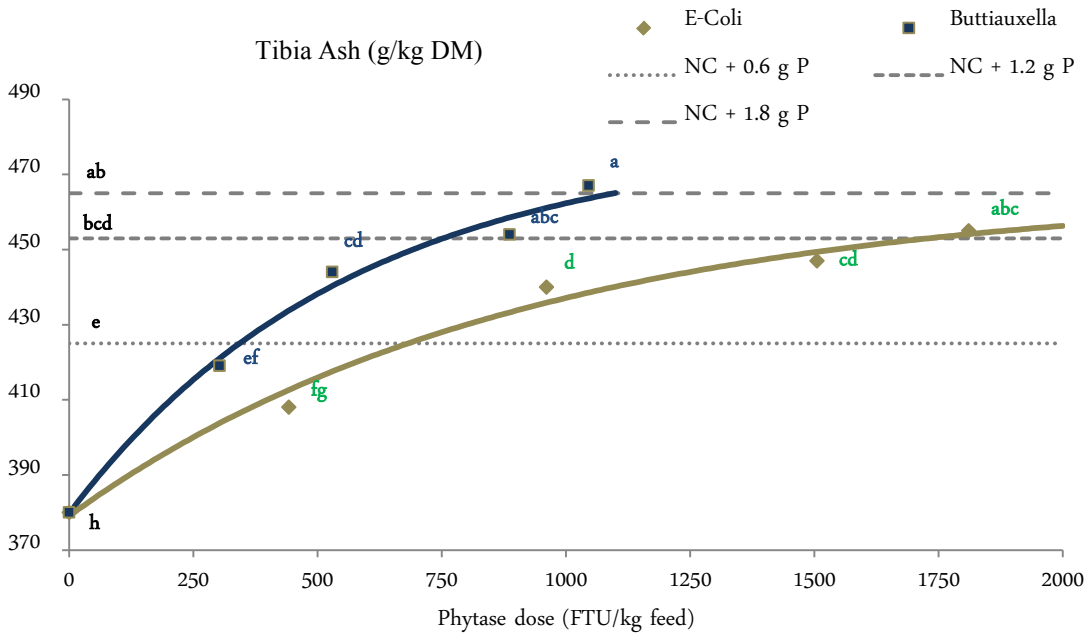

Figure 4 - Tibia ash in response to increasing phytase dose and inorganic P levels (a,b,c: significant difference at P<0.05).

IV. DISCUSSION

Selle et al. (2012) reviewed that phytate can reduce AA absorption due to the following mechanisms: 1) bind to AA, forming binary protein-phytate complexes or ternary protein-phytate complexes at pH levels below or above the isoelectric point of proteins and are refractory to pepsin digestion; 2) may increase mucin secretions into the gut and increase endogenous AA losses; 3) may interfere with starch and AA absorption by compromising Na(+)-dependent transport systems and the activity of the Na pump. A phytase that is more active at low pH and wide pH range will break down phytate more quickly and completely in the stomach or gizzard, and reduce these anti-nutritional effects of phytate, result in increased digestibility of AA and AME. This study showed a clear extra-phosphoric effect with increasing *Buttiauxella* phytase dose to 1050 FTU/kg, which resulted in an increased digestible AA, AMEn and feed efficiency. A greater efficacy with *Buttiauxella* phytase was observed compared to the *E. coli* phytase, which might be explained by the different pH optima of the phytases. Compared to the NC diet, 1050 FTU *Buttiauxella* phytase increased ileal digestibility of total AA (sum 17 AA) by 5.3 g/kg, whereas the increase was 2.6 g/kg for 1810 FTU *E. coli* phytase. Based on the linear regression analysis, with corn/SBM based diets used in this study, 1000 FTU *Buttiauxella* phytase could increase 5.9 g/kg ileal total digestible AA (sum of 17 AA), whereas the increase would be 1.5 g/kg for the *E. coli* phytase at 1000 FTU.

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NET ENERGY DRIVES FEED INTAKE OF BROILERS WHEN ADEQUATE APPARENT METABOLISABLE ENERGY IS SUPPLIED IN FEED

S.B. WU[1], R.A. SWICK[1] and M. CHOCT[1,2]

Energy supplied through carbohydrates and fat in feed are critical to broiler growth. How the feed intake (FI) of broilers responds to feed energy content, especially net energy (NE) and heat increment (HI), has not been fully investigated. In the present study, the roles of feed apparent metabolisable energy (ME), NE and HI on FI of broilers were analysed with diets formulated to different energy levels. Growth, FCR, FI, ME and NE of diets were measured in 16 closed circuit calorimeter chambers from 25 to 28 d (Swick, et al., 2013).

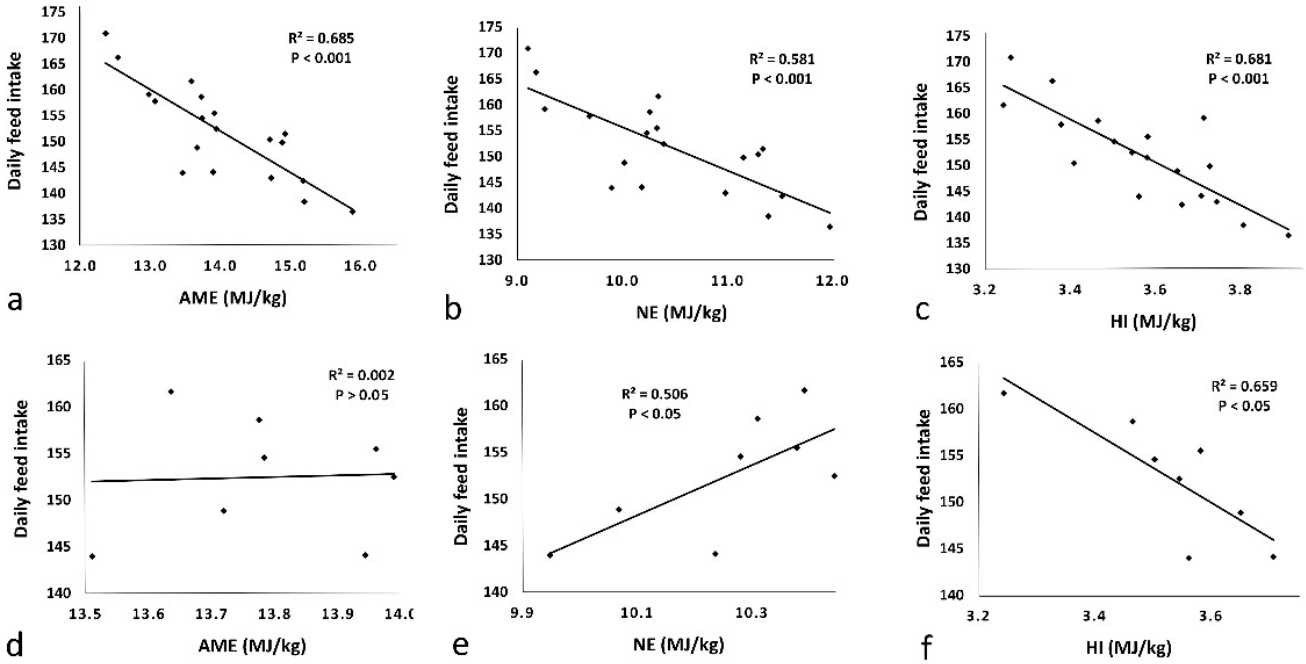

Figure 1 - Correlations between feed intake and energy contents, i.e., AME, NE and HI of feed with feed AME values in wide and narrow ranges. a, b and c: AME ranged from 12.36 MJ/kg to 15.87 MJ/kg (DM); d, e and f: AME ranged from 13.46 MJ/kg to 13.94 MJ/kg (DM).

The results showed that FI was negatively regulated by ME ($R^2 = 0.685$, $P < 0.001$), NE ($R^2 = 0.581$, $P < 0.001$), and HI ($R^2 = 0.681$, $P < 0.001$) while the diets used had a large range of AME values (from 12.36 MJ/kg to 15.87 MJ/kg) (Fig. 1). However, FI of birds did not respond to ME ($R^2 = 0.002$, $P > 0.05$) and was positively correlated to NE ($R^2 = 0.506$, $P < 0.05$) but negatively correlated to HI ($R^2 = 0.659$, $P < 0.05$) when the range with AME values was restricted from 13.46 MJ/kg to 13.94 MJ/kg. This indicated that while FI of birds is generally based on ME levels in the feed, the FI of birds is driven by NE of the feed when it contains appropriate energy sufficient for optimal growth and balance to amino acid content. At these levels, NE and HI of feed affect FI. Birds choose to consume feed with higher NE but lower HI possibly to maximize the use of the energy through minimizing heat production.

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STANDARDIZED ILEAL DIGESTIBILITY OF NUTRIENTS IN BROILER CHICKENS FED DIETS CONTAINING VARYING LEVELS OF RAW FULL-FAT SOYBEAN MEAL AND MICROBIAL PROTEASE

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Summary

A 2 by 3 factorial study, with 3 levels of raw full-fat soybean meal (RSBM) (commercial soybean meal (SBM) was replaced by RSBM at 0, 15, or 25 %) and 2 levels of protease (0 or 15000 PROT/kg) was used to evaluate the effects of RSBM and protease supplementations on ileal nutrient digestibility of broilers. A nitrogen-free diet (NFD) was included to enable calculation of N and amino acid flows at the ileum, and estimate standardized ileal digestibility of these nutrients. The content of trypsin inhibitors (TI) in the test diets ranged between 1940 and 10193.4 TIU/g. Rising levels of RSBM in diets increased ($P < 0.001$) the loss of ileal undigested CP while apparent and standardized ileal digestibilities (AID and SID, respectively) of CP, measured at 24 d of age were reduced ($P < 0.01$). Losses of most ileal undigested amino acids (AA) increased in line with increasing levels of RSBM, resulting in a reduction of the AID and SID of all AA (except methionine) at 24 d of age. When diets were supplemented with microbial protease, the AID and SID of CP were significantly ($P < 0.05$) improved due to a reduction in loss of undigested ileal CP. The AID ($P < 0.05$) and SID ($P < 0.05$) values of lysine, at 24 d of age were significantly improved due to supplementation with protease. This study showed that the ileal loss and ileal digestibility of nutrients were adversely affected by RSBM in diets but protease supplementation slightly reduced the negative effect of the ingredient.

I. INTRODUCTION

Commercial SBM is generally recognised as one of the best sources of proteins for poultry feeding but the nutritive value of raw, full-fat soybeans is poor due to the presence of antinutritive factors (ANF), including, but not limited to, protease (trypsin) inhibitors, lectins and phytate (Pettersson and Pontoppidan, 2013; Erdaw *et al*., 2016). The presence of dietary trypsin inhibitors (TI) in legumes, such as soybeans causes a substantial reduction in the digestibilities (up to 50 %) of proteins and AA and protein quality (up to 100 %) in non-ruminant animals (Gilani *et al.*, 2012). Nitrogen retention can also be negatively affected by TI and result in an increase in endogenous N excretion (Dourado *et al*., 2011). However, there are conflicting reports on the effects of RSBM on endogenous protein losses and AA digestibility (Barth *et al*., 1993; Clarke and Wiseman, 2005) who reported that AA digestibility in both commercial SBM and raw, full-fat SBM did not correlate with the concentration of TI.

Although the effects of TI are reduced by heat treatment, some ANFs in soybean, for example, phytate, are not heat-labile. Such factors would require other interventions and supplementation with relevant microbial enzymes can improve the quality of the diet containing soybean but it is not certain how much impact this will have on RSBM. The objectives of this study were to evaluate the AID

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and SID of CP and AA of broiler chicken diets containing RSBM and supplemented with or without protease.

II. MATERIALS AND METHODS

The test raw soybean seeds were obtained from a local farmer in northern New South Wales, Australia and hammer-milled before mixing in complete diets. A 2*3 factorial study, with 2 levels of *Nocardiopsis prasina* protease (0 or 15000 PROT/kg diet, DSM, Europe) and 3 levels of RSBM (commercial SBM was replaced by RSBM at 0, 15 and 25 %, equivalent to 0, 45 and 75 g/kg of diets, respectively) was conducted. Each diet was uniformly supplemented with microbial phytase (2000 FYT/kg diet, DSM, Europe), and each was replicated six times, with eight birds per replicate. A seventh group, which was used to calculate the N and AA flows at the ileum, was fed the commercial diets (starter and grower) until 19 days and then transferred to a (NFD) and allowed to feed for the next 5 consecutive days. The birds were raised on sawdust litter, in a climate-controlled room, and were offered corn-soybean-based starter (0-10 d) and grower (10-24 d) diets formulated to Aviagen standard (2009) for Ross 308 broiler. Samples of ileal digesta were collected on day 24 from all treatment groups, including the NFD, to calculate AID and SID.

III. RESULTS AND DISCUSSION

Undigested CP loss in the ileum increased ($P < 0.001$) with rise in dietary RSBM level, resulting in a reduction in AID ($P < 0.01$) and SID ($P < 0.01$) of CP, at 24 d of age. There were significant losses in undigested AA from the ileum and a reduction in the AID and SID of all AA (except methionine), at 24 d of age, with increases in the level of RSBM in the diet. When diets were supplemented with microbial protease, the AID and SID of CP were improved ($P < 0.05$), following a reduction (up to 6.5 %) in loss of undigested CP from the ileum. Protease supplementation also reduced the loss of undigested CP at the ileum by up to 4.5 %, resulting in marginal improvements in the AID and SID of AA. The AID and SID of lysine, at 24 d of age were improved ($P<0.05$) due to supplementation with protease.

There are conflicting reports on the effect of TI on nutrient digestibility. de Coca-Sinova *et al*. (2008) reported that the apparent digestibility of N and AA in broilers varies between SBM samples, with greater values corresponding to lesser TI, which is similar to the results obtained in the current study. In contrast, Clarke and Wiseman (2005) reported that the AID and SID of AA did not correlate with TI levels, indicating that other factors also affect the AA digestibility of full-fat and extracted SBM in broiler diets. Additionally, Barth *et al*. (1993) explained that the ingestion of food containing TI affects the nitrogen balance by increasing the outflow of amino acids from endogenous secreta rather than through the loss of dietary AAs. Importantly, it is possible to reduce these losses and improve digestibility using the type of protease product that was tested in this study. Microbial proteases are generally able to improve the digestibility of CP and AA (Murugesan *et al*., 2014) but usually not when high levels of TI are present.

Table 1- Effects of supplementing varying levels of raw full-fat soybean meal and protease on AID of CP and AA of broilers at 24 d.

Main effects			Indispensable amino acids									Dispensable amino acids			
Protease PROT/kg	RSBM g/kg	CP	His	Arg	Thr	Lys	Met	Val	Ile	Leu	Phe	Ser	Gly	Ala	Pro
0		0.772	0.806	0.887	0.746	0.832^{b}	0.944	0.758	0.765	0.802	0.802	0.764	0.725	0.787	0.783
15000		0.786	0.819	0.888	0.757	0.848^{a}	0.948	0.773	0.781	0.808	0.809	0.770	0.730	0.793	0.783
	0	0.794	0.830^{a}	0.899^{a}	0.767^{a}	0.854^{a}	0.946	0.794^{a}	0.805^{a}	0.828^{a}	0.828^{a}	0.793^{a}	0.759	0.815^{a}	0.804^{a}
	45	0.778	0.816^{b}	0.889^{ab}	0.755^{ab}	0.843^{ab}	0.949	0.768^{b}	0.777^{b}	0.805^{ab}	0.807^{ab}	0.766^{b}	0.725	0.785^{b}	0.781^{b}
	75	0.766	0.792^{b}	0.874^{b}	0.733^{b}	0.823^{b}	0.942	0.735^{c}	0.737^{c}	0.782^{b}	0.781^{b}	0.741^{c}	0.699	0.769^{c}	0.765^{c}
Sources of variation															
RSBM		**	***	**	***	**	NS	***	***	***	***	***	***	***	***
Protease		*	0.08	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSBM x protease		*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; pooled standard error of means; NS= not significant; *P < 0.05; ** P < 0.01; ***P < 0.00; 1RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25 %, equivalent to 0, 45 and 75 g/kg of diet, respectively).

Table 2 - Effects of supplementing varying levels of raw full-fat soybean meal and protease on the SID, CP and AAs at 24 d (as-is basis).

Main effects			Indispensable amino acids									Dispensable amino acids			
Protease PROT/kg	RSBM g/kg	CP	His	Arg	Thr	Lys	Met	Val	Ile	Leu	Phe	Ser	Gly	Ala	Pro
0		0.800	0.861	0.915	0.833	0.871^{b}	0.963	0.819	0.819	0.841	0.843	0.831	0.789	0.844	0.831
15000		0.814	0.872	0.915	0.841	0.884^{a}	0.967	0.831	0.832	0.847	0.850	0.836	0.793	0.849	0.831
	0	0.822	0.884^{a}	0.927^{a}	0.855^{a}	0.891^{a}	0.967	0.853^{a}	0.857^{a}	0.868^{a}	0.869^{a}	0.859^{a}	0.819^{a}	0.871^{a}	0.851^{a}
	45	0.805	0.869^{a}	0.917^{b}	0.838^{b}	0.879^{b}	0.967	0.827^{b}	0.829^{b}	0.844^{b}	0.848^{b}	0.831^{b}	0.790^{b}	0.843^{b}	0.829^{b}
	75	0.794	0.847^{b}	0.902^{c}	0.818^{b}	0.861^{c}	0.961	0.796^{c}	0.791^{c}	0.821^{b}	0.822^{c}	0.809^{c}	0.765^{c}	0.827^{b}	0.814^{b}
Sources of variation															
RSBM		**	***	***	***	**	NS	***	***	**	***	***	***	***	***
Protease		*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS
RSBM x protease		*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a,b,c] Means bearing uncommon superscripts within a column are significantly different; SEM= pooled standard error of means; NS= non-significant; *p<0.05; **p<0.01; ***P < 0.001; 1RSBM= raw soybean meal meal (SBM was replaced by RSBM at 0, 15 and 25 %, equivalent to 0, 45 and 75 g/kg of diet, respectively).

IV. CONCLUSION

This study demonstrated the negative effects of dietary TI on CP and AA digestibility but also the possibility of reducing these effects through supplementation with the test protease.

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DIETARY ENERGY, DIGESTIBLE LYSINE AND AVAILABLE PHOSPHORUS LEVELS INFLUENCE GROWTH PERFORMANCE AND CARCASS TRAITS OF BROILERS

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Energy (E) and amino acids (AA) are two of the most expensive components in broiler diets. There is no general consensus regarding the interaction of E and AA on broiler performance and this requires further investigation. Phosphorus (P) is the third most expensive diet component after E and AA (Woyengo and Nyachoti, 2011). Phosphorus plays a vital role in E and AA metabolism, and protein synthesis while P requirement has not been established with certainty. It was hypothesized that the requirements of digestible lysine (dLys, based on the ideal ratio as suggested by Baker and Han, 1994), AMEn and available P (avP) for broilers are not in the same proportion and these nutrients may interact with each other to affect broiler performance. To test this hypothesis, an experiment was conducted using a 3-factor-3-level Box-Behnken design that included dLys (9.5, 10.5, 11.5 g/kg), AMEn (12.77, 13.19, 13.61 MJ/kg) and avP (3.0, 4.0, 5.0 g/kg) generating a total of 15 treatments with 5 replicates of 12 birds. A total of 1050 d-old Ross 308 male broiler chicks were fed a common starter diet (dLys 12.0 g/kg, AMEn 12.77 MJ/kg, avP 4.5 g/kg) up to d 14 and allocated to treatment diets from d 14-34. Response surface was fitted by first, second or third degree polynomial regressions in JMP statistical software v. 12.0.1.

Body weight gain (BWG) was described by a third-order equation (adj. $R^2 = 0.80$, $P < 0.001$) and was affected by dLys (linear and quadratic) AMEn (linear) and AMEn × avP. Increase in dLys increased BWG but increase in AMEn decreased BWG in the birds fed the low avP diet but had no effect on BWG in those fed the high avP diet. High dLys, low AMEn and low avP maximised BWG during 14-34 d. Similarly, FCR was described by a third-order equation (adj. $R^2 = 0.92$, $P < 0.001$) and was affected by dLys (linear and quadratic), AMEn (linear), avP (linear) and AME × avP. Increase in dLys decreased FCR but increase in AMEn decreased FCR in the birds fed the low avP diet but had no effect on FCR in those fed the high avP diet. High dLys, low AMEn and high avP minimised FCR during 14-34 d. dLys had greatest influence on breast yield (adj. $R^2 = 0.47$, $P < 0.001$) where increasing dLys increased breast yield (linear) and breast yield percentage (linear and quadratic) but increasing AMEn decreased breast yield (linear) and breast yield percentage (linear). Similarly, increase in dLys decreased abdominal fat percentage but increase in AMEn increased abdominal fat percentage at both low and high avP levels with a more distinct effect on high avP level ($R^2 = 0.50$, $P < 0.001$). In conclusion, dLys had the greatest influence on performance and carcass traits in broilers from 14-34 d post-hatch. Interactions between AMEn and avP for BWG, FCR and abdominal fat percentage were detected, however, no interactions were detected between dLys and AMEn or dLys and avP for these parameters. These results indicate that increasing dLys levels above current industry standard would improve broiler performance irrespective of AMEn of the diet.

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ADJUSTING INCUBATION TEMPERATURE TO ALTER MEAT CHICKEN HATCH TIME AND LEG STRENGTH

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Up to 30% of late growth meat chickens demonstrate reduced levels of locomotion (Knowles et al 2008), which is cause for concern for both the industry and chicken meat consumer. Egg incubation, which represents up to 30% of the existence of a meat chicken, and in particular, egg shell temperature (EST), has been shown to have an effect on leg health of fast growing broilers (Groves and Muir, 2016). We have previously reported that, in a meat chicken parent line, early incubation at EST below 37.8^{0}C (the recommended incubation temperature) improved bone parameters, including a higher femoral bone ash (FBA) at hatch, and, a longer latency to lie (LTL) time at 6 weeks of age, when compared to chicks incubated at 37.8^{0}C EST (Groves and Muir, 2014). The current experiment evaluated the effect of a lower EST during early incubation, on chick hatch window, percent hatch, chick quality, bone ash and 5 week latency to lie, in two commercial strains of meat chicken (strain A and B).

Fertile eggs for both strains were obtained from breeder flocks aged 30-35 weeks at the time the eggs were laid. Each egg was identified with a number prior to the start of incubation. Incubation EST treatments were identified as Standard incubation (37.8^{0}C EST from setting to day 18 incubation), or, Slow start incubation, (a gradual increase from 36.9^{0}C EST at setting to 37.8^{0}C at day 16 incubation). EST was measured every minute, on one egg near the centre of each tray, using a Netic® temperature sensor. At 18 days incubation each egg was moved into an individual hatching cell in a hatching tray, and all chicks were hatched under the same temperature regime. Chick hatch was observed every 6 hours from 468 (19.5 days) until 516 hours (21.5 days) of incubation, enabling determination of chick hatch window and percent hatch. At 516 hrs incubation chicks were removed from the incubator (take – off; TO) and weighed. A subset of chicks, chosen at random, were utilized to determine chick length and FBA at TO. Remaining chicks were grown to 5 weeks of age, at which time the standing ability of all visibly male birds was assessed in a LTL test.

Under both EST incubation treatments, the mean hatch time of strain A birds was earlier than strain B. Compared to the Standard incubation, Slow start incubation delayed mean hatch time and increased chick weight at TO in both strain A and B. However the Slow start reduced percent hatch in strain B. Strain B had significantly higher FBA at TO, with a moderate interaction (P=0.07) of incubation and strain on FBA. From day 7 to day 35 of age there were no significant differences in mean chick weight. However, growth rate from hatch to day 7 only, was highest for chicks incubated under the Standard treatment compared to Slow start incubation. The LTL of both strain A and B birds when 5 weeks of age, was significantly longer in chicks exposed to the Slow start incubation compared to the Standard.

In both strains, a lower EST during the first 16 days of incubation delayed chick hatch time, increased chick weight at TO and generated an extended standing time at 5 weeks of age. However despite the latter benefit, the detrimental effect of the Slow start incubation on the percent hatch of strain B highlights the need to assess such alterations in each strain of bird.

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A RAPID AND SPECIFIC METHOD FOR THE DETECTION OF *C. HEPATICUS*, THE AGENT RESPONSIBLE FOR SPOTTY LIVER DISEASE IN AUSTRALIA

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Summary

Spotty liver disease (SLD) in chickens was first described over 60 years ago and there have been sporadic reports of the disease throughout the intervening decades. Recently it has become of increasing concern in Australia as outbreaks of the disease have occurred more frequently. It is characterised by multiple, grey/white spots in the liver and causes significant egg production losses and mortality in free range and barn layer flocks, and broiler breeders housed in deep litter barns. However, the cause of the disease has long been a mystery. We have recently identified the causative agent of SLD; a new species of *Campylobacter* that we have named *C. hepaticus*. In this study, a PCR assay has been developed and proven to be specific to the SLD organism. This assay can be used for epidemiological investigations of SLD in the field.

I. INTRODUCTION

The poultry industry plays an important role in the national economy of Australia. In 2013-14, Australia produced 1,084,000 tonnes of chicken meat (http://agriculture.vic.gov.au/) and 434.6 million dozen eggs were produced in 2015 (http://www.aecl.org/). It is important to protect poultry from serious endemic diseases and emerging diseases which have the potential to disrupt the poultry industry.

Spotty Liver Disease (SLD) is an emerging disease. It is characterised by multiple, grey/white spots in the liver and has been reported to cause significant egg production losses, and mortality (Crawshaw and Young, 2003; Grimes and Reece, 2011). First reports of the disease came from the United States in the 1950s and reports of what appears to be the same disease have come from Canada, the United Kingdom and Germany (Tudor, 1954; Truscott and Stockdale, 1966; Crawshaw and Irvine, 2012). The disease has been seen in the Australian poultry industry for many years but recently the incidence has dramatically increased. It is now causing significant problems with both mortalities and egg production. Therefore, it is important to understand the aetiology and epidemiology of the disease to enable us to find ways to control the disease.

The cause of the disease has long been a mystery. There were early suggestions that a "vibrio-like" organism was associated with disease cases. Later *Campylobacter jejuni, Campylobacter coli* and *Helicobacter pullorum* have been suspected, but without any definitive experimental verification. Recently Crawshaw and colleagues isolated a novel *Campylobacter* from cases of SLD that occurred in free-range laying flocks in England (Crawshaw et al., 2015). They used the bacteria that they isolated to infect four week old specific pathogen free birds and were able to identify microscopic lesions in the liver, however, no gross lesions were observed and no mortality occurred.

We have isolated a similar organism from SLD outbreaks in Australia. We went on to fully characterise the organism, using biochemical and molecular methods, and formally named it as a new bacterial species, *Campylobacter hepaticus* (Van et al., 2016a). We then demonstrated that *C. hepaticus* can induce SLD in experimentally infected layer birds (Van et

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al., 2016b). The study reported here aimed to develop a PCR assay for specific detection of *C. hepaticus*. This assay can be used for epidemiology investigations of SLD in the field, and can be applied to samples taken from liver and caecum.

II. MATERIALS AND METHODS

C. hepaticus strains were isolated from 11 SLD outbreaks from different farms in Victoria, Queensland, South Australia, and New South Wales as described by Van *et al.* (2016a). Other *Campylobacter* species (*Campylobacter jejuni ATCC 81116, Campylobacter coli* NCTC 11366T*, Campylobacter concisus* ATCC 51562*, Campylobacter mucosalis* ATCC 43264T*, Campylobacter lari, Campylobacter upsaliensis, Campylobacter sputorum, Campylobacter pullorum),* and *Helicobacter pullorum,* and *Enterococcus cecorum* were used to test the specificity of the SLD primers designed in this study.

DNA from bacterial cultures were prepared by suspending a single colony in 100 µL dH_2O and boiled for 10 min at 100ºC. DNA samples from liver and caecum of SLD affected and healthy birds were prepared using the Isolate fecal DNA kit (Bioline).

To identify areas of the *C. hepaticus* genome that could be targeted for design of specific PCR primers, we interrogated the genome sequence of C. *hepaticus* NCTC 13823^T (=CIT 111092^T) (GenBank accession no. LUKK01000000) and compared it to the published genome sequences of other *Campylobacter* species and other related bacteria. Primers were designed based on a glycerol kinase gene region found to be unique to *C. hepaticus*. The primer sequences selected were G2F3: CAGGAGTTTTACCACAATTC and G2R2: CAAGCTAAAACAGGTTTGG, with an expected amplicon size of 463 bp.

PCR was carried out using an Eppendorf Mastercycler pro PCR instrument with the cycling conditions; 98°C for 1 min, 35 cycles of 98°C for 10 s; 57°C for 30 s and 72°C for 30 s, final extension at 72°C for 10 min. Selected products were Sanger sequenced (Micromon, Monash University, Victoria, Australia). DNA derived from a culture of known CFUs was diluted in 10–fold increments to determine the limit of detection of the PCR. The PCR was tested for specificity for *C. hepaticus* and the assay was then applied to assess liver and caecum samples from the field.

III. RESULTS

PCR amplification was obtained with boiled DNA samples from each of the 11 independently isolated *C. hepaticus* strains originally recovered from SLD affected birds (including the type strain, *C. hepaticus* NCTC 13823^T). No amplification was seen with any of the other *Campylobacter* species or other bacteria. The PCR is species-specific. The limit of detection of the assay was found to be $10^{0.7}$ (≈ 5) CFU.

The PCR assay was then applied to the investigation of tissue samples recovered from field cases of clinical SLD and samples from healthy birds. Liver and caecum samples from 12 birds from a layer farm with no history of SLD were used as negative control. As expected, *C. hepaticus* could not be isolated from any of these samples and PCR of the liver and caecum DNA was negative. Amplification of DNA from liver and caecum samples of diseased birds gave the expected specific product of 463 bp. From 45 sampled birds from eight SLD outbreaks *C. hepaticus* was isolated from 35 liver samples and confirmed by colony PCR. The specific PCR detected *C. hepaticus* in all 35 caecum samples from these same birds and in 33 liver samples. In the remaining 10 sampled birds *C. hepaticus* could not be isolated due to contamination with other bacteria on the putative isolation plates. However, *C. hepaticus* could be detected by PCR in the liver and caecum samples from all 10 birds. Thirteen samples from birds without any sign of SLD but housed on the same farms as the SLD birds were examined for *C. hepaticus* infection by bacterial isolation and PCR assay.

Samples from nine birds were negative for *C. hepaticus* isolation and PCR of the liver and caecum DNAs. For the remaining four birds, PCR of liver and caecum sample DNAs were positive, and *C. hepaticus* was isolated from one liver sample but not from the other three samples. Sequencing of a sample of the PCR products demonstrated that the amplicons had the expected sequence.

IV. DISCUSSIONS

The identification of *Campylobacter* at the species level using isolation and subsequent biochemical and molecular testing is laborious due to the slow growing nature of *Campylobacter* spp. Therefore, a rapid DNA-based method would be of value. Several genes, such as *cadF, ceuE, glyA, hipO,* and *lpxA*, have previously been used for the differential identification of *C. jejuni* and *C. coli* (Cloak and Fratamico, 2002; Adzitey and Corry, 2011; Shams et al., 2016). The PCR assay designed in this study targeted a glycerol kinase gene. We have shown that it could differentiate C. *hepaticus* from other *Campylobacter* species and from other bacteria.

C. hepaticus was isolated from all the SLD outbreaks tested. Failure to isolate the bacterium from individual birds within an outbreak was mainly due to contaminants overgrowing the cultures rather than absence of the organism. Of 10 birds from which *C. hepaticus* could not be isolated because of contamination with other bacteria, *C. hepaticus* could be detected by PCR in all the liver and caecum samples. Of the birds without any sign of SLD but housed in the same farm as the SLD birds, most of them did not contain *C. hepaticus* (9/13); in the remaining four samples, *C. hepaticus* was detected by PCR from all caecum and liver DNA samples, but it was only isolated from one of four samples. This indicated that the PCR assay is more sensitive than the bacterial isolation process from the liver samples. *C. hepaticus* could not be detected in any of the samples derived from birds from a layer farm with no history of SLD.

C. hepaticus can be readily isolated from liver samples because it is generally present as a monoculture, however, there is currently no specific media that can be used to isolate *C. hepaticus* from bacteriologically complex samples such as from the gut or environment. Therefore, this PCR is the only rapid method to identify the bacterium in gut or environmental samples. It provides a tool that can be applied within the poultry industry for quick and accurate detection of *C. hepaticus* bacteria within flocks and for epidemiology investigations of SLD.

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SCENARIO TREES TO ASSESS THE RISK OF AVIAN INFLUENZA EXPOSURE AND SPREAD WITHIN AUSTRALIAN COMMERCIAL CHICKEN FARMS

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Summary

The risk of avian influenza (AI) exposure and spread to Australian commercial chicken farms has been quantified using scenario tree mathematical modelling following the World Organization for Animal Health (OIE) methodology for risk assessment. Input values for the models were sourced from an on-farm survey conducted during 2015 and 2016 of Australian commercial chicken farms located in New South Wales (NSW) and Queensland. Scientific literature, branching process models and an expert opinion workshop held in 2015 were also used. Outputs from the models revealed that the probability of AI virus exposure to Australian commercial chicken farms from one wild bird at any point in time is extremely low. Across the five farm types (non-free range meat chicken, free range meat chicken, cage layer, barn layer and free range layer farms), free range layer farms had the highest probability of exposure (0.00075; 5% and 95%, 0.00057 – 0.0010). The proportion of wild birds that are waterfowl on the farm, the presence of waterfowl in the range and feed storage areas, and the prevalence of LPAI in wild birds are the most influential inputs for the exposure model. After exposure to the virus, it is most likely that no establishment and hence no spread of the virus will occur. However, both free range egg and meat chicken farms are more likely to experience low pathogenic AI (LPAI) spread compared to other farm types due to their greater probability of direct exposure to the virus. Layer farms in general are more likely to have high pathogenic AI (HPAI) spread due to their longer production cycle compared to meat chicken farms and hence higher probability of mutation of LPAI to HPAI. Sharing equipment between sheds was the most likely pathway of shed-to-shed spread of AI. Pickup trucks for both dead and alive birds, egg trays and egg pallets were important pathways for farm-to-farm spread of AI.

I. INTRODUCTION

a. Avian Influenza Virus in the Australian Context

There has been a significant expansion of both free-range egg and meat chicken production due to consumer demand in Australia in recent years (ACMF 2011; AECL 2015). The low pathogenic form of AI (LPAI) is currently circulating in Australian wild birds at an approximate 2% prevalence most commonly in waterfowl and shorebird types (Grillo et al., 2015). There is concern the expansion of free-range chicken production could lead to more AI virus introductions on farms as more chickens will have access to the outdoors. Once poultry are infected, LPAI virus subtypes H5 and H7 can mutate to highly pathogenic AI virus (HPAI) which has caused devastating losses in poultry production worldwide. Morbidity and mortality rates of HPAI virus can reach up to 100% in chickens (Saif et al., 2009). Australia has experienced seven HPAI outbreaks in poultry farms all of which occurred in the three eastern states; Victoria (three separate outbreaks), Queensland (one

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outbreak), and New South Wales (three separate outbreaks) from 1976 to 2013 and all involved chickens (CFFR 2014; Swayne 2008). All viruses isolated from these HPAI outbreaks were of subtype H7 and were unique Australian lineages. The risk of introduction of an exotic AI virus into Australia has been deemed low due to the nomadic rather than migratory nature of Australian wild waterfowl. Future HPAI outbreaks are more likely to occur from AI virus circulating in local Australian wild bird populations (East et al., 2008).

b. Risk Assessment of Avian Influenza to Australian Commercial Chicken Farms

There was a need to quantify the level of risk of AI virus introduction and spread to Australian commercial chicken farms especially given the expansion of free range poultry production and to calculate how these risks can be mitigated. This research project commenced in 2015 to address these knowledge gaps. The exposure and partial consequence assessments from the OIE methodology for risk analysis were followed in this project (OIE 2010). Scenario tree mathematical models were developed (Martin et al., 2007); these were graphical representations of all the potential pathways by which AI can be introduced and spread to and between sheds and farms. The scenario tree models considered Australian commercial chicken farms of all types; cage, barn and free range of both egg and meat chicken sectors. This paper describes the methodology and results obtained from the scenario tree models including the results of the probabilities obtained for AI introduction and spread to and between Australian commercial chicken farms.

II. METHODS

a. Risk Assessment Model

The exposure and consequence assessment steps in the OIE methodology for risk assessment were followed for the scenario tree modelling. All potential pathways by which chickens situated in a commercial layer or meat chicken farm can be exposed to AI from wild birds were considered in the exposure assessment. A partial consequence assessment was used rather than a full consequence assessment because it considered all pathways by which AI can spread between sheds and farms but does not consider the consequences of these events. Scenario trees were illustrated using Microsoft Excel (PC/Windows 7, 2010). The probabilities of these pathways occurring were calculated using Monte Carlo stochastic simulation modelling using the program @RISK 7.0 (Palisade Corporation, USA). Each simulation consisted of 50,000 iterations sampled using the Latin hypercube method with a fixed random seed of one. Sensitivity analysis was also used to find the most influential parameters of each model.

b. Data sources

Input values for the scenario tree models were obtained from data collected from a cross-sectional survey of commercial chicken farms in Australia, expert opinion, scientific literature and branching process model work. The survey involved on-farm interviews on 73 commercial chicken farms; nine cage layer, nine barn layer, 25 free range layer, 15 non-free range meat chicken and 15 free range meat chicken farms. The farms were located in the Sydney basin region in New South Wales (NSW) and in South East Queensland. The interviews involved a comprehensive questionnaire with questions relating to biosecurity practices performed on farm, wild bird and animal presence and general farm information.

An expert opinion workshop was held in 2015 to obtain data on the many unknowns of the AI virus, including the probability of mutation. The workshop hosted 10 experts with

varying expertise in the poultry industry, AI virus behavior and wild bird ecology. Branching process model work was also conducted within the research team. This work involved more complex mathematical modeling relating to AI virus behavior at a shed, farm and industry level. The probability of establishment of the virus in a shed obtained from the branching process model work was used for the scenario tree modeling.

III. RESULTS AND DISCUSSION

a. Probability of exposure

Outputs from the scenario tree modeling reveal that the probability of an Australian commercial chicken farm being exposed to LPAI virus from one wild bird at any point in time is extremely low. Across the five farm types, free range layer farms have the greatest median probability of exposure (0.00075; 5% and 95%, 0.00057 – 0.0010) followed by barn meat chicken farms (0.00037; 0.00020 – 0.00064), both free range meat chicken farms (0.00032 (0.00018 – 0.00057) and cage layer farms (0.00032; 0.00015 – 0.00063) and barn layer farms (0.00030; 0.00014 – 0.00058). These probabilities can be used to describe particular scenarios; if there are 100 free range layer farms with 1000 wild birds visiting the farm across any time period, in 46% of farms it was estimated there would be no exposure to the virus. In 35%, 15%, 3.5% and 0.5% of farms, it was estimated there would be 1, 2, 3 and 4 exposures occurring on those farms respectively. Fewer exposures were estimated for the other farm types in the same scenario.

The probability of exposure in three different seasons; winter (June to August), summer (December to February) and autumn and spring combined (March to May and September to October respectively) were also assessed. The season with the greatest average median probability across the farm types was winter (0.000516) followed by autumn and spring (0.000509) and summer (0.000204). These differences are due to differences in the prevalence of AI in wild birds at certain times of the year and the level of access of free range chickens to the outside in certain weather conditions.

Sensitivity analysis revealed that the most influential pathways contributing to the overall probability of exposure were the proportion of wild birds on the property that are waterfowl, the probability of wild waterfowl in feed storage areas and the probability of wild waterfowl on the range.

b. Probability of spread

The spread model revealed that in most cases after AI exposure, no establishment will occur as this model also considered the probability of infection and establishment after exposure. Both free range egg and meat chicken farms are more likely to experience low pathogenic AI (LPAI) establishment and spread compared to other farm types (0.068; 0.033 – 0.12 and 0.056; 0.0071 – 0.12 respectively). This is due to their greater probability of direct exposure to AI virus from going on the range; the probability of infection is higher after direct exposure compared to indirect exposure.

Layer farms in general are more likely to have high pathogenic AI (HPAI) spread due to their longer production cycle compared to meat chicken farms and hence higher probability of mutation of LPAI to HPAI after LPAI established in a flock (0.00022; 1.00 x 10^{-5} – 0.0019, 0.00017; 7.33 x 10-6, 0.00037; 1.65 x 10^{-5} – 0.0031 for cage layer, barn layer and free range layer farms respectively). Sharing equipment between sheds was the most likely pathway of shed-to-shed spread of both LPAI and HPAI (average median probabilities 0.016 and 5.76 x 10^{-5} respectively). Pickup trucks for both dead and alive birds had the greatest average median probability of LPAI spread between farms across all farm types

(average median probability 0.0072) and egg trays for HPAI (average median probability 3.70 x 10^{-5}). Egg pallets were also an important pathway for the spread of HPAI between farms.

IV. IMPLICATIONS AND CONCLUSIONS

The modelling work has provided detailed descriptions of pathways, relative probabilities and the most influential parameters of AI virus exposure and spread in Australian commercial chicken farms. Biosecurity considerations can be developed from this work and be applied on-farm to mitigate these risks.

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YOLK MINERAL LEVELS DURING INCUBATION AND THREE DAYS POST HATCH

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Summary

This study observed the levels of Ca, Sr, Na, K, Mg, Mn, Cu, Zn, Fe and P in the yolk of fertile Cobb 500 broiler eggs during incubation, and the levels of these minerals in the residual yolk sac of the birds hatched from remaining eggs. Correlations were found between several minerals.

I. INTRODUCTION

The yolk contains the majority of minerals required for chick embryonic development. The residual yolk sac is absorbed, prior to hatch, into the chick's abdominal cavity, acting as a nutrient reserve. Via this reserve birds can survive several days without feed or water, in practice until placement in a grow-out shed. Chick embryos regulate the intake of minerals from the yolk via the yolk sac membrane (Richards, 1997). Recent work indicated the possibility that hatched chicks were absorbing residual yolk P while Ca was being left in the yolk (Hopcroft et al 2016). Results from this trial can be used to quantify yolk mineral absorption rates.

II. MATERIALS AND METHODS

Fertile Cobb 500 broiler eggs, laid by a 53 week old breeder flock, were obtained from a commercial hatchery. Yolk (including the yolk sac membrane) from 36 eggs were randomly sampled for fresh yolk weight (FYW) and mineral analysis, described below. Remaining eggs were incubated in six Multiquip E3 incubators at an EST of 37.8^{o}C throughout the first 18 days of incubation. At 6.5, 13.5 and 17.5 embryonic days (ED) 6 eggs were sampled from each incubator for FYW and yolk mineral analysis. At ED 17.5, the remaining eggs were transferred into individual cells on hatching trays, and placed in a randomized fashion into one Aussieset 2000 egg capacity incubator (Bellsouth Pty Limited, Victoria, Australia).

One day before placement (P-1) (ED 20.5), 36 hatched chicks were sampled (6 from each initial incubator). Their yolk sacs were removed, weighed and used for later mineral analysis. At placement (P0) (ED 21.5), remaining chicks were taken off from the hatcher. 77 chicks were selected representatively across hatching times. The yolks of these chicks were sampled as at P-1. Remaining chicks were distributed into cages. All chicks received a standard starter crumble ration until day 3. Three days from placement (P+3), 64 chicks were randomly selected. The yolk remnants from these chicks were sampled in the same manner as above. Additional chicks were sampled at P0 and P+3 to fulfil other sampling requirements.

III. MINERAL ANALYSIS

To perform mineral analysis, yolk samples were homogenised and freeze dried. Samples were weighed after freeze drying to give the freeze dried yolk weights (DYW). 0.5 g (or 0.1g for P+3) of powdered sample was placed into a 50 ml digestion vessel. 5 mL (0.2 mL P+3) of nitric acid was added, and samples were heated on a heat block at 50°C for two hours, with a watch glass on top. Temperature was then raised to 90°C for 30 minutes. Samples were cooled to room temperature and 2 mL (0.1 mL P+3) of hydrogen peroxide was added.

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Samples were then heated back to 90°C for 10 minutes, then heat was increased to 120°C for 30 minutes. The sample was diluted with distilled water to 30 mL(10 mL P+3) and sent to the UNSW analytical lab for inductively coupled plasma atomic emission spectroscopy (ICP-AES), measuring concentrations of Ca, Cu, Fe, K, Mg, Mn, Na, P, Sr and Zn. Yolk mineral concentrations were multiplied by DYW to determine the total amount of mineral in each yolk. DYW was subtracted from FYW, to give approximate water content (AWC). Data from infertile eggs was not included in the analysis. The results were subjected to ANOVA and Tukey's test for significance. Pearson's correlation was performed on mineral data, and selected mineral pairs reported at $P < 0.001$. The rate of absorption of total P over time was graphed.

IV. RESULTS

Fresh yolk weight and the concentration of yolk minerals over time is shown in Table 1. Dried yolk weight, approximate water content and yolk mineral total amounts are shown in Table 2. FYW increased from ED0 to ED6.5. It then decreased until P+3. DYW significantly decreased at each sample point, with the exception of at ED13.5.

By P0, yolk [Ca] significantly increased compared to ED0. Total Ca initially remained constant, then began to rise at ED13.5, peaking at ED17.5 and P-1, then dropped back to ED0 levels at P+3. [Sr] did not significantly vary until P+3, when it rose. Total Sr had an initial decrease at ED6.5, then increased back to ED0 levels until P0, when it decreased. [Ca and Sr], and total Ca and Sr had correlation coefficients of 0.96 and 0.84 respectively, over the entire sampling period.

Yolk [Na and K], and total yolk Na and K increased from ED0 to ED6.5. [Na and K] then decreased from ED13.5 until P+3, when they increased. Total K and Na decreased from ED6.5 to P+3. [Na and K] and Na and K had correlation coefficients of 0.73 and 0.86.

Yolk [Mg and Mn] were constant until P+3, when they increased. Total Mg did not change until P-1, when it began to decrease. Total Mn gradually decreased until P-1, after which it remained constant. [Mg and Mn], and total Mg and Mn had correlation coefficients of 0.77 and 0.75.

Yolk [Cu and Zn] were constant until ED17.5, when they decreased. Total yolk Cu and Zn decreased over time. At P+3, [Cu] and total Cu increased. [Cu and Zn] had a correlation coefficient of 0.40, and total Cu and Zn 0.98. [Fe] gradually decreased until ED17.5, and then remained constant between P-1 and P0. It increased to pre-hatch levels by P+3. Total Fe significantly decreased until P-1, and thereafter did not significantly vary. [P] and total P decreased over time. [Fe and P] and total Fe and P have correlation coefficients of 0.70 and 0.96.

Table 1 - Fresh yolk weight and mineral concentration in the yolk.

Sample Period	Yolk Location	Fresh yolk weight (g)	Yolk Ca (mg/kg)	Yolk Sr (mg/kg)	Yolk Na (mg/kg)	Yolk K (mg/kg)	Yolk Mg (mg/kg)	Yolk Mn (mg/kg)	Yolk Cu (mg/kg)	Yolk Zn (mg/kg)	Yolk Fe (mg/kg)	Yolk P (mg/kg)
ED0	Egg	23.03^{a}	2676^{a}	2.61^{a}	954^{a}	1771.5^{ab}	233.8^{a}	2.52^{a}	2.77^{ab}	67.1^{a}	116.3^{a}	10426^{a}
ED6.5	Egg	27.20^{b}	2823^{a}	2.69^{a}	3894^{b}	3074.1^{cd}	277.5^{a}	2.55^{a}	3.07^{ab}	70.5^{a}	110.2^{a}	10917^{a}
ED13.5	Egg	18.61^{c}	4494^{ab}	3.41^{a}	2248^{a}	2608.9^{bc}	315.7^{a}	2.08^{a}	2.56^{bc}	66.3^{a}	71.4^{b}	9510^{ab}
ED17.5	Egg	15.86^{d}	6946^{ab}	4.51^{a}	1439^{a}	2317.0^{ac}	338.0^{a}	2.13^{a}	1.35^{cd}	46.9^{b}	38.3^{bcd}	7596^{bc}
P-1	Chick	6.76^{e}	13562^{ab}	7.36^{a}	1260^{a}	1392.2^{a}	349.6^{a}	2.44^{a}	1.27^{d}	40.0^{b}	22.8^{c}	6233^{cd}
P0	Chick	4.56^{f}	18126^{b}	8.41^{a}	1597^{a}	1443.8^{a}	345.5^{a}	4.43^{a}	1.30^{d}	38.5^{b}	28.0^{c}	6204^{c}
P+3	Chick	0.83^{g}	49886^{c}	24.99^{b}	3498^{b}	3097.5^{d}	907.6^{b}	15.34^{b}	3.01^{a}	39.5^{b}	41.2^{d}	4504^{d}

Table 2 - Freeze dried yolk weight, approximate water content and total mineral content in the yolk.

Sample Period	Hours of Incubation	Dried yolk weight (g)	Approx. Water Content (g)	Yolk Ca (mg)	Yolk Sr (mg)	Yolk Na (mg)	Yolk K (mg)	Yolk Mg (mg)	Yolk Mn (mg)	Yolk Cu (mg)	Yolk Zn (mg)	Yolk Fe (mg)	Yolk P (mg)
ED0	0	11.17^{a}	11.86^{a}	29.9^{abc}	0.029^{ab}	10.65^{a}	19.78^{a}	2.615^{a}	0.028^{a}	0.0310^{a}	0.750^{a}	1.298^{a}	116.4^{a}
ED6.5	156	8.76^{b}	18.72^{b}	24.8^{ab}	0.024^{c}	33.77^{b}	26.89^{b}	2.457^{a}	0.023^{b}	0.0269^{b}	0.619^{b}	0.982^{b}	96.2^{b}
ED13.5	324	8.35^{bc}	10.86^{a}	36.6^{acd}	0.028^{b}	18.62^{b}	21.63^{a}	2.623^{a}	0.017^{b}	0.0211^{c}	0.536^{c}	0.595^{c}	79.1^{c}
ED17.5	420	7.43^{c}	8.43^{c}	48.8^{d}	0.032^{b}	10.05^{a}	16.47^{c}	2.406^{a}	0.015^{bc}	0.0096^{d}	0.332^{d}	0.274^{d}	53.6^{d}
P-1	492	3.65^{d}	3.26^{d}	48.0^{d}	0.026^{abc}	4.49^{c}	5.00^{d}	1.262^{b}	0.009^{cd}	0.0046^{e}	0.145^{e}	0.085^{e}	23.2^{e}
P0	516	2.41^{e}	2.15^{e}	42.1^{cd}	0.020^{c}	3.81^{c}	3.36^{d}	0.854^{c}	0.010^{d}	0.0030^{ef}	0.090^{f}	0.067^{e}	14.9^{f}
P+3	588	0.40^{f}	0.43^{f}	20.0^{b}	0.010^{d}	1.40^{d}	1.24^{e}	0.363^{d}	0.006^{d}	0.0012^{f}	0.012^{g}	0.016^{e}	1.8^{g}

^{abcdefg} Means without common superscripts differ significantly ($P < 0.05$)

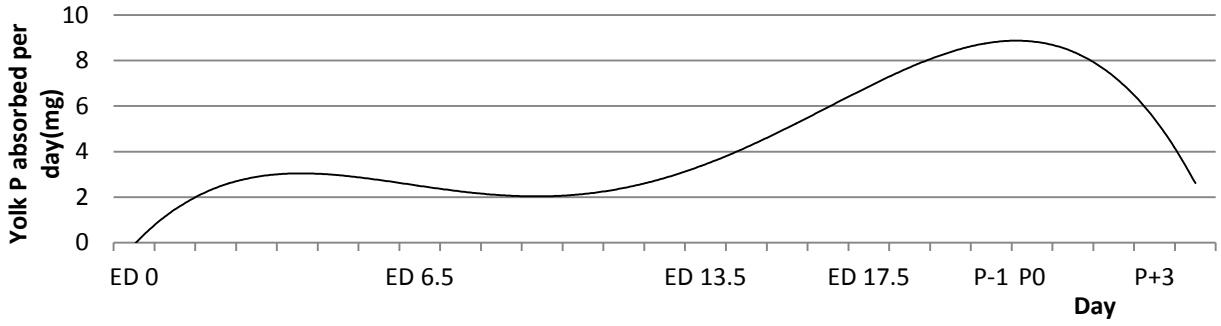

Figure 1 - Absorption of yolk P per day.

V. DISCUSSION/CONCLUSION

The two tables of results can be used to calculate a range of values. The change in yolk mineral total over time allows calculation of yolk mineral uptake per day, as shown for yolk P absorption in Figure 1. Maximum yolk P absorption occurred between P-1 and P0, while chicks were in the hatcher unfed. The increase in FYW, AWC, and yolk K and Na at ED6.5 is due to the drawing in of the albumen, which Richard and Packard (1996) found to be the major store of these nutrients. High correlation values between minerals may indicate relationships between those minerals. Ca and Sr increase in the yolk at ED13.5. In the second week of incubation, the chorioallantoic membrane is formed and transportation of Ca from the eggshell into the yolk has begun (Rocky and Tamao, 1986). The 0.96 correlation between yolk [Ca and Sr] suggest Sr may be mobilised via this pathway, in competition and proportion with Ca, supported by work done by Browning and Cowieson (2015), which showed increased dietary Sr decreased bone Ca. Ninety percent of yolk Fe is localised to phosvitin, a yolk protein high in P (Greengard et al 1964). Over 90% of yolk zinc is bound to lipovitellin (Tupper et al 1954), another yolk protein, which also binds copper. Determining mineral levels in the embryonic and residual yolk sac leads to better knowledge of the mechanisms by which minerals are supplied to the developing chick. This is fundamental to ensure maximum utilisation of the yolk sac.

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GLYCINE SUPPLEMENTATION OF LOW PROTEIN DIETS IN BROILERS

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Soybean meal and meat and bone meal are the primary protein meals used in poultry diets. The high cost of soybean meal and nutrient variability of meat and bone meal suggests the industry needs to reduce protein meal dependence. High dietary protein is also associated with high water consumption, having a negative impact on litter quality and bird health (Alleman and Leclercq, 1997). The poultry industry currently supplements diets with methionine, lysine and threonine to reduce some dependence on protein meals. There is evidence that the supplementation of glycine in poultry diets can improve performance in low protein diets (Dean et al., 2006). Glycine is involved in a diverse range of metabolic pathways, including synthesis of proteins and purines. Although glycine is categorized as a non-essential amino acid, it may become limiting under certain circumstances (Corzo et al., 2004). The aim of this study was to measure the effect of glycine supplementation in low protein diets in broilers. Male day-old Ross 308 chicks (n = 546) were raised in 42 floor pens, 13 birds per pen. On d7 the birds were distributed to ensure there was no significant difference in starting weight between treatments. All birds were fed a standard wheat-sorghum-soybean meal based starter diet from d0-10. On d10, birds were assigned to one of seven wheat-sorghum-soy based treatments in a randomized design and provided *ad libitum* food and water. The control treatment contained 21.7/19.8% CP for the grower and finisher phases respectively, and the remaining treatments contained either 20/18% CP, 18.5/16.5% CP or 17/15% CP. Glycine was added to equal the total level of glycine in the control treatment and all essential amino acids were supplemented when limiting. The feed conversion ratio (FCR) of birds aged d10-35 was calculated based on total feed intake divided by body weight gain per pen, taking into account any mortalities. The results showed that the addition of glycine improved feed conversion by 4.71% (1.438 versus 1.509; $P < 0.001$) in birds fed the diet with 20/18% CP and by 4.78% (1.473 versus 1.547; $P < 0.05$) in birds fed the diet with 18.5/16.5% CP, but had no significant effect in birds fed the diet with 17/15% CP. Glycine addition also improved BWG from d10-35 in birds fed the diet with 20/19% CP by 10.8% (2234 versus 2017 g/bird; $P < 0.05$) and by 13.9% (2076 versus 1822 g/bird; $P < 0.05$) with the 18.5/16.5% CP diet; no significant effect on BWG was observed at 17/15% CP. These results suggest that the supplementation of glycine could significantly enhance performance in broilers fed low protein diets.

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FISHMEAL AND CORN STARCH INCLUSIONS IN SORGHUM-SOYBEAN MEAL DIETS HAVE DIFFERING IMPACTS ON THE PERFORMANCE OF BROILER CHICKENS

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Summary

The partial substitution of soybean meal by fishmeal had a more profound effect on broiler performance as fishmeal inclusions significantly improved weight gain by 12.1% and FCR by 8.13%. Fishmeal significantly increased starch digestibility coefficients in four small intestinal segments and fishmeal increased digesta retention time in the small intestine from 210 to 289 minutes ($P < 0.001$). Starch digestibility was significantly correlated with weight gain, FCR and energy utilisation (AME, ME:GE ratios, AMEn) and these parameters were all significantly enhanced by the fishmeal substitution.

I. INTRODUCTION

Starch and protein are critical nutrients in poultry diets with starch from feed grains providing the largest energy source and various protein meals contributing a large proportion of the amino acid profile. Presently least-cost formulations of broiler diets largely overlook the rate, site and extent of glucose and amino acid absorption along the small intestine which is probably a rate limiting step to broiler performance. Therefore, an understanding of the digestive dynamics of starch and protein is important to cost effective and sustainable chicken meat production. The objective of this study was to examine the influence of starch and protein digestive dynamics on growth performance and nutrient utilisation of corn starch and fishmeal in broiler diets based on sorghum and soybean meal.

II. MATERIALS AND METHODS

One hundred and twenty 15-day old Ross 308 chicks were weighed and assigned to four dietary treatments with 5 replicates of 6 birds per cage. The dietary treatments were based on sorghum and soybean meal and formulated to be iso-energetic (12.97 MJ/kg ME) with similar ideal protein ratios but with differing levels of fishmeal and corn starch as shown in Table 1. This study comprised a 2×2 factorial array of dietary treatments offered to male Ross 308 broiler chicks from 15 to 28 days post-hatch. The treatments consisted of a sorghum-soybean meal diet (12.97 MJ/kg ME) in which either sorghum was partially substituted by corn starch (200 g/kg) or soybean meal was partially substituted by fishmeal (175 g/kg) or both substitutions were completed. Birds were fed the steam-pelleted dietary treatments from 15 to 28 days with 15 hours daily feed access. Initial and final body weight and feed intake were determined at 15 and 28 days post hatch and used to calculate FCR. Total excreta were collected from 25 to 27 days post-hatch to determine parameters of nutrient utilisation (AME, ME:GE ratios, N retention, AMEn) by standard procedures. Apparent starch digestibility coefficients and starch disappearance rates (g/bird/day) in four small intestinal segments using Celite as the inert dietary marker and digesta retention times in four small intestinal segments were determined.

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Table 1 - Dietary composition, calculated nutrient specifications and analysed concentrations in experimental diets.

Dietary composition	Control	Fishmeal	Corn starch	FM + C. starch
Sorghum	555.8	639.4	307.5	424.7
Soybean meal	327.5	135.0	379.7	152.5
Fishmeal	0.0	175.0	0.0	175.0
Corn starch	0.0	0.0	200.0	200.0
Canola oil	53.0	17.0	50.0	10.5
Limestone	5.0	3.1	5.0	3.3
Salt	2.0	0.0	2.5	0.0
Sodium bicarbonate	4.6	3.1	3.9	3.0
Dicalcium phosphate	22.5	1.5	23.0	4.0
Lysine	2.4	0.0	1.3	0.0
Methionine	3.5	2.3	3.6	2.7
L-Threonine	1.1	0.5	1.0	0.8
L-Arginine	0.0	0.9	0.0	1.1
Choline chloride 60%	0.6	0.3	0.6	0.5
Celite™	20.0	20.0	20.0	20.0
Vitamin-mineral px	2.0	2.0	2.0	2.0
Nutrient specifications				
ME (MJ/kg)	12.97	12.97	12.97	12.97
Protein	214.7	235.8	213.5	222.8
Starch	417.6	476.6	431.4	515.7
Fat	72.3	52.7	62.6	40.3
Fiber	33.6	25.5	30.5	21.2
Calcium	8.7	14.5	8.7	15.0
Total phosphorus	8.5	10.0	7.9	9.7
Available phosphorus	4.4	7.3	4.4	7.5
Lysine[1]	11.5	11.5	11.5	11.5
Methionine	6.2	6.5	6.2	6.7
Methionine + cystine	8.7	8.7	8.7	8.7
Threonine	7.7	7.7	7.7	7.7
Tryptophan	2.4	2.3	2.5	2.2
Arginine	12.3	12.3	13.2	12.3
Analysed[2]				
Starch	317.1	370.0	336.0	417.0
Protein (N × 6.25)	226.9	232.5	229.3	219.3

[1]Standarlised digestible amino acids [2]As-is basis

III. RESULTS

As shown in Table 2, fishmeal inclusions significantly improved weight gain and FCR. Broiler chickens offered diets with 175 g/kg fishmeal had higher weight gains by 12.1% (1260 versus 1124 g/bird; $P < 0.001$) and improved FCR by 8.13% (1.299 versus 1.414; $P < 0.001$). Interestingly, fishmeal inclusions enhanced starch digestibility coefficients in all four intestinal segments to highly significant extents ($P < 0.001$). As shown in Table 3, this was associated with increases in AME of 0.59 MJ (14.49 versus 13.90 MJ/kg; $P < 0.001$), ME:GE ratios by 8.57% (0.717 versus 0.717; $P < 0.001$) and AMEn of 0.67 MJ (13.48 versus 12.81 MJ/kg; $P < 0.005$).

Table 2 - The influence of dietary corn starch and fishmeal concentrations on growth performance from 15 to 28 days post-hatch and 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 broiler chickens at 28 days post-hatch.

Treatment		Growth performance			Starch digestibility coefficients				Starch disappearance rates (g/bird/day)			
Corn starch (g/kg)	Fishmeal (g/kg)	Weight gain (g/bird)	Feed intake (g/bird)	FCR (g/g)	PJ	DJ	PI	DI	PJ	DJ	PI	DI
0	0	1126	1565	1.389	0.354	0.582	0.691	0.784	14.3	23.6	28.0	31.7d
0	175	1256	1634	1.301	0.586	0.802	0.892	0.926	28.8	39.3	43.7	45.4b
200	0	1122	1613	1.439	0.349	0.595	0.734	0.793	15.3	26.3	32.4	34.9c
200	175	1264	1638	1.296	0.608	0.817	0.912	0.948	33.5	45.1	50.3	52.3a
SEM		17.6	24.8	0.0203	0.0414	0.0155	0.0161	0.0148	1.93	1.01	0.96	0.80
Main effects: starch												
0		1191	1600	1.345	0.470	0.692	0.791	0.855	21.5	31.4b	35.8b	38.5
200		1193	1625	1.367	0.478	0.706	0.823	0.870	24.4	35.7a	41.3a	43.6
Fishmeal												
0		1124b	1589	1.414a	0.351b	0.588b	0.712b	0.788b	14.8b	25.0b	30.2b	33.3
175		1260a	1636	1.299b	0.597a	0.809a	0.902a	0.937a	31.2a	42.2a	47.0a	48.8
Significance (P =)												
Corn starch (CS)		0.904	0.319	0.297	0.840	0.360	0.066	0.316	0.154	< 0.001	< 0.001	< 0.001
Fishmeal (FM)		< 0.001	0.076	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
CS x FM interaction		0.723	0.392	0.198	0.754	0.952	0.476	0.663	0.343	0.156	0.268	0.035

ab, Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Fishmeal accelerated starch disappearance rates in all four small intestinal segments to significant extents ($P < 0.001$). Fishmeal inclusions increased retention time in the small intestine by 37.6% (289 versus 210 minutes; $P < 0.001$). Corn starch inclusions did not influence starch digestibilities, but significantly increased starch disappearance rates in the three posterior segments (subject to an interaction in the distal ileum). Corn starch also improved ME:GE ratios by 2.17% (0.755 versus 0.739; $P < 0.05$) as shown in Table 2.

Table 3 - The influence of dietary corn starch and fishmeal concentrations on nutrient utilisation parameters and total retention times in four small intestinal segments

Treatment		Nutrient utilisation				
Corn starch (g/kg)	Fishmeal (g/kg)	AME (MJ/kg DM)	ME:GE ratio	N retention (%)	AMEn (MJ/kg DM)	Retention time (minutes)
0	0	13.73	0.710	54.26	12.67	221.8
0	175	14.47	0.767	51.91	13.30	291.9
200	0	14.06	0.723	55.80	12.95	199.0
200	175	14.51	0.787	48.33	13.65	286.2
SEM		0.149	0.008	3.363	0.186	9.58
Main effects: corn starch						
0		14.10	0.739b	53.08	12.99	256.9
200		14.29	0.755a	52.06	13.30	242.6
Fishmeal						
0		13.90b	0.717b	55.03	12.81b	210.4b
175		14.49a	0.777a	50.12	13.48a	289.0a
Significance (P =)						
Corn starch (CS)		0.228	0.049	0765	0.107	0.156
Fishmeal (FM)		< 0.001	< 0.001	0.164	0.003	< 0.001
CS x FM interaction		0.335	0.675	0.457	0.860	0.387

ab, Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

IV. DISCUSSION

The partial substitution of soybean meal by fishmeal enhanced growth performance and, surprisingly, starch digestibility coefficients and starch disappearance rates; this was reflected in significantly better energy utilisation (AME, ME:GE ratios, AMEn). Moreover, starch digestibility was significantly correlated with weight gain and FCR. Interestingly, fishmeal inclusions increased significantly increased small intestinal retention times and this may have been the genesis of the enhanced starch digestibility. It is tempting to suggest that the fat component of fishmeal may have activated the 'ileal brake'; thereby, increasing small intestinal retention times intestine as avian gastrointestinal motility can be influenced by intraluminal lipids (Martinez et al., 1995),

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BRANCHED-CHAIN AMINO ACIDS: RINGMASTERS OF AMINO ACID CATABOLISM IN ENTEROCYTES?

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Summary

It is not clear if branched-chain amino acids (BCAA) play a central role in determining the relative extents to which glucose or amino acids are catabolised in the gut mucosa for energy provision. Nevertheless, they demand close attention especially given the intense interest in low-protein diets with high synthetic amino acid inclusions. Additions of synthetic BCAA will modify digestive dynamics immensely, quite possibly with pivotal implications for the successful development of low-protein diets for chicken-meat production.

I. INTRODUCTION

A substantial proportion of amino acids that are absorbed along the avian small intestine fail to enter the portal circulation because they are utilised by the gut mucosa via anabolic or catabolic pathways. This of itself means that apparent or true ileal amino acid digestibilities of amino acids may not be sufficiently indicative. Amino acids, predominantly glutamate and glutamine, and glucose are both catabolised in enterocytes to approximately similar extents to meet the copious energy demands of the gut in rats (Fleming et al., 1997). Equally, glucose, glutamine and glutamate are the 'preferred fuels of respiration' in avian enterocytes (Watford et al., 1979). Therefore, uncertainties exist as to the extent to which the balance of amino acids are catabolised in the gut mucosa and whether the 'glucose:amino acid catabolic ratio' is subject to manipulation by dietary formulations or feeding strategies to advantage poultry performance. Interestingly, there are some indications that this may be the case in broiler chickens (Enting et al., 2005). The question posed in the title of this mini-review is an open one. It stems from the assertions of Chen et al. (2009) that 'intestinal branched-chain amino acids catabolism may play an important role in regulating the balance of dietary amino acids that enter the portal vein and may have enormous nutritional and physiological significance'. And 'knowledge about intestinal amino acid metabolism is crucial for understanding and improving the efficiency of utilisation of dietary protein'. Thus, this paper is an attempt to assess whether or not BCAA are indeed the ringmasters of amino acid catabolism in the gut mucosa.

II. BACKGROUND

The physiology and multi-dimensional functionality of the gut wall has been reviewed by van der Meulen and Jansman (1997) and Bannink et al. (2006). The subject is complex because the amino acids in enterocytes that enter the portal circulation may be of luminal or arterial origin; dietary amino acids are not the only source. Moreover, amino acids in enterocytes may be synthesised into proteins to maintain gut integrity or serve as precursors for digestive enzymes, mucin, nucleotides, polyamines and amino acids (Wu, 1998). Nevertheless, Reeds et al. (2000) concluded that amino acids are critical energy sources for the intestinal mucosa and the possibility that they are subject to nutritional regulation remains uninvestigated. Thus the identification and proportion of amino acids that are catabolised for energy provision to the gut is justified. Instructively, it appears that the net portal outflow of ammonia (NH_3)

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accounts for 18% of the total dietary intake of amino acid nitrogen in young pigs (Stoll et al., 1998). In rats, the small intestine utilises nearly all absorbed dietary glutamic acid and circulating glutamine, but 60% of glutamate/glutamine is catabolised to CO_2 and represents 'an important contribution to the energy requirement of the gut' (Windmueller and Spaeth, 1975). Thus, the catabolism of amino acids in the gut mucosa is substantial; furthermore, energy is derived more efficiently from glucose than glutamate/glutamine (Fleming et al., 1997). Consequently, if this ratio is subject to manipulation, it would be advantageous to tip the glucose:amino acid catabolic ratio in favour of glucose by any relevant strategies that are practically feasible. Given this outcome, not only would the provision of energy for the gut be more efficient but the post-enteral availability of amino acids would be enhanced for potential protein accretion.

III. DISCUSSION

One of the proposed mechanisms underpinning the slowly digestible starch concept is that it spares amino acids from catabolism in the gut mucosa (Weurding et al., 2003). Enting et al. (2005) added amino acids as glutamine and casein to broiler diets based on unprocessed or hydrothermally treated maize and peas as processing accelerates starch digestion rates. In rapidly digestible starch diets, the addition of amino acids improved FCR by 4.66% (1.638 versus 1.718). In contrast, there was not a tangible FCR response (0.60%) when amino acids were added to slowly digestible starch diets indicating that amino acid utilisation was enhanced by slowly digestible starch. This prompted the researchers to suggest that slowly digestible starch might 'prevent the use of amino acids as an energy source for the gut wall'. Usually, starch is almost completely digested and absorbed in the anterior small intestine thus effectively forcing enterocytes in more posterior segments to catabolise amino acids for energy provision. Thus slowly digestible starch may provide enterocytes in the posterior small intestine with an alternative energy source, thereby sparing amino acids from catabolism.

The underlying data is complex, conflicting and complicated by the fact that the majority of it stems from pigs and poultry data is extremely limited. However, it does appear that the properties of dietary starch influence the glucose:amino acid catabolic ratio in the gut mucosa. The likelihood is that glucose and amino acids may effectively compete for co-absorption with sodium via Na^+-dependent transport systems into the gut mucosa (Murer et al., 1975; Vinardell, 1990). Interestingly, Yin et al. (2010) compared maize and sticky rice starch in pig diets. The proximal jejunal digestibility of sticky rice starch (0.819) was noticeably higher than maize starch (0.472) and it was more completely digested (0.998 versus 0.931) in the distal ileum. However, sticky rice starch supported higher mean ileal digestibility coefficients of 12 essential amino acids by 7.59% (0.822 versus 0.764) and higher free amino acid concentrations in the systemic circulation than maize starch.

From an earlier study, van der Meulen et al. (1997), it is evident that dietary starch has the capacity to manipulate the net portal flux of amino acids in pigs. The transition from rapidly (maize) to slowly (pea) digestible starch increased the net portal flux of 12 essential amino acids by 22.9% (224.4 versus 182.6 mmol; $P = 0.030$). Alternatively, expressed as a proportion of ileal digestible amino acids, slowly digestible starch increased the net portal flux by 16.1 percentage units (96.0 versus 79.9%; $P = 0.019$) in 40 kg pigs. The generated percentage unit increases for BCAA ranged from 12.6 for leucine (99.0 versus 86.4%; $P = 0.071$), 14.4 for isoleucine (97.7 versus 83.3%; $P = 0.066$), to 17.4 for valine (84.0 versus 66.6%; $P = 0.091$). While the outcomes are somewhat contradictory, the two studies suggest that starch influences both the digestibility and post-enteral availability of amino acids.

Evidence that BCAA may be pivotal is provided by the study of Zhang et al. (2013). Weaner pigs were offered a high protein (209 g/kg) maize-soy diet, a low protein diet (171 g/kg) and the low protein diet supplemented with 3.4 g/kg valine, 1.9 g/kg isoleucine and 1.0 g/kg leucine. Perhaps the outstanding aspect of this study is that the transition from high to low protein diets substantially reduced BCAA concentrations in the systemic circulation; however, BCAA additions to the low protein diet restored these concentrations to those of the high protein diet with remarkable precision. The high to low protein diet transition tangibly compromised 14-days post-weaning performance of pigs in terms of weight gain (174 versus 303 g/day) and FCR (1.73 versus 1.21). However, BCAA addition to the low protein diet generated improvements of 64.4% in gain (286 versus 174 g/day) and 22.5% in FCR (1.34 versus 1.73). While these performance improvements were attributed to a BCAA-induced up-regulation of intestinal expression of amino acid and peptide transporters in weanling pigs, this does not preclude the possibility that BCAA were attenuating amino acid catabolism in the gut mucosa. Interestingly, BCAA tended to increase glutamate plus glutamine serum concentrations (597 versus 567 μmol/L) which could imply that relatively more glucose was being catabolised in the gut mucosa for energy provision. In a subsequent study, Zhang et al. (2016) added 4.0 g/kg isoleucine to a 175 g/kg maize-soy diet for weaner pigs. This single amino acid addition significantly improved weight gain by 29.5% (114 versus 88 g/day), FCR by 10.4% (1.63 versus 1.82) and tended to reduce systemic glucose serum concentrations. Also, isoleucine substantially increased sodium-dependent glucose transporter (SGLT-1) concentrations in the duodenum, jejunum and ileum; thus, isoleucine may be impacting on sodium and glucose co-absorption along the small intestine.

In poultry, Ospina-Rojas et al. (2014) found that combined additions of valine and isoleucine to a low protein (190 g/kg) maize-soy broiler diet significantly increased weight gain by 11.0% (867 versus 781 g/bird) from 1-21 days post-hatch. The gain of birds offered this treatment was statistically similar to those fed the high protein (220 g/kg) control, but significant FCR responses to valine and isoleucine were not observed. In addition, birds were offered diets containing 190 and 160 g/kg protein from 22-42 days post-hatch. The transition from high to low protein diets compromised weight gain (1578 versus 1817 g/bird) but the tandem addition of valine plus isoleucine significantly improved weight gain by 11.7% (1762 versus 1578 g/bird). Similarly, FCR was compromised (2.048 versus 1.816) but, individually, valine and isoleucine significantly improved FCR by 5.03 and 4.00%, respectively. In tandem, valine and isoleucine significantly improved FCR by 6.84% (1.908 versus 2.048). These outcomes suggest that synthetic BCAA are pivotal to poultry performance.

There is considerable interest in the successful development of low-protein diets for chicken-meat production and, axiomatically, these diets will contain increasing inclusions of synthetic amino acids. This is despite the consensus that the performance of pigs offered low-protein diets is superior to poultry for reasons that have yet to be clarified. In this quest, there is a real focus on the so-called non-essential amino acids including glycine, serine and proline. The digestion rates of protein-bound BCAA are relatively slow partially due to their hydrophobicity; therefore, the inclusion of synthetic BCAA will accelerate digestion rates markedly and cause a proximal shift in sites of absorption (Liu et al., 2013). Exogenous phytases are routinely included in broiler diets and phytase has a pronounced impact on proximal jejunal digestibility coefficients as respective increases of 53.8, 47.7 and 64.8% for protein-bound isoleucine, leucine and valine have been documented (Truong et al., 2015). Instructively, it has been reported in rats (Daenzer et al., 2001) that increases in post-prandial plasma BCAA, relative to other essential amino acids, are higher when fed unbound (synthetic) as opposed to being protein-bound (casein). This suggests that the post-enteral availability of BCAA will be enhanced when they are more rapidly absorbed from more anterior small intestinal sites.

In conclusion, whether or not BCAA are ringmasters of amino acid catabolism in the gut mucosa remains an open question. Chen et al. (2009) found that BCAA are catabolised in the gut mucosa but the basis of his assertions as to their central role is perhaps obscure. Nevertheless, the data generated by Zhang et al. (2013, 2016), albeit in pigs, and Ospina-Rojas et al. (2014) in poultry, provides justification for consideration to be given to BCAA. In a practical context, this applies to the development of low-protein diets in the short-term and role of synthetic BCAA in this quest. Antagonistic interactions between BCAA have been long recognised and may be relevant (Smith and Austic, 1978) and BCAA are functional amino acids; for example, leucine activates the mammalian target of rapamycin to stimulate protein synthesis (Wu, 2013). As there are indications that the rapid absorption of synthetic BCAA, relative to protein-bound BCAA, may be beneficial, it is vital that the question posed in the title of this paper should be addressed, if this is confirmed.

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DIGESTIBLE VALINE REQUIREMENT OF BROILERS: ESTIMATION BY META-ANALYSIS

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Summary

A meta-analysis was conducted to estimate the Val requirement of broilers in the concept of the ideal protein. Among 28 dose-response studies to Val, eight experiments were selected for modelling with non-linear regression models. Estimated Val requirements varied between 78.6 and 95.1% standardized digestible (SD) Val:Lys depending on the model used and the growth performance criteria. By evaluating the different requirements and responses, it is concluded that 80% SD Val:Lys ratio is sufficient to ensure optimal growth and feed efficiency of broilers.

I. INTRODUCTION

Thanks to the development of knowledge on amino acid (AA) nutrition (ideal protein concept, digestibility systems…) and to increased availability of feed grade AA (DL-Met, L-Lys, L-Thr, L-Trp and L-Val), crude protein (CP) levels in broiler diets have been reduced over the last decades. Protein-bound AA were replaced by feed grade AA to cover AA requirements while maintaining performance. Lowering dietary CP helps to improve the global sustainability of animal production by reducing feed costs, mitigating environmental impact (Belloir et al., 2016), lowering the incidence of foot pad lesions (de Jong et al., 2013) and reducing bacterial proliferation of pathogens (Widyaratne, 2012). When formulating diets without a minimum constraint on the CP level, the extent to which CP can be reduced is dependent on the next limiting AA in the diet. After Thr, whose requirement was recently updated by meta-analysis (Lambert et al., 2015), Val is the fourth limiting AA in corn-soybean meal broiler diets (Corzo et al., 2007). Digestible Val requirement have been recently investigated (Schedle et al., 2013, Tavernari et al., 2013) but the use of different strains, sexes, ages, AA systems and statistical models, has led to the discrepancy in the Val recommendations. A meta-analysis was therefore conducted to evaluate broiler Val needs based on the method by Simongiovanni et al. (2012).

II. MATERIALS AND METHODS

Valine dose-response trials from literature were entered in the database if they 1) were published after 1990, 2) provided specific information such as age, sex and diet composition and 3) tested a basal diet supplemented with at least three levels of L-Val. The database consisted of 28 trials from 18 publications. The dietary energy, CP and standardized digestible (SD) AA levels were recalculated based on INRA tables (Sauvant et al., 2004). In order to estimate the Val requirement expressed as a ratio to Lys based on dose-response study design, dietary digestible Lys should not be in excess and all indispensable dietary AA levels (Met, Thr, Ile, Arg, His, Leu, Phe…) must cover the needs of the broilers. The trial selection for Lys level was based on the Aviagen (2014) and Cobb (2012) recommendations while the ideal AA profile was taken from Rostagno (2011). In order to describe the response of average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) to increased Val level, a linear-plateau (LP) and a curvilinear-plateau (CLP) models were used:

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$$\text{LP: } Y_{ij} = A_i\left(1 + U\left(R - X_{ij}\right)\right) + \varepsilon_{ij} \text{ for } X_{ij} < R \text{ ; } Y_{ij} = A_i + \varepsilon_{ij} \text{ for } X_{ij} \geq R$$

$$\text{CLP: } = Y_{ij} = A_i\left(1 + U\left(R - X_{ij}\right)^2\right) + \varepsilon_{ij} \text{ for } X_{ij} < R \text{ ; } Y_{ij} = A_i + \varepsilon_{ij} \text{ for } X_{ij} \geq R$$

where Y_{ij} is the response criterion (i.e. ADG, ADFI, G:F) for observation j of experiment i, X_{ij} is the SD Val:Lys supply, A_i is the maximum response for experiment i (i.e. plateau), R is the minimum SD Val:Lys supply required to reach the plateau and U is the parameter describing the broiler response to the SD Val:Lys supply before the plateau value. The modelling was performed with the PROC NLIN procedure of SAS 9.2.

III. RESULTS AND DISCUSSION

In total, 50% of the 28 trials were coming from Brazil, 29% from the USA and 18% from Europe. Among the 28 trials, 17 presented excessive Lys levels and three of the remaining trials were deficient in one or more AA. Eight trials were at the end selected (Table 1). In the studies estimating the Val requirement in ratio to Lys (6 trials), recommendations from the authors varied from 76 to 85% SD Val:Lys and averaged 80% SD Val:Lys.

Table 1 - Age and genetic of the broilers, model used to estimate valine requirement and conclusion from the authors on the valine need for the eight selected trials.

Publication	Corzo et al. (2008)	Tavernari et al. (2013)	Schedle et al. (2013)	Dusel (2016)	Corzo et al. (2007)	Duarte et al. (2014)	Tavernari et al. (2013)	Corzo et al. (2004)
Age (d)	0-14	8-21	8-22	8-35	21-42	22-42	30-43	42-56
Genetic	Ross	Cobb	Ross	Ross	Ross	Cobb	Cobb	Ross
Model[1]	QR	LP	CLP	LR	QR	QR	LP	QR
Val needs[2]	0.91% dVal	78% dVal:Lys	84% SD Val:Lys	85% SD Val:Lys	78% dVal:Lys	80% dVal:Lys	76% dVal:Lys	0.67% dVal
Graph ref.[3]	1	2	3	4	5	6	7	8

[1]QR = quadratic regression, CLP = curvilinear-plateau model, L = linear-plateau model, LR = linear regression. [2]SD = standardized digestible (standardization based on INRA tables); d = digestible, Note: different digestibility systems were used in the different publications. [3] Reference used in the legend of Figure 1.

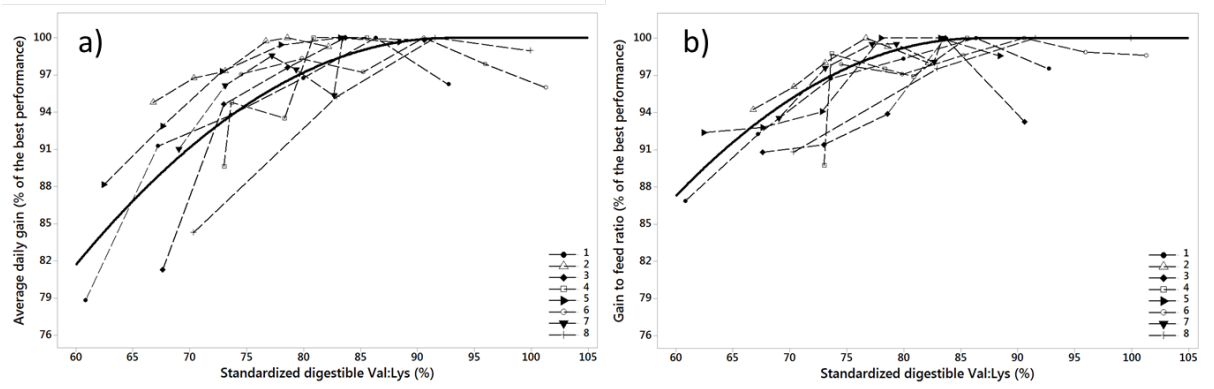

Figure 1 - a) Average daily gain (ADG) and b) gain to feed ratio (G:F) according to the dietary standardized digestible (SD) Val: Lys level. Dashed lines = 8 selected trials, solid line = curvilinear-plateau model.

In Figure 1, ADG and G:F according to SD Val:Lys levels of the eight selected experiments are presented. In all trials, best performance for ADG and G:F were observed between 80 and 92% SD Val:Lys. Being deficient at 75% SD Val:Lys (compared to the Val

level required to reach the best performance) results in a loss of performance between 2 and 12% for growth and between 2 and 10% for feed efficiency.

Estimates of the model parameter *R* and approximate standard errors using the CLP and LP models are presented in Table 2. The response criterion ADFI with the LP model could not be modelled. The other response criteria to dietary Val level showed that being deficient at a level of 75% SD Val:Lys (compared to *R*-values estimates), the loss of ADG and G:F is 6.2 and 2.3% respectively, on average of both models. However, for either the CLP or the LP models, deterioration of feed efficiency at 80% SD Val:Lys is very limited (0 to 0.8%). As a conclusion, 80% SD Val:Lys is sufficient to safely ensure best performance.

Table 2 - Parameter estimates, error of estimates and calculated responses of the curvilinear-plateau (CLP) and linear-plateau (LP) model for average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio (G:F) as response criteria (n=8 trials).

Model[1]	CLP			LP		
Performance criteria[2]	ADG	ADFI	G:F	ADG	ADFI	G:F
R (%)[3]	92.8	95.1	86.6	86.0	NA	78.6
Approximate standard error	167	418	180	76	NA	72
U (Slope)	-0.00017	-0.00006	-0.00018	-0.00573	NA	-0.00581
A (Plateau)	69.4	127.6	0.60	69.4	NA	0.60
Loss of performance (%)						
From *R* to 80% SD Val:Lys[4]	-2.9	-1.4	-0.8	-3.6	NA	-0.0
From *R* to 75% SD Val:Lys[5]	-5.7	-2.5	-2.4	-6.7	NA	-2.2

[1]CLP = curvilinear-plateau model, LP = linear-plateau model [2]ADG = average daily gain, ADFI = average daily feed intake, G:F = gain to feed ratio, [3]NA = not applicable (PROC NLIN procedure failed to converge), R = minimum SD Val:Lys supply required to reach the plateau [4]80% SD Val:Lys is the average of the published recommendations expressed in ratio to Lys (Table 1), [5]75% SD Val:Lys is the Aviagen (2014) and Cobb (2012) recommendation for 0-10d broilers

With the CLP model, significant response to Val was found for the criterion ADFI; on the contrary to Thr to which no response of ADFI was observed (Lambert et al., 2015). Valine is a particular AA as it was demonstrated that young piglets could sense the deficiency in dietary Val and reduce their feed intake in a short time (Gloaguen et al., 2012). According to the present results, broilers are not capable of compensating Val marginal deficiency by increased feed intake.

In the present study, Val requirement estimates are found to be greater with a CLP than with a LP model. Nevertheless, the CLP model is considered to be more suitable to estimate AA requirements of a growing population of animals: 1) it better estimates the response of a population as a whole (Hauschild et al., 2010); 2) it better takes into account the dynamic response of a growing population (Pomar et al., 2003) and 3) it usually better depicts the external AA dose-response experiments (Simongiovanni et al., 2012).

Statistically, it was possible, based on eight trials, to estimate a theoretical digestible Val requirement for broiler chickens, which varies between 78.6 and 95.1% SD Val:Lys. However, the approximate standard error is relatively high (between 72 and 418%) compared to the Thr meta-analysis by Lambert et al. (2015), where approximate standard error varied from 2.9 to 8.0% using the same models. In Lambert et al. (2015), the Thr requirement was estimated based on 21 selected trials, 13 trials more than in our study. Once more data on broilers response to dietary Val level are available, statistical models can be strengthened and Val requirement of broilers can be fine-tuned.

In addition, besides its role in body protein synthesis, Val is also involved in immune response and bone mineralization (Farran and Thomas, 1992, Foroudi and Rezaman, 2014). In starter broilers, Val requirements were found to be greater for maximal antibody

production against Newcastle virus and for maximal bone Ca mineralization than for maximal growth.

IV. CONCLUSION

Dietary Val requirement was confirmed to be at least 80% SD Val:Lys to maximize broilers growth performance. This new knowledge can be applied in practical feed formulation to successfully reduce dietary CP without affecting broiler performance and to benefit from the positive impacts of low CP diets. Next challenge in broiler AA nutrition will be to fine-tune the evaluation of the requirements of the next limiting indispensable AA (Arg, Ile, His, Trp…) and to take into account the interactions with the semi-indispensable and dispensable AA (Gly, Cys, Pro…).

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AMINO ACIDS AND INTESTINAL BARRIER FUNCTION: A CASE TO BE STUDIED IN REDUCED PROTEIN DIETS

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Summary

Optimum functionality of the epithelium of the intestine is important in controlling permeability in which amino acids may play a role. While there is evidence for such effect in other species, there is clear lack of data for poultry. In addition, much research has been done on low-protein, amino acid supplemented diets but there is little scientific evidence on their impact on intestinal health and function. The aim of a new project is to investigate the role of key amino acids along with reduction of dietary protein in intestinal function. This paper highlights the background and importance of the role of amino acids and needs for more research, particularly in the area of intestinal barrier function.

I. INTRODUCTION

Maintaining intestinal health and growth remains a concern as demands increase to limit the use of antibiotics in poultry production. There is also an interest to feed meat chickens with lower protein content diets using more synthetic amino acids to maintain productivity while reducing the environmental impact of poultry production. In that situation, closer attention needs to be given to those amino acids that maintain the intestinal barrier, decrease the variability of nutrient utilization and absorption, and make the birds less vulnerable to a physiological stress while not compromising performance. Optimum function of the epithelium of the intestine is important in controlling permeability. Poorly digested diets, fasting, endotoxins and several forms of stress have been shown to adversely affect intestinal barrier function (Vicuña et al. 2015, Gilani et al. 2016). In addition to their usual role in the synthesis of proteins, amino acids are regarded as key regulators of fluxes through several major metabolic pathways with additional roles in maintaining gut health (Jacobi and Odle 2012). Threonine, arginine (Arg), and glutamine (Gln) are regarded as the three most critical amino acids involved in metabolism, function, integrity and health of the intestinal tract across different species. There are therapeutic roles documented for specific amino acids including Gln, glutamate (Glu), Arg, glycine (Gly), Lys, Thr, and sulphur amino acids in gut-related disorders and disease.

II. INTESTINAL BARRIER FUNCTION

The small intestine serves as an organ for nutrient absorption. The mucosa of the small intestine has finger like projections known as the villi. A single layer of epithelial cells (enterocytes) covers these villi. The enterocytes are linked with each other through complex proteins known as adherens junctions (AJ), tight junctions (TJ) and desmosomes. Adherens junctions and desmosomes create the mechanical link between enterocytes e.g. Zonula Adherens. Tight junctions including Claudin, Occludens and Jams play an important role in paracellular permeability as reviewed by Groschwitz and Hogan (2009). These junctions not only have a fundamental role in absorbing nutrients, but also prevent entry of microbes and

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toxins into the body. However, during stress or disease when these junctions are disrupted, the barrier function of the gut is compromised leading to increased intestinal permeability (IP) (Chen et al. 2015). Increased IP can lead to compromised health, bacterial and toxin translocation, lameness and compromised performance (Gilani et al., 2016). Increased IP in chickens has not been studied in detail so far and needs further research. A few models have been studied in this regard e.g. dextran sodium sulphate and feed withdrawal for increasing IP (Gilani et al. 2016). In addition to the limited knowledge of increased IP models in chickens, very little has been researched regarding the biomarkers to evaluate the changes in increased IP. Tight junctions mRNA expression and Ussing chamber have been used in the past, however, information regarding the biomarkers to assess increased IP in live birds is limited. Gilani et al. (2016) have concluded in their review that two-sugar methods can effectively be used to evaluate the changes in IP in live chickens. These sugar methods include lactulose, rhamnose, mannitol and fluorescein isothiocyanate dextran (FITC-d). The modulation of IP through nutrition warrants further research.

III. KEY AMINO ACIDS INVOLVED IN INTESTINAL FUNCTION

Amino acids are involved in major metabolic pathways as regulators in addition to their normal roles as the building blocks of protein synthesis. Additional roles of amino acids in gut function and integrity has been reviewed by Wang et al. (2009). In this regard, only a few major amino acids are mentioned with specific focus on their role and their interactions on intestinal function.

There are also growing evidences that there are dietary requirements for non-essential amino acids (NEAA) including Gln to support optimum animal growth (Wu 2014). Typical plant protein sources often do not contain adequate amount of NEAA. Glutamine has been traditionally considered as a NEAA being the most abundant amino acid in blood plasma. It is established, mainly in other species, that Gln is the main energy source facilitating proliferation of intestinal enterocytes and activated lymphocytes (Chen et al. 2009). Several studies have confirmed that Gln is vital in maintaining the functional integrity of the gut as it plays a nourishing role for rapidly dividing intestinal epithelial cells, enterocytes and lymphocytes (Soares et al. 2014). This maintenance role is directly related to tight junctions, mucosal cell proliferation and differentiation (Soares, et al. 2014). Glutamine is involved in mucin synthesis. N-acetylglucosamine is a glycoprotein, a component of the mucin that protects mucosal surfaces and its formation is wholly dependent on Gln (Coster et al. 2004). Glutamine may be considered a conditionally essential amino acid when animal suffers from stress, injury or malnutrition. Under such circumstances the requirement may exceed the capacity for endogenous synthesis required to maintain gut integrity and reduce inflammation because during an immune response a marked increase in the uptake of plasma Gln by immunocytes occurs (Coster, et al., 2004). Beneficial effects of Gln on growth performance and gut development in broiler chickens have been previously demonstrated (Murakami et al. 2007). Nevertheless, its effectiveness in regards to enhancement of gut barrier function and more importantly its interaction with other nutrients with similar regulatory effects are still largely unknown for poultry.

Arginine has known roles in the urea cycle, in transport, storage, and excretion of nitrogen and eventually disposal of ammonia. Enterocytes can synthesize Arg from Gln and this is required to support optimum growth and intestinal function (Wu and Knabe 1995). Synthesis of nitric oxide (NO) is dependent on Arg as an essential substrate. Production of NO along with enterocyte migration is crucial for restoration of epithelial continuity (Jacobi and Odle, 2012). Arg is known to preserve intestinal barrier integrity and reduce bacterial translocation in mice (Viana et al, 2010). It has also been shown that Arg can attenuate

inflammatory response in broiler chickens in response to lipopolysaccharide injection (Tan et al. 2014b). Similar results have been reported for broilers subjected to a coccidial vaccine challenge (Tan et al. 2014a).

From the arginine family of amino acids, Arg and Gln have some similar pivotal functions. It has been reported that dietary supplementation with these two amino acids can improve porcine intestinal immunity and growth performance. It appears that there is complementary mode of action. Gln and Arg have been shown to decrease gut permeability through regulation of TJ (Ulluwishewa et al. 2011). Wu et al. (2013) showed that exogenous Arg and Gln could attenuate the adverse effect of deoxynivaleonol stress and immune related cytokines in piglets. They found that Gln and combinations of Arg and Gln reduced the alkaline phosphatase levels in pigs indicating an improved detoxification function of the liver (Wu et al. 2013). Lack of information in poultry warrants further research in this area.

The role of Thr in synthesis of mucin and maintenance of intestinal function is well documented in literature (Horn et al. 2009). Deficiency of Thr impairs the mucosal integrity evidenced mainly by differences observed in intestinal morphology and mucin production. In regard to paracellular permeability, no data are available for poultry. However, research in piglets has shown that a moderate deficiency of Thr (30% reduction) increased paracellular permeability in the ileum assessed by Ussing chambers (Hamard et al. 2010). Expression of genes related to ZO1, cingulin and MLCK controlling the paracellular permeability were also modified by low Thr in the diet of piglets (Hamard et al. 2010).

IV. DIETARY PROTEIN CONTENT

Dietary protein and therefore balance of amino acids plays a major role in animal health and performance. There is an interest to feed birds with low protein diets to reduce cost, environmental impact and wet litter. However, this practice has often resulted in impaired performance and unbalanced supply of amino acids. Supplementation of amino acids have been extensively researched in relation to the reduced protein diets and the order of limiting amino acids are very much known (Ospina-Rojas et al. 2012). Glycine and serine have been shown to improve the bird performance fed low protein diets. A very recent report has also shown the effect of Gly for permeability in enterocytes in piglets (Li et al. 2016) that need to be confirmed for poultry. In the concept of intestinal gut health, the role of specific amino acids and their relationship together for intestinal integrity and development are still not fully understood when dietary protein is reduced. However, Chen et al. (2016) recently showed that reducing dietary protein exacerbated the effect of aflatoxin in broiler performance and nutrient utilisation along with a numeric tendency to increase intestinal permeability. These researchers found that increasing dietary protein to 26% completely restored the adverse effect of aflatoxin on bird performance.

V. CONCLUSION

There is a clear need to investigate the role of key amino acids including Gln, Arg and Thr for intestinal barrier function. Although some supporting evidence has emerged on positive effects of some amino acids, such as Gln, Gly and serine, traditionally regarded as non-essential, their inclusion in practical poultry diets may not be economically and practically feasible without reduction of the protein content of the diets; a practice that may potentially have some implications for the bird performance and intestinal function. A new project supported by RIRDC Chicken Meat in Australia aims to investigate the role of selected amino acids along with reduction of dietary protein for intestinal health with particular emphasis on intestinal barrier function and permeability.

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ASSESSMENT OF NUTRITIONAL STRATEGIES TO REDUCE DIETARY CRUDE PROTEIN IN COMMERCIAL BROILERS

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Summary

Well over half a century of amino acid work has laid the groundwork for an understanding of feeding low CP diets to broilers with respect to amino acid "requirements", antagonisms, and imbalances. But the sensitivity to amino acids in modern commercial strains in terms of feed intake and whole body protein accretion continues to change. As such, many nutritionists rely on the ideal protein concept with constant evaluation of amino acid needs in modern broiler strains, amino acid content of feed ingredients, and their respective digestibility values. However, feed grade sources of valine have now entered formulation in some locations, which has led to a global discussion of the need of the amino acids beyond valine and the CP point of diminishing return in practical diets. Data presented herein from research at the Arkansas Agriculture Experiment Station, the University of New England, and elsewhere indicate large variations in CP (four percentage points) in growing broilers may be too extreme, or the work failed to satisfy a critical amino acid or nitrogen need. But marginal reductions in CP, while paying more attention to the so-called "conditional essential amino acids", such as glycine and serine, as well as relaxing less limiting amino acid needs (i.e., arginine, valine, and isoleucine), warrants more attention due to tremendous cost savings. Clearly, reducing amino acid needs from intact protein sources improves the environment.

I. INTRODUCTION

The addition of each feed grade amino acid in poultry diets necessitates knowledge of its estimated need "requirement", as well as adequate knowledge of the need of the next limiting amino acid. Hence, the first limiting amino acid, that constitutes its least cost minimum from intact protein sources, sets dietary CP in linear programming. Four essential and primary limiting feed grade amino acids (i.e., synthetic methionine, and crystalline lysine, threonine, and valine) have been included in poultry diets in commercial settings. In addition, feed grade crystalline and synthetic tryptophan was included in poultry diets in the 1990's. Availability of these feed grade amino acids has enabled the poultry industry to produce diets that are nutritionally balanced and economically viable. However, the need to reduce nitrogen excretion into the environment and to spare matrix space for the inclusion of less energy dense ingredients in feed formulation begs for a systematic look of CP level of poultry diets. Experimentally, a clear framework for all feed grade amino acids to be included in a semi-synthetic-low CP diet that supports good live performance has not been established. These proceedings include an overview of low CP research in broilers, recent low CP research from our laboratories, and opportunities, as well as limitations, going forward.

II. LOW CP/AA NEEDS

Research with methionine (Moran et al., 1994) and lysine (Acar et al., 1991; Bilgili et al., 1992), but not threonine (Kidd et al., 2004), has been shown to vary in terms of amino acid responses as a function of amino acid level and broiler strain. Work by Almquist (1972)

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demonstrated that the arginine requirement of chicks could differ over 100% as a function of strain. In the past it seemed that faster growing broilers required more amino acids whereas now it seems that strains with increased whole body protein from breast deposition to fat ratio are more responsive to amino acids. Some recent research at the Arkansas Agriculture Experiment Station Poultry Research Farm has been focused on low protein diets in broilers. The broiler strain utilized in our laboratory has been limited to the fast feathering Cobb 500 broiler.

A titration of CP from 16.0 to 20.0% of diet in increments of 0.8% CP was conducted to pinpoint the sensitivity of less limiting or nonessential amino acids in diets fed to Cobb 500 male broilers. Diets were formulated to be equal in total sulfur amino acids, lysine, threonine, valine, isoleucine, arginine, and tryptophan. Whereas less limiting essential and non-essential amino acids were allowed to fall to the designated CP level. Quadratic responses occurred for breast tenders (*Pectoralis minors*) and total breast yield (Figure 2) relative to live BW with maximal responses approximating 19% CP. As CP increased, however, a linear reduction in thigh yields was observed (data not presented). Trends for BW gain (Figure 1) were not significant, but feed conversion (Figure 1) improved linearly with progressive CP increases. Similarly, protein efficiency ratio was improved linearly as CP was increased (data not presented).

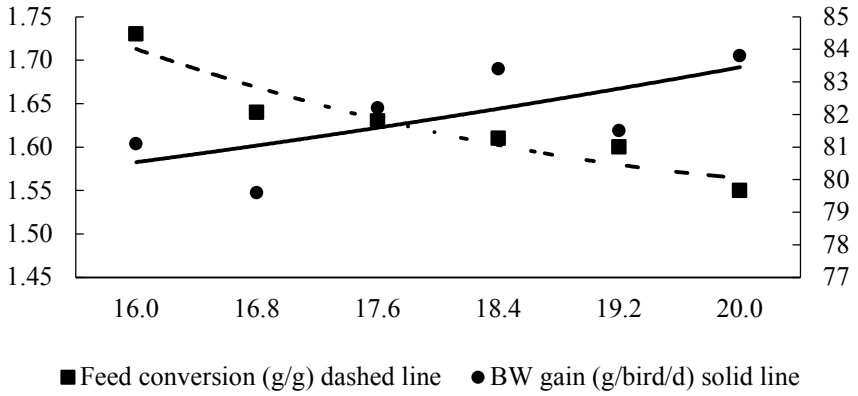

Figure 1 - Feed conversion and body weight gain of Cobb 500 male broilers fed diets balanced in amino acids but varying in CP (16.0 to 20.0% of diet) from 14 to 35 d of age.

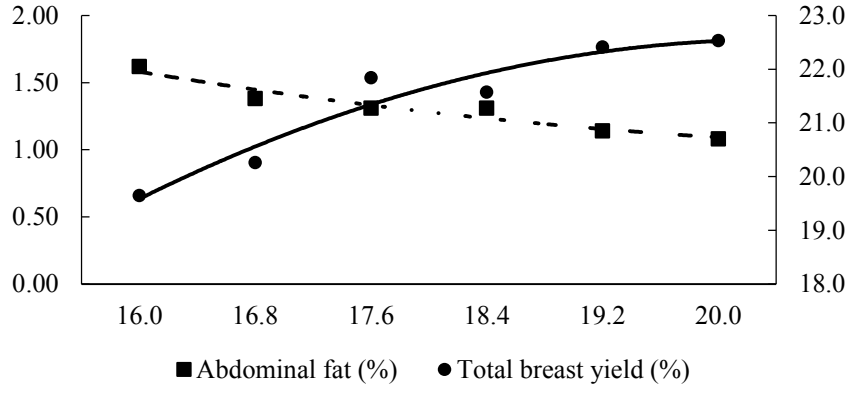

Figure 2 - Abdominal fat and breast meat yields (Pectoralis major and minor) of Cobb 500 male broilers fed diets balanced in amino acids but varying in CP (16.0 to 20.0% of diet) from 14 to 35 d of age.

In a companion study, utilizing the same diets (20 vs 16% CP), a mixture of glutamine, glycine, and leucine (++AA) was added to the 16% CP diet, in addition to total sulfur amino acids, lysine, threonine, valine, isoleucine, arginine, and tryptophan (+AA). The

addition of essential amino acids and the addition of leucine and the non-essential amino acids failed to support live performance equal to the control (Figures 3 and 4) or processing attributes (data not presented).

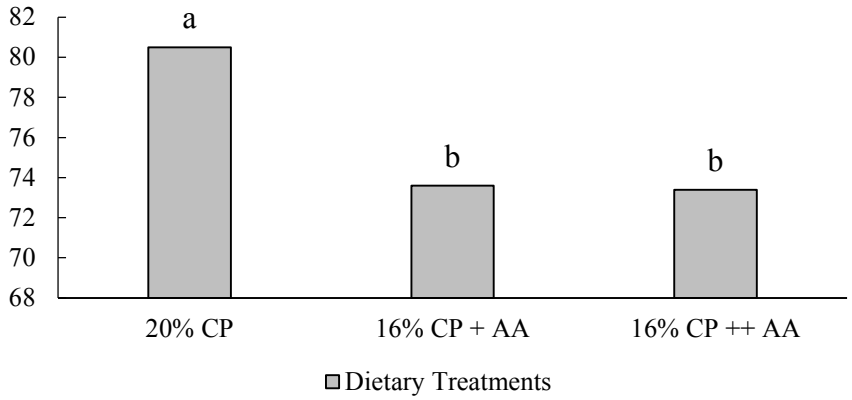

Figure 3 - BW gain (g/bird/d) of Cobb 500 male broilers fed diets differing in amino acids from 14 to 35 d.

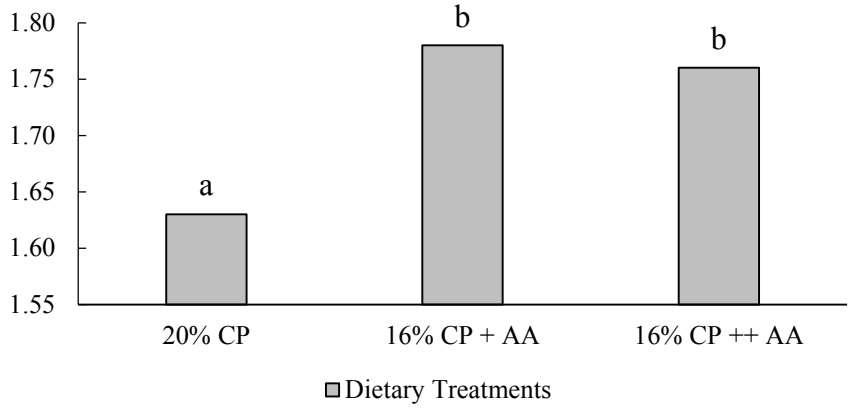

Figure 4 - Feed conversion (g/g) of Cobb 500 male broilers fed diets differing in amino acids from 14 to 35 d.

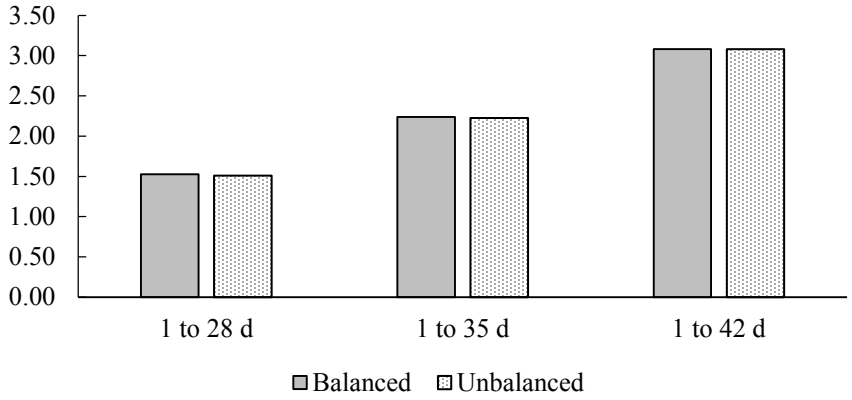

Figure 5 - BW gain (kg/bird) of Cobb 500 male broilers fed diets differing in amino acid balance from 1 to 28, 1 to 35, and 1 to 42d.

An amino acid density study was conducted using two diets: a diet balanced in all essential amino acids and a diet balanced in the most limiting essential amino acids with 5% reductions in valine, isoleucine, and arginine. The diets were fed to sex separate Cobb 500

broilers from 1 to 28, 1 to 35, and 1 to 42 d of age. Diet did not impact BW gain in any period (Figure 5). However, feed conversion (Figure 6) was impaired with unbalanced diets in the 1 to 35 d period, but not the 1 to 28 or 1 to 42 d periods. Unbalanced diets had more abdominal fat in 42-day old females, but not males, indicating their sensitivity of fat over lean accretion as they age (data not presented). Males had more woody breast than females, but diet effects on woody breast did not occur (Figure 7).

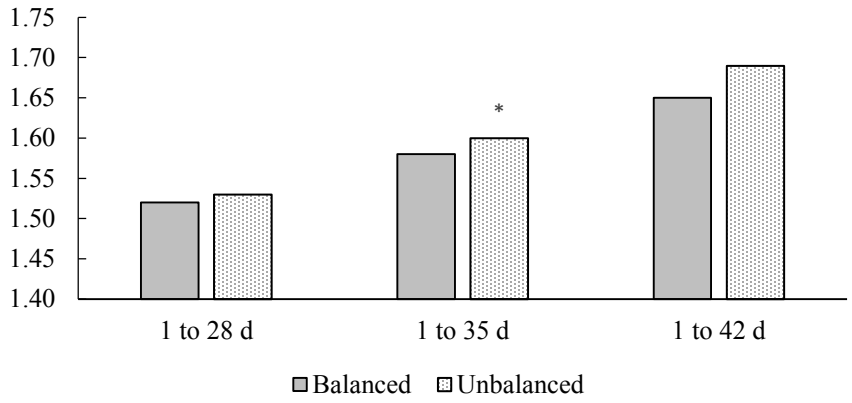

Figure 6 - Feed conversion (kg/kg) of Cobb 500 male broilers fed diets differing in amino acid balance from 1 to 28, 1 to 35, and 1 to 42d (* = $P \leq 0.05$).

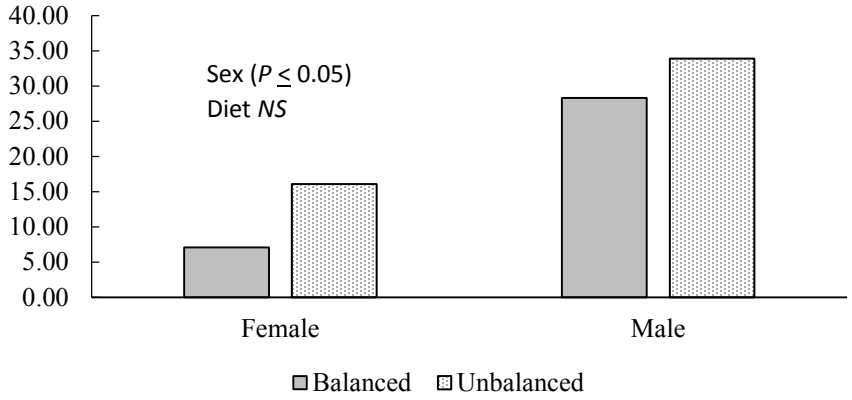

Figure 7 - Woody breast incidence (%) in male and female broilers (d 42) fed diets differing in amino acid balance from 1 to 42d.

Ospina-Rojas et al. (2014) fed reduced CP diets (3%) to Cobb 500 broilers from 1 to 21 and 22 to 24 d of age. The diets were adequate in methionine, lysine, and threonine as achieved by feed grade amino acids and intact protein, and individual or combined treatments of valine, isoleucine, arginine, and glycine were implemented. To sustain good performance and reduce N excretion, both valine and glycine were needed in the starter period, but valine, isoleucine, arginine, and glycine were needed in the growing period. Indeed, recent studies (Kriseldi 2016) demonstrated breed standard performance for the starter period in birds fed diets 20.9% CP. The diets were adequate in most of the essential amino acids (i.e., total sulfur amino acids, lysine, threonine, valine, isoleucine, arginine, and tryptophan), as well as glycine.

III. LOW DIETARY CP CONSIDERATIONS: ECONOMIC, WELFARE AND ENVIRONMENTAL IMPACTS

A decade of chick research at the University of Illinois was conducted to validate a reference diet that supported satisfactory chick growth and allowed for amino acid need determination (Klain et al., 1960a,b). The need for glycine (Greene et al., 1960) and proline (Greene et al., 1962) was found in the reference diet. Amino acids found necessary for satisfactory growth were: arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, methionine, cysteine, leucine, isoleucine, valine, glycine, proline, and glutamic acid (Dean and Scott, 1965). During this time vertical integration of broilers in the U.S. was increasing, and resultant success to present is chiefly attributable to primary breeder strain selection, and to a lesser extent for nutrition, management, or housing design. But it is widely known that going forward optimal resource (i.e., feed ingredients) efficiency for profitability and environmental stewardship are paramount, with both areas requiring close attention to and optimization of dietary amino acid digestion, absorption, and utilization.

The economic benefits of using low CP diets come from a reduction in energy expenditure on excreting excess N as uric acid, sparing of matrix space in feed formulation for inclusion of less energy dense ingredients, which can reduce feed cost. Indeed, feeding low CP diets to broilers reduces nitrogen excretion, ammonia emissions, litter moisture and litter pH. At the University of New England in Australia, Sharma et al. (2016) formulated a low CP diet to provide all the required amino acids without excesses. The diets were formulated to contain the same ratio of soybean, canola and meat meals and similar levels of metabolizable energy and digestible amino acid contents. The ratios between the protein meals were similar in both the diets to minimize the effect of protein source on odor but the protein levels differed by 5 percentage points in the starter diet and 4.5 percentage points in the grower and finisher diets between the high and low CP treatments. The results showed that the birds fed the low CP diet produced a significantly lower flux of dimethyl amine, trimethyl amine, hydrogen sulfite, ammonia and phenol in litter compared to those fed the high CP diet. In addition, the birds fed the low and high CP diets had markedly different litter moisture levels (31.8% vs 38.3%). A lower litter moisture often means less footpad dermatitis in broiler flocks (Taira et al., 2014), which, in turn, is an important outcome for bird welfare.

IV. CONCLUSIONS

Research presented herein shows inconsistencies of reduced CP diets to support growth of commercial broilers. However, reduced CP diets reduced N excretion and compounds associated with odor. With four to five of the most limiting amino acid being available to commercial nutritionists in feed grade form, the precedence is set to redefine overall amino acid needs and balance in commercial broilers to allow for the most economical and environmental friendly diets as possible. Scientists at amino acid companies and researchers at universities still have much work to complete to unlock the key to low CP-feed grade amino acid diet regimes for commercial broilers.

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NECROTIC ENTERITIS, WET LITTER AND ODOUR: THEIR INTERRELATIONSHIP

N.K. SHARMA[1], C. KEERQIN[1], N. MORGAN[1], S.B. WU[1], T. WALKER[2], M. CHOCT[2] and R.A. SWICK[1]

Necrotic enteritis (NE) affected flocks have poor enteric health and nutrient digestibility leading to decreased performance and increased excretion of nutrients (Barekatain et al., 2013; Hofacre et al., 2003). Evidence suggests that there is an association between diarrhea, wet litter and NE (Williams, 2005) but it is unclear whether the occurrence of NE leads to wet litter or vice-versa (Hermans and Morgan, 2007). If NE affected birds produce sticky droppings, diarrhea or wet litter, then NE may exacerbate odour emissions. This study was conducted to investigate the effect of sub-clinical NE and wet litter on litter headspace concentration of odorants in a broiler house.

A total of 160 day-old Ross 308 male broiler chicks were assigned to four dietary treatments each with 4 replicates of 10 birds/pen (pen size: 1.2 m × 0.65 m up to d 10 and 0.84 m × 0.60 m thereafter) with fresh pine shavings as bedding material. A litter collection tray measuring 0.46 m × 0.29 m × 0.065 m was placed in each pen away from the feeder and drinker before litter was spread over the pens covering the tray. A 2 × 2 factorial arrangement of treatments was employed in a completely randomized design to study the effect of NE challenge (no, yes), dietary Na level (1.6 g/kg, 4.0 g/kg) and their interaction on litter headspace concentration of odorants. The high Na diet contained a high level of salt to produce wet litter condition. On d 20, odorants were collected from litter headspace with a flux hood and measured using selective ion flow tube mass spectrometry (SIFT-MS). Data were subjected to two-way ANOVA and means were separated by Tukey's HSD test at $P < 0.05$ using SAS JMP v.8 software. Odorant concentrations were log transformed before analysis. On d 33, while challenge did not lead to higher mortality, it reduced feed intake by 5.48 % ($P < 0.05$), body weight gain by 9.02 % ($P < 0.01$) and increased FCR by 5 points ($P < 0.01$) indicating the presence of subclinical necrotic enteritis in challenged birds. Challenge increased ($P < 0.01$) litter moisture and litter headspace concentrations of dimethyl sulfide ($P < 0.05$), propyl mercaptan ($P < 0.05$), total butanols ($P < 0.05$), acetoin ($P < 0.01$), skatole ($P = 0.05$), butyric acid ($P < 0.05$), methyl amine ($P < 0.05$) and tended to increase concentrations of ethyl mercaptan ($P = 0.07$), carbon disulfide ($P = 0.09$), indole ($P = 0.10$) and formic acid ($P = 0.10$) compared to the unchallenged group. The birds fed high Na diet produced higher litter moisture ($P < 0.01$), wet litter and higher litter headspace concentration of sulfur compounds and phenol ($P < 0.01$) compared to those fed normal Na diet. In the birds fed high Na diet (that produced wet litter), challenge increased the litter flux of some additional odorants which included 2,3-butanedione ($P < 0.05$), acetic acid ($P < 0.01$), propionic acid ($P < 0.01$), isobutyric acid ($P < 0.01$), isovaleric acid ($P < 0.01$), pentanoic acid ($P < 0.05$), 2-butanone ($P < 0.05$) and 3-methyl-1-butanol ($P < 0.05$).

These findings suggest that both wet litter and sub-clinical NE may increase the odour nuisance potential of broiler farms. Sub-clinical NE affected broilers may further increase odour emissions if the disease comes together with wet litter condition.

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EMISSIONS OF VOLATILE ODOROUS METABOLITES BY *CLOSTRIDIUM PERFRINGENS* - *IN VITRO* STUDIES USING TWO BROTH CULTURES

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Clostridium perfringens (*Cp*) is a gram-positive anaerobic spore-forming rod-shaped bacterium that was first described to cause necrotic enteritis (NE) in poultry by Parish (1961). One of the major gross pathological changes in NE affected broilers is the presence of highly odorous brown fluid content in the intestine (Helmboldt and Bryant, 1971). The volatile metabolites produced by *Cp* causing the foul odour during NE infections have not been investigated. Research has shown that *Clostridium spp* produce various short chain fatty acids, dimethyl disulfide, 2,3-butanediol, isopentanol and acetoin (Stotzky and Schenck, 1976). This study was an *in vitro* measurement of odorants produced by the growth of NE inducing *Cp* type A field strain in two commonly used culture media, thioglycollate broth (USP-alternative) with peptone and starch supplementation (TPS) and brain heart infusion broth (BHI).

A total of six treatments each with three replicates were arranged in a 2 × 3 factorial to study the effect of two broth media (TPS, BHI), three *Cp* concentrations (0, 10^3 cfu/ml, 10^6 cfu/ml), and their interaction on media/bacterial culture headspace concentration of odorants. The culture media with and without *Cp* were freshly prepared on the day of odorant measurements and the emissions were measured using selective ion-flow tube mass spectrometry (SIFT-MS) using methods previously described (Sharma et al., 2016). Data were subjected to two-way ANOVA and means were separated by Tukey's HSD test at $P < 0.05$ using SAS JMP v.8 program. At a concentration of 10^6 cfu/ml, *Cp* increased the culture headspace concentration of methyl mercaptan, 1-propanethiol and dimethyl sulfide regardless of the media type ($P < 0.01$) compared to a 0 cfu/ml. There were *Cp* × media interactions in the culture headspace concentration of most odorants. In TPS medium, 10^6 cfu/ml of *Cp* increased the culture headspace concentrations of carbonyl sulfide by 90 fold ($P < 0.01$), ethyl mercaptan by 2.5 fold ($P < 0.01$), hydrogen sulfide by 19 fold ($P < 0.01$), 2,3-butanedione by 3.2 fold ($P < 0.01$), acetoin by 35.4 fold ($P < 0.01$), dimethylamine by 5.3 fold ($P < 0.01$), trimethylamine by 3.8 fold ($P < 0.01$), acetic acid by 13.7 fold ($P < 0.01$), propionic acid by 13.5 fold ($P < 0.01$), butyric acid by 100 fold ($P < 0.01$), isobutyric acid by 23 fold ($P < 0.01$), pentanoic acid by 2.8 fold ($P < 0.01$), hexane by 2.6 fold ($P < 0.01$), indole by 3.9 fold ($P < 0.01$) and 1,4-diaminobutane by 2.3 fold ($P < 0.01$) compared to the medium with no *Cp* but *Cp* had no effect on the concentration of these odorants in BHI medium ($P > 0.05$).

In conclusion, *Cp* produced a wide range of volatile odorous metabolites belonging to the group of sulfur compounds, alcohols, ketones, amines and carboxylic acids the concentrations of which varied according to the media type and *Cp* concentration. Thus, *Cp* contributes to the production of a wide range of odorous metabolites that can impart noxious smell during infection.

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EFFECTS OF FREE CHOICE OAT HULLS ON BROILER PERFORMANCE AND GUT MICROFLORA DURING NECROTIC ENTERITIS CHALLENGE

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Structural fiber in broiler diets have been observed to enhance performance, intestinal function and modify the composition and quantity of the microbial population in the gastrointestinal tract (Kheravii et al., 2016; Jiménez-Moreno et al., 2016 and Choct 2009). Jiménez- Moreno et al. (2011) reported that the inclusion of oat hulls in broiler diets reduced the number of necrotic enteritis (NE) causative agent (*Clostridium perfringens*). The present study assessed the impact of free choice oat hulls (OH) on the performance and gut microbiota in broilers challenged with NE. A total of 240 day-old male Ross 308 chicks were assigned to 24 cages in 2 rooms, in a 2 × 2 factorial arrangement of treatments. Factors were: challenge - or +; and OH - or +. The NE challenge method followed the study reported previously (Rodgers et al., 2015). Feed conversion ratio (FCR), weight gain and feed intake were measured for 0-16 d. Nine groups of bacteria, i.e., *Lactobacillus* spp., *Bifidobacterium* spp., *Bacillus* spp., *Ruminococcus* spp., *Bacteroides* spp., Enterobacteriaceae, *Clostridium perfringens*, *Salmonella* spp., and total bacteria, were quantified from the caecal digesta on d 16 using qPCR with 16S ribosomal primers (Wu et al., 2014).

On d 16, challenged birds had lower weight gain and feed intake ($P < 0.05$) compared to non-challenged birds (Table 1). The FCR in challenged birds tended to be higher ($P = 0.052$) compared to non-challenged birds. On day 16, birds given OH had lower feed intake ($P < 0.05$) compared to those without access to OH. Birds with access to OH tended ($P = 0.062$) to have lower FCR compared to those without OH. No challenge by OH performance interactions were observed ($P > 0.05$).

On d 16, the challenge led to significant changes in the counts of caecal microflora. Increased numbers of *C. perfringens* ($P < 0.001$) and reduced numbers of *Lactobacillus* and *Salmonellae* were observed ($P < 0.05$) in the caecal contents of challenged birds (Table 2). However, no changes were observed in caecal *Bifidobacteria*, *Bacillus*, *Ruminococcus*, *Bacteroides* or total bacteria as a result of challenge ($P > 0.05$). OH had no effect on caecal bacterial counts on d 16. An OH by challenge interaction was observed for Enterobacteriaceae group counts ($P < 0.001$). In the unchallenged birds, those without OH had lower counts of Enterobacteriaceae compared with those accessed to OH but no effect of OH was observed in challenged birds. However, the unchallenged birds without OH had lower counts of Enterobacteriaceae than challenged birds without accessed to OH. This study suggests that OH improves performance by enhancing FCR and decreasing feed intake.

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Table 1 - Impact of free choice OH on the performance in broilers challenged with NE at d 16.

	Main effect	FCR	Weight gain (g/bird)	Feed intake (g/bird)
Challenge	No	1.167	612[a]	715[a]
	Yes	1.187	569[b]	675[b]
OH	No	1.187	600	712[a]
	Yes	1.167	582	679[b]
P values	Challenge	0.052	0.008	0.013
	OH	0.062	0.241	0.037
	Challenge × OH	0.607	0.844	0.599

[a,b] Means in the same column sharing the same superscripts are not significantly different (P < 0.05)

Table 2 - Impact of free choice OH on caecal microflora (log10 CFU) in broilers challenged with NE at d 16.

Treatments	Lacto-bacillus	Bifido-bacteria	Bacillus	Ruminococcus	Bacteroides	Entero-bacteriaceae	C.perfringens	Salmonella	Total bacteria
Unchallenged - OH	8.40	8.46	8.67	9.39	4.52	6.54[b]	0.00	6.97	10.00
Unchallenged + OH	8.54	8.71	8.96	9.30	4.62	7.19[a]	0.00	6.92	9.99
Challenged - OH	8.26	8.70	8.74	9.43	4.56	7.39[a]	9.58	6.77	10.06
Challenged + OH	8.15	8.58	9.03	9.29	4.98	6.98[ab]	9.49	6.75	10.03
Main effect									
Challenge									
No	8.47[a]	8.59	8.81	9.34	4.57	6.86[b]	0.00[b]	6.94[a]	9.99
Yes	8.21[b]	8.64	8.88	9.36	4.77	7.18[a]	9.54[a]	6.76[b]	10.05
OH									
No	8.33	8.58	8.71	9.41	4.54	6.96	4.79	6.87	10.03
Yes	8.34	8.64	8.99	9.30	4.80	7.09	4.75	6.84	10.01
P value									
Challenge	0.035	0.666	0.764	0.765	0.185	0.023	< 0.001	0.026	0.282
Oat hulls	0.913	0.601	0.214	0.132	0.087	0.327	0.853	0.658	0.649
Challenge × OH	0.304	0.133	0.988	0.723	0.290	0.001	0.853	0.861	0.859

[a,b] Means in the same column sharing the same superscripts are not significantly different (P < 0.05)

DIETARY SUPPLEMENTATION OF ARABINOXYLO-OLIGOSACCHARIDES IMPROVES GROWTH PERFORMANCE OF NECROTIC ENTERITIS-CHALLENGED CHICKENS

C. KEERQIN[1], N. K. MORGAN[1], S. WU[1], R. SWICK[1], B. SVIHUS[2] and M. CHOCT[1]

The effect of dietary supplementation of arabinoxylo-oligosaccharides (AXOS) and arabinoxylans on growth performance of necrotic enteritis (NE)-challenged broiler chickens was examined. Male day-old Ross 308 chicks (n = 180) were placed in 30 individual floor pens with five replications per treatment, six birds per pen. From d 0-10, birds were fed a wheat-soy based commercial standard starter crumble. On d 10, birds were allocated a standard wheat-soy grower diet, of which 2% of the wheat was replaced by either arabinoxylans (AX), AXOS or arabinoxylans + xylanase (AX+E) until d 21. Necrotic enteritis was induced to half of the birds at d 9 by a one-off oral dose of 1 ml *Eimeria* species (*E. acervulina*, 5,000 oocytes; *E. maxima*, 5000 oocytes; and *E. brunetti*, 2500 oocytes), which was followed by an oral dose of 2 ml of freshly prepared field strain of *Clostridium perfringens* type A (approx. 10^7 CFU/ml) (CSIRO, Australian Animal Health Laboratory) at d 14. Necropsies were undertaken on dead birds to determine the cause of death. Weight gain, feed intake and mortality-corrected feed conversion ratio (FCR) were measured and calculated for d 10-21.

Necrotic enteritis challenge suppressed ($P < 0.001$) feed intake and weight gain at d10-16 and d10-21 and FCR at d10-16. Birds fed the AXOS-diet had significantly better FCR ($P=0.043$) compared to those fed the AX-diet at d10-16 (Table 1). There were numerical differences in FCR between birds fed different diets at d10-21, highlighting that FCR was improved in birds fed AXOS and AX+E compared to those fed AX, but they were not statistically different due to the small number of replicates. No interactions between dietary treatment and NE challenge were observed in this study. These findings suggest that AXOS potentially had a beneficial effect on intestinal microbiota balance and hence bird health and performance in the presence of NE challenge. Future studies need to investigate the mechanisms by which AXOS exhibit their advantageous effects on broilers challenged with NE.

Table 1 - Weight gain, feed intake and FCR of birds fed AX, AXOS or AXOS+E with or without NE challenge.

	d 10-16			d 10-21		
Main effects on parameters	Feed intake (g)	Weight gain (g)	FCR[1]	Feed intake (g)	Weight gain (g)	FCR[1]
Diet						
AX	409.3	269.9	1.565[a]	950.0	694.5	1.372
AX+E	406.4	266.5	1.587[ab]	936.7	712.6	1.318
AXOS	409.4	281.0	1.500[b]	920.2	701.9	1.319
SEM	5.8	5.8	0.024	16.2	16.0	0.024
Necrotic Enteritis Challenge						
Unchallenged	459.4[a]	339.3[a]	1.356[b]	990.1[a]	757.8[a]	1.310
Challenged	357.4[b]	205.7[b]	1.744[a]	881.2[b]	648.2[b]	1.362
SEM	4.8	4.7	0.019	13.2	13.1	0.019
P values						
Diet	0.920	0.195	0.043	0.441	0.726	0.202
Challenge	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.068
Diet*Challenge	0.736	0.857	0.213	0.689	0.747	0.343

[1]Feed conversion ratios were as per pen basis and calculated including the mortalities and sampled bird weights; [a, b] Means within the same column, within the same age group, with no common subscript, differ significantly ($P \leq 0.05$).

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INCREASED BIOAVAILABILITY OF COPPER RESULTS IN GREATER INTESTINAL HEALTH AND PRODUCTION THAN COPPER SALTS

X.U. ARBE[1] and M.S. BEKKER[1]

Summary

Trace minerals have different roles in the performance of the birds. Copper is involved in the synthesis of haemoglobin, synthesis and activation of oxidative enzymes, synthesis of connective tissue like collagen and it is a co-factor of many enzymes. It is a common practice to add additional copper in broilers diets to cover the basic requirements of this trace mineral, around 8 to 20 ppm. However, there is also research showing that adding higher levels of copper, 125 to 250 ppm will result in improved gut health and performance in birds. Copper metabolism is the key to understanding the benefits of using elevated levels.

I. INTRODUCTION

Copper is absorbed and accumulated in the liver. From the liver it passes into the blood stream where it reduces oxidative stress (Yen and Nienaber 1993) or stimulates the production of growth hormone (Zhou 1994). Copper also has a major role in immune activity, inflammatory agents (e.g. lipopolysaccharides) stimulate copper uptake by inducing the expression of the CTR1 copper importer at the plasma membrane. Cytoplasmic copper is delivered via the ATOX copper chaperone to the ATP7A copper pump, which undergoes trafficking to the phagolysosomal compartment, into which it loads copper. The NADPH oxidase (NOX) produces superoxide, which spontaneously generates hydrogen peroxide, the bactericidal potency of which is augmented by conversion to the hydroxyl radical via Cu(I)-catalyzed Fenton chemistry. Copper may also function as a bactericidal agent by disruption of iron (Fe) – Suphur (S) clusters (Hodgkinson and Petris 2012). This immune activity has an impact on gut health, the addition of high levels in the diet will have an antimicrobial effect, such as the reduction of E.coli and Clostridium and the increase of intestinal lymphocytes (Arias and Koutsos, 2006). This immune activity will be achieved by copper both before it is absorbed and also by the copper conjugated in the bile resulting in greater villous to crypt ratio (Zhao et al 2007). The increase of copper in bile has been shown to increase the activity of lipases and the digestibility of fats (Luo and Dove 1996).

The addition of high levels of inorganic copper has shown improvements in performance however, high levels of copper from inorganic sources reduces tibia ash and phosphorous retention (Banks et al 2004). HMTBa chelation technology makes trace minerals more bioavailable than mineral salts due to the ability of the molecule to place a larger percentage of the metal ion (copper in this case) directly into the tissue and bloodstream of the animal at the wall of the small intestine. This results in greater bioavailability than inorganic trace minerals (Richards et al 2007) and also deposition (Richards et al 2010).

Greater bioavailability allows a nutritionist to use lower levels of equivalent mineral salts and have better performance (Zhao 2010). The effect of HMTBa chelated copper in broilers has been studied in different trials compared to high levels of inorganic copper. It has been reported that lower levels of HMTBa chelated copper can reduce intestinal E.coli and enterobacteria levels in broilers and increase the level of copper in bile as compared to inorganic copper sulphate. (Butkeraitis, 2007).

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II. MATERIALS AND METHODS

Two trials were conducted to evaluate the performance of broilers fed reduced levels of HMTBa chelated Copper (Mintrex® Cu) compared to higher levels of inorganic copper sulphate.

Trial 1: A total of 1,080 Lohmann day old broilers were housed in closed wire housing and assigned to 4 treatments with 15 pens per treatment and 18 chickens per pen. Data was analysed as factorial in randomized complete block design using the General Linear Models procedure of SAS® and the significant differences between treatment groups were detected by Duncan's multiple range test. The differences among the treatment were considered significant at P-value≤ 0.05. Test diets included: T1 (-ve control): a diet free of additional copper; T2: T1 with 15 ppm of copper from copper sulphate; T3: T1 with 120 ppm of copper from copper sulphate and T4: T1 with 30 ppm of copper from HMTBa chelated copper (Mintrex® Cu).

Trial 2: A total of 1,050 Cobb 500 male day old broilers were housed on the floor and they were assigned to 5 treatments with 7 pens per treatment and 30 chickens per pen. Data was analyzed as factorial in randomized complete block design using the General Linear Models procedure of SAS® and the significant differences between treatment groups were detected by Duncan's multiple range test. The differences among the treatments were considered significant at P-value≤ 0.05. Test diets were wheat and soya based, with no enzymes or antibiotic growth promoters. The treatments were, T1: 10 ppm of copper from copper sulphate; T2: 120 ppm of copper from copper sulphate; T3: 10 ppm of Copper from HMTBa chelated Copper (Mintrex® Cu), T4: 20 ppm of Copper from HMTBa chelated Cu (Mintrex® Cu), and T5: 30 ppm of Copper from HMTBa chelated copper (Mintrex® Cu).

III. RESULTS AND DISCUSSION

Table 1 - Adjusted Feed Conversion Ratios for Trial 1

Copper addition	adj FCR (1-21 days)
no additional Cu	1.3
Cu SO_4 15ppm	1.293
Cu SO_4 120ppm	1.283
Cu HMTBa Chelate 30ppm	1.285

Table 2 - Adjusted Feed Conversion Ratios for Trial 2

Copper addition	adj FCR (1-35 days)
Cu SO_4 10ppm	1.476
Cu SO_4 120ppm	1.474
Cu HMTBa Chelate 10ppm	1.485
Cu HMTBa Chelate 20ppm	1.474
Cu HMTBa Chelate 30ppm	1.455

Trial 1. There were no significant differences in body weight gain and mortality between treatments at 21 days, however, the FCR of those diets with 120ppm inorganic copper and HMTBa chelated copper was significantly better than the control diet and the diet with inorganic copper at 15ppm. In summary, high levels of inorganic copper can be replaced by lower levels of HMTBa chelated Copper for equivalent performance.

Trial 2. Bodyweight gain at 41 days showed no significant difference between treatments but the FCR in these broilers fed the chelated copper at 30ppm showed a

significant improvement compared to the other treatments including the treatment with high levels of inorganic copper.

IV. CONCLUSION

As antibiotic growth promoters are removed as a mainstream feed additive, alternative solutions and their application must be fully understood. Copper is an ideal candidate with proven effectiveness in practical production yet environmental considerations must be kept in mind, and any opportunity to reduce overall mineral load in manure and soils must be sought.

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EFFECTS OF BLEND OF ORGANIC ACIDS SUPPLEMENTATION WITH DIFFERENT LEVELS OF DIETARY PROTEIN ON PERFORMANCE, CARCASS TRAITS AND MICROBIOTA PROFILE IN BROILER CHICKENS

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Summary

The experiment was conducted to investigate the effects of organic acids supplementation on growth performance, carcass traits and microbiota profile of broiler chicken fed different levels of protein. A 4 × 2 factorial arrangement of treatments including four inclusion rates of dietary organic acids (0, 0.10, 0.15, and 0.20%) and two levels of protein (90 and 100% National Research Council (NRC; 1994) recommended level) were used. Organic acid supplementation had no effect on weight gain of broilers fed 100% protein level recommended by NRC, but had significant effect ($P < 0.05$) in broilers fed low protein diets, with higher weight gain in 0.15 and 0.20% organic acid supplementation. Birds fed 100% level of protein showed significantly higher feed intake compared to those fed 90% protein recommended by NRC. Feed conversion ratio (FCR) improved ($P < 0.05$) in birds fed low protein level with 0.15% organic acid supplementation, while in birds fed 100% level of protein 0.2% organic acid had better FCR compared to 0 and 0.15% organic acid supplementation. Dietary treatments had no significant effect ($P > 0.05$) on microbial population and carcass characteristics. However, birds fed 100% protein level showed higher ($P < 0.05$) relative breast weight compared to those fed 90% protein level.

I. INTRODUCTION

The use of antibiotics as growth promoters in chicken nutrition has been banned by the European Union (EU) since 2006. So many attempts have been made to use an alternative for antibiotics in broiler nutrition to enhance the performance of broiler chicken. One such alternative was the use of organic acids as feed additives in the poultry industry. The use of organic acids has been reported to increase the nutrient utilisation and growth performance of broiler chickens (Denil et al., 2003; Garcia et al., 2007). The organic acids may penetrate the bacteria cell wall and disrupt the normal physiology of certain types of bacteria (Langhout, 2000). Apart from the antimicrobial activity, they reduce the pH of digesta, increase the pancreatic secretion, and have trophic effects on the mucosa of gastro-intestinal tract (Dibner and Buttin, 2002). Acidification with blend of organic acids has been reported to decrease the production of toxic components by the bacteria and colonisation of pathogens on the intestinal wall, thus inhibiting the damage to epithelial cells and subsequently increase the digestibility of proteins and minerals (Langhout, 2000).

Feed constitutes the majority of costs (70-75%) associated with the production of poultry. A large portion of feed cost involves meeting the amino acid requirements of the birds (Corzo et al., 2004). By reducing the level of crude protein in the diet it is possible to achieve significant cost saving. In addition to reducing feed cost, the ability to lower crude protein in the diet can result in decreased nitrogen excretion (Ferguson et al., 1998), thereby decreasing the chance of environmental pollution. Reduction of nitrogen and phosphorous in

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animal waste will be one of the primary consideration in the diet formulation in the near future (Si et al., 2004a,b).

Therefore the study reported herein was carried out to evaluate the response of broiler chickens, in terms of performance, carcass trait and and microbiota profile with dietary supplementation of organic acids and two protein levels.

II. MATERIALS AND METHODS

The experiment had a completely randomised design with a 4 × 2 factorial arrangement of treatments evaluating four inclusion rates of organic acid (0, 0.10, 0.15, and 0.20%) and two levels of protein (90 and 100% NRC recommended level). Experimental procedures were conducted in accordance with the Islamic Azad University of Qaemshar, Iran Animal Ethics Committee guidelines. A total of 320 Day-old male broilers (Ross 308), obtained from a commercial hatchery and were allocated to 32 floor pens (10 chicks/pen). Each of the eight dietary treatments was randomly assigned to four pens. The birds were kept in pens for the experimental period of 42 days. Each pen was equipped with a separate feeder and a manual drinker. Feed intake and body weight gain of each pen was measured at the end of each phase. Feed conversion ratio (FCR) for each pen was calculated by dividing feed intake by body weight gain. At the end of the experiment, 4 birds per pen were killed and the carcass traits were measured. For microbial populations, 4 birds per treatment were selected, weighed, and killed by cervical dislocation. The intestinal content of each bird was removed and samples of fresh digesta (1 to 2 g) from the ileum (Meckel's diverticulum to 1 cm proximal to the ileocecal junction) and ceca were collected and gently placed in sterile sampling tubes. Samples were put on ice until they were transported to the laboratory for enumeration of microbial populations. The populations of total aerobic bacteria, *Escherichia coli* and *Lactobacilli* were estimated as the log 10 of colony forming units (cfu) per gram of ceca and ileal digesta contents (Eftekhari et al., 2015). Pen was considered as experimental unit for performance and digestibility data. Data were analysed as a two-way factorial arrangement of treatments using the General Linear Models procedure of SAS (2004). Differences were considered to be significant at $P < 0.05$.

III. RESULTS AND DISCUSSION

During whole trial period (d 1-42) supplementation of organic acid in diets with 100% protein level recommended by NRC had no effect on weight gain, while birds fed low protein diets with 0.15 and 0.20% organic acid had higher weight gain compared to those fed 0 and 0.10% organic acid supplementation (Table 1). The results of the present study regarding weight gains are consistent with other studies (Yeo and Kim, 1997; Adil et al., 2010) who reported that the supplementation of organic acids in broiler chicken improved the body weight gain compared to un-supplemented diets. Adil et al. (2010) suggested that the improved body weight gain is probably due to the beneficial effect of organic acids on the gut flora. The organic acids may affect the integrity of microbial cell membrane or cell macromolecules or interfere with the nutrient transport and energy metabolism causing the bactericidal effect. However in current study organic acid supplementation had no effect on E. coli and lactobacilli population.

Table 1 - The effects of dietary treatments on growth performance and microbiota profile (cfu/g) of broiler chickens (d 1-42).

Dietary protein	Organic acid (%)	Parameters				
		Weight gain	Feed intake	FCR	E.coli	Lactobacilli
90% NRC	0	45.36[b]	86.69	1.91[ab]	5.51	5.23
	0.10	45.29[b]	86.75	1.92[a]	5.31	5.38
	0.15	48.19[a]	87.10	1.80[e]	5.78	5.43
	0.20	49.25[a]	90.99	1.85[cd]	5.96	5.03
100% NRC	0	49.97[a]	93.55	1.87[bc]	5.80	5.01
	0.10	50.50[a]	93.78	1.86[cd]	5.23	5.32
	0.15	50.79[a]	95.56	1.88[abc]	5.23	5.50
	0.20	50.43[a]	92.34	1.83[de]	5.14	4.85
SEM[1]		0.80	1.51	0.01	0.31	0.26
Main effects						
Dietary protein						
90%		47.02	87.88[b]	1.87	5.64	5.27
100%		50.42	93.80[a]	1.86	5.35	5.17
Organic acid						
0		47.66	90.12	1.89	5.66	5.12
0.10		47.90	90.26	1.89	5.27	5.35
0.15		49.49	91.33	1.84	5.50	5.46
0.20		49.84	91.67	1.84	5.55	4.94

[a,b,c,d,e] Means in a column not sharing a common superscript are significantly different ($P < 0.05$).
[1] Pooled standard error of mean.

Feed intake was influenced by dietary protein levels ($P < 0.05$). Birds fed 100% level of protein showed significantly higher feed intake compared to those fed 90% protein recommended by NRC. As low dietary protein diets tended to decrease most amino acids, it is possible that some essential amino acid become limiting and unbalanced amino acids profile occurs, which could cause lower feed intake.

The interaction between organic acid and protein level was observed ($P < 0.05$) for FCR. Feed conversion ratio improved in birds fed low protein level with 0.15% organic acid supplementation, while in birds fed 100% level of protein 0.2% organic acid had better FCR compared to 0 and 0.15% organic acid supplementation. The results of organic acid on FCR are consistent with the result of Runho et al. (1997).

The main effects of organic acid and protein level and also the interaction between them were not significant ($P > 0.05$) for microbial population and carcass traits. But protein level had significant effect on relative breast weight of broiler chicken during whole trial period. Birds fed 100% level of protein had higher relative breast weight compared to those fed lower protein diets. The results are in agreement with the results of Adil et al. (2010) who found no significant effect of organic acids supplementation on carcass characteristics in broiler chickens.

In conclusion, dietary organic acids may be exploited as growth promoters in the broiler diets with low protein level in finisher phase. The results showed that with standard protein level the relative breast weight increased compared to low protein level irrespective of organic acid supplementation.

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ASSESSMENT OF EGG QUALITY OVER TIME OF HENS RANKED AS HIGH OR LOW FEED EFFICIENCY

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Summary

The present study was conducted to investigate the associations between feed conversion ratio (FCR) and the quality characteristics of stored eggs. From an initial screening phase using 140 Isa Brown layers (28 week old), 10 most efficient (FCR < 1.94) and 10 least efficient (FCR > 2.51) hens were identified and designated as high feed efficiency (HFE) and low feed efficiency (LFE) groups respectively. For quality assessment, eggs (*n* = 10 per group) were stored in a cool room at 10 ^{0}C for 0, 14 and 28 d. Eggs were collected on 3 consecutive days for each time point from each group of hens. At 0, 14 & 28 d, the albumen weight, albumen height, Haugh unit and albumen: yolk ratio of eggs from the HFE group were significantly higher than those of the LFE group, whereas eggs from the LFE group of eggs had heavier yolk than the HFE group. After 28 d storage, the yolk colour score of the LFE group was lower than that of the HFE group. No significant changes were found in external egg and shell quality measurements as well as in albumen width and albumen pH, yolk height and yolk pH of freshly laid and stored eggs. The findings of this study indicate that HFE birds have greater egg quality parameters related to albumen both at lay and after storage. Furthermore, the yolk colour score of HFE became lower at a slower rate during storage.

I. INTRODUCTION

The feed efficiency of the hen and the quality and shelf life of the egg are important production considerations in laying hen flocks. The contributing factors to individual feed efficiency are varied and complex. Leeson and Morrison (1978) indicated that differences in feed efficiency of laying hens are associated with fasting metabolic rate, while Bottje et al. (2006) suggested that mitochondrial function has been linked to feed efficiency in poultry. The mitochondrial formation of reactive oxygen species (ROS) makes the mitochondrion a major source of oxidative stress. Research suggests that increased oxidative stress in inefficient birds may be causal of poor feed efficiency, while other studies demonstrate a need for antioxidant supplement strategies to improve egg quality and storage life (Surai and Fisinin, 2012). Cherian et al. (1996) stated that incorporation of antioxidants in layer diets could provide oxidative stability and increase the quality of both eggs and productivity. Therefore, as feed efficiency and egg quality may both be influenced by similar biological processes, a relationship between feed efficiency and the quality and shelf-life of the egg may exist. The purpose of this study was to characterize the egg quality and egg shelf-life of birds which were ranked as having high or low feed efficiency.

II. MATERIALS AND METHODS

From a preliminary screen phase of 140, 28 week old Isa Brown hens, 10 most efficient (FCR < 1.94) and 10 least efficient (FCR > 2.51) hens were identified and designated as high feed efficiency (HFE) and low feed efficiency (LFE) groups respectively. Feed conversion ratio

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was calculated from daily egg production and weekly feed intake over 4 weeks to verify the feed efficiency of each group. Eggs (n = 10 per group) were stored in a cool room at 10°C for 0, 14 and 28 d. Eggs were collected on 3 consecutive days for each time point from each group of hens. For quality assessment, each egg was weighed, the height and width were measured using a digital caliper and broken on a flat glass slide for measuring the height of the thickest part of the albumen and yolk. Albumen height was measured using an albumen height gauge TSS (Technical Services and Supplies). Albumen and yolk width were measured using a digital caliper. Yolk height was measured by a tripod micrometer. Albumen and yolk were separated and weighed. Yolk color was measured using a DSM Yolk Color Fan and yolk lightness (L*), redness (a*) and yellowness (b*) values were determined using the CIELAB method with a Minolta Lab CR-10 colourimeter. Albumen pH and yolk pH in each egg were estimated by digital pH meter. Egg shell thickness was measured using a digital caliper. The ratio of albumen and yolk was calculated by dividing the mean albumen weight by the yolk weight. Haugh unit (HU) values were calculated using the formula: $100 \times \log (h - 1.7 \times w^{0.37} + 7.6)$ where h = albumen height (mm), w = egg weight (g) (Sekeroglu & Altuntas, 2008). Data from 3 days collection at each separate time point were pooled and analysed using the generalised linear model procedure of SAS (SAS Institute) with feed efficiency group as the main effect. Means were separated using the Tukey-Kramer method. All data are presented as least square means ± standard error of the mean (SEM). The probability value which denotes statistical significance was $P < 0.05$.

III. RESULTS

Over the course of this study, the HFE group had a lower FCR when compared with the LFE group (1.94 VS 2.51; SEM 0.05; P<0.001). Egg quality data are presented in Table 1 and Figure 1, 2, 3 and 4. No significant differences were found between groups in external and shell quality (data not presented) measurements of fresh and stored eggs.

Table 1 - The effect of feed efficiency group on quality characteristics of eggs during storage.

Measurement	Storage time (d)	HFE	LFE	SEM	*P value*
Egg weight (g)	0	67.5	65.0	1.21	0.15
	14	67.0	64.5	1.21	0.14
	28	68.2	65.2	1.21	0.09
Albumen weight (g)	0	40.5	36.5	0.96	0.005
	14	39.4	35.3	0.96	0.003
	28	38.9	34.8	0.96	0.004
Yolk weight (g)	0	24.7	27.9	0.58	0.0002
	14	26.3	28.7	0.58	0.004
	28	26.3	29.5	0.58	0.0002

HFE – High feed efficiency group, LFE – Low feed efficiency group; SEM - standard error of the mean

Similarly, feed efficiency had no significant influence on egg weight (Table 1) albumen width, albumen pH and yolk height, yolk pH (data not presented) but significant effects were observed in albumen weight and yolk weight in both fresh and stored eggs (Table 1).

There was a significant effect of FE group on albumen height during storage of eggs for different time intervals; with HFE group producing eggs with higher albumen height with the LFE group at 0, 14 & 28 d of storage (Figure 1). Haugh unit (HU) was also significantly

affected by feed efficiency and HFE group showed better HU value during the entire storage period (Figure 2).

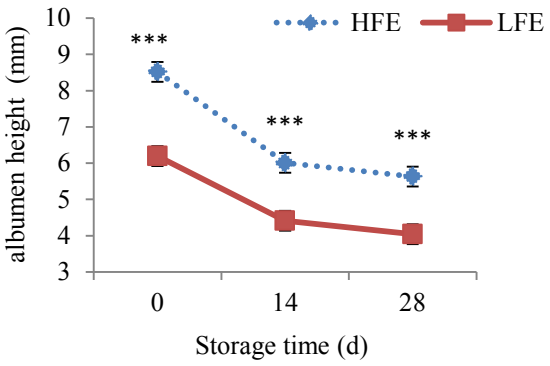

Figure 1 - The effect of feed efficiency group on albumen height of eggs during storage (mean ± SEM n = 10); HFE – High feed efficiency group, LFE – Low feed efficiency group.

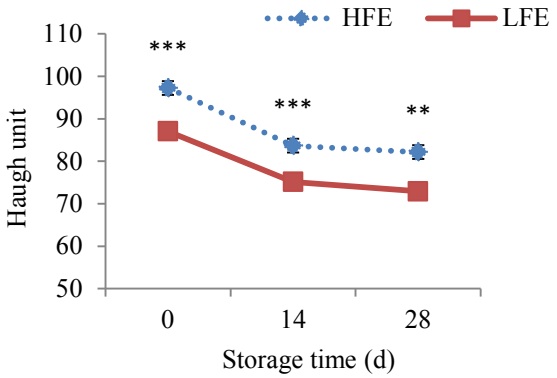

Figure 2 - The effect of feed efficiency group on Haugh unit of eggs during storage (mean ± SEM n = 10); HFE – High feed efficiency group, LFE – Low feed efficiency group.

Although freshly laid eggs obtained from both HFE and LFE groups did not show any significant difference in yolk colour score, the eggs from LFE group showed significantly lower yolk colour score (Figure 3) at the end of the storage (28 d). Colour measurements for lightness (L*), redness (a*) and yellowness (b*) values of yolks obtained from freshly laid or stored eggs were unaffected by FE group (data not presented).

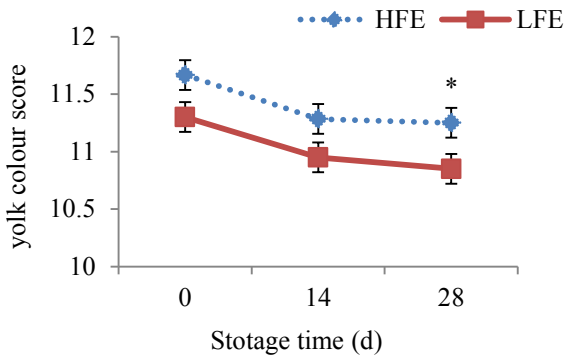

Figure 3 - The effect of feed efficiency group on yolk colour score of eggs during storage (mean ± SEM n = 10); HFE – High feed efficiency group, LFE – Low feed efficiency group.

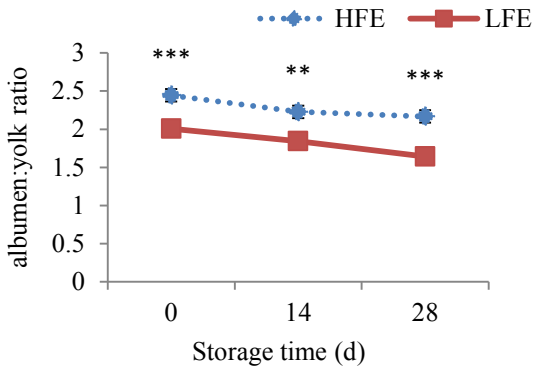

Figure 4 - The effect of feed efficiency group on albumen and yolk ratio of eggs during storage (mean ± SEM n = 10); HFE – High feed efficiency group, LFE – Low feed efficiency group.

In the current study, the albumen: yolk weight ratio (Figure 4) was significantly higher in fresh and stored eggs produced from the HFE group of hens.

IV. DISCUSSION AND CONCLUSION

The main objective of this study was to investigate whether there was any association with feed efficiency and egg storage life. Results of this study showed that HFE group of eggs had greater albumen weight, albumen height and HU during storage at 0, 14 and 28 d compared with the LFE group. Voltolini et al. (2014) reported that the mitochondria of LFE quail

produced more H_2O_2, which is associated with higher protein oxidation. Higher level of oxidized proteins might contribute to the LFE phenotype due to an increase in cell energy requirements for repairing such proteins, as well as the reduction or impairment of the function of the damaged proteins which might have negative effect on egg protein synthesis. A significantly higher yolk weight value in eggs produced from LFE group might be due to higher fat deposition in the body of the birds as well as in the eggs (Pym and Solvyns, 1979). Interestingly, at the end of the storage (28 d), eggs from the HFE group showed higher yolk colour score and maintained higher albumen: yolk weight ratio when compared with those from the LFE group. The results of lower yolk colour score in LFE group might be due to presence of reactive oxygen species (ROS) in yolk which may cause oxidative damage of yolk pigments. Yolk pigments are associated with the lipid molecules of the yolk membranes (Martino et al., 2014). Without antioxidant protection, ROS can cause oxidative degradation of yolk lipids (Bottje et al., 2006) and its pigments (Martino et al., 2014). The results obtained in this study suggest a strong association between feed efficiency and stored egg quality. The role of oxidative stress status in the bird and the level of oxidation of associated eggs is an area which merits further research to collectively improve both feed efficiency and egg quality.

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HOW MUCH COLD PRESSED CANOLA MEAL CAN WE USE IN LAYER DIETS?

M.M. BHUIYAN[1] and R.A. SWICK[1]

Canola meal has been available for the layer feed industry in Australia for over 30 years. However, very little has been used due to the concerns of "fishy taint" in eggs. This problem has now been solved by work of the layer breeders, selecting against the gene responsible for the problem. Therefore we now need to know how much canola meal can be used in layer diets, while maintaining high levels of performance. Also we now have a relatively new type of canola meal, called cold pressed canola meal (CPCM). This meal was not previously available in large quantities. This meal is subjected to low processing temperatures, maximum 60°C, compared with over 140°C for both expeller and solvent meal. This lower temperature means less damage to the protein, as shown in consistently higher reactive lysine assays for this meal. The CPCM contains 110 g/kg oil, a high ME level of 11.1 MJ/kg, and total lysine of 20 g/kg. The current trial was conducted to test the inclusion of cold pressed canola meal at 100 and 200 kg/MT in layer diets.

Ninety Nine (99) Hy-Line Brown pullets aged 21 weeks at the start and housed in single cages were used in the trial. Diets containing 0, 100 and 200 g/kg CPCM (from Cootamundra Oilseeds Ltd) were fed. Diets were wheat, soybean meal, cold pressed canola meal, 11.72 MJ/kg ME, 155 g/kg protein, 7.6 g/kg digestible lysine, 38 g/kg calcium and 3.0 g/kg available phosphorus. Pullets were at 50 % egg production at the start and the trial lasted for 20 weeks. Measurement of egg production parameters and egg quality were made, data was analysed with SPSS version 22. Some results are shown in Table 1.

Table 1 - Effect of cold pressed canola meal on egg production (21-41 weeks of age).

Cold pressed canola meal (g/kg)	0	100	200	SEM[1]	P value
Feed intake, g/d	117.5	117.8	116.0	0.85	0.665
Feed conversion ratio, g/g	1.90	1.90	1.92	0.01	0.734
Hen day egg production (%)	97.6	97.4	97.2	0.27	0.853
Egg size, g	63.7	63.9	62.4	0.36	0.189
Egg yolk sensory evaluation	no ft[2]	no ft	no ft	-	-
Mortality, %	0	0	0	-	-

[1] SEM = standard error of mean;
[2] No fishy taint

Including CPCM at 100 and 200 g/kg had no effects on egg production parameters and maintained the extremely high levels of production of the control diet. Sensory evaluation on samples of eggs (n=99) from the 3 treatments did not reveal the presence of "fishy taint" in any treatment. Measurement of external and internal egg quality showed no differences ($P > 0.5$) in transparency score, shell reflectivity, breaking strength, deformation, shell weight, shell %, shell thickness, albumen height, haugh units and yolk colour score.

CPCM may be successfully used at levels of at least 200 g/kg in layer diets. The problem of "fishy taint", which previously prevented use of canola meal at high levels, was not apparent in CPCM fed to Hy-Line brown layers at 100 and 200 g/kg. This research was conducted within the Poultry CRC, established and supported under the Australian Government's Cooperative Research Centres Program.

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EFFECT OF CANOLA MEAL SOURCE ON BROWN EGG PRODUCTION PARAMETERS

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Canola meals (CM) are produced in Australia by three different methods; cold pressed (CPCM), expeller (ECM) and solvent (SCM). Recent research showed that CPCM can be used up to 150-200 g/kg in layer diets while maintaining high levels of performance (Bhuiyan et al., 2016). As CPCM is processed at lower temperature (max. 60°C) compared to 140°C for both ECM and SCM, it was of interest to compare performance of hens fed these meals.

Meals CPCM, ECM and SCM contained 360, 360 and 370 g/kg crude protein, 110, 80 and 20 g/kg oil, AMEn of 11.1, 10.0 and 8.4 MJ/kg, and 17.8, 16.3 and 16.3 g/kg SID lysine respectively. They were formulated into layer diets at 150 g/kg taking differences in nutrient content into consideration. Iso-caloric and iso-nitrogenous diets based on wheat, soybean meal and CM with 11.72 MJ/kg AMEn, 155 g/kg protein, 7.3 g/kg digestible lysine, 36 g/kg calcium and 3.0 g/kg available phosphorus were fed to 96 Hy-Line Brown hens, 46 weeks of age and at 90% production for 20 weeks. Hens were housed in single cages and 32 replicate cages were fed the CPCM, ECM and SCM diets. The CPCM, ECM and SCM were freshly obtained from Cootamundra Oilseeds Ltd., MSM Milling and Cargill respectively. Body weight, egg number, egg weight, FI, FCR, shell breaking strength, shell deformation, albumen height, yolk colour, shell thickness, sensory evaluation in eggs and mortality were measured. Data were analysed with SPSS and some results are shown in Table 1.

Table 1 - Effect CM source at 150 kg/MT inclusion levels on egg production from 45-65 weeks of age.

	CPCM	ECM	SCM	SEM	P value
Feed intake (FI), g/d	112.8^{b}	115.0^{ab}	117.7^{a}	0.75	<0.02
FCR, g/g	1.93^{b}	1.92^{b}	2.06^{a}	0.02	<0.01
Hen day egg production, %	93.8	95.1	92.0	0.57	0.08
Egg size, g	62.9	63.2	63.5	0.41	0.83
Fishy taint evaluation	no ft[1]	no ft[1]	no ft[1]	0	>0.05
Mortality, %	0	0	0	0	>0.05

CPCM = cold pressed CM; ECM = expeller CM; SCM = solvent CM; [1] no ft = no fishy taint

Inclusion of SCM at 150 g/kg increased feed intake ($P < 0.02$) and FCR ($P < 0.01$) compared to the other two diets. Hen day egg production tended to be lower in the birds fed SCM relative to other diets ($P = 0.08$). Egg size was not affected by diet ($P > 0.05$). No differences ($P > 0.05$) in egg transparency, shell reflectivity, breaking strength, deformation, shell weight, shell %, shell thickness, albumen height or Haugh units were observed between diets. Yolk colour score was higher ($P < 0.03$) in eggs from hens fed ECM and SCM compared to CPCM diets (11.47, 11.23, and 10.87 respectively). Sensory evaluation of eggs revealed no "fishy taint" from hens fed any diet ($P > 0.05$). This study indicates that any of the canola meals examined may be successfully incorporated in Hy-Line Brown layer diets at 150 g/kg. This may reduce feed cost without the possibility of "fishy taint". This research was conducted within the Poultry CRC, established and supported under the Australian Government's Cooperative Research Centres Program.

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WHAT ARE THE LIMITS OF EGG PRODUCTION IN THE MODERN LAYER?

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A group of ninety six (96) Hy Line strain pullets (21 weeks of age) were used in trials for evaluation of canola meal. Hens were housed in single cages with 2750 cm^2 floor space and height of 50 cm with 2 nipple waterers. This compares to the post 2001 floor space recommendations of Primary Industries Model Code (SCARM, 2002) of 1000 cm^2 floor and 45 cm H. This paper compares performance levels obtained relative to published HyLine standards and suggests reasons for these differences with hen welfare considerations.

Feed consisted of wheat, soybean meal, canola meal, dicalcium phosphate, limestone, xylanase and phytase fed as mash. Diets had calculated nutrient values of 11.72 MJ/kg ME, 155 g/kg protein, 7.62 g/kg digestible lysine, 38 g/kg Ca and 3.0 g/kg available P. Performance results in comparison to HyLine standards (2016) are shown in Table 1.

Table 1 - Hy-Line brown egg production performance from 21-78 weeks age (57 weeks).

Items	Observed	HyLine Standard	Difference
Age at 50% production, weeks	21	20	-1 week
Hen Day Egg Production, %	93.0	87.8	+6%
Hen Day Egg Production, number	371.1	343.1	+28.0
Feed consumption, g/d	115	109	+6 grams
FCR, g/g	1.95	2.08	+6.3%
FCR, kg/dozen eggs	1.47	1.52	+3.3%
Weeks above 95%	50	7	+43 weeks
Weeks above 90%	56	24	+32 weeks
Egg size, (g/egg)	63.3	63.9	-0.6 grams
Cumulative egg mass, kg	23.5	21.7	1.8 kg
Mortality, %	0	4.6	+4.6%
Total egg production from the group	35,623	32,938	+2,685 eggs

The hens had remarkably high levels of production, exceeding the strain standards in hen day and hen house production, FCR, weeks above 95% and 90%, daily egg mass, cumulative egg mass, mortality and in total eggs produced by the group. At the end of the period birds were fully feathered and healthy. These production levels were achieved with a simple wheat-based diet with no animal protein. This work suggests a diet with balanced protein based on 7.62 g/kg digestible lysine, 38 g/kg calcium, 3.0 g/kg P avail meets the nutritional requirements of the modern layer. Such performance, feather cover and lack of mortality suggests that large single bird battery cage rearing systems with no enrichment other than an additional water nipple supports high hen welfare. Assessment of hen welfare in various production systems should include objective measures of mortality, performance and feather score.

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METABOLISABLE ENERGY OF INGREDIENTS IN PEAK LAYERS

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Layer nutritionists typically rely on AME data generated in broilers as layer AME data are less available. The bioassays are typically conducted using 20- to 27-d-old broilers and values obtained for AME are adjusted to zero nitrogen retention (AME_n) ,(Bourdillon, et al., 1990).

A bioassay study was conducted in laying hens to measure the AME and AME_n of maize, SBM, wheat, and wheat plus xylanase using the substitution method. It was hypothesised that xylanase would have relatively small benefit in layers. Test diets contained 300 g/kg of test ingredient and approximately 657 g/kg of reference diet. The test diets were formulated to have equivalent levels of vitamins, minerals and amino acids to the reference diet. Econase XT 25® (AB Vista) was included at a rate of 50 mg/kg in the test diet with added xylanase. A completely randomised design was used to analyse the data using the GLM procedure. Sixty 42-week old Hy-Line Brown hens were used, 2 per cage with six replicates per diet. Birds were fed their respective diets for seven days for adaptation and another three days for AME bioassay using total collection. Dry matter, gross energy and nitrogen content of feed, test ingredients and excreta were determined. Results are given in Table 1 and show that addition of xylanase increased the AME and AMEn of the wheat by 1.19 and 1.06 MJ/kg respectively, making it essentially equivalent to maize. As expected, correction of AME to zero nitrogen retention decreased the measure in SBM to a greater extent (6.0%) than maize (2.0%) or wheat (2.6%).

Table 1 - Assayed dry matter, crude protein, AME and AMEn.

Ingredient	Maize	SBM	Wheat	Wheat + xylanase
Nutrient				
Dry matter, g/kg	880	902	896	896
AME, MJ/kg, as is	14.47[a]	10.10[c]	13.70[b]	14.89[a]
AMEn, MJ/kg, as is	14.18[a]	9.50[c]	13.34[b]	14.40[a]

[abc]Means within rows with different superscripts are different ($P < 0.05$)

The AMEn values obtained in layers at peak production were slightly higher than those obtained using prediction equations for broilers published by (Janssen, 1989) except for soybean meal. The excess protein and crude protein to energy ratio of the soybean meal test diet is the likely reason for this. Further work is required to examine AME measures of this ingredient at a range of inclusion levels, and the validity of correction to zero nitrogen retention as layers in peak production retain around 50% of N intake.

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ROLE OF SALTBUSH ON FREE RANGE LAYER FARMS

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Old man saltbush (*Atriplex nummularia*) was identified as a potentially useful plant on free range poultry farms in Australia, primarily for shelter and shade (de Koning 2015). There is only limited published information on the effects of layers fed saltbush (eg. Abd-el-Galil et al. 2014) and there is no published information on the *in situ* use of saltbush on free range farms. Like many plant species, saltbush contains anti nutritive factors and is high in NaCl. Therefore it is important to determine if there are negative consequences for poultry that may eat saltbush. The objective of this project was firstly to determine if free range laying hens eat saltbush and secondly what are the effects on production, welfare and product quality should free range laying hens consume saltbush?

Firstly, it was found that layer hens do eat saltbush. An estimated 5% of their dietary dry matter intake was saltbush (n-alkane analysis) when hens were provided with fresh saltbush on the range. Secondly, the consequences of eating saltbush were determined in a feeding trial. Saltbush was incorporated into Ridley Barastoc Top Layer Crumbles at the following levels; 0%, 5%, 10%, 15% and 20%. Hy-line Brown layer hens were fed the diets for 28 days (32 – 35 weeks old). There were 15 individually housed hens per diet. The saltbush had no impact on egg production, hen live-weight and feed intake (Table 1). Excreta moisture increased significantly with increased saltbush in the diet. Furthermore, high saltbush eggs (20%) had stronger egg yolk colour (10.1) compared to the control eggs (8.1, Roche fan, p = 0.0057). Under commercial free range conditions it is unlikely that layer hens would be able to eat enough fresh saltbush to impact their production.

Table 1 - Average live-weight of Hy-Line Brown hens, feed intake, egg production and percentage moisture in excreta for hens at peak lay fed air dried saltbush at 0%, 5%, 10%, 15% and 20% in a commercial layer pellet diet.

Diet treatment	Live weight (kg) @ 35 weeks	Average feed intake g/hen/day	% Egg production	% moisture in excreta (day 28)
0% saltbush	1.900	143.30	99.3	73.69 ^{c}
5% saltbush	1.895	142.26	100.0	77.25 ^{b}
10% saltbush	1.973	141.31	99.6	78.55 ^{b}
15% saltbush	1.947	148.44	98.2	79.97 ^{ab}
20% saltbush	1.941	141.99	95.7	81.73 ^{a}
P- value	*P=0.7285*	*P=0.7534*	*P=0.6182*	*P<0.001**

*Means in the same column with different superscript letters show significant diet effects at P<0.001.

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TOTAL SULFUR AMINO ACIDS AND LINOLEIC ACID ON CHICKEN EGG PRODUCTION

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Eggs produced by young layers up to 42 weeks old are innately small. This period covers up to early post peak hen day egg production and accounts for about 40% of total eggs laid by a flock. Increasing egg size of the young flock can potentially provide economic gains in layer operations. Studies have shown that increasing egg size during the early laying phase is influenced by dietary levels of total sulfur amino acids and linoleic acid (Leeson and Summers, 2005). Experiments were conducted to determine the effective dietary combination of total sulfur amino acids (TSAA) and linoleic acid (LA) that will enhance egg size of young layer chickens (29-34 weeks old). Experimental diets were corn-soya based, isocaloric (11715 kj ME/kg) and had approximately equal crude protein (15% CP). Dietary levels of TSAA were adjusted by DL-methionine supplementation while the main ingredient for adjusting LA levels was full fat soy bean meal.

A factorial experiment in completely randomized design used 4 levels of TSAA (0.63, 0.66, 0.69 and 0.72%) and 3 levels of LA (1.85, 2.0 and 2.15%), with 10 replicates per treatment and 4 birds per replicate (n = 120). Proportion of small size eggs was higher ($P < 0.05$) in groups fed 1.85% LA (43.86%) than for 2.15% LA (35.38%). Regression analysis of egg weight (EW) at each level of TSAA with LA as the independent variable was highly significant ($P < 0.01$, R^2 = 25%) for the equation: EW g = 42.11 + 7.18LA at 0.72% TSAA. FCR per dozen eggs at 1.85% LA (1.28 kg) was lower ($P < 0.01$) than groups fed 2.0 and 2.15% LA (both at 1.31 kg). Consequently, income over feed cost tended to decrease ($P < 0.10$) as egg size increased, as affected by LA. Evaluation of egg quality (n = 72) showed egg shell proportion at 0.72% TSAA (9.96%) was lower ($P < 0.01$) than for the other levels of TSAA (average of 10.25%). Haugh Units score was higher ($P < 0.05$) at 0.72% TSAA (99.33) than at 0.63% TSAA (98.01). Dietary treatments had no effect on livability, hen day egg production, feed consumption and feed per kg egg mass.

Body weight change (BWc) and abdominal fat pad (AFP) as affected by extreme levels of TSAA (0.63 and 0.72%) and LA (1.85 and 2.15%) were evaluated (n = 12). AFP decreased ($P < 0.001$) as TSAA increased from 0.63% (4.04%) to 0.72 % (2.90%). Conversely, AFP increased ($P < 0.05$) corresponding to the levels of LA at 1.85% (3.20%) and at 2.15% LA (3.74%). No differences ($P>0.05$) were observed in BWc.

In conclusion, egg size or EW during the early laying stage of egg production can be enhanced by increasing the level of LA from 1.85 to 2.15% at 0.72% TSAA without adversely affecting laying performance and egg quality. EW increased by 1g for every 0.15% increase in LA at 0.72% TSAA. TSAA at 0.72% improved albumen quality and reduced abdominal fat pad. However, increase in egg size tended to reduce hen day egg production and increase FCR per dozen eggs resulting in a tendency to have lower income over feed cost.

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THE EFFECT OF *ASCARIDIA GALLI* ON PERFORMANCE AND EGG QUALITY OF FREE RANGE LAYING HENS

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Ascaridia galli is the most prevalent gastrointestinal helminth parasite found in free range poultry (Gauly et al., 2007). Infections with this parasite have been associated with reduced body weight gain, egg production and an increased incidence of infectious diseases (Dahl et al., 2002). However, the effect of infection levels with *A. galli* on hen performance and egg quality has not been reported. Therefore, the aim of this study was to determine the effect of different infection levels of *A. galli* on performance and egg quality in free range laying hens.

A total of 200 Lohmann brown laying hens were allocated to four treatment groups (n=50 per group) each with 5 replicate pens of 10 birds. Three treatment groups were orally inoculated at 19 weeks of age with three levels of *A. galli* eggs: low (250 *A. galli* eggs), medium (1000 *A. galli* eggs), and high (2500 *A. galli* eggs). The fourth treatment group served as control group and was sham inoculated with saline. The impact of *A. galli* infection on hen performance was assessed by measuring feed intake, body weight, number and weight of eggs produced when hens were 25, 30, 35, and 40 weeks of age. Internal and external egg quality was also examined when hens were 30 and 40 weeks of age. Statistical analysis was performed using the GLM procedure in SAS software to compare all three infection levels with the control, as well as to compare the combined treatment groups with the control group (Version 9.3, SAS Institute, Cary, NC, USA). A P value of <0.05 was considered significant.

Levels of *A. galli* infection had no effect on excreta egg count, intestinal worm count, feed intake, body weight and feed conversion ratio (FCR) in hens. However, egg production (P=0.002) and egg mass (P=0.003) were significantly lower in the low infection group birds as compared to control group birds. When comparing the control group to all infected hens without differentiation of the infection levels (Table 1), overall hen day egg production was significantly lower in infected birds (P<0.05). There was a tendency for higher FCR in infected birds (P=0.07; Table 1). No differences were observed in egg quality parameters.

Table 1 - The effect of *A.galli* infection on laying hen performance (control vs. infected).

	Egg weight (g)	Egg mass (g/bird/day)	Egg production (% hen day)	Feed intake (g/hen/day)	FCR	Body weight (g)
Control (n=50)	63.4 ±0.86	59.6 ±1.39	94.0 ±0.0150	121 ±4.27	2.03 ±0.09	1971 ±0.02
Infected (n=150)	63.9 ±0.48	57.2 ±1.12	89.5 ±0.0162	127 ±3.00	2.22 ±0.08	2001 ±0.02
P-value	0.192	0.146	0.050	0.354	0.066	0.17

In conclusion, the present study demonstrated that *A. galli* infection had no effect on egg quality, but decreased egg productivity though the extent of this effect is unknown. Further studies will be required to confirm the influence of different infection levels on performance.

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THE EFFECTS OF PECKING STONES, HOUSING SYSTEM AND AGE ON PLUMAGE CONDITION AND MORTALITY OF FREE RANGE EGG LAYING HENS

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Vigorous or severe feather pecking (SFP) is forceful pecking or pulling of feathers which can lead to plumage deterioration, skin lesions and in severe cases, death (Hartcher et al., 2015). SFP can trigger cannibalism, predisposing serious welfare problems, whilst the cause is considered to be multifactorial (Savory, 1995). In some experiments environmental enrichment has been shown to reduce feather pecking. Recently pecking stones (containing minerals Ca and P) were used as a tool for reduction of feather pecking, and therefore improved welfare practice (El-Lethey et al., 2000). The aim of this research was to investigate the effects of pecking stones, housing system and flock age on plumage condition and health parameters of free range laying hens.

In total 18 flocks housed in 18 sheds (fixed sheds n=10; mobile sheds n=8) located on two commercial farms were investigated. Both farms housed Hy-line brown laying hens at different flock sizes: 19,500 hens / each fixed shed, 2,500 hens / each mobile sheds).Hens from the control flocks (n=9) were housed under the same conditions without pecking stones, whilst treatment flocks (n=9) were provided one pecking stone / 1,000 hens every ten weeks. All 18 flocks were investigated from the time of placement (16 weeks) in 10 week intervals until the end of lay (66 weeks). For each flock and time point, 50 hens per flock were randomly selected and individually evaluated for body weight, beak length and scored for feather condition. Plumage condition of individual hens was evaluated on a scale from 1 (no feathers) to 4 (full feather coverage) (Tauson et al., 2005). Mortalities were recorded daily by the farm management. Data were analysed using a general linear model with a 2 (fixed shed vs mobile shed) x 2 (treatment vs control) x 6 (time point) arrangement. Means were compared using Tukey's range test. All statistical analyses were performed using SPSS ver. 2.0.

Feather scores of the back, vent and tail feathers were higher ($p < 0.05$) in fixed sheds (3.62 ± 0.11, 3.80 ±0.07, 3.62 ±0.09) compared to mobile sheds (3.25 ±0.12, 3.61 ±0.08, 3.31 ±0.10). However, breast feather scores and cumulative mortality respectively, were higher ($p < 0.05$) in mobile sheds (3.24 ±0.08, 5.60% ±0.79) compared to fixed sheds (2.66 ±0.07, 4.36% ±0.53). While feather score worsened from 16 to 66 weeks of age ($p < 0.05$), there were no effects of pecking stones ($p > 0.05$) on any parameters investigated. Significant ($p < 0.05$) interactions between farm and time points were observed in feather scoring on all body parts. In conclusion, farm management and age of hens as well as their interactions were key factors affecting mortality and plumage deterioration.

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FEATHER-EATING HENS SHOW SPECIFIC ESSENTIAL AMINO ACID APPETITES IN A DOUBLE-CHOICE MODEL

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Nutritional balance is one of the major considerations to prevent severe feather pecking in layer hens (Kjaer and Bessei, 2013). Individual variation in digestive and metabolic efficiencies and nutrient requirements may explain differences in specific appetitive behaviours. For example, dietary nutrient deficiencies or imbalances, particularly in amino acids, may increase the risk of severe feather pecking (Van Krimpen et al., 2005). Taste plays a crucial role in identifying the nutritional content of foods by detecting dietary nutrients (what has been referred to as nutritional chemosensing) (Roura et al., 2013). We hypothesise that severe feather pecking may be partially explained as a specific nutrient-driven appetitive behaviour. Therefore, this study aimed to elucidate whether there are differences in the specific amino acid appetite between feather eating and non-feather eating laying hens.

A total of 12 amino acids (lysine, methionine, cysteine, tryptophan, glutamine, arginine, histidine, glycine, proline, serine, tyrosine, and alanine) were selected based on potential involvement in severe feather pecking from existing literature. Each amino acid was tested at three concentrations (0.2, 1 and 5%) using double –choice tests (offering a carrier alone –ground wheat- or mixed with the amino acid/level under study) in 96 mature laying hens (ISA Brown). At the end of the trial the hens were euthanised and feather consumption assessed. The consumption of the two feeds in each test (control vs treatment) was analysed as a subtract (consumption of treatment – consumption of control feed) and as a standard preference index (% of treatment consumption over total consumption). Preference value were compared to the random choice value of 50%, while the subtraction was contrasted to 0g. The two groups of birds (feather eaters versus feather non-eaters), were compared using the GLM procedure of SAS.

Feather eating and non-feather eating birds showed significant ($P < 0.05$) differences in amino acid preferences. Feather eating birds showed a higher preference for methionine and lysine than non-feather eating birds ($P < 0.05$). These two are the most limiting amino acids in laying hens. These finding is consistent with previous findings that a dietary deficiency of lysine and methionine + cysteine increased severe feather pecking (Elwinger et al., 2002). We speculate that the specific methionine appetite may be related to our previous finding which showed that feather eating birds had shorter beaks. Keratin, is the main protein in the beak, and is very rich in sulphur amino acids (i.e. methionine and cysteine). Thus, there may be a relationship between slow beak development, feather eating and methionine/lysine specific appetites.

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A PRELIMINARY INVESTIGATION INTO THE RELATIONSHIP BETWEEN FEATHER-PECKING AND INTEGUMENT MICROFLORA

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Summary

Severe feather-pecking (SFP) is thought to be one of the greatest welfare concerns impacting the non-cage-egg industry. Despite a multitude of studies on the topic, the underlying causes and motivations behind this behaviour remain unclear. This experiment investigated the bacteriological relationship between feather-pecked and non-feather-pecked birds upon an outbreak of feather pecking. Feather-pecked birds had twice the amount of bacteria on the rump, uropygial gland and vent compared to non-feather-pecked birds.

I. INTRODUCTION

Severe feather-pecking, whereby birds peck at and pull out the feathers of conspecifics, is a major welfare concern in laying hens (Bestman et al., 2009). Upon the initial outbreak, feather-pecking is difficult to control, particularly due to the social transmission of the abnormal behaviour in non-cage housing systems (Zeltner et al., 2000). The underlying causes are multi-factorial, complex and influenced by many different environmental stimuli (Rodenburg et al., 2004; Janczak & Riber, 2015). Studies have suggested severe feather pecking develops as a redirected behaviour, for example due to the inability of birds to satisfactorily dust-bathe (Vtergaard and Lisborg, 1993), with preen oil also serving as a possible attractant for feather-pecking (McKeegan and Savory, 2001; Sandilands et al., 2004). Furthermore, it has been suggested dust-bathing controls plumage lipid quality and therefore bacterial species on the skin (Jacob and Ziswiler, 1982). The objectives of this preliminary study were (1) to limit dust-bathing behaviour in non-caged hens and (2) investigate the bacteriological relationship between feather-pecked and non-feather-pecked birds upon outbreak initiation.

II. MATERIALS AND METHODS

A total of 528 ISA Brown pullets (laser beak-trimmed at day old) were purchased from a commercial supplier at 14 weeks of age. Pullets were transported to the Free-Range Research Facility at the University of Sydney, Camden campus and divided randomly across 8 pens (66 birds per pen) measuring 3.66 m x 3.25 m. A thin layer of wood shavings (~2-3 mm depth) was spread to partly cover the concrete floor to minimise dust-bathing behaviour. Pens were cleaned out weekly to ensure adequate hygiene and to prevent litter and dust build up. After cleaning, new wood shavings were provided. At 26 weeks of age, one pop-hole from each pen was opened and birds were given continuous access to the free-range measuring 1.83 m x 10 m.

At 27 weeks of age a feather-pecking event occurred in pen 5 and birds were feather-scored using a method adapted from Tauson et al., (2005) (1 indicating no damage and 4 signifying a denuded body region). Non-invasive skin swabs were taken to collect bacterial samples from the breast, rump, uropygial gland (UPG) and vent of fifteen hens randomly selected from pen 5 (n = 10 feather-pecked, n = 5 non-feather-pecked) and five birds randomly selected from pen 8 (n = 5, non-feather-pecked). Colonies were quantified based on

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their morphology and the streak plate method was applied to further isolate and purify organisms on agar. Pure culture colonies were then studied using the gram staining technique and 16s rRNA gene sequencing will be applied to identify and classify bacteria to the genus or species level. As this was a preliminary study, data were analysed using descriptive statistics. The University of Sydney Animal Ethics Committee approved all housing procedures and experiments.

III. RESULTS AND DISCUSSION

The feather-pecked birds had double the amount of bacteria on the rump, UPG and vent compared to non-feather-pecked birds, while the opposite was true for the breast samples (Figure 1). A possible explanation for this result is that the breast is the most thoroughly oiled area during preening (van Liere, 1992) and is frequently exposed to litter when lying compared to other regions on the body (van Liere et al., 1991). Non-feather-pecked birds exhibited no plumage damage (score 1). In comparison, feather-pecked birds had a minimum score ranging from 2 - 3 on the rump. In the present study, it was not certain whether the apparent differences in the bacterial population variables were due to, or as a result of, the feather-pecked status of the hens. Further research is required for clarification.

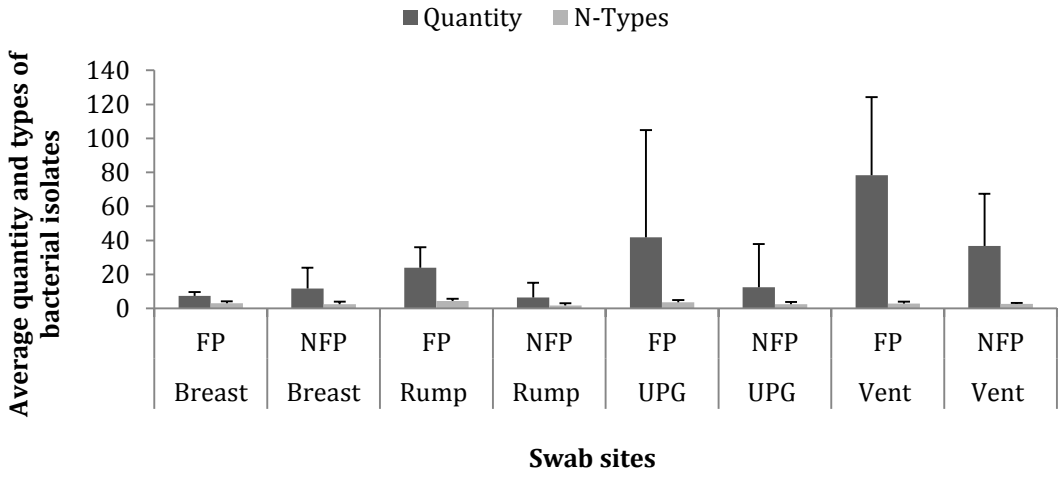

Figure 1 - Mean bacterial populations (+SD) derived from swab samples taken from four sites on the skin of 10 feather-pecked (FP) and 10 non-feather-pecked (NFP) birds. The dark grey columns ('Quantity') refer to number of bacterial colonies, and the light grey columns ('N-Types') refer to the number of different types based on morphology of colonies grown on the agar plates.

Furthermore, it remains unclear why some birds are more prone to developing feather damage from SFP than others. Previous studies have shown feather lipids will accumulate and become stale on plumage when birds are deprived access to a dust-bathing material (van Liere and Bokma, 1987; van Liere 1991). Thus, preen oil may serve as a possible attractant for feather pecking (McKeegan and Savory, 2001; Sandilands et al., 2004). The current trial was a preliminary investigation and future studies aim to further investigate the potential relationships between these variables in detail. In addition, the authors aim to develop a reliable model to initiate feather-pecking to determine whether SFP is related to dust-bathing deprivation, increased stress, or both.

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MEASURING FEATHER APPETITE AND FEATHER DIGESTIBLITY IN ISA BROWN HENS

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Summary

There are potential nutritional motivations behind feather pecking and feather eating behaviour in ISA Brown hens. Although feather eating behaviour has been previously studied, the nutritional benefit to the bird is unknown. To determine this, the appetite for and digestibility of feathers were investigated, and the effect of ground and whole feather consumption in ISA Brown birds on feather pecking was observed. Increased feed intake and protein digestion, with significant increases in the amount of methionine and cysteine digested associated with ground feather intake, could be responsible for an increase in latency to peck at feathers presented on an artificial substrate and an effective decrease in feather pecking motivation.

I. INTRODUCTION

Feather pecking and cannibalism are major behavioural and welfare concerns associated with layer production. Although widely studied, motivation for feather pecking remains unclear. Feather pecking and cannibalism have been linked to protein and amino acid deficiencies (Ambrosen & Petersen, 1997). Feather eating behaviour performed by hens suggests potential nutritional motivations; however, the nutritional benefit from feather consumption is unknown. Consumption of whole feathers is known to increase feed passage rate in layer hens, however its effect on feed intake and nutrient digestibility requires further investigation (Harlander-Matauschek et al., 2006). Similarly, dietary inclusion of ground feathers at 5% inclusion rate has been found to positively influence the presence of keratinolyic microbes in the gut of layer hens, which may allow birds to digest feathers (Meyer et al., 2012). The aims of this experiment were to determine appetite for ground and whole feathers in ISA Brown laying hens, and to determine the effects of ground and whole feather consumption on the digestibility of nutrients and feather pecking behaviour.

II. METHOD

A total of 60 individually housed 56-week-old ISA Brown hens classified as feather-eaters were selected for use in this study from a trial cohort studied previously. Birds were randomly allocated into one of three dietary treatment groups: commercial diet (Control), commercial diet + pelleted diet with 15% ground feathers (Ground), and commercial diet + whole feathers (Whole). Feed was presented in two separate feeders and placed in front of individual cages. Feathers were ground through a 1mm cutting mill sieve (Cutting Mill SM 100, Retsch GmbH) and incorporated into commercial diet at 15% inclusion level before being cold-pelleted. Twenty whole semi-plume feathers measuring 4-6 cm were provided daily in feeders to Whole treatment group birds. Birds were fed ad-libitum and feed intake from both feeders was measured weekly. Celite was added at 2% inclusion for all dietary treatments.

Apparent metabolisable energy and nitrogen retention were determined using total excreta collection from all birds over 48 h after 13 days on treatment diet. A total of four

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birds per treatment were randomly selected and euthanised for ileal content collection to determine dry matter and amino acid digestibility.

Each hen was individually observed daily for 14 days, and the latency of the bird to peck was recorded when presented with 10 semi-plume feathers mounted on a perforated piece of clear flat plastic hung in front of the cage. If the hen did not peck within 30 s the observation concluded.

Average feed intake, feed conversion efficiency, apparent metabolisable energy, nitrogen retention, protein and amino acid digestibility and digested nutrient content for each treatment was compared using ANOVA in Genstat 16 (VSN International, 2015). Kaplan Meier estimates of mean latency to peck at feathers presented on artificial substrates were compared using survival analysis, and survival curves were compared using non-parametric tests.

III. RESULTS

Overall daily feed intake was significantly higher in the Ground treatment group compared to Control and Whole treatment groups (Table 1). Average ground feather component consumption throughout the trial was ~5.3% (range 0.2-10.7%) of total feed intake in the Ground treatment group. Consumption of whole feathers from feeders was low, and only 3 of 20 birds consistently consumed all 20 feathers presented in the feeders throughout the trial. Six birds consumed no feathers when presented in the feeders.

Table 1 - Average daily feed intake (FI)(g/day), feed conversion efficiency per kg of egg mass (FCE), apparent metabolisable energy (AME) (KJ/kg of feed), nitrogen (N-) retention (%), and dry matter (DM) digestibility (Dig. %) of Control, Ground and Whole treatment groups over 48 hours.

Parameter	Control	Ground	Whole	p-value
Daily FI	111.8[b]	125.9[a]	114.7[b]	0.018
FCE	1.721[b]	1.938[a]	1.693[b]	0.005
AME	11.03	10.50	10.47	NS
N-retention%	31.0	31.2	25.1	NS

[ab] Means with different superscripts between columns indicate differences ($P < 0.05$)

Feed conversion efficiency per kg of egg mass was significantly poorer in the Ground treatment group when compared to Control and Whole treatment groups (Table1). There were no differences in AME or N-retention% between treatment groups over 48 h.

Table 2 - Mean ileal dry matter digestibility coefficient (%), mean ileal methionine (Met) and cysteine (Cys) digestibility coefficients (%) and amount digested (mg/g of feed intake) by Control, Ground and Whole treatment groups.

Parameter	Control	Ground	Whole	p-value
DM Dig. %	84.7	84.7	86.0	NS
Cys Dig. %	0.57	0.526	0.55	NS
Cys digested	1.2	3.02	1.15	0.009
Met Dig. %	0.792	0.825	0.812	NS
Met digested	1.584	1.951	1.624	<0.001

[ab] Means with different superscripts between columns indicate differences ($P < 0.05$)

Dietary treatment did not affect ileal amino acid digestibility coefficients for the majority of amino acids (Table 2). However, Ground treatment birds digested significantly higher amounts of methionine and cysteine compared to Control and Whole treatment groups. In addition, dry matter digestibility was unaffected by dietary treatment.

Approximately 75% of birds consistently pecked at the feathers presented on the substrates within 30 s of feather presentation. Provision of ground and whole feathers in the diet significantly decreased the likelihood of birds to peck at presented feathers with 14.3%, 32.2% and 23.9% of Control, Ground, and Whole treatment group birds not pecking at the substrate within 30 seconds respectively (Figure 1).

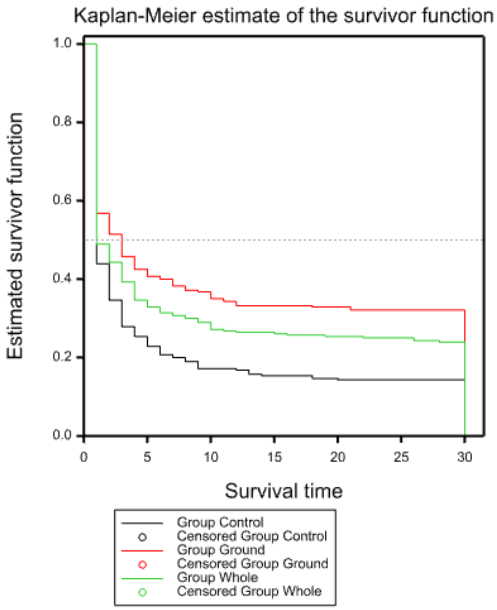

Figure 1 - Plot of Kaplan-Meier curve of survivor function estimate of latency to peck (s) in Control, Ground and Whole treatment birds.

IV. DISCUSSION

Whole feather consumption, when presented in the feeder, was highly variable and relatively low for the majority of birds in this trial which was reflected in the lack of significant differences between the Control and Whole treatment groups. Ground feather consumption also varied between individual birds; however individual consumption was more consistent throughout the trial.

Consumption of Ground treatment diet significantly decreased feather pecking motivation towards artificially presented feathers. This was likely related to the observed increase in feed intake for this treatment group. Qaisrani et al. (2013) found that dietary dilution with fibre could effectively reduce feather pecking behaviour due to increased feeding time and feed intake. Ground feathers may have acted as a dietary diluent, resulting in a compensatory increase in feed intake by Ground treatment birds and subsequent decrease in feather pecking behaviour. Feather pecking motivation may have also been reduced through increased protein digestion by Ground treatment birds when compared to other treatments. This supports the findings of Ambrosen and Petersen (1997) who observed a reduction in feather pecking behaviour and improvement in plumage condition with increasing dietary protein content.

The combination of increased feed intake and increased protein content of the Ground diet allowed Ground treatment birds to digest significantly higher amounts of methionine and cysteine than other treatments. Methionine and cysteine are both major limiting amino acids for egg production and feather development (Leeson and Walsh, 2004). However, there has been limited research into their roles on feather pecking behaviour. Kjaer and Sorensen (2002) studied the impact of dietary methionine and cysteine content on mortality and

integument condition, but no significant effect was found when methionine was supplemented.

Significant increases in the amount of methionine and cysteine digested by Ground treatment birds due to ground feather consumption suggests ISA Brown layers are capable of obtaining nutrients from the feathers. This may be possible through the action of keratinolytic microbes which have been found to be positively influenced by ground feather inclusion at 5% (Meyer et al, 2012).

A decrease in feather pecking behaviour associated with increased methionine and cysteine digestion suggests feather pecking may be at least in part nutritionally motivated, and satiation of methionine and cysteine requirements beyond maintenance may decrease feather pecking motivation. Further study is required to determine the effect of methionine and cysteine supplementation on feather pecking behaviour.

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FEED REFUSAL OF LAYING HENS- A CASE REPORT

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Summary

This case study reports the event of a severely reduced voluntary feed intake in free range laying hens. While some hens had access to only one feed source, others were offered choice feeding with Black Soldier Fly Larvae (BSF), a product of high protein and fat content. Decreased feed intake resulted in a severe reduced laying performance, egg mass and reduced body weight. Hens with the option to choice feed could partially compensate for the reduced standard diet intake. Several potential reasons for the refused diet were investigated, but a cause could not be identified. A change in feed resulted in an improved feed intake and subsequently improved performance data.

I. INTRODUCTION

Feed intake of laying hens can be affected by a variety of factors. Physical presentation of the feed (e.g. colour, particle size, smell) is known to affect feed selection (Forbes, 2000; Forbes and Covasa, 2005; Forbes, 2006). Sudden changes of the physical feed presentation (for example mash feed to pelleted feed), or the level of feed de-mixing have been reported to reduce feed acceptance (McCoy et al., 1994). A reduced hygienic status of feed due to bacterial or mycotoxin contamination can result in a limited voluntarily feed intake (Sakthivelan and Sudhakar Rao, 2010).

II. MATERIALS & METHODS

A total of 160 ISA brown free-range laying hens (20 hens/pen) were housed with access to indoor feeders, drinkers, perches, and nesting boxes. Hens had access to the range daily from 9 am – 7 pm. All hens received *ad lib* a typical Australian wheat-soy based layer diet formulated according to breed recommendations. While hens of four pens were housed under standard conditions (control group), hens of four pens (treatment group) were provided with an additional *ad lib* feed source using biosecure outdoor feeders filled with dried Black Soldier Fly (*Hermetia illucens)* larvae (BSF). The layer diet was mixed on two different time points (one month apart) by the same people from the same batch of ingredients. Mix 1 was offered to the hens during the adaptation period when hens were 38-43 weeks of age. Feed intake during this adaptation period was around 116 g/hen/day and is displayed for the last week (Week 0) in Table 1. Feed was switched from mix 1 to mix 2 with beginning of the choice feeding (Week 1). During the next few weeks, feed intake decreased dramatically to 72.0 ±2.77 and 79.5 ±6.73 g/hen/day for the control and choice fed groups, respectively. Subsequently, the laying performance, egg weight, egg mass, and body weight decreased dramatically with lowest levels observed in Week 4 (Table 1). After changing the diet to a newly mixed batch (mix 3), feed intake and production data recovered until reaching standard breed recommendations in Week 6.

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Table 1 - Performance data of laying hens that were fed the standard diet (control) and hens that had an additional feed source (choice fed) after replacing the initial standard diet (mix 1) by the same diet mixed at a different time point (mix 2).

Week	Treatment	Laying performance (%)	Egg weight (g)	Egg mass (g)	Control diet intake (g/day)	Choice feed intake (g/day)	Total feed intake (g/day)	Feed conversion ratio	Body weight (kg)
0	Control	94.6 ±0.64	67.9 ±0.35	64.3 ±0.46	117.3 ±0.31	0	117.3 ±0.31	1.80 ±0.017	2.04 ±0.018
	Choice fed	97.6 ±1.10	68.4 ±0.15	66.5 ±1.02	115.8 ±1.54	0	115.8 ±1.54	1.74 ±0.034	2.05 ±0.009
1	Control	95.2 ±1.25	67.9 ±0.35	64.8 ±1.17	108.7 ±2.26	0	108.7 ±2.26	1.68 ±0.019	2.02 ±0.014
	Choice fed	91.7 ±1.40	68.0 ±0.37	60.7 ±0.94	91.1 ±4.05	16.6 ±3.26	107.7 ±2.29	1.73 ±0.020	2.05 ±0.016
2	Control	89.4 ±0.96	67.4 ±0.31	60.2 ±0.93	97.3 ±2.23	0	97.3 ±2.22	1.62 ±0.056	1.95 ±0.022
	Choice fed	91.8 ±1.28	67.2 ±0.52	61.8 ±1.63	91.1 ±4.05	16.6 ±2.01	84.5 ±4.26	1.37 ±0.061	2.02 ±0.021
3	Control	78.5 ±4.65	64.5 ±0.30	48.3 ±3.50	72.0 ±2.77	0	72.0 ±2.77	1.45 ±0.053	1.79 ±0.024
	Choice fed	82.7 ±3.94	65.3 ±0.55	54.2 ±2.95	58.2 ±4.38	21.2 ±2.50	79.5 ±6.73	1.44 ±0.055	1.92 ±0.022
4	Control	39.7 ±4.53	62.5 ±0.19	24.8 ±2.86	85.8 ±1.25	0	85.8 ±1.25	3.94 ±0.496	1.82 ±0.004
	Choice fed	60.4 ±5.61	64.3 ±0.31	35.7 ±5.89	88.1 ±5.14	14.9 ±0.93	103.0 ±5.34	3.06 ±0.494	1.90 ±0.009
5	Control	46.3 ±2.17	64.7 ±0.42	30.1 ±1.96	77.3 ±2.27	0	77.3 ±2.27	2.56 ±0.124	2.03 ±0.006
	Choice fed	64.1 ±3.20	65.2 ±0.22	41.9 ±2.16	76.3 ±8.25	15.5 ±2.76	91.8 ±10.9	2.17 ±0.209	2.06 ±0.009
6	Control	80.7 ±1.85	66.9 ±0.42	54.6 ±1.90	94.7 ±3.94	0	94.7 ±3.94	1.70 ±0.038	2.03 ±0.012
	Choice fed	88.3 ±1.19	67.6 ±0.19	59.6 ±0.81	86.8 ±4.47	6.49 ±0.72	93.3 ±5.06	1.53 ±0.082	2.05 ±0.007

Table 2 - Results of mycotoxin testing of the refused feed (mix 2) and the newly mixed feed (mix 3).

	mg/kg on dry weight basis	
Nutrient content	Mix 2 – refused feed	Mix 3 – new feed
Aflatoxin B1	< 0.001	< 0.001
Aflatoxin B2	< 0.001	< 0.001
Aflatoxin G1	< 0.001	< 0.001
Aflatoxin G2	< 0.001	< 0.001
Deoynivalenol	< 0.05	< 0.05
Nivalenol	< 0.05	< 0.05
HT2 Toxin	< 0.05	< 0.05
T2	< 0.05	< 0.05
Ochratoxin A	< 0.001	< 0.001
Patulin	< 0.05	< 0.05

As analysed results, scan by LC—MS Symbiolab, Brisbane.

III. RESULTS

Physical evaluation of fed quality was performed by sensory testing. The refused feed was dry, smelled aromatic without foreign odours, was of product typical colour, homogenous, macroscopic free from contamination, moult, mites, or others. Dry matter (% w/w) was 89.8 for the refused feed (mix 2) and 91.5% for the new mixed feed (mix 3). The feed was evaluated for mycotoxins, results are displayed in Table 2.

In order to exclude a mixing error or de-mixing of the feed, chemical feed analysis of macro and micro ingredients was performed. Results are displayed in Table 3:

Table 3 - Feed composition and nutrient content of the refused feed (mix 2) and the newly mixed feed (mix 3).

	g/kg dry matter as analysed	
Nutrient content	Mix 2 – refused feed	Mix 3 – new feed
Crude protein*	183.6	162.3
Histidine†	4.6	4.8
Serine†	9.4	9.9
Arginine†	12.0	12.6
Glycine†	8.7	9.1
Aspartic Acid†	16.6	17.8
Glutamic Acid†	41.5	42.9
Threonine†	6.8	7.2
Alanine†	7.7	8.1
Proline†	13.0	13.3
Lysine†	9.2	9.8
Tyrosine†	4.5	4.7
Methionine†	3.8	3.7
Valine†	9.2	9.7
Isoleucine†	7.8	8.2
Leucine†	13.8	14.5
Phenylalanine†	9.2	9.7
Ether extract*	32.4	25.6
Calcium*	44.8	41.3
Phosphorus*	5.03	0.43

As analysed results,*University of New England, Armidale; †Symbiolab, Brisbane.

IV.DISCUSSION

Hens of groups that had access to choice feeding with Black Soldier Fly Larvae were able to partially compensate the reduced feed intake of the standard diet. This becomes especially evident when comparing laying performance and egg mass of the choice fed group to the control hens. Especially in Week 4, when standard feed intake of the control and choice fed group was comparably low (85.8 ±1.25 g/hen/day and 88.1 ±5.14 g/hen/day, respectively), total feed intake differed by 17.2 g/hen/day (85.8 ±1.25 g/hen/day and 103.0 ±5.34 g/hen/day, respectively). Duncan and Hughes (1972) investigated the motivation of hunger in domestic fowl for their willingness to work for it. A large individual variation of their ten tested hens failed to show a clear hunger motivation. The large individual response towards the challenged feed situation can be observed in this case report when evaluating the SEM. While the SEM for total feed intake at week 0 was as low as ±0.31 (control group), it increased up to ±10.9 in week 5 (choice fed group). In contrast to the study of Duncan and Hughes, hens in this case study showed an overall strong willingness to work for feed, as indicated by the additional BSF intake which was only available in feeders on the range in 15 m walking

distance. Intake of the BSF increased gradually until week 3, and decreased continuously as soon as the newly mixed standard diet (mix 3) was offered.

Voluntary feed intake can be influenced by many factors, including negative feedback from the consequences of eating a meal (Forbes, 2000). After introducing the hens of this report to a newly mixed feed (mix 3), feed intake gradually increased. We can therefore conclude that these animals were not affected by negative feedback.

Despite physical and chemical testing of the feed, the reason for the feed refusal remains unknown. During the entire time period, hens did not show any clinical symptoms of a disease and appeared healthy and active. Therefore, feed refusal due to a reduced health status of the flock seems unlikely. All feed mixing was performed by the same individual, the same equipment, and the same batch of raw ingredients was used. A reduced hygenic status of one or more feed ingredients or a missing essential component in the premix can therefore be excluded (Table 2). The analysis of the essential amino acids (Table 3) and micro minerals demonstrated a high mixing accuracy and could not detect a lack of an essential component. Cross-contamination with an unknown substance during feed manufacturing seems to be the only possible explanation for the feed refusal.

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ASSESSING ORGANIC SELENIUM SOURCES

Y.G. LIU[1], P.A. GERAERT[2] and M. BRIENS[2]

Summary

Modern intensive production system brings about more stresses to livestock and poultry thus it is important to supplement effective antioxidants through diets. Recent research has well illustrated the role of seleno-methionine (SeMet) and seleno-cysteine (SeCys) in the antioxidant system. In view of many selenium products available on the market, this article provides clarity on the various chemical forms of organic selenium sources; introduces a new bio-assay method to assess the efficacy of various Se products by measuring Se deposition rate in the breast muscle of broiler birds on day 7 post-hatch. Our chemical analyses on a number of seleno-yeast products (SeY) showed these products contain 0-65% Se as SeMet with considerable variation. The bio-assay on Se deposition in the muscle reflects well the level of SeMet present in the SeY products. In contrast, seleno-proteinates and seleno-amino acid complexes do not appear to provide SeMet thus are similar to mineral selenium.

I. INTRODUCTION

Today the animal industry has a growing interest in using selenium based products as *in vivo* antioxidant, not only for breeding stock but also for commercial animals. There is a wide range of selenium products available: from inorganic, such as sodium selenite, coated or not, even nanoparticles, to organic forms like selenized yeasts (SeY), chelates of selenomethionine (SeMet), pure forms of SeMet or hydroxy-selenomethionine (OH-SeMet or HMSeBA), as well as Se complexes through mixing mineral Se with glycinates, proteinates, etc.

Recent research has revealed that selenium is the key component of up to 25 different seleno-proteins in the mammal, with a majority of these proteins being enzymes. In the body, Se functions through selenocysteine (SeCys) as an essential amino acid in these functional proteins acting as antioxidants, such as glutathione peroxidases, thioredoxine reductases, methionine sulfoxide reductase etc. However, body is unable to synthesize SeCys that has to be synthetized *de novo* in the cells rather than supplying through diets. Dietary SeCys undergoes complete transformation to selenide (H_2Se) to synthesize *de novo* SeCys (Surai and Fusinin, 2014).

In terms of bio-efficacy of various selenium chemical forms, numerous studies have confirmed the organic forms of selenium, namely Se-Met, is better absorbed and deposited in the body tissues thus more efficient in serving selenium needs than the mineral selenium (Briens et al. 2013, 2014; Jlali et al. 2013). Simon et al. (2013) demonstrated that the efficacy of SeY products can only be compared based on their SeMet contents in spite of their levels of total Se being the same. Since analyses on selenium and SeMet are rather sophisticated and not done in routine laboratories, yet chemical analysis does not appear to be sufficient in determining selenium bioavailability, how to compare selenium sources remains a problem in the industry.

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II. CHEMICAL ASSESSMENT ON SELENIUM-YEASTS

Using ICP-MS, UT2a Lab in France analysed 12 SeY products belonging to 8 commercial brands, collected from end users in the Asia Pacific region. The results (Table 1) revealed that SeY products carry large variations in terms of total Se contents and the proportion of Se as SeMet. For a number of samples, SeMet counts on approximately 60% of total Se. However, certain brands carry extremely high variation (Se-yeast 5) or even zero % SeMet (Se-Yeast 6), being a simple mixture of yeast and mineral selenium.

Table 1 - Lab Analyses on Various Se-Yeast Products (UT2A Lab, France).

Product	Specification Se, mg/kg	Sample Code	Se total, mg/kg	SeMet, mg/kg	SeMet, %
Se-yeast 1	3000	1	2408	1531	63.58
Se-yeast 2	3000	1	3164	1817	57.43
Se-yeast 3	3000	1	2783	1951	70.10
Se-yeast 4	2000	1	2361	1550	65.65
		2	2067	1229	59.50
Se-yeast 5	2000	1	2146	465	21.67
		2	1905	289	15.17
Se-yeast 6	2000	1	2508	0.98	-
Se-yeast 7	4000	1	3864	179	4.63
Se-yeast 8	3000	1	2941	1823	62.3
		2	2974	1825	61.4
		3	2848	1832	64.3

The process to produce SeY products is to grow a yeast on mineral Se to facilitate the yeast to integrate the mineral Se into SeMet. Managing such fermentation process for optimum yeast growth, while feeding the yeasts with mineral Se that is toxic, is already rather tricky and often gives rise to large variability, leading to variations in the SeMet contents, which is the major active part in the end product. Measuring the total Se content of the yeasts is relatively easy, but determining SeMet content is not usually performed in routine laboratories and certainly not at the user's level. Depending on the deposition of this SeMet, it is even more difficult to get a precise measurement.

A specific SeY (Se-yeast 7 in Table 1) is produced based on *Torula* yeast. Its specificity lies on the fact that it does not contain mainly SeMet but Se-homolanthionine (Se-HLan). However, such a yeast seems difficult to analyse on a batch to batch basis in that most laboratories do not possess calibration sample for this Se-HLan. Its quality guarantee will thus only be based on total Se contents. Moreover, scientists compared pure SeMet and Se-HLan on rats, and concluded Se-HLan being only 60% of SeMet efficiency (Anan and Ogra, 2013). More recent works compared Se-*Torula* yeast with other Se-yeasts, without measuring their SeMet contents, suggesting similar efficacy between those yeasts. It further points out the importance of complete characterization of the Se-yeasts when comparing efficacy.

Apart from SeY, there are Se products called Se-chelates or complexes with either proteins or amino acids, without specifying their exact molecules. Users would have no means to assess such type of products except analysing total Se contents. The only option of evaluation is bio-assay.

III. USING RAPID BIO-ASSAY TO EVALUATE SELENIUM EFFICACY

It is well-understood that SeMet is the reserve form in the tissue. Under stressful conditions, this SeMet can be mobilised and re-synthesized to its functional form SeCys, being the key element of selenoproteins. Hence, SeMet and SeCys should thus be the best predictor of the efficacy of the selenium source (Surai and Fusinin, 2014).

The objective of the dietary Se supplementation is definitely to enhance tissue Se deposition. For day-old chicks, the Se contents in the breast muscle depend solely on the Se supply in their breeder's diets. If not supplemented with Se or only with sodium selenite, the chicks show a decrease of their muscle Se content at day 7 post-hatch and thereafter remain at the similar level till day 21 (Couloigner *et al*. 2015). The results in Figure 1 clearly showed the Se levels in the chick muscles are directly related to the chemical form of Se supplements.

Using this method the authors developed a rapid bio-assay to evaluate biological value of different organic Se sources. Figures 1 & 2 clearly show that, after feeding 0.2 ppm Se, the Se deposition rates in the broiler muscle reflect their SeMet contents in the respective yeasts. The samples of SY1 and SY2 were analysed to contain 33% and 74% Se as SeMet, respectively. These analytical results are well in line with the range of SeMet contents (Table 1), depending on product brands or between different batches of the same product.

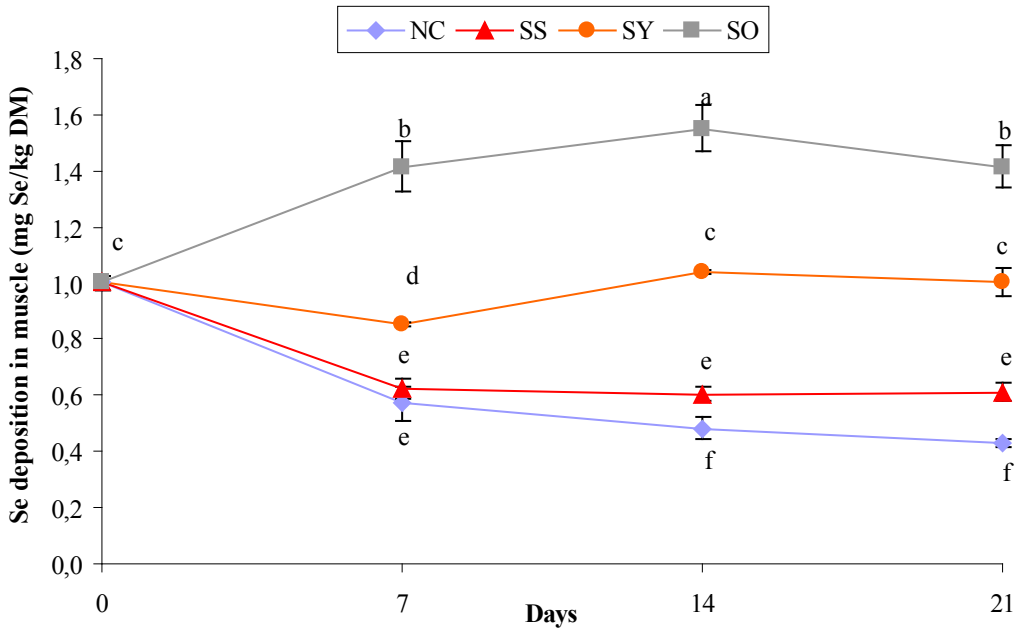

Figure 1 - Changes of Se contents in breast muscle of chicks. NC, Negative Control without Se supplementation; SS, sodium selenite 0.2 ppm; SY, Se-yeast 0.2 ppm; SO, OH-SeMet 0.2 ppm.

As the chelates of certain minerals (Zn, Cu, Mn, Fe etc.) show better bioavailability, producers have also tried to develop so-called Se chelates or complexes. Notwithstanding, Se is a metalloid and cannot be chelated. The only true available chelate existing today is a chelate of selenomethionine with Zn, thus a source of both SeMet and Zn. The bio-assay indicates an elevated Se deposition (SM in Figure 2) comparing with SeY. However, methionine and selenomethionine are absorbed through the same transporters. Chelating methionine or SeMet with Zn, may enhance Zn absorption, but will most likely reduce SeMet absorption as shown in Figure 2 (SM). Moreover, chelation has often been promoted to enhance stability of the molecule, it is however suspected that such weak bonding cannot improve the stability. Anyway, measuring the stability of such chelate in feed processing is almost impossible through conventional analyses and this rapid *in vivo* test is the only way to determine the bioavailability of such a molecule.

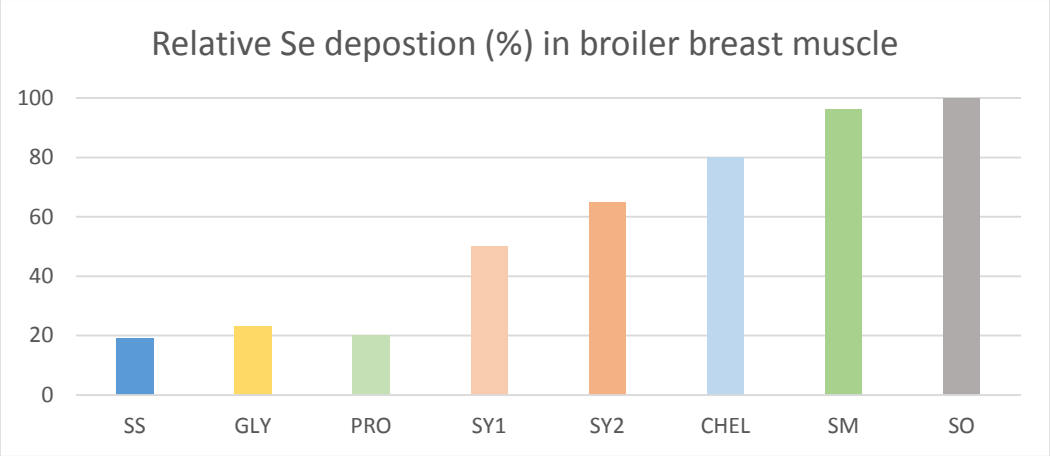

Figure 2 - Comparative Se deposition in the muscle of broiler after feeding various Se forms (0.2 ppm) for 7 days post-hatch. SS, sodium selenite; GLY, Se-glycinate, PRO, Se-proteinate; SY1 and SY2, Seleno-yeast (SY1 was analyzed to have 33% of its Se as SeMet; and SY2 was analyzed to have 74% of its Se as SeMet); CHEL, chelate of SeMet and zinc; SM, pure SeMet and SO, pure OH-SeMet (Selisseo®, SO).

Another type of selenium sources is often promoted as organic Se: the glycinates, proteinates or complexes. These sources are usually obtained through mixing mineral Se (sodium selenite) and amino acids or soya protein hydrolytes. As for chelates, Se being a metalloid is not prone to complexation. The bio-assay test presented above (Figure 2) confirmed that those products have a similar efficacy to sodium selenite.

Hydroxy-selenomethionine (OH-SeMet or HMSeBA) is a new molecule, recently developed through a specific chemical synthesis (EU Registration 3b814, Selisseo®). It is water soluble and adsorbed on silica carrier, with high consistency as pure HMSeBA and stable under various feed processing conditions. Studies have demonstrated that this molecule is fully metabolised into SeMet and SeCys in the animal's digestive system with high rate of deposition in animal tissues (SO in Figure 2). When comparing HMSeBA with SeY, tissue Se deposition increased by more than 40% in either broilers (Briens et al. 2013, 2014), layers (Jlali et al., 2013) or swine (Jlali et al., 2014).

In conclusion, despite a large variety of selenium sources on the market, many suppliers only provide total selenium content either with or without a vague indication on its chemical form. Regulatory constraints and most of the nutritional recommendation as of today only consider total Se, although it is now very well established that the chemical form of Se largely affects its efficacy. Using chemical analysis on SeMet proportion followed by the 7-d broiler bioassay can provide a clear and reliable picture on the quality and bio-efficacy of various Se sources, either organic or mineral form.

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FAST AND SLOW-GROWING BROILER CHICKENS SHOW DIFFERENT APPETITE FOR LIMITING AND NON-ESSENTIAL AMINO ACIDS

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One of the most important challenges that the broiler chicken industry deals with is variability in growth. Non-uniformity of growth has a robust association with variability in feed consumption (Gaya *et al*., 2006) which is controlled by hunger-satiety mechanisms. These mechanisms are mediated by nutrient sensors monitoring the nutritional environment in the gastro-intestinal tract and plasma. Such nutrient sensing mechanisms in turn, trigger the signals to the brain related to nutrient abundance/deficiency (Efeyan *et al*., 2015). We hypothesize that low growth rates are associated with a higher sensitivity of the nutrient signalling mechanisms which, in turn, relate to an earlier onset of satiety in broiler chickens.

In order to test our hypothesis, we designed a dietary manipulation based on supplemental amino acids (AA). A total of 96 slow (<800g) and 96 fast-growing chickens (>1000g) were selected from a large commercial shed (Ross 308) at the age of 21d and transferred to individual cages. Four combinations of AAs (73% supplemented) were offered against control feed (commercial feed with CP: 19.3%, ME: 13.3 MJ/KG) in a double-choice scenario to compare the preference/avoidance in both fast and slow growing chickens. Treatments were: feed/feed (T1); feed/feed+ Met-Lys-Thr (T2); feed/feed+ Ala-Asp-Asn (T3); feed/feed+ Cys-Ser-His (T4). Treatment and control intake was measured daily for two weeks. Preferences values were calculated using the equation Preference%= treatment intake (g)/ control intake (g) + treatment intake (g) x 100, and data were analysed using proc GLM of SAS considering body weight at week 4 as a covariate effect.

Results showed that slow growers compared to fast growers consumed more non-essential AA (T3) than fast growers ($P < 0.05$). Moreover, slow growing chickens consumed more T3 than T2 (essential AA) ($P < 0.05$). In conclusion, fast growing chickens were less responsive to AA supplementation of a balanced diet compared to slow growing chickens. Also, slow growing chickens may have a specific appetite for non-essential AAs which are not tightly controlled when formulating commercial broiler diets. However, the experiment was done by mixed-sex chickens, so the results probably have been affected by confounding effect of sex.

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Table 1 - Analysis of covariance: comparison of treatments in fast and slow growing chickens

	Fast growers				Slow growers				P values			
Group	T1	T2	T3	T4	T1	T2	T3	T4	Group	Treatments	Group × Treatment	BW4 Covariate
F(g) (SE)	1813 (174)	1586 (162)	1793 (165)	1514 (174)	1475 (172)	1597 (175)	1370 (169)	1493 (142)	0.41	0.74	0.22	0.56
T(g) (SE)	1067^{b} (139)	1184^{ab} (129)	1129^{b} (132)	1355^{ab} (139)	1683^{ab} (137)	1335^{b} (14)	1847^{a} (13)	1573^{ab} (113)	0.02	0.12	0.01	0.00
P% (SE)	47.1 (5.0)	42.4 (4.6)	39.0 (4.7)	46.0 (5.0)	55.4 (4.9)	47.0 (5.0)	59.3 (4.8)	51.6 (4.0)	0.06	0.58	0.05	0.11

^{a,b,c}: means with different letters in each row are significantly different at $P < 0.05$;
F: control intake;
T: treatment intake;
P: total preference;
T1: control;
T2: Met-Lys-Thr;
T3: Ala-Asp-Asn;
T4: Cys-Ser-His;

RESPONSE OF BROILER CHICKENS TO DIETS CONTAINING VARIED LEVELS OF SODIUM AND SUPPLEMENTED WITH MICROBIAL PHYTASE

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Summary

A 3 x 2 factorial experiment was designed to investigate the effect of different levels of dietary sodium (Low, 0.15%; Mid, 0.25%; or High, 0.35%) and microbial phytase supplementation (0 and 500 FTU/kg) on the performance of broilers. There was no effect of sodium (Na) and phytase on overall performance and tibia bone development. High dietary Na increased the excreta dry matter content. The total tract retention of Ca, P and Na was reduced with high Na diet, which was counteracted by phytase supplementation. The diets containing 0.25% improved the activities of Na-K-ATPase in the jejunum. The results indicate that birds are capable of withstanding a wide range of dietary Na levels, regardless of phytase inclusion.

I. INTRODUCTION

Phytase supplementation of poultry diets increases the availability of phytate-bound minerals (Ca and P), protein and other nutrients and therefore improves the performance of birds. Phytase achieves this result by reducing the phytate-induced hyper-secretion of gastric hydrochloric acid and sodium bicarbonate, and subsequently improves the Na utilisation of broiler chickens (Ravindran *et al.*, 2008). It has been reported that high Na concentration could mute the anti-nutritive effect of phytate, resulting in a lower phytase response on nutrient digestibility (Ravindran *et al.*, 2008; Adeola & Cowieson, 2011). Besides, an increased concentration of Na in the intestinal lumen may compromise Na+,K+-ATPase activity and Na+-dependent transport systems, which are involved in intestinal uptake and absorption of different nutrients, particularly glucose and amino acids (Selle *et al.*, 2012). Therefore, the present study was designed to evaluate the effect of different levels of dietary Na on phytase activity and subsequent impact on broiler performance.

II. MATERIALS AND METHODS

A total of 360 day-old male Ross 308 broilers were offered six maize-soybean meal-based diets containing three levels of Na (Low, 0.15; Mid, 0.25 or High, 0.35 %) and with (500 FTU/kg) or without phytase (Quantum Blue, AB Vista, Marlborough, UK). Each diet was replicated six times, with ten 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 specification (Aviagen, 2009) except Na (0.16-0.23% Na for starter and grower and 0.16-0.23% Na for finisher). The Ca, P, Na and DEB in the diets are presented in table 1. Birds were provided feed and water *ad libitum*.

III. RESULTS AND DISCUSSION

The results of the study are presented in Table 2. Dietary Na and phytase had no effect ($P > 0.05$) on performance and tibia bone development. This result is in agreement with a previous study (Goodgame *et al.*, 2011). High dietary Na adversely affected ($P < 0.001$) the excreta

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DM content and resulted in watery excreta, which is inconsistent with findings of Ravindran *et al.* (2008). In addition, phytase supplementation to the high Na diet significantly increased ($P < 0.01$) the excretion of ammonia.

Birds offered the high Na diet exhibited a reduced total tract retention of Ca, P and Na. This negative effect was countered with phytase inclusion (Na x phytase, $P < 0.001$). The reduced retention of Na due to high dietary Na is in agreement with Ravindran *et al.* (2008). However, in contrast, Ravindran et al. (2008) reported no change in the retention of Ca and P due to varying level of DEB or Na. Variation in diet compositions between the studies may be the cause of this discrepancy. Diets containing mid-range Na levels improved ($P < 0.001$) the activity of Na-K-ATPase in the jejunum. Supplementation of phytase also improved ($P < 0.01$) the activity of this enzyme, but the interaction between Na and phytase was not significant. The positive effect of phytase on this enzyme is in support with the findings of Liu *et al.* (2008).

IV. CONCLUSIONS

The results imply that the dietary Na level used in this study had no negative effect on phytase response. However, a high Na diet without phytase supplementation negatively influenced nutrient utilisation and the intestinal enzyme activity of birds, regardless of the effect on production performance.

ACKNOWLEDGEMENTS: Funding was provided by AB Vista, UK and UNE.

Table 1 - Analysed Ca, P, Na (g/kg) and calculated DEB (mEq/kg) values in the diets.

	Diets					
	0.15% Na	0.25% Na	0.35% Na	0.15% Na + 500 FTU phytase	0.25% Na + 500 TFU phytase	0.35% Na + 500FTU phytase
Starter						
Ca	10.7	11.2	11.0	11	11.1	10.9
Total P	7.9	7.4	7.5	6.2	6.4	6.2
Phytate-P	2.84	2.83	2.81	2.88	2.87	2.85
Na	1.5	2.4	3.5	1.3	2.3	3.3
DEB	244.4	286.8	330.3	244.4	287.6	330.9
Grower						
Ca	9.0	9.2	8.9	8.9	8.8	8.6
Total P	7.2	7.0	7.1	5.7	5.9	5.9
Phytate-P	2.69	2.68	2.66	2.73	2.71	2.7
Na	1.5	2.6	3.5	1.4	2.4	3.4
DEB	220.0	264.1	307.8	220.0	263.8	307.3
Finisher						
Ca	8.6	8.7	8.9	8.4	8.8	8.6
Total P	7.2	7.4	7.0	5.8	5.8	5.4
Phytate-P	2.68	2.67	2.65	2.77	2.73	2.71
Na	1.4	2.5	3.6	1.5	2.3	3.3
DEB	207.5	250.5	294.1	222.7	257.1	301.1

Table 2 - Influence of different levels of dietary Na with or without phytase on gross performance, tibia bone development, excreta DM %, total tract retention of nutrient and intestinal enzyme activity of broiler chickens at 24 days of age.

	Diets[1]						P value	
	0.15% Na	0.25% Na	0.35% Na	0.15% Na+phy	0.25% Na+phy	0.35% Na+phy	Na X phytase	SEM
Gross performance								
FI	1645	1632	1640	1689	1678	1663	NS	59.6
BWG	1230	1246	1262	1257	1333	1304	NS	48.7
FCR	1.33	1.32	1.30	1.34	1.26	1.27	NS	0.06
Tibia bone quality								
Ash %	47.9	48.8	49.3	49.1	49.3	49.1	NS	0.58
Ca %	38.7	38.7	38.4	38.5	38.4	38.4	NS	0.17
P %	17.9	18	17.9	18.1	18.1	18.0	NS	0.14
Litter quality								
DM % [2]	24.8	23.4	20.7	25.0	22.6	20.1	NS	1.23
Ammonia (mg/L)[3]	19.8^{c}	33.3^{ab}	30.1^{b}	39.4^{a}	34.9^{ab}	37.9^{a}	< 0.01	0.63
Total tract retention[2,3]								
N	0.64^{bc}	0.61^{ab}	0.50^{d}	0.67^{a}	0.65 ^{ab}	0.67^{a}	<0.001	0.05
Ca	0.60^{a}	0.50^{a}	0.27^{c}	0.58^{a}	0.56^{b}	0.60^{a}	<0.001	0.03
P	0.56^{b}	0.49^{c}	0.26^{d}	0.62^{a}	0.55^{b}	0.63^{a}	<0.001	0.02
Na	0.64^{b}	0.41^{d}	0.19^{f}	0.73^{a}	0.47^{c}	0.13^{e}	<0.001	0.02
Enzyme activity in jejunum (mmol/mg protein/min) [3,4]								
Na-K-ATPase	49.2	73.6	65.3	86.4	98.7	85.4	NS	1.09

[a,b,c,d,e,f] Means within a row without common superscript are statistically significant at the level indicated.
[1] phy-phytase;
[2] Na effect at $P < 0.001$;
[3] Phytase effect at $P < 0.001$;
[4] Na effect at $P < 0.01$.
NS- not significant

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RESPONSE OF BROILER CHICKENS TO HIGH INCLUSION LEVELS OF COTTON SEED MEAL SUPPLEMENTED WITH COMPOSITE MICROBIAL ENZYMES

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Soybean meal (SBM) is the premier plant protein source used by the poultry industry around the world. The price of soybean meal fluctuates but is generally high, particularly in importing countries. There are other vegetable protein sources of local importance around the world, such as canola seed meal (CM) and cottonseed meal (CSM). Some of these alternative sources are comparable to SBM in nutritive value but contain factors that reduce their quality for birds (Aftab, 2009). CSM contains approximately 222.0 to 560.2 g crude protein per kg and 7.4 to 11.99 MJ metabolisable energy per kg. The use of CSM in poultry diets is limited by the presence of some anti-nutritional factors. Various efforts have been made to increase the incorporation of CSM in broiler diets including physical treatments and supplementation with microbial enzymes (Nagalakshmi et al., 2007). A new composite xylanase and beta-glucanase product (Danisco Animal Nutrition, Marlborough, UK), which was developed for use on fibrous vegetable protein sources such as CSM, was tested in the current study.

In this study, the performance and uniformity of broiler chickens, pellet durability and nutrient digestibility were studied from hatch to 35d of age when fed isocaloric and isonitrogenous diets containing three levels of CSM (None), Low (5, 10, 15%) or High (6, 12, 18%) in the starter, grower, and finisher phases, respectively). The diets were supplemented with three levels of the enzyme (0, 100, or 150 mg/kg). Pellet durability index (PDI) linearly increased with increasing levels of CSM, with PDI values 0.88, 0.91, 0.91, 0.92 and 0.94 for diets containing 0, 10, 12, 15 and 18 % CSM, respectively. Each of the nine dietary treatments was randomly assigned to 6 replicates, with 10 birds per replicate. Feed intake (FI) results obtained at d35 but not at the starter and grower phases decreased ($P < 0.05$) linearly with increase in enzyme level. During 1-10 and 1-24 d, birds fed CSM-supplemented with either 100 or 150 mg/kg microbial enzyme gained more ($P < 0.05$) weight than those grown without the microbial enzyme, with the heaviest birds observed in the low CSM group supplemented with 100 mg/kg enzyme. The enzyme supplement decreased ($P < 0.01$) the FCR at 1-10, 1-24d and 1-35 d, with both levels of the enzyme producing about the same results. There was no significant difference ($P > 0.05$) in flock uniformity between treatments, with all groups recording 92.0 % BW uniformity. The microbial enzyme improved ($P < 0.05$) apparent ileal crude protein digestibility. High inclusion levels of CSM decreased ($P < 0.05$) the digestibility of starch, but this was improved ($P < 0.05$) by the enzyme supplement. Gross energy digestibility was not affected ($P > 0.05$) by CSM levels or the enzyme. There was an interaction between CSM and microbial enzyme on the digestibility of starch. It can be concluded that increasing dietary CSM levels to 18% does not adversely affect broiler performance. Furthermore, enzyme inclusion significantly improved FCR throughout the production cycle. Nutrient digestibility decreased with increasing dietary CSM level but this was improved or restored by supplementation with the test enzyme product.

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PHOSPHORUS-HOW LOW CAN WE GO IN BROILER DIETS?

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Phosphorus (P) is essential for all forms of life. Dietary P content either in excess of, or below requirements may adversely affect broiler performance. The P requirement of broilers has been the subject of numerous investigations. However, the requirement for this nutrient has not been adequately established; in part, due to its interactions with calcium and vitamin D. Previous work by the authors showed the P requirement of broilers is lower than NRC (1994) recommendations.

The current study investigated the growth response of Ross 308 male broilers to graded levels of dietary available phosphorus (AvP) and total Ca from day 1 to 21 post-hatch housed in floor pens. The experimental mash diets, based on wheat and sorghum, were formulated to contain 2.0, 2.5 and 3.0 g/kg AvP; each with 3.5, 4.5, 5.5 and 6.5 g/kg total Ca. All other nutrients met the requirements of birds (NRC, 1994). Phytase (Axtra PHY 10,000 TPT at 0.05 g/kg, Feedworks, Australia) was supplemented to all diets. Each diet was fed to 5 pens with 10 birds per pen; Starter diet from day 1 to 14 and Grower diet from day 15 to 21. Live weight and feed intake were measured weekly and feed conversion ratios (FCR) were calculated.

Birds fed the diet containing 2.0 g/kg AvP and 4.5 g/kg total Ca had higher ($P<0.05$) live weight than birds consuming the same AvP level with other Ca levels at day 21. There were no significant differences in live weight between the other Ca levels with 2.0 g/kg AP. For AvP concentrations of 2.5 and 3.0 g/kg, live weight numerically increased as dietary total Ca increased, ($P = 0.094$ and $P = 0.217$, respectively). Broilers fed diets containing 3.5 g/kg total Ca had poorer growth rate ($P = 0.011$) and FCR ($P = 0.047$) than those fed the higher levels of Ca regardless of AvP levels in the diets. There were no significant differences in feed intake among all treatment groups.

The results show that broilers fed diets containing of 2.0 g/kg AvP and 4.5 g/kg total Ca with supplemental phytase had superior growth performance and FCR at day 21. Therefore the poultry industry should consider reducing the concentrations of both AP and Ca in broiler diets from day 1 to 21.

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COMPARISION OF WHEAT AND MAIZE-BASED DIETS ON GROWTH PERFORMANCE AND MEAT QUALITY OF BROILER CHICKENS

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Summary

Maize and wheat are major sources of energy in broiler diets, however there are few comparative studies undertaken on the effect of these cereal types on chicken meat quality. In this study, the response of meat quality and growth performance to diets based on maize or wheat was evaluated. A total of 72, 10-day old Ross 308 broilers male chicks were randomly allocated to 2 dietary treatments with 6 birds per cage and 6 replications in each treatment. Experimental diets were formulated to be iso-calorific and iso-nitrogenous. Body weight (BW), average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (FCR) from 10-38 d, and *pectoralis* muscle drip loss rate, colour, temperature, pH, and shear force at 38 d were evaluated. Results of this study show that ADFI and FCR values of birds from 25- 38 days of age were lower in the maize-based diet ($P<0.001$). Drip loss rate was higher in maize fed group as compared to those from wheat fed group ($P<0.01$). Wheat-based diet increased the lightness (L*; $P<0.001$) and redness (a*; $P<0.05$) values, whereas maize-based diet markedly increased the yellowness (b*; $P<0.001$) value in meat. The shear force and pH values of muscle were unaffected by dietary cereals. The results of this study indicate that dietary cereals had significant effects on ADFI, FCR, and meat drip loss and meat colour of broiler chickens.

I. INTRODUCTION

Maize and wheat are a major source of energy in broiler diets. Generally, wheat-based diets are offered to broiler chickens in Europe, Australia and New Zealand while maize-based diets are widely used in the US and Asia. Studies have demonstrated that dietary cereal source have a significant influence on bird performance (Mateo and Carandang, 2006; Abdollahi et al., 2010) however the type of cereal used has received little attention as a factor affecting chicken meat quality. Del Puerto et al. (2016) reported that dietary cereals may affect pH and colour of meat. Moreover, Kennedy et al. (2005) have shown that while meat from birds fed maize-based diets may benefit from dietary vitamin E supplementation to improve the sensory value of meat, there was no advantage in supplementing wheat-based diets. This observation suggests a gap in the meat quality between the two cereal types. Therefore, the objective of this study was to measure some meat quality and growth performance parameters of broiler chickens offered diets where maize or wheat provided the major energy source.

II. MATERIALS AND METHODS

All procedures used in this study were approved by the Animal Ethics Committee of University of Sydney. Experimental birds were housed in battery cages. Broilers (Ross 308, male) were placed on a common wheat-based standard starter diet until day 10. From 10 to 17 days and from 18 to 38 days, broilers were offered grower and finisher diets respectively which were based on either maize or wheat as a principle source of carbohydrate, and satisfied all nutrient requirements as suggested by the breeder manual. All experimental diets

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provided similar calculated metabolizable energy (ME kcal/kg) and crude protein (%). Energy levels were adjusted with soybean oil and digestible amino acid levels with soybean meal, canola meal and synthetic amino acids. Exogenous enzymes were not included. Chicks had *ad libitum* access to water and feed. Average individual body weights (BW) and average daily gain (ADG), and cage average daily feed intake (ADFI) and feed conversion ratio (FCR) were recorded on a weekly interval. The FCR values were corrected for the body weight of any bird that died during the course of the experiment. At 38 days of age, birds were killed according to the recommendations for euthanasia of experimental animals. Meat quality was determined on 1 bird per cage. Drip loss was determined by the weight difference of suspended *Pectoralis* muscle samples stored at 4°C at 0, 24, 48 & 72 h post-slaughter. Meat colour change was determined daily over 7 days using the CIELAB method for lightness (L*), redness (a*) and yellowness (b*) in the *Pectoralis* muscle using a Minolta Lab CR-10 colourimeter. The pH was determined at 0 and 1 day of *post mortem* in the *Pectoralis* muscle using a glass electrode (TPS ionode) attached to a portable pH meter (TPS LC80A pH-mv-TEMP) which is temperature compensated.. Shear force value of *Pectoralis* muscle was determined with a Warner Bratzler Shear attachment on a Stable Micro Systems TAXT2 Texture Analyser. Data were analysed using the generalised linear model procedure of SAS. Meat colour data were analysed by repeated measures using the mixed procedure of SAS. The experimental units were pooled cage means for growth performance and 1 bird per cage for meat quality. Data are presented as least squared means ± standard error of the mean (sem). Differences were considered significant at $P < 0.05$.

III. RESULTS AND DISCUSSION

The effects of feeding wheat and maize-based diets on growth performance are presented in Table 1 and meat quality is presented in Figure 1 & 2 and Table 2. Dietary cereals had no effect on body weight (BW), average daily gain (ADG), feed conversion ratio (FCR), and average daily feed intake (ADFI) of broilers chicken from 10-17 and 17-24 d of age. Similarly, BW and ADG of birds from 25 - 38 days of age were not influenced by wheat or maize-based diet, but the birds fed on maize-based finisher diet had lower FCR ($P<0.001$) and ADFI ($P<0.001$) compared to those offered wheat-based diets. These outcomes are in agreement with Peng *et al.* (2003) who stated that a maize-based diet significantly improved FCR when compared with a wheat-based diet. There was no significant effect of dietary cereals type on mortality rate of broiler chicken over the entire growth period.

The breast meat of broilers offered the maize-based diet had a higher drip loss rate than the wheat-based diet ($P<0.05$; Figure 1). These results are confirmed by Ao and Choct (2004) who reported that wheat-based diet fed group of birds had a lower drip loss value than the maize. An increase in drip loss in the maize-fed group also agrees with the observations of Kennedy et al. (2005) who observed an improvement in the eating quality of maize-fed, but not wheat-fed chicken meat when supplemented with vitamin E. In the present study, meat from the birds fed the wheat-based diet had ($P < 0.001$) higher lightness (L*) value (Figure 2) which might be due to a lack of pigments otherwise present in maize (Smith et al., 2002). Similarly, wheat-based diet fed birds produced meat with ($P < 0.05$) a higher redness (a*) value (Figure 2). These findings are in disagreement with Smith et al. (2002) who stated that wheat-based diets decreased redness (a*) value compared to maize-based diets. In contrast, the yellowness (b*) value (Figure 2) of meat from maize-based diet was ($P < 0.001$) higher than the meat from wheat-fed birds which might be because of pigments (carotene and xanthophylls) present in yellow maize.

Table 1 - Effect of wheat and maize-based diets on growth performance of broiler chickens from 10-38 days post-hatch.

Parameter	Wheat-based Diet	Maize-based Diet	SEM	*P*
Day 10-17				
Body weight(g/bird) at day 17	875	877	16.205	0.933
Average gain (g/bird/day)	68.5	70.8	1.525	0.314
Average feed intake (g/bird/day)	73.9	73.5	0.595	0.596
Feed conversion ratio (FCR)	1.07	1.04	0.0221	0.408
Day 18-24				
Body weight(g/bird) at day 24	1570.62	1573.01	19.821	0.934
Average gain (g/bird/day)	99.38	98.67	1.362	0.719
Average feed intake (g/bird/day)	162.55	157.70	3.0626	0.293
Feed conversion ratio (FCR)	1.64	1.61	0.0341	0.592
Day 25-38				
Body weight(g/bird) at day 38	3184	3155	37.916	0.600
Average gain (g/bird/day)	115	113	1.881	0.419
Average feed intake (g/bird/day)	229[a]	193[b]	4.0456	0.001
Feed conversion ratio (FCR)	1.99[a]	1.71[b]	0.0201	0.001
Mortality (%)	2.0	1.7	0.500	0.100

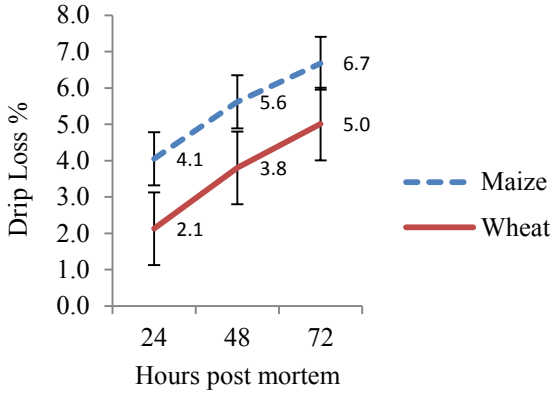

Figure 1 - Effect of wheat and maize-based diet on drip loss % of broiler chicken meat over time.

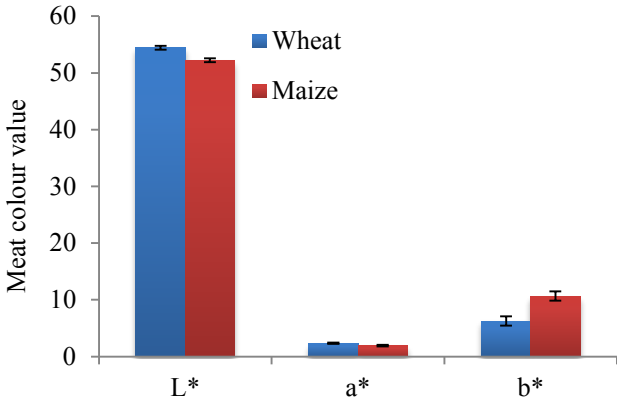

Figure 2 - Effect of wheat and maize-based diet on meat colour of broiler chicken over 72 h post slaughter; L*- Lightness, a* - Redness, b* - Yellowness.

In this study, the shear force value of breast meat was not (P > 0.05) influenced by dietary wheat or maize. These results are in disagreement with Lyon et al (2003) who got significantly higher shear force value in meat from wheat-based diet compared with maize diet. Similarly, dietary cereals had no significant effects on the pH value of breast meat. These findings are supported by Perlo et al. (2010); Osek et al. (2010); Garcia et al. (2013) who did not notice any significant effect of dietary cereals on pH value of broiler chicken meat.

Table 2 - Effect of maize and wheat-based diets on meat quality of broiler chickens.

Parameter	Wheat-based diet	Maize-based diet	sem	*P*
Shear force (N)	35.81	35.32	3.789	0.929
Initial pH 0h	6.82	6.84	0.0765	0.845
Final pH 24h	5.82	5.9	0.0259	0.068

IV. CONCLUSION

To our knowledge, there has been little research conducted to evaluate the response of meat quality to broiler diets based on maize or wheat. In this study, birds fed wheat-based diets had poorer feed efficiency and higher feed intake in the final week (from 25-38 days of age) only. Meat from maize-based diets was more yellow whereas birds offered a wheat-based diet had more red and lighter-coloured meat and a higher water holding capacity. The results of this study indicate that variations in dietary cereal use as a major carbohydrate may be related to differences in meat quality properties. Further evidence is merited exploring the evidence and explanation for variation in meat quality between diets based on these major cereals.

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THE RESPONSE OF GROWTH PERFORMANCE AND MEAT QUALITY OF BROILER CHICKENS TO AN EXPERIMENTAL MODEL OF CYCLICAL HEAT STRESS

Y. AKTER[1] C.J. O'SHEA[1], D. MOORE[2] and C. HUTCHISON[3]

Summary

Due to rapid growth rates and body weight gain, broiler chickens are sensitive to high temperature that causes heat stress. This study was undertaken to investigate the effects of cyclical high temperature on growth performance and meat quality of broiler chickens from 21 to 35 d of age. A total of 80 day old male Ross 308 broiler chicks were used for this study. All chicks were kept in metabolism cages at standard brooding temperatures until 21 days of age and then exposed to either a standard temperature (22 ± 1°C; RH 60%); or cyclical high temperature (32 ± 1°C; 8 h; 80-90% RH and 16 h at 22 ± 1°C; RH 60%);) with 8 replicate cages with 5 birds in each. Birds were offered common wheat, soybean meal-based diet containing endogenous selenium only. Average individual body weight (BW), average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (FCR) from 21-35 d, and *pectoralis* muscle shear force, colour, temperature, pH, and drip loss rate at 35 d were evaluated. Results of this study show that ADFI, ADG and BW of birds from 28-35 d of age were significantly lower in the cyclical high temperature group. The breast meat of the birds from the cyclical high temperature group had significantly higher shear force, lightness (L*) and yellowness (b*) values as well as a lower redness (a*) value compared to birds from the standard temperature group. The results of this study show that cyclical high temperature significantly reduced growth performance, increased meat shear force value and reduced meat colour, and eventually reduced meat quality. These findings provide targets to improve broiler productivity in systems affected by cyclical heat waves.

I. INTRODUCTION

Modern poultry genotypes are more sensitive to high ambient temperature because of their normally elevated body temperature, rapid metabolism and lack of sweat glands (Geraert et al. 1993). When the birds are exposed to heat stress it alters behavioural, physiological and immunological responses (Lara and Rostagno, 2013). Heat stress can alter blood pH and disrupt muscle membrane integrity, which may be associated with changes in breast muscle glycolytic metabolism, leading to changes in ultimate meat pH and quality (Sandercock et al., 2001). Meat quality traits, such as tenderness and colour, are critical aspects for consumer acceptance (Song and King, 2015); and meat producers strive to produce product with the appropriate colour to avoid appearance defects, which negatively impact on product selection and/or price (Fletcher, 2002). Enhancing the knowledge on how heat stress affects meat quality traits will help to focus on nutritional and management strategies in broiler production in tropical regions. Therefore, the aim of the present study was to investigate the effect of cyclical high temperature on growth performance and meat quality of broiler chickens from 21 to 35 d of age.

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II. MATERIALS AND METHODS

All procedures used in this study were approved by the Animal Ethics Committee of the University of Sydney. A total of 80 Ross 308 male day old broiler chickens were used for this study. All chicks were raised at standard brooding temperatures until 21 days age and then they were reared under two different temperatures: either standard temperature (ST) or a cyclical high temperature pattern (CHT). Each group consisted of 8 replicates cages containing 5 broilers each. From 21 days until 35 days of age, the broilers in ST group were raised in thermoneutral conditions (22 ± 1°C; RH 60%) while the CHT treatment was exposed to cyclical high temperature (8 h at 32 ± 1°C; RH 80-90% and 16 h at 22 ± 1°C; RH 60%). Water and feed were provided *ad libitum* to both groups. Broilers were offered a starter-grower diet from 0 to 14 days of age and a grower-finisher diet from 15 to 35 days of age. Supplementary selenium was excluded to amplify the response to cyclical high temperature. Endogenous selenium levels were 0.387 and 0.583 mg/kg diet in the starter-grower, grower-finisher diets, respectively. Body weights (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were recorded at weekly intervals. At 35 days of age, birds were euthanised. Meat quality was determined on one bird per cage. Shear force value of *Pectoralis* muscle was determined with a Warner Bratzler Shear attachment on a Stable Micro Systems TAXT2 Texture Analyser. Meat colour was determined using the CIELAB method lightness (L*), redness (a*) and yellowness (b*) for the *Pectoralis* muscle using a Minolta Lab CR-10 colourimeter. The pH was determined at slaughter and 24 h *post mortem* in the *Pectoralis* muscle using a glass electrode (TPS ionode) attached to a portable pH meter (TPS LC80A pH-mv-TEMP) which was temperature compensated. Drip loss was determined by the weight difference of suspended *Pectoralis* muscle samples stored at 4°C at 24 h post-slaughter. Data were analysed using the generalised linear model procedure of SAS. Meat colour data were analysed by repeated measures using the mixed procedure of SAS. The experimental units were pooled cage means for growth performance and one bird per cage for meat quality. Data are presented as least squared means ± standard error of the mean (SEM). Differences were considered significant at $P < 0.05$.

III. RESULTS AND DISCUSSION

The effects of cyclical high temperature on growth performance are presented in Table 1 and meat quality is presented in Figure 1 and 2 and Table 2. Although cyclical high temperature had no significant effect on bird performance from 21-28 d of age (Table 1), it significantly affected average final body weight (BW) and average daily gain (ADG) and average daily feed intake (ADFI) of broiler chickens from 28 - 35 d of age (Table 1). The average final BW, ADFI and ADG of broilers exposed to cyclical high temperature (CHT) were significantly lower than that of the standard temperature (ST) group birds. The results of this study are confirmed by Lu et al. (2007); Zhang et al. (2012) who reported that high ambient temperatures significantly affect broiler performance, especially from 28-42 d of age. The average mortality rate of this study was 9.2% throughout the entire experimental period and not affected by treatment group (data not presented).

Table 1 - Effect of cyclical high temperature on growth performance of broiler chickens from 21-35 d of age.

Parameter	ST	CHT	SEM	P-value
Day 21-28				
Bodyweight (g)	1707	1668	31.3	0.38
Average daily feed intake (g/bird)	154	147	5.16	0.35
Average daily gain (g/bird)	89.8	84.8	2.95	0.25
Feed conversion ratio	1.72	1.74	0.02	0.47
Day 28-35				
Final bodyweight (g)	2364	2169	61.7	0.04
Average daily feed intake (g/bird)	226	179	7.6	0.001
Average daily gain (g/bird)	107	83	7.7	0.04
Feed conversion ratio	2.21	2.20	0.16	0.96

ST- Standard temperature; CHT - Cyclical high temperature

In the present study the shear force value (Figure 1) was significantly higher in the CHT group which might be because of the higher *post-mortem* muscle temperature (0 h). Lee et al. (1976) reported that the shear force value in breast meat may be increased due to heat exposure which is in agreement with our results. Another important factor affecting meat shear force value is the activity of proteases (tenderising enzymes which exhibit optimal activity at approximately neutral pH). Lower (tended towards significance) muscle pH at 24 h of *post-mortem* in CHT group might have had a negative effect on proteases activity and the tenderness value in meat.

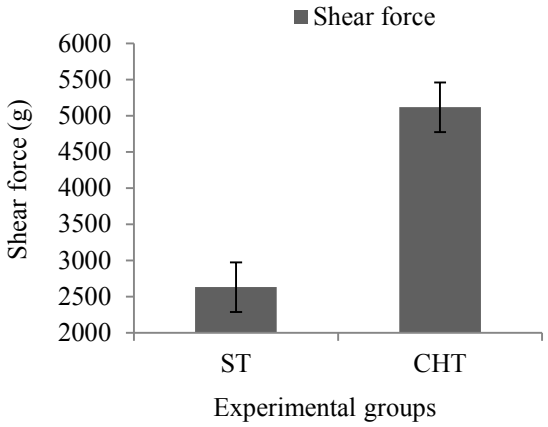

Figure 1 - Effect of cyclical high temperature on shear force value of broiler chicken meat; ST – Standard temperature; CHT - Cyclical high temperature.

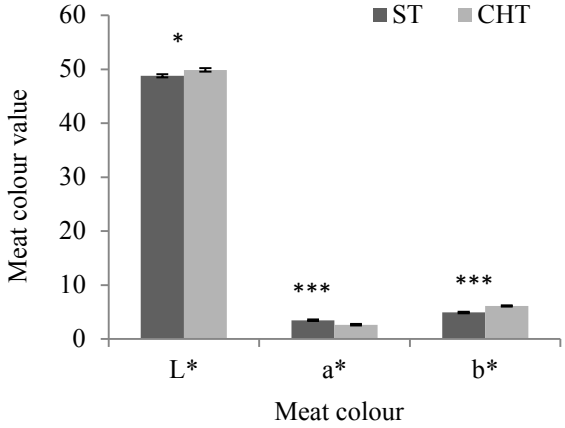

Figure 2 - Effect of cyclical high temperature on meat colour of broiler chicken; L*- Lightness, a* - Redness, b* - Yellowness; ST – Standard temperature; CHT - Cyclical high temperature.

In the present study, CHT broilers had significantly higher lightness (L*) and yellowness (b*) values, and lower redness (a*) values than the ST group (Figure 2). Higher L* values in heat-exposed broilers are confirmed by Lu et al. (2007) who reported that chronic heat stress increased the L* value in meat. Similarly, a higher yellowness (b*) value in meat in CHT group might be due to heat exposure. These findings are in agreement with Tang et al. (2013) who observed higher b* value in meat in heat-stressed broiler chickens. The results of lower redness (a*) value in meat in CHT group also indicates that there were more oxidized myoglobin in birds' exposed to the high temperature. High temperature accelerates biochemical reactions, metabolic rates and production of reactive oxygen species

(ROS) in cells. Reactive oxygen species (ROS) act to disrupt cellular components, such as proteins, DNA and lipids and generally alter cellular integrity. In such cases, antioxidants supplementation could be beneficial for maintaining cellular integrity and providing colour stability of muscular tissues (Ripoll et al., 2011). There was an effect of time on meat colour in this study. The L* and a* values of meat were decreased whereas the b* value was increased when the meat samples were stored (4 ^{0}C) up to 7 d of *post mortem* (data not presented).

Table 2 - Effect of cyclical high temperature on meat quality of broiler chickens.

Parameter	ST	CHT	SEM	P-value
Initial pH 0h	6.6	6.8	0.10	0.10
Final pH 24h	6.0	5.8	0.04	0.05
Temperature 0h (^{0}C)	31.8	39.4	0.48	0.001
Temperature 24h (^{0}C)	6.7	6.8	0.32	0.89
Drip loss rate 24h	7.2	6.1	0.72	0.34

ST- Standard temperature; CHT - Cyclical high temperature

Although the high temperature significantly increased initial (0 h) muscle temperature (Table 2) in the CHT group, it had no significant influence on 24 h muscle temperature, pH (0 h; 24 h) and drip loss rate (24 h) of meat (Table 2).

IV. CONCLUSION

Cyclical high temperature and the likely heat stress was detrimental to the growth and meat quality of broiler chickens. This study may provide some useful targets to improve the outcomes of broiler chickens exposed to heat stress.

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EFFECT OF A NOVEL CARBOHYDRASE COMPLEX ON PERFORMANCE OF BROILERS FED WHEAT-BASED DIETS WITH DIFFERENT LEVELS OF AMINO ACIDS

D. WU[1], R.M. NETO[2], P. COZANNET[2] and A. PREYNAT[2]

The use of non-starch polysaccharide (NSP) degrading enzymes in poultry diets has been shown to improve performance in birds fed wheat-based diets. These enzymes exert their performance promoting effect by degrading water soluble arabinoxylans, and releasing nutrients that are encapsulated in cell wall polysaccharides. The NSP content and nutritive level varies greatly in different raw materials. Therefore, it is of interest to see how birds respond to the addition of an NSP enzyme when they are fed diets containing different nutrient densities.

The objective of this study was to evaluate the effect of the addition of a carbohydrase complex (CC) enzyme on the performance and nutrient deposition of broiler chickens fed wheat-based diets with different digestible amino acid (DAA) levels. Feed and water were provided ad libitum. Six control diets were formulated with different DAA content (−7.5%, −5.0%, −2.5%, 0.0%, +2.5%, and +5.0%) based on Rhodimet nutrition guide (Adisseo, 2013), but with the same ideal protein profile. Energy and other nutrients were formulated to meet the recommendation of the breeder company. Six treatment diets were prepared by adding CC (Rovabio Advance L) to the 6 control diets reduced in AME (85 kcal/kg or 2.8% of average energy reduction) to isolate the energy effect. Each of the 12 treatments included 8 replicate pens of 15 male Ross PM3 birds. Performance parameters, efficacy of energy and protein deposition (calculated as g of BWG for each cal of AME or mg of dig. Lys ingested) and carcass and cuts yield were calculated for the period between 0 and 42 d. Data was subjected to General Linear Regression, taking enzyme addition effect into consideration in a co-variance analysis of the regression.

The reduction of DAA linearly compromised ($P<0.05$) all performance, nutrient deposition and carcass parameters. Carcass and cuts yield were not affected by CC addition ($P>0.05$). No interaction was found between DAA content and the addition of the CC. The addition of the CC significantly improved ($P<0.001$) BWG (+3.2%), FCR (−2.7%), and deposition of energy (+5.7%) and protein (+2.8%). In conclusion, the reduction of DAA linearly degrades growth performance, nutrient deposition and carcass yields. The addition of CC can increase growth performance and deposition of nutrients, regardless of DAA level in wheat-SBM-based diets.

Table 1 - Performance during overall experimental period (d 1-42), energy and protein deposition, and carcass yield of broilers fed diets with or without carbohydrase complex (CC).

	Performance			Efficacy of deposition		Carcass yield		
CC addtion	BWG	FI	FCR	Energy[1]	Protein[2]	Carc[3]	Breast[4]	Leg[5]
Without	2917	5111	1.754	0.185	56.69	72.13	30.05	13.51
With	3009	5129	1.706	0.196	58.27	72.69	30.72	13.46
P-values	< 0.01	0.7	< 0.01	< 0.01	< 0.01	0.62	0.12	0.86

[1] Calculated as g of BWG for each calorie of AME ingested in the same period.
[2] Calculated as g of BWG for each g of Dig. Lysine ingested in the same period.
[3] Calculated as carcass weight compared to body weight (presented in %).
[4] Calculated as entire breast weight (with muscle and bones) compared to carcass weight (in %).
[5] Calculated as one entire leg weight (with muscle and bones) compared to carcass weight (in %).

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USING SUNFLOWER MEAL TO REPLACE SOYBEAN MEAL IN BROILER DIETS WITH THE ADDITION OF A CARBOHYDRASE COMPLEX

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Sunflower seeds are extensively grown for oil production for human consumption. Therefore, a great amount of sunflower meal becomes available for use in feed industry. While sunflower meal is a good protein source for monogastric animals, their high indigestible carbohydrate contents should not be overlooked. This study aimed to evaluate the effect of the addition of a commercial enzyme complex on growth performance of broilers from d 0 to d 35 fed corn-based diets in which soybean meal (SBM, 48% CP) was partially replaced by sunflower meal HiPro (SMH, 48% CP).

Nine hundred and thirty-six d-old male broiler chicks (Arbor Acres Plus) were randomly allocated to 6 treatments according to a randomized complete block design experiment with 12 replicates/treatment and 13 chicks/replicate. Tested treatments included: (T1) regular nutritional levels (breeder's recommendation), corn-SBM; (T2) regular nutritional levels, corn-SBM-SMH, with a SBM to SMH inclusion ratio of 75/25 (d 1-14) and 50/50 (d 15-35); (T3) T2 reduced apparent metabolisable energy and digestible amino acids by 3% and calcium and phosphorus by 0.12%; (T4) T2 added with the enzyme complex (Rovabio Max Advance L, a multi-carbohydrase complex combined with a 6-phytase, 200 mL/ton); (T5) T3 added with the enzyme complex (200 mL/ton); (T6) T3 added with the enzyme complex at double dose (400 mL/ton). Feeds and water were provided ad libitum. Body weight gain, feed intake, feed conversion ratio and mortality were subjected to one-way ANOVA.

In the overall period, the partial replacement of SBM by SMH (T1 vs. T2) significantly impaired FCR (1.448 vs. 1.371, +5.6%, $P < 0.01$) without significant differences for BWG (2674 vs. 2693 g, $P > 0.05$). The reduction of nutritional levels of feed with SMH (T2 vs. T3) compromised BWG (2462 vs.2674 g, -7.9%, $P < 0.001$) and FCR (1.448 vs. 1.458, $P < 0.05$). The addition of enzyme complex at 200 mL/ton improved FCR in both diets with SMH (regular and reduced nutritional levels) by -3.2% (1.402 vs. 1.448, $P < 0.01$) and -3.0% (1.414 vs. 1.458, $P < 0.01$), respectively. BWG were improved by the enzyme complex in diets with reduced nutritional levels (2462 vs. 2667 g, +8.3%, $P < 0.001$). No significant difference in any performance parameter was observed between T5 and T6 (200 vs 400 mL/ton).

In conclusion, the replacement of SBM by SMH impaired feed efficiency. In addition, the supplementation of the evaluated commercial enzymatic complex improved performance (BWG and FCR) of broilers fed diets with SMH regardless the nutritional level, but it did not fully recover all FCR impairment caused by the meal replacement.

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EFFECTIVENESS OF A DOUBLE CHOICE TEST TO ASSESS DIETARY TASTE PREFERENCES IN BROILER CHICKENS

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Investigations into the taste system of chickens can help to improve poultry feeding strategies (Roura et al., 2013). However, a comprehensive study on dietary taste preferences for broilers is lacking. Dietary preferences are conventionally tested by presenting a choice of two diets simultaneously to individual or grouped chickens (double choice test) followed by a comparison of the intakes of both diets. Current double choice methods allow to test only a few nutrients and are carried out over relatively long periods of time. Using wheat as a delivery matrix, Cho *et al*. (2016) demonstrated that laying hens can show preferences in 1 hour tests. To validate this method in broilers, the current study aimed at optimizing the number of birds and feed withdrawal time prior to the test.

Two hundred and twenty four 21 day-old Ross-308 broilers were assigned to six experimental groups following a 2 × 3 factorial arrangement, with 2 levels of feed withdrawal (0 hr vs 2 hr) and 3 group sizes (1, 2 or 4 birds/cage). Pens were divided into 8 blocks of 12 pens each resulting in 2 replications per treatment per block. Broilers were trained for 5 days to habituate to a double choice feeding regime. Subsequently, for each pen, 4 tastants (NaCl, monosodium glutamate (MSG), citric acid (CA) and Alanine (Ala) at 3 inclusion levels (0.1%, 1% and 10%) were added to wheat and tested against a wheat only control during 10 days with two tasting sessions per day. The differences in consumption between treatment and control were tested against 0 g (implying no difference). In addition, the percentage of tastant intake relative to the total intake of the two feeders (preference) was compared with the neutral value of 50% using the t-test. To see the effect of withdrawal and number of birds per pen on the differences in intakes, the data was analysed using the GLM procedure of SAS.

Broilers preferred complete feed to wheat ($P<0.05$) regardless of group size and withdrawal. The wheat-based treatments experienced very low intakes. For instance the overall intakes of 0.1% NaCl, 0.1% MSG, 0.1% CA and 0.1% Ala were respectively 1.9±0.6 g, 1.9±0.5 g, 2.6±0.5 g and 2.2±0.6 g. Similarly the intakes of 1% inclusion level of NaCl, MSG, CA and Ala were 2.2±0.6 g, 2.0±0.6 g, 1.7±0.6 g 1.3±0.5 g. The intakes for 10% inclusion levels of NaCl, MSG, CA and Ala were 1.7±0.5, 2.4±0.5, 1.6±0.5 and 1.9±0.5 respectively in the first test. Similar intakes were observed in the second test. Based on t-tests against 50% neutral value, rejections could be detected for 0.1% CA in 1 and 2 birds/cage ($P<0.05$) in the first test. Similarly 1% and 10% Ala, 10% MSG were ($P<0.05$) rejected at the first test in the group size with 1 bird per cage and 10% NaCl was rejected by the group with 2 birds per cage in the second test. In conclusion, broilers could show their preferences in a short term double-choice model. However, the use of wheat as a delivery matrix resulted in an unexpected low intake. Group size did not influence the intakes, this was also observed for feed withdrawal period. The use of delivery systems alternative to wheat should be explored to improve double-choice tests in broiler chickens.

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AGE OF INTRODUCTION AND XYLANASE SUPPLEMENTATION ON GROSS PERFORMANCE AND NUTRIENT DIGESTIBILITY OF BROILER CHICKENS OFFERED WHOLE SORGHUM GRAIN

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The main problem with introducing whole-grain diets (WGD) to newly hatched chicks is the kernel size (Biggs *et al*., 2009) and this has led to such diets being delayed by at least 5 days and up to as many as 11-14 days (Hetland *et al*., 2002). However, several studies have offered WGD as early as hatch (Wu *et al*., 2004) and some as late as 24 days of age. The interest in increasing the size and activity of the gizzard through feeding whole grain has developed from research reports that showed improved morphology of the intestinal tract, which in return enhances nutrient digestibility and utilization. Sorghum is attractive due to its small kernel size which can be fed to young birds without difficulties in swallowing. A total of 324 day-old male broiler chickens were randomly assigned to a 3 (age of introduction) × 2 (with or without xylanase) factorial arrangement in a completely randomized design having six replicates, with nine birds per replicate. A whole sorghum inclusion level of 50 % with or without xylanase was offered to birds at hatch (HWG), 11 d (WG11) or 25 d (WG25). Feed intake (1-35 d) was numerically higher in the HWG group than in the WG11 and WG25 groups. However, final body weight was poorest ($P < 0.01$) in the WG11 group than in the two other groups. Final body weight was not improved by enzyme supplementation. Overall FCR was best ($P < 0.1$) in groups in which the introduction of WGD was delayed and without enzyme supplementation. The interactions between the two factors were not significant except for feed intake between hatch and 10 days of age. The AME of the diets was higher ($P < 0.001$) when WGD was introduced at hatch and at 11 d than when it was introduced at 25 d. The ileal digestibility of energy and CP was numerically improved by early introduction of WGD.

The results obtained in this study showed that early introduction of WGD to broiler chickens increases feed consumption but may not be beneficial for growth and feed efficiency.

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INCORPORATION OF CALCIUM PIDOLATE INTO BROILER FEED- THE EFFECT ON PERFORMANCE IN LOW DENSITY PRODUCTION SYSTEM IN TROPICAL CONDITIONS

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Summary

Musculoskeletal disorders in commercial broilers have increased in frequency, and this has impacted on the economic return as well as, the welfare of the birds. There are multi factorial causes for these disorders such as genetics, litter quality, stock density as well as nutrition. Musculoskeletal deformation as well as the associated pain can affect the ability to access resources (feeders and drinkers) especially for the more seriously affected animals (Arnoult et al, 2011).

Several studies under European conditions (stocking density of 20 birds/m^2) have shown that Calcium Pidolate improves the skeletal structure in broilers, leading to improved performance at slaughter. This experiment aimed to study the effect of Calcium Pidolate on broiler chickens in lower stocking densities of animals (12 broilers/m^2) under tropical conditions.

Calcium Pidolate was included in broiler diets from day 0-21. There were 12 replicates of 12 male Arbor Acre Plus broilers for the treatment and control groups. Quantitative parameters (weight, feed intake, FCR) were studied until day 35.

The incorporation of Calcium Pidolate significantly improved feed intake (+2.4%, $p< 0.05$) .The bodyweight gain had a tendency to be significant ($p=0.05$) at 35 days, while maintaining the same FCR.

This experiment shows that even at a low stocking density under tropical condition the incorporation of Calcium Pidolate during the 3 first weeks can provide a nutritional means to improve growth performances in broiler chickens to 35 days of age.

I. INTRODUCTION

The high frequency of musculoskeletal disorders represents a major cause of pain and discomfort in fast growing broilers in commercial farms. With multifactorial origins, those disorders provoke walking abnormalities which are probably associated with pain. These conditions affect the broiler's ability to access resources (feeders and drinkers), especially for the most seriously affected individuals (Arnoult et al, 2011, 2005 and 2007).

According to some studies in intensive farms, 75% to 90% of the birds have an altered gait, and 26% to 30% have a seriously altered gait (Kestin et al 1992, Sanotra et al 2001), which can have a negative effect on feed conversion ratio and a reduction of growth rate (Bizeray et al 2004).

Calcium Pidolate (calcium supplement and stimulator of collagen deposition, Moczar et al., 1979) has been shown to improve the skeletal structure of European broilers (stocked at 20 birds/m^2) leading to improved performance at slaughter (Roulleau et al, 2015).

The experiment reported here studied the effect of Calcium Pidolate on broiler performance in a low density context (12 birds/m^2) under tropical conditions.

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II. MATERIALS AND METHODS

Arbor Acres Plus® male chicks were weighed at Day 0 and separated randomly into groups of 12 birds. There were 2 treatments, control and Calcium Pidolate. The Control group were given a standard feed formulated according to the genetic company's recommendation. The second group received Calcium Pidolate (PIDOLin® PCa, organic calcium salt patented by DIETAXION) incorporated into feed from 0 to 21 days at 300 ppm in substitution of 300 ppm of calcium carbonate. There were 12 replicates for each treatment.

The experiment was conducted in a closed house system with tunnel ventilation and evaporative cooling system. Birds were raised on solid concrete floor pens using rice hulls as litter material. Each pen measured 1.0 m x 1.0 m and was equipped with a tubular feeder and three nipple water drinkers. Feed and water were provided *ad libitum*. Slaughter age was 35 days. All birds were vaccinated for Newcastle and Infectious Bronchitis diseases at 7 days of age and Gumboro disease at 14 days of age.

Body weight, feed intake and water intake as pen basis were measured for growth calculation at 18 and 35 days of age. Mortality was recorded daily.

The equality of variances of the data was checked with the F-TEST and variables were then subjected to a Student t-test.

III. RESULTS AND DISCUSSION

Table 1 shows that there was no significant difference between the Control and Calcium Pidolate group at day 18- the midway point of the trial. However, there was a tendency towards higher feed intake and weight gain in the Calcium Pidolate group.

Table 2 shows that Calcium Pidolate included in feed from day 0-21 significantly increased feed intake to day 35 ($p<0.05$). The incorporation of Calcium Pidolate to day 21 also significantly increased the final bodyweight at 35 days ($p<0.10$). There was no significant difference in feed conversion ratio or livability between the 2 treatments.

Table 1 - Effect of Calcium Pidolate on broiler performance at Day 18.

Calcium Pidolate	Initial body weight	Final body weight	Body weight gain	Feed intake	FCR	Livability
(g/t)	(g)	(g)	(g)	(g)	(g)	(%)
0	42	846	804	986	1.233	99.3
300	42	858	816	1004	1.233	100.0
P value, 5%		*0.3204*	*0.1451*	*0.1757*	*0.9764*	*0.3332*

Table 2 - Effect of Calcium Pidolate on broiler performance at Day 35

Calcium Pidolate	Initial body weight	Final body weight	Body weight gain	Feed intake	FCR	Livability
(g/t)	(g)	(g)	(g)	(g)	(g)	(%)
0	42	2667 [c]	2625	3850 [b]	1.497	96.5
300	42	2727 [d]	2685	3943 [a]	1.491	97.9
P value, 5%		*0.0517*	*0.0522*	*0.0372*	*0.7061*	*0.5151*
P value, 10%		*0.0690*				

[a,b] Means within column with no common superscript differ significantly ($p<0.05$).
[c,d] Means within column with no common superscript differ significantly ($p<0.1$).

IV. CONCLUSIONS

This work showed that the feed intake of the Calcium Pidolate fed birds was significantly higher than the control group ($p<0.05$). This was reflected in higher final bodyweight ($p<0.1$).

Previous work under European conditions (i.e. higher density per m² but with temperate temperature and humidity) has shown improvement of broiler performance with Calcium Pidolate occurring during the end of fattening (Roulleau et al, 2015). In comparison with these previous studies, the body weight gains observed in this work were lower, but were significant.

The feeding of calcium Pidolate (even though limited to the first 3 weeks) improved feed intake and bodyweight gain in this trial.

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INFLUENCE OF RATIO OF UNSATURATED TO SATURATED FATTY ACIDS ON THE PERFORMANCE AND, APPARENT METABOLISABLE ENERGY AND TOTAL TRACT RETENTION OF FAT IN BROILERS FED WHEAT-BASED DIETS

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Summary

An experiment was conducted to investigate the influence of unsaturated to saturated fatty acid ratio (U:S ratio) on the performance and, apparent metabolisable energy (AME) and total tract retention of fat in broilers fed wheat-based diets. Six wheat-based diets were formulated and supplemented with varying proportions of animal fat and soybean oil (animal fat: soybean oil, 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100, respectively) which corresponded to dietary U:S ratios of 1.30, 1.64, 2.10, 2.74, 3.73 and 5.43, respectively. Over the whole trial period (d1 to 35), weight gain and feed per gain were unaffected ($P > 0.05$) by dietary treatments. Feed intake tended ($P = 0.053$) to decrease linearly with increasing U:S ratios. Total tract fat retention increased linearly ($P < 0.001$) with increasing U:S ratios. A positive correlation (R^2=0.63; $P < 0.001$) was observed between the U:S ratio and AME of fat. Overall, the present data suggests that increasing the U:S ratio through the blending of animal fat with soybean oil improved the total tract retention and AME of fat, but had no effect on broiler performance.

I. INTRODUCTION

Fat is usually included in diet formulations to meet the high energy requirements of broiler chickens, as the energy value of fat is at least twice as high as those of carbohydrates and protein. However, it is known that the efficiency of utilisation of fats varies depending on the source of fat. Tancharoenrat et al. (2013) showed that the digestibility of tallow was lower than that of soybean oil due to the high concentration of long chain saturated fatty acids, which are nonpolar and thus difficult to digest and absorb (Krogdahl, 1985). It is well accepted that the digestion of saturated fats can be increased by the addition of small amounts of unsaturated fat (Muztar et al., 1981). Changes in the unsaturated to saturated fatty acid ratio (U:S ratio) through the blending of saturated fats with unsaturated fats has been shown to increase the digestibility and AME of blended fats (Wiseman and Lessire, 1987; Tancharoenrat et al., 2013), which sometimes may be synergistic (Lall and Slinger, 1973; Muztar et al., 1981). Manipulation of U:S ratios, through blending of saturated and unsaturated fats, may be a potential strategy to improve the utilisation of fat in broiler diets. However, studies evaluating the effect of U:S ratios have used wheat-maize-based (Wiseman and Lessire, 1987) or rye-based (Danicke et al., 2000) diets. These researchers reported that increasing U:S ratios increased the digestibility of fat. No studies to date have examined the effect of U:S ratios in birds fed wheat-based diets. Hence, the objective of the present study was to determine the effect of U:S ratios on the performance and AME and retention of fat in broilers fed wheat-based diets.

II. MATERIALS AND METHODS

Six diets, based on wheat and soybean meal, were formulated to meet the Ross 308 strain

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recommendations for the major nutrients (Ross, 2007; Table 8.1). The experimental diets were formulated to contain 60 g/kg fat, using different proportions of animal fat and soybean oil (animal fat: soybean oil, 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100, respectively) which corresponded to dietary U:S ratios of 1.30, 1.64, 2.10, 2.74, 3.73 and 5.43, respectively. Each diet was fed to six replications of 8 birds each from day 1 to 35 post hatching in mash form. The animal fat used in this experiment was a mixture of 850 g/kg sheep and beef tallow with 150 g/kg lard. All diets were supplemented with a commercial xylanase (Avizyme®, Danisco Animal Nutrition, Marlborough, UK) as per standard commercial practice. All diets were formulated to be isocaloric and isonitrogenous. The calculated differences in the AME between animal fat and soybean oil were overcome by the inclusion of cellulose and maize starch. Body weight and feed intake (FI) were recorded on cage basis at d 1 and 35. From d 17 to 20 post-hatch, FI and excreta output was measured quantitatively per cage for the determination of AME and total tract retention of fat.

III. RESULTS AND DISCUSSION

Over the whole trial period, weight gain and feed per gain were unaffected by the U:S ratio (Table 1). These findings are in accordance with those of Wongsuthavas et al. (2007) who fed broilers diets with different U:S ratio using blends of tallow with soybean oil. These researchers reported that weight gain, feed intake and feed per gain of broilers were unaffected by dietary treatments. Based on these observations, it may be concluded that the U:S ratios has no effect during the grower period as the physiological ability of broilers to utilise fat is fully developed.

The present results showed that increasing the U:S ratio increased ($P < 0.001$) total tract retention of fat (Table 1). Increasing the U:S ratio from 1.30 to 1.64, 2.10, 2.74, 3.73 improved the retention by 3.8, 4.1, 6.3 and 16.7%, respectively. The observed improvements in the fat retention may be reflective of increasing concentrations of unsaturated fatty acids in the blend. It is known that the utilisation of saturated fatty acids is improved in the presence of high concentrations of unsaturated fatty acids (Garrett and Young 1975). Similarly, Danicke et al. (2000) reported that total tract fat retention of blends of tallow and soybean oil increased with increasing proportions of soybean oil. The absorption of long chain saturated fatty acids is limited by their incorporation rate into micelles (Friedman and Nylund, 1980). Saturated fatty acids are also less rapidly incorporated into micelles because of their non-polarity, which makes them rely on the presence of adequate amount of bile salts and unsaturated fatty acids for efficient emulsification (Polin et al., 1980; Danicke, 2001). Increasing concentration of unsaturated fatty acids will increase the formation of mixed micelles and absorption of saturated fatty acids (Scheele et al., 1997). In the current study, a linear positive correlation was observed between U:S ratio and AME of fat in birds fed wheat-based diets, with the AME of fat increasing as the U:S ratio increased (Table 2).

Wiseman and Lessire (1987) evaluated the linear regression for blends two fat sources. These researchers observed improvements in the AME of fat when broilers were fed wheat-maize-based diets supplemented with five ratios of tallow and rape oil (100:0, 95:5, 90:10, 80:20 and 0:100). Although the fat sources and proportion of blending of fat were different, the regression equation obtained in the present study (Figure 1) showed similar trends as that reported by Wiseman and Lessire (1987). The regression equation generated in the present study may be used, based on the U:S ratio, to predict the AME of blends of animal fat and soybean oil in wheat-based diets. However, it is noteworthy that the equation reported in the current study may not be valid to predict the AME of blends of fat sources other than animal fat and soybean oil. Factors such as content of free fatty acids, chain length, position of fatty acids, quality of fat and age of birds should also be considered to accurately

predict the AME of fat blends. Overall, the present data suggest that the manipulation of the U:S ratio, through blending of fat sources, may be a potential approach to improve the utilisation of fat.

Table 1 - Influence of U:S ratio on the weight gain (g/bird), feed intake (g/bird), feed per gain (g feed /g gain), and total tract fat retention coefficient of broilers[1].

	Performance (d 1 to 35)			
U:S ratios	Weight gain	Feed intake	Feed per gain	Fat retention
1.30	2409	3483	1.464	0.607
1.64	2486	3587	1.447	0.630
2.10	2407	3373	1.432	0.632
2.74	2424	3465	1.429	0.645
3.73	2414	3399	1.444	0.708
5.43	2425	3420	1.436	0.744
SEM[2]	37.7	46.9	0.0109	0.0152
Probability				
Linear	NS	0.053	NS	***
Quadratic	NS	NS	NS	*

NS, not significant; * P < 0.05; *** P < 0.001
[1]Each value represents the mean of six replicates.
[2] Pooled standard error of mean.

Table 2 - Influence of the U:S ratio on apparent metabolisable energy of the fat[1] (MJ/kg).

U:S ratios	Gross energy	AME of fat[2]
1.30	39.17	23.77
1.64	39.12	24.67
2.10	39.07	24.69
2.74	39.02	25.21
3.73	38.97	27.60
5.43	38.92	28.98

[1]Calculated by multiplying total tract retention of fat with gross energy of fat (MJ/kg).
[2] Each value represents the mean of six replicates.

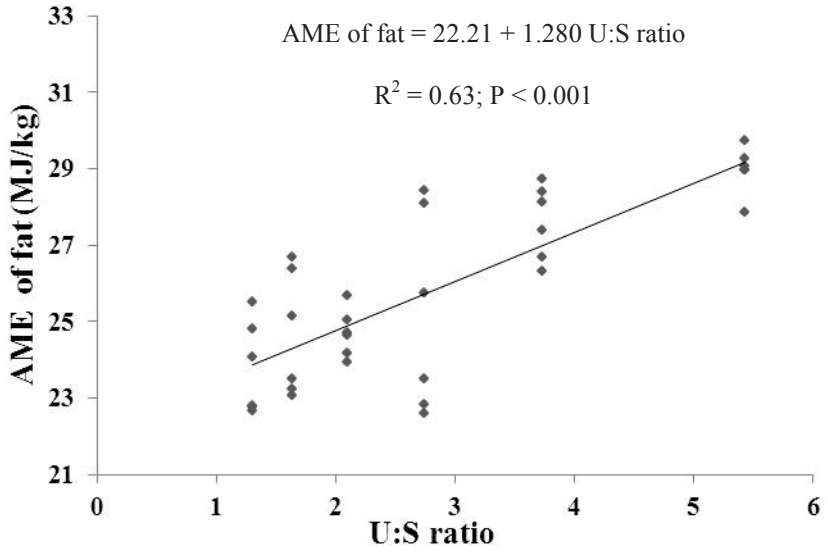

Figure 1 - Relationship between unsaturated to saturated fatty acid ratio (U:S ratio) and apparent metabolisable energy of fat blends.

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DIET COMPOSITION AND POLYPHENOLS IMPROVES PERFORMANCE IN HEAT STRESSED BROILERS

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Summary

The dietary supplementation of polyphenols show varying response depending on the diet composition. The present study was carried out to assess the impact of polyphenols and diet composition on the performance of heat stressed broilers. Polyphenols were extracted from pomegranate peels (PPE). A total of two hundred and forty day-old coloured broiler chicks were randomly divided into six groups, 1: fed corn soybean meal diet without PPE supplementation, 2: fed corn-soybean meal supplemented with 50 ppm PPE, 3: fed corn-soybean meal supplemented with 100 ppm PPE, 4: fed rice-sorghum-soybean meal based diet without PPE, 5: fed rice-sorghum-soybean meal based diet with 50 ppm PPE and 6: fed rice-sorghum-soybean meal based diet with 100 ppm PPE. The birds were maintained under standard management conditions for six week in a hot humid environment (Temp: 29-36°C; RH: 69-80%). Fortnightly body weight and feed intake were recorded and feed efficiency was determined. The body weight gain was significantly ($P < 0.05$) higher in rice-sorghum based diet than the corn diet. Supplementation of polyphenols significantly increased body weight gain compared to the unsupplemented group with more prominent results with rice-sorghum based diets. No significant ($P > 0.05$) difference was observed in feed intake and feed efficiency except in the starter phase. It could be concluded that supplementation with 50 ppm PPE improved broiler's performance in rice-sorghum-soybean meal based diet under hot-humid environment conditions.

I. INTRODUCTION

Use of active compounds of phytogenic origin has received extensive attention due to their wide spectrum of functions. The nutritional significance of polyphenolic compounds has increased in recent years due to their antioxidant activity (Scalbert and Williamson, 2000). These polyphenols are concentrated on the pericarp of fruit or seed and act as outer protective layers which possess various biological activities including anti-oxidant properties, anti-inflammatory activity and potential to modify gut microflora. Similarly, polyphenols derived from *Punica granatum* possess anti-oxidant, anti-inflammatory and anti-carcinogenic properties and can also promote wound healing. They can also act as potent inhibitors of pathogenic organism including *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella enteritidis* (Al-Zoreky, 2009). Poultry diets are formulated according to the bird's physiological stage and availability of raw materials on a least cost basis. Similarly, gut development, microbial community and function will be modulated by the composition of the diet and this inturn affects the bird's performance. Manach *et al.* (2004) reported that in addition to their diversity, polyphenols associated with various carbohydrates and organic acids can interact which affects their biological activities. The response of birds to any additives of phytogenic origin cannot be expected to be the same for different feed formulations and could be a reason for the diverse results in the literature (Cao *et al.,* 2005; Chamorro *et al*., 2013; Kristina *et al.,* 2014). The effects of the diet composition on the bioavailability of polyphenols are not clear as it involves two interactions; directly between

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polyphenols and components of the diet, such as binding to proteins and polysaccharides and indirectly, by the alterations of diet on gut physiology, pH, intestinal fermentations, biliary excretion and transit time. In the current study, the performance of broilers fed diets using different cereal grain sources supplemented with different amounts of polyphenols was assessed.

II. MATERIALS AND METHODS

A total of 240-day old coloured broiler (CARIBRO DHANARAJA) chicks were randomly allocated to six treatment groups and reared for 42 days under standard management practices in a hot-humid environment (Temp: 29-36°C; RH: 69-80%). The first group was fed the corn-soybean meal diet with no polyphenol supplementation. A second group was fed the corn-soybean meal diet supplemented with 50 ppm of pomegranate peel extract (PPE), while a third group was fed the corn-soybean meal diet supplemented with 100 ppm of PPE. The fourth group was fed a rice-sorghum-soybean meal based diet with no added PPE, with the fifth and sixth groups fed the rice-sorghum-soybean meal based diet supplemented with 50 ppm and 100 ppm PPE, respectively. The polyphenols were extracted from pomegranate peels as per the method of Althunibat *et al.* (2010) using methanol as the solvent. The total phenolic content (TPC) of the extracts was determined by the colorimetric method of Cam *et al.* (2010). The birds were fed with pre-starter, starter and finisher diets formulated as per ICAR (2013) recommendations (Table 1). Fortnightly, body weight and feed intake were recorded and from these feed efficiency determined. All mortalities were recorded. The data were subjected to 2 by 3 factorial analysis using SPSS version 20.0 to assess the treatment effects. Where effects were significant individual treatment differences were compared using Tukey's range test. Significance was determined at $P < 0.05$.

III. RESULTS AND DISCUSSION

The body weight and weight gain showed significant ($P < 0.05$) difference due to dietary treatments (Table 2). Higher body weight and weight gain was observed in group 5 which were fed with 50 ppm PPE supplemented rice-sorghum-soybean meal diet. The treatment groups (2, 3 and 5) supplemented with PPE (at both levels) had significantly higher body weight and weight gain than the respective unsupplemented groups. Similar to the present findings Masek *et al.* (2014) fed a combination of phenolic compounds in a corn-soybean meal based diet to broilers and observed significantly higher final body weight, body weight gain and feed utilization in the tannic acid supplemented groups compared to the control and thymol and gallic acid groups. The tannic acid's ability to significantly reduce the cecal pH was attributed to be the reason for the differences in growth performance. Even though, the present findings showed a similar trend a more pronounced effect was observed with rice-sorghum based diet. The improved performance in the rice-sorghum supplemented diet could be due to; the rice having higher starch and lower fibre content, that stimulate relatively higher glucose and insulin postprandial responses and which might increase the bird's feed intake and in turn the weight gain (Vicente *et al.*, 2008). The higher anti-oxidant activity due to the higher phenolic content in sorghum grains could exhibit an additive effect with the supplemented polyphenols compared with the pigment corn grain which exhibits only moderate activity (Guajardo-Flores *et al.,* 2006). Hosseini-Vashan *et al.* (2016) reported that feeding dried tomato pomace reduced the detrimental effects of heat stress in broilers due to its anti-oxidant property. The supplementation of 50 ppm PPE showed better growth performance than the control and 100 ppm in the rice-sorghum diet.

Differences in feed intake and feed efficiency were not significant except in the starter phase where PPE supplementation improved feed efficiency except for treatment 6 (Table 3).

Similar to the present findings, Cao *et al.* (2005) and Brenes *et al.* (2010) observed no significant ($P > 0.05$) effect on the feed intake and feed efficiency in broilers fed grape pomace concentrate and green tea polyphenols, respectively. Since the added polyphenols did not contain protein and metabolizable energy and supplemented at very low concentrations, could be reasons for there being no effect on feed utilization in the present study.

Table 1 – Ingredient and chemical composition of the basal diets.

Corn-Soybean meal diet				Rice-Sorghum-Soybean meal diet			
Ingredients (%)	Pre-starter	Starter	Finisher	Ingredients (%)	Pre-starter	Starter	Finisher
Maize	54.60	54.20	57.62	Broken Rice	34.29	34.61	36.01
Soybean meal	39.58	37.80	32.58	Sorghum	21	21.9	19
Rice bran oil	02.12	04.24	05.86	Soybean meal	37.8	35.7	35
Calcite	01.54	01.52	01.73	Calcite	1	1	1.8
Di-Cal Phosphate	0.90	0.95	1.10	Di-Cal Phosphate	1.7	1.5	1.3
Salt	0.18	0.18	0.18	Rice bran Oil	3.7	4.8	6.6
Lysine	0.30	0.15	0.17	Methionine	0.269	0.262	0.194
Methionine	0.30	0.28	0.27	Lysine	0.18	0.161	0.033
Phytase	0.015	0.15	0.15	Phytase	0.015	0.015	0.015
B-Complex vitamins	0.015	0.15	0.15	Salt	0.018	0.018	0.018
Vitamin AD_3EK	0.014	0.14	0.14	B-Complex vitamins	0.015	0.015	0.015
Coccidiostat	0.01	0.01	0.01	Vitamin AD_3EK	0.014	0.014	0.014
Toxin binder	0.05	0.05	0.05	Coccidiostat	0.01	0.01	0.01
Total	100	100	100	Toxin binder	0.05	0.05	0.05
				Total	100.00	100.00	100.00
Nutrient composition (%)							
Crude protein	22.65	21.65	19.70	Crude protein	22.45	21.58	19.80
ME (kcal/kg)	3000	3125	3250	ME (kcal/kg)	3000	3120	3247
Calcium	0.96	0.95	0.90	Calcium	0.96	0.94	0.92
Available Phosphorus*	0.45	0.46	0.46	Available Phosphorus*	0.45	0.46	0.44
Lysine*	1.42	1.25	1.14	Lysine*	1.42	1.25	1.10
Methionine*	0.62	0.59	0.55	Methionine*	0.64	0.59	0.52

*Calculated values.

Table 2 - Body weight and weight gain in broilers fed different cereals sources and polyphenols.

Cereal source	PPE level (ppm)	Overall weight	Pre-starter	Starter gain	Finisher gain	Overall gain
Corn	0	1736±17 ^{c}	327±4	599±13 ^{b}	799±12 ^{c}	1695±18 ^{c}
	50	1827±18 ^{b}	313±5	669±12 ^{a}	841±16 ^{b}	1787±16 ^{b}
	100	1846±22 ^{b}	314±7	671±16 ^{a}	864±15 ^{b}	1804±19 ^{b}
Rice-Sorghum	0	1834±20 ^{b}	311±5	641±15 ^{b}	820±16 ^{bc}	1793±19 ^{b}
	50	1900±24 ^{a}	311±6	697±15 ^{a}	911±18 ^{a}	1858±17 ^{a}
	100	1828±23 ^{b}	312±6	699±17 ^{a}	830±17 ^{b}	1786±20 ^{b}
P-value		0.001	0.107	0.001	0.001	0.001
Effect of cereal sources						
Corn		1803±11 ^{b}	319±3	647±9 ^{b}	834±9 ^{b}	1768±12 ^{b}
Rice-Sorghum		1854±13 ^{a}	312±3	699±10 ^{a}	854±9 ^{a}	1813±12 ^{a}
P-value		0.003	0.154	0.001	0.044	0.004
Effect of PPE levels (ppm)						
	0	1823±5	321±4	639±11 ^{b}	831±5 ^{b}	1787±4 ^{b}
	50	1830±4	313±4	696±11 ^{a}	857±6 ^{a}	1798±5 ^{a}
	100	1837±5	313±4	683±11 ^{a}	847±6 ^{a}	1800±7 ^{a}

P-value	0.098	0.298	0.001	0.041	0.024

Table 3 - Feed efficiency in broilers fed different cereals sources and polyphenols.

Cereal source	PPE level (ppm)	Pre-starter	Starter	Finisher	Overall period
Corn	0	1.46±0.03	2.12±0.06 [a]	2.34±0.37	2.32±0.19
	50	1.42±0.07	1.67±0.05 [b]	2.17±0.08	1.86±0.04
	100	1.53±0.04	1.75±0.07 [b]	2.20±0.15	1.95±0.11
Rice-Sorghum	0	1.55±0.14	1.60±0.08 [b]	2.17±0.28	1.98±0.22
	50	1.57±0.13	1.61±0.08 [b]	2.26±0.21	1.88±0.13
	100	1.56±0.09	1.77±0.13 [ab]	2.42±0.26	2.11±0.16
P-value		0.807	0.001	0.481	0.299
Effect of cereal sources					
Corn		1.47±0.03	1.85±0.06 [a]	2.37±0.14	2.04±0.09
Rice-Sorghum		1.56±0.07	1.66±0.06 [b]	2.31±0.14	1.99±0.10
P-value		0.215	0.035	0.776	0.665
Effect of PPE levels (ppm)					
	0	1.51±0.07	1.86±0.06 [a]	2.45±0.17	2.05±0.11
	50	1.49±0.07	1.64±0.06 [b]	2.22±0.17	1.87±0.11
	100	1.55±0.07	1.76±0.06 [ab]	2.36±0.17	2.03±0.11
P-value		0.839	0.040	0.621	0.200

IV. CONCLUSION

Supplementation of polyphenols as 50 ppm pomegranate peel extract improved the performance of broilers maintained under hot-humid environment conditions.

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SYNTHETIC CAROTENOIDS ALTERS PERFORMANCE AND BLOOD BIOCHEMICAL PROFILES IN HEAT STRESSED BROILERS

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Summary

One hundred and sixty day old broiler (CARIBRO-Vishal) chicks were allocated to five treatments with four replicates, a control treatment and 4 treatments supplemented with a combination of two synthetic carotenoids (Canthaxanthine (CAN) and Apocarotenoid (APO). Broiler chicks were fed with a control basal diet, the control diets and CAN and APO both at 25 mg/kg (T1), the control diet and CAN 50 mg/kg and APO 25 mg/kg (T2), the control diets and CAN 25 mg/kg and APO 50 mg/kg (T3) and the control diet with CAN 50 mg/kg and APO 50 mg/kg (T4). Fortnightly body weight and feed intake were recorded and feed efficiency determined. Blood was collected and shank colour determined at day 42 of age. The overall body weight and finisher phase body weight gain was significantly higher ($P < 0.05$) in T1 when compared to all other dietary treatment groups. The overall feed conversion efficiency was significantly ($P < 0.05$) better in T1 and T2 supplemented groups than the other groups. Dietary inclusion of synthetic carotenoids significantly ($P < 0.05$) altered the concentrations of AST, phosphorus, triglycerides, total protein and total cholesterol. The shank colour showed significant ($P < 0.01$) improvement due to supplementation of synthetic carotenoids. In conclusion, supplementation of canthaxanthine and apocarotenoids each at 25 mg/kg improved the performance of broilers under hot-dry season.

I. INTRODUCTION

There are more than 750 naturally occurring carotenoid pigments mostly found in plants, algae and some micro-organisms (Britton *et al.,* 2004). Carotenoids have antioxidant, pigmenting, immunomodulatory and pro-vitaminic activities (Surai *et al.,* 2003). Carotenoids had the activities of potent lipid soluble anti oxidant and free radical scavangers (Rengel *et al.,* 2000). Colour of chickens greatly influences the consumer's preference (Han *et al.,* 1987; Fletcher, 1999). Moreover, peoples perceive that brightly red coloured meat has more nutritive value. Corn, a common ingredient of chicken feed, is the major source of carotenoids responsible for pigmenting egg-yolks and meat. Carotenoids accumulate mainly in the liver, skin and leg shanks of poultry (Allen, 1988). Since carotenoids are not synthesised by chickens, they need to be supplied in feed for pigmentation to occur (Dua *et al*., 1967). Studies have shown that carotenoids had synergistic effects when supplied in combination. Most of the experiments using pigments have used extractions of phytogenic origin which contain other compounds in addition to the pigments. In the present study, the effect of feeding a combination of relatively cheap synthetic Canthanxanthine (CAN) and Apocarotenoid (APO) pigments on broiler performance and carcass appearance was investigated.

II. MATERIALS AND METHODS

A total of one hundred and sixty day old (CARIBRO-Vishal) broiler chicks were allocated to five treatments. Each treatment consisted of four replicates of 8 birds in each and reared for

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42 days during the hot-dry summer season (Temperature 35.4°C; RH 46.94%) using standard management practices. The control group was fed a basal pre-starter, starter and finisher diet without any carotenoid supplementation. Broiler chicks were fed with a control basal diet, the control diets and CAN and APO both at 25 mg/kg (T1), the control diet and CAN 50 mg/kg and APO 25 mg/kg (T2), the control diets and CAN 25 mg/kg and APO 50 mg/kg (T3) and the control diet with CAN 50 mg/kg and APO 50 mg/kg (T4). The pre-starter, starter and finisher diets where formulated as per ICAR (2013) nutritional recommendations (Table 1). Fortnightly, body weight and feed intake were recorded and the feed efficiency was calculated. The mortality was recorded. Blood samples were collected from a wing vein at 42 days of age for blood plasma biochemical estimation using Span diagnostic kit, Surat, India. The shank colour was measured by using a DSM broiler colour fan. The collected data were subjected to one-way ANOVA using SPSS version 20.0. The means were compared for its significance ($P < 0.05$) using Tukey's range test.

Table 1 - Ingredient and chemical composition of the basal diets.

Ingredients (%)	Pre-starter	Starter	Finisher
Maize	54.60	54.20	57.62
Soybean	39.58	37.80	32.58
Rice bran oil	02.12	04.24	05.86
Calcite	01.54	01.52	01.73
Di-Calcium Phosphate	0.90	0.95	1.10
Salt	0.18	0.18	0.18
Lysine	0.30	0.15	0.17
Methionine	0.30	0.28	0.27
Phytase	0.015	0.15	0.15
B-Complex vitamins	0.015	0.15	0.15
Vitamin AD_3EK	0.014	0.14	0.14
Coccidiostat	0.01	0.01	0.01
Toxin binder	0.05	0.05	0.05
Total	100	100	100
Nutrient composition (%)			
Crude protein	22.65	21.65	19.70
ME (kcal/kg)	3000	3125	3250
Calcium	0.96	0.95	0.90
Available Phosphorus*	0.45	0.46	0.46
Lysine*	1.42	1.25	1.14
Methionine*	0.62	0.59	0.55

*Calculated values

III. RESULT AND DISCUSSION

The final body weight and finisher phase body weight gain was significantly higher for the T1 treatment compared to other dietary treatment groups (Table 2). However, no significant difference was observed in body weight and body weight gain during the pre-starter and starter phases. Studies by European Food Safety Authority (2014) and Rosa *et al.* (2012) observed no significant difference in body weight or body weight gain during a 0-6 weeks feeding period when supplementation was with 60 mg canthaxanthine/kg. However, in this present study, the higher body weight gain and finisher body weight observed could be due to supplementation of synthetic carotenoids helping to alleviate heat stress effects through their anti-oxidant properties. The effect of heat stress will be more serve during the finisher phase when broilers are at their heaviest.

Table 2 - Body weight and body weight gain in broilers fed with combination of synthetic carotenoids.

Group	Hatch weight	Body weight (g)			Body weight gain (g)			
		Pre-starter	Starter	Finisher	Pre-Starter	Starter	Finisher	Overall
Control	44 ±1	386 ±5	826 ±16	1650^{b} ±32	342± 5	440±15	824^{b} ±24	1606^{b}±41
T1	46 ±2	380 ±7	846±21	1725^{a} ±36	334 ±16	467±14	879^{a} ±21	1679^{a}±37
T2	45 ±1	372 ±7	798±25	1617^{b} ±60	327±7	426±13	819^{b} ±28	1573^{b}±39
T3	44 ±1	363 ±5	794±15	1644^{b} ±49	320 ±5	431±17	849^{b} ±16	1600^{b}±42
T4	43 ±1	376 ±6	846±16	1654^{b} ±56	334 ±6	469±13	808^{b} ±30	1612^{b}±32
P-Value	0.300	0.066	0.169	0.051	0.172	0.331	0.015	0.012

^{a,b} Means within column with different superscript differ significantly ($P < 0.05$)

The feed intake was not different among the treatment at all the three growth phases and over the full production periods. The feed conversion efficiency (feed/ gain) was significant different during the pre-starter ($P < 0.01$) and overall treatment period ($P < 0.05$) (Table 3). During the pre-starter phase, the feed efficiency was better in all other treatment groups other than the T3 treatment. During the entire growth period supplementation with the carotenoid combination in the T1 and T2 treatments significantly improved the feed efficiency, compared to the control treatment ($P < 0.05$), but this was not the case with the combinations used in the T3 and T4 treatments which were similar to the control treatment. . The feed efficiency did not different significantly during starter and finisher phases ($P > 0.05$). Our present findings agreed with observations of EFSA (2014) who observed a no-significant difference in feed efficiency in broiler fed with different levels of canthaxanthine.

Table 3 - Feed efficiency in broilers fed with combination of synthetic carotenoids.

Group	Pre-starter	Starter	Finisher	Overall
Control	1.19^{b}±0.02	1.66±0.03	1.95±0.10	1.91^{a}±0.08
T1	1.11^{b}±0.04	1.60±0.03	1.98±0.07	1.82^{b}±0.08
T2	1.17^{b}±0.07	1.72±0.04	1.97±0.07	1.86^{b}±0.09
T3	1.37^{a}±0.09	1.61±0.08	2.01±0.02	1.88^{ab}±0.05
T4	1.23^{b}±0.04	1.65±0.05	1.98±0.01	1.92^{a}±0.07
P-value	0.005	0.464	0.136	0.036

^{a,b} Means within column with different superscript differ significantly ($P < 0.05$)

The blood plasma biochemical measures and leg shank colour are presented in the Table 4. The total cholesterol concentration was significantly higher in T2 treatment. Compared to other treatment groups. The plasma triglycerides concentration was significantly lower with carotenoid. The total protein and aspartate transaminase was significantly ($P < 0.01$) higher in T3 and T4 treatments, respectively. The shank colour was significantly more intense ($P < 0.05$) with the combination of canthoxanthine and apocarotenoids supplementation. In contrast to our present findings, EFSA (2014) reported that feeding of canthaxanthine at 200mg/kg of feed reduced the total protein and increased cholesterol and triglycerides concentration. Feeding of canthxathine at higher dose rate (60mg/kg of feed) resulted in significantly higher shank colour compared to unsupplemented treatment group (Rajput *et al.,* 2012).

Table 4 - Blood biochemical profile of broilers fed with combination of synthetic carotenoids.

Treatment	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	Total protein (g/dl)	Phosphorus (mg/dl)	AST (IU/L)	Shank colour
Control	165^{b}±10	165^{a} ±14	4.90^{b} ±0.10	5.93^{ab} ±0.36	169^{bc}±11	104.00^{c}±0.00
T1	165^{b} ±5	110^{b} ±2	4.93^{b} ±0.31	5.61^{b} ±0.64	122^{d}±20	105.00^{a}±0.00
T2	204^{a}±12	116^{b} ±3	5.42^{ab} ±0.20	6.05^{ab} ±0.43	160^{c}±5	104.33^{bc}±0.17
T3	159^{b}±10	113^{b} ±2	5.80^{a} ±0.18	5.10^{b} ±0.11	197^{b}±16	104.67^{ab}±0.17
T4	173^{b}±11	104^{b} ±2	5.13^{b} ±0.40	7.46^{a} ±1.26	287^{a}±8	104.67^{ab}±0.17
P-value	0.00	0.00	0.00	0.00	0.00	0.00

^{a,b,c,d} Means within column with different superscript differ significantly ($P < 0.05$)

IV. CONCLUSION

The supplementation of synthetic carotenoids canthaxanthine and apocarotenoids in combination at 25 mg/kg feed improved the performance and shank colour of broilers under hot-dry summer season.

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EFFECT OF DIFFERENT SEMEN DILUTORS ON FERTILIZING CAPACITY OF CHICKEN SEMEN STORED FOR 24 H

J. MOHAN[1], S.K. SHARMA[1], G. KOLLURI[1], M. GOPI[1], J.S. TYAGI[1] and J.M. KATARIA[1]

Summary

Sixty healthy adult males and females from the same hatch of White Leghorn (WL) chickens were taken randomly and maintained in individual cages under uniform husbandry conditions. Under this study, 2 experiments were carried out. In the first experiment, fertilizing capacity of chicken semen stored for 24 h was examined using CARI poultry semen diluent at different storage temperatures (3, 7 and 11°C). Good quality pooled semen samples was collected and diluted 1:2 with CARI poultry semen diluent. Immediately after dilution samples were divided equally into three aliquots and stored in 5 ml round bottom glass tubes (length=7cm, diameter=1 cm) at different storage temperatures (3±1, 7±1 and 11±1^{0}C) for 24 h before insemination of hens. Artificial insemination of stored semen was carried out using 70-100 million sperm per hen. The results of this study indicated that using the 24 h-stored semen, high fertility (89.6±0.79%) was achieved at 7±1°C followed by 11±1°C (54.5±2.01%) and 3±1°C (49.1±1.66%) in WL hens in the 5 d after insemination. Overall, the pattern of fertility after 10 d was also high (79.2±1.08%) in 7±1°C stored semen in comparison to 11±1°C (36.2±2.72%) and 3±1°C (35.0±1.47%).Our studies indicate that chicken semen can be stored for 24 h at 7°C using CARI poultry semen diluent and will express good fertility similar to the freshly ejaculated semen (90.0±1.58% fertility). In the second experiment, effect of commonly used diluents (i.e. BPSE and Lake's) was compared with CARI poultry semen diluent on the fertilizing capacity of spermatozoa. After the first 5 d of fertile period maximum fertility (84.3±2.02%) was noticed using CARI poultry semen diluent, followed by Lake's (70.2±2.77%) and BPSE (58.2±3.37%). The same pattern was found after 10 d. Overall, during the 10 d after insemination, CARI poultry semen diluent expressed superior fertility (69.1±2.62%) of chicken spermatozoa over the others. From this study it can be concluded that CARI poultry semen diluent expressed nearly 90% fertility at 7°C during 24 h storage, equivalent to the freshly ejaculated semen. Further, it was concluded that CARI poultry semen diluent is superior to other commonly used poultry semen diluents like BPSE and Lake's.

I. INTRODUCTION

Poultry semen is highly concentrated, containing 6 (roosters) to 12 (toms) billion spermatozoa /ml (Donoghue and Wishart, 2000). The risk of sperm death by dehydration at room temperature is likely in highly concentrated low volume semen unless it is diluted. Hence, the dilution of semen is needed to utilize a low volume of highly concentrated chicken/poultry semen from a valuable or proven sire for inseminating a greater number of females through the application of artificial insemination (A.I.). In this way the diluent will increase semen volume and thereby permit the uniform distribution of spermatozoa in media resulting in improved fertility by A.I. in hens. In addition, the diluent prolongs the sperm survival of semen *in vitro*. Using the poultry semen diluent, the services of superior males can be used maximally by A.I. technique. Without diluent successful application of A.I. is not possible. In past several workers have developed various semen diluents to preserve the

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fertilizing capacity of chicken semen for up to 24 h at low temperature with varying pattern of fertility (Lake, 1960; Van Wambeke, 1967; Sexton, 1977; Sexton and Fewlass, 1978, Lake and Raive, 1979, Tselutin *et al.*1995).Limited success was achieved with sustaining good sperm fertility after 24 h storage. Therefore, we have made the attempts to develop CARI poultry semen diluent that preserve the fertility of chicken semen for 24 h at low temperature comparable with freshly ejaculated semen. Further, attempts have also been made to compare the fertilizing ability of CARI poultry semen diluent with world famous poultry semen diluents (BPSE and Lake's).

II. MATERIALS AND METHODS

Sixty healthy adult males and females from the same hatch of White Leghorn (WL) were taken randomly and maintained in individual cages under uniform husbandry conditions. Two experiments were carried out under this study. In the first experiment, effect of CARI poultry semen diluent (Mohan *et al*. 2015) was observed on fertilizing ability of 24 h-stored chicken spermatozoa under various storage temperatures (3, 7 and 11°C).Good quality semen samples were collected (Burrows and Quinn, 1937), pooled and diluted 1:2 with CARI poultry semen diluent. Immediately after dilution samples were divided equally into three aliquots and stored in 5 ml capacity round bottom glass tubes (length=7cm, diameter=1cm) at different storage temperatures (3±1, 7±1 and 11±1°C) for 24 h before insemination of hens. A.I. was carried out by intravaginal insemination (70-100 million sperm/hen) using A.I. gun (IMV, France) in three different groups containing 20 hens in each. No further inseminations were given and fertile eggs were collected for the following 10 d.

In the second experiment, we have compared the fertilizing ability of CARI poultry semen diluent with commonly used poultry semen extenders like BPSE (Sexton, 1977) and Lake's (Lake, 1960). This experiment was carried out using the same number of males and females under similar experimental conditions to the first experiment. Pooled semen of WL was diluted with various semen dilutors such as CARI poultry semen diluent (Mohan *et al*. 2015), Beltsville Poultry Semen Extender (BPSE), (Sexton, 1977) and Lake's diluent (Lake, 1960). One part of good quality of semen was taken in 5 ml round bottom glass tubes and mixed with two parts of the respective diluent. In this way three groups (CARI, BPSE and Lake's) of diluent mixed semen were prepared targeted for A.I. at 24 h. A.I. was carried out in 20 hens for each diluent in the similar manner as described in experiment 1. Fertility of birds was assessed by incubating the eggs (99.5^{o}F temperature and 55-60% relative humidity) laid by hens 1 to 10 d after a single intravaginal insemination. Obtained data were analysed as per the standard method of Snedecor and Cochran (1989).

III. RESULTS AND DISCUSSION

In the first experiment, effect of CARI poultry semen diluent at different storage temperatures (3, 7 and 11°C) was observed on the fertilizing ability of spermatozoa during 24 h storage. The result of this study (Table 1) indicated that high fertility (89.6±0.79%) was achieved at 7±1°C followed by 11±1°C (54.5±2.01%) and 3±1°C (49.1±1.66%) in WL hens during the 5 d after insemination. Overall, the pattern of fertility over the 10 d of fertile egg collection was also high (79.2±1.08%) in 7±1°C stored semen in comparison to 11±1°C (36.2±2.72%) and 3±1°C (35.0±1.47%). The freshly ejaculated semen revealed 90.1±1.58% fertility. Our studies indicated that chicken semen can be stored for 24 h at 7°C using CARI diluent which will then express good fertility similar to the freshly ejaculated semen. However, earlier studies indicated that chicken semen can be stored at 0-5°C (Lake, 1960, Van Wambeke, 1967; Sexton, 1977; Lake and Ravie, 1979). This indicated that the composition of the CARI

poultry semen diluent may differ from others that express better fertility at 7±1°C instead of 0-5°C.

Table 1 - Effect of different storage temperatures on fertilizing capacity of chicken (WLH) spermatozoa stored for 24 h using CARI poultry semen diluent (Mean±SEM, n= 20).

Day	Storage temperature		
	3±1ºC	7±1ºC	11±1ºC
Day1	47.75±2.88	87.57±1.42	61.67±2.31
Day2	53.30±2.41	87.93±1.15	53.33±2.84
Day3	53.90±2.91	91.61±1.13	58.50±3.27
Day4	43.65±2.07	91.00±1.20	51.50±2.48
Day5	47.05±2.50	89.64±1.30	47.33±3.38
Average Day 1-5	49.13 ± 1.66^{A1}	89.55 ± 0.79^{A2}	54.47 ± 2.01^{A3}
Day6	32.50±3.37	85.96±1.20	33.67±1.90
Day7	20.00±1.86	77.86±1.63	13.33±1.12
Day8	19.30±1.59	68.68±1.76	18.00±2.30
Day9	17.15±1.02	65.32±1.92	13.00±0.67
Day10	15.50±1.04	46.82±2.17	11.67±0.54
Average Day 6-10	20.89 ± 1.38^{B1}	68.93 ± 1.58^{B2}	17.93 ± 1.77^{B1}
Total	35.01 ± 1.47^{C1}	79.24 ± 1.08^{C2}	36.20 ± 2.72^{C1}

Mean values bearing different super script in columns (A, B, C) differs significantly (P ≤0.05).
Mean values bearing different super script in rows (1, 2, 3) differs significantly (P ≤0.05).

In the second experiment, fertility of CARI poultry semen diluent was compared with two of the most commonly used diluents (i.e. BPSE and Lake's) and the results are presented in Table 2. During the first 5d after insemination maximum fertility (84.3±2.02%) was noticed using CARI diluent, followed by Lake's (70.2±2.77%) and BPSE (58.2±3.37%). The same pattern was found in the following 5 d (6-10 d) of fertile period. Overall, during the 10 d after insemination CARI diluent expressed superior fertility (69.1±2.62%) than others. This study indicated that CARI poultry semen dilutor was superior to others. Similar observations were reported by Shinde *et al*. (2013) in Kadaknath chicken and Mohan *et al*. (2015) in guinea fowl. Future work should be directed at the development of new diluents designed specifically for the storage of semen for 48 h and longer.

Table 2 - Effect of various semen dilutors on fertilizing ability of chicken (WL) spermatozoa after24 h storage at low temperature (Mean±SEM, n= 20).

Day	Dilutor		
	Cari	BPSE	Lake's
Day1	74.00±1.74	69.67±5.95	79.17±1.61
Day2	92.83±2.27	65.33±4.11	75.17±5.95
Day3	91.17±2.06	62.83±5.70	66.67±3.91
Day4	84.50±1.97	49.50±2.14	69.67±3.74
Day5	79.17±3.83	43.67±3.98	60.50±3.84
Average Day 1-5	84.33 ± 2.02^{A1}	58.20 ± 3.37^{A2}	70.23 ± 2.77^{A3}
Day6	59.00±3.23	42.50±5.02	52.83±4.59
Day7	65.33±1.66	30.00±3.51	44.50±5.66
Day8	62.00±3.23	22.00±2.80	24.50±2.84
Day9	47.83±3.82	12.50±2.75	32.33±4.07
Day10	35.00±2.71	20.00±3.21	23.17±3.66
Average Day 6-10	53.83 ± 2.78^{B1}	25.40 ± 2.90^{B2}	35.47 ± 3.39^{B3}
Total	69.08 ± 2.62^{C1}	41.80 ± 3.07^{C2}	52.85 ± 3.14^{C3}

Mean values bearing different super script in columns (A, B, C) differs significantly (P ≤0.05).
Mean values bearing different super script in rows (1, 2, 3) differs significantly (P ≤0.05).

IV. CONCLUSION

From these studies it can be concluded that for chicken semen stored for 24 h, CARI poultry semen dilutor expressed highest fertility at 7±1ºC. Further, it was noted that CARI poultry semen dilutor is superior to the BPSE and Lake's semen diluents.

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EVALUATION OF SUGAR CANE BAGASSE AND PARTICLE SIZE ON BROILER GROWTH PERFORMANCE, LITTER CONDITION AND CONTACT DERMATITIS UNDER A WET LITTER CHALLENGE MODEL

S.K. KHERAVII[1,2], R.A. SWICK[1], M. CHOCT[1] and S.B. WU[1]

It is well documented that structural components of feed, such as coarse fibre, improve broiler performance by modulating of gastrointestinal tract development and function, gut health and litter quality (Kheravii et al., 2016; Jiménez-Moreno et al., 2016; Xu et al., 2015; and van der Hoeven-Hangoor et al., 2014). In addition, certain fibre sources may help maintain litter quality and thus contact dermatitis (foot pad dermatitis and hock burns) of broilers. A study was conducted to evaluate the effect of sugar cane bagasse and corn particle size on growth performance, litter quality and bird contact dermatitis under a wet litter challenge model.

A total of 672 day-old Ross 308 male broilers were allocated to 48 pens using a 2 × 2 × 2 factorial arrangement of treatments with 2 particle sizes (coarse or fine) and 2 levels of a sugar cane bagasse (0 or 20 g/kg) and 2 levels of Na (1.6 or 4.0 g/kg). Salt was used to adjust Na of the diets (and this also increased dietary Cl). Each treatment had 6 replicate pens of 14 birds. The method of Allain et al., (2009) was used to score footpad dermatitis, where 0 indicated no lesions and 9 the most macroscopic deep lesions. Whereas, the method of Kjaer et al. (2006) was used to score hock burns: 1, no lesion; 2, minor lesion; 3, severe lesion.

Results indicated that birds fed 20 g/kg sugar cane bagasse had higher weight gain, feed intake ($P < 0.001$) and lower FCR ($P < 0.05$) compared to those given no bagasse from 0 to 35 d. Birds fed coarsely ground corn had lower FCR ($P < 0.05$) than those fed finely ground corn from 0 to 35 d. A corn particle size x bagasse interaction was observed for FCR ($P < 0.05$) from 0 to 35 d, where birds fed coarsely ground corn with sugar cane bagasse had lower FCR ($P < 0.05$) than all other treatment groups. At 35 d, higher litter moisture ($P < 0.001$) was observed in birds fed 4.0 g/kg Na (79 %) as compared to those fed 1.6 g/kg (32 %). Sugar cane bagasse and particle size had no effect on litter moisture content ($P > 0.05$) at 35 d. The birds fed the 1.6 g/kg Na diet had a lower ($P < 0.001$) incidence of foot pad dermatitis (mean = 0.44) compared to those fed the 4.0 g/kg Na diet (mean = 2.57) at 35 d. There was a tendency for higher incidence of hock burns ($P < 0.056$) in the birds fed the 4.0 g/kg Na diet compared to those fed the 1.6 g/kg Na diet. Corn particle size and bagasse had no effect on contact dermatitis ($P > 0.05$). No significant 2 way or 3 way interactions were observed between particle size, bagasse and Na level on contact dermatitis ($P > 0.05$) at 35 d. These findings suggest that coarsely ground corn and bagasse independently improve broiler performance, and the combination of both is more beneficial for improving broiler performance without any adverse effect on litter quality and bird contact dermatitis.

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ISOQUINOLINE ALKALOIDS LOWER THE PREVALENCE OF SALMONELLA HEIDELBERG IN BROILER CHICKENS

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Worldwide, tens of millions human cases are reported every year for salmonellosis. This makes it one of the most common foodborne diseases, which can end lethally, depending on the salmonella strain and host factors. More than 2,600 different serovars are known nowadays. Poultry meat is a potential carrier for the animal-food-human transmission route. In North America, *Salmonella enterica* serovar Heidelberg is one of the most common serovars isolated from people suffering from salmonellosis (Patchanee et al., 2008). Furthermore, *Salmonella enterica* may carry antimicrobial resistance genes adding further to a potential risk for animals and humans. The objective of the study was to investigate the effect of a standardized blend of plant-derived isoquinoline alkaloids (IQs, Sangrovit® G Premix) in broiler chickens infected with *Salmonella enterica* serovar Heidelberg.

1,200 male day-old chicks (Ross 308) were split into two groups: 1) Control (CON): infected, no feed additive 2) Feed additive (IQ): infected, IQs (120 ppm throughout the study). At study initiation, fifty broiler chicks were allocated to twenty-four floor pens (n=12) with fresh litter in a modified conventional poultry house. Three rations were used: starter (d 0 - 14), grower (d 14 - 28), and finisher (d 25 - 35). Feed formulations consisted of non-medicated commercial-type broiler feed and birds had *ad libitum* access to feed and water. The challenge in this study was a natural seeder bird method where 13 birds (25%) per pen were orally gavaged with 4 x 10^5 CFU/ml of a nalidixic-acid resistant *Salmonella enterica* serovar Heidelberg at four days of age. The gavaged birds were tagged. Bootsocks swab samples were collected for *Salmonella* environmental contamination determination from all pens on d 14 and 35. Furthermore, cecal sampling was completed on d 35 for evaluating *Salmonella* counts using MPN (Most Probable Number). Ten non-tagged birds were taken from each individual pen, euthanized by cervical dislocation and ceca aseptically removed. After removal the cecal samples were placed in sterile plastic bags, labeled, stored on ice, and analyzed. Generalized estimating equations logistic models were applied for statistics, where $P < 0.05$ was considered significant. MPN values were log-transformed prior to statistical analysis. On d 35, no significant differences were observed for feed intake and average weight gain between CON and IQ. Birds fed IQs showed a significantly improved adjusted FCR compared to control birds (1.744 and 1.799, respectively). IQ treated birds showed a numerical reduction in *Salmonella* prevalence (boot sock samples) compared to birds of the control group (87.5% and 100%, respectively). Furthermore, cecal *Salmonella* MPNs for culture-positive ceca samples were lower in IQ fed birds than in untreated birds (0.40 and 0.55; $P > 0.05$).

The use of IQs reduced the prevalence and *Salmonella* level in the positive ceca. This will decrease the pathogen pressure for the next growout as chicks are exposed to less *Salmonella*. Consequently, this can lead to a significant *Salmonella* reduction in the broiler house over time. Furthermore, a beneficial significant effect on FCR was observed in challenged chicks if IQs were applied, improving economics.

In conclusion, the use of a standardized blend of plant-dervied isoquinoline alkaloids offers a promising solution to support broiler chickens challenged with *Salmonella* and contribute to food safety and an economical broiler production.

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BROILERS PERFORM BETTER WITH INTERMITTENT LIGHTING PROGRAMS

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Roles of certain parts of the digestive tract of chickens seems to have been lost with the evolution of time and commercial practices. Gizzards no longer serve their grinding purposes, having become more like transit organs and crops have lost their function with the abandonment of discontinuous feeding systems. The extremely fast transit time in the anterior digestive tract is regarded as a limitation to the performance of exogenous enzymes. Manipulation of feed retention time in these gut segments via meal feeding vs. *ad libitum,* lighting management programs, presence of structural components in the diet and coarse vs. fine feed particles may further enhance the efficacy of exogenous enzymes.

Following an adaptation starter period until d10, six-hundred twenty four one-day-old ROSS 308 male broilers were subjected to two different lighting programs (referred to as 'continuous' – 18L:6D or 'intermittent' – 1L:3D:1L:3D:1L:3D:1L:3D:2L:6D) and fed isoenergetic (on an ME basis) and isonitrogenous wheat-, sorghum- and soybean meal-based diets (Table 1) with or without the supplementation of phytase and xylanase over 34 days. Chicks were randomly allocated to eight treatments (2x2x2) with six replicates per treatment. Data were analysed using ANOVA (SPSS Statistics, ver. 24). Means were compared using the Tukey multiple range test.

Table 1 – Chemical composition of experimental diets, as calculated.

	AME, Kcal/kg	CP, %	dLys, %	dMet+Cys, %	dMet, %	dThr, %	Ca, %	Na, %	Cl, %	AvP, %
Grower (d10 - d24)	3023	20.89	1.08	0.80	0.51	0.70	0.88	0.16	0.23	0.44
Finisher (d25 - d34)	3120	20.21	1.00	0.76	0.48	0.65	0.79	0.16	0.23	0.39

At d10, after the initial adaptation period, body weight (BW) of chicks was virtually the same for both treatments (290 g vs. 288 g, for continuous lighting (CL) and intermittent lighting (IL) groups, respectively). At d34, birds submitted to CL were numerically heavier than those in IL (2,224 g vs. 2,159 g, $P = 0.08$) and presented statistical significant higher mortality-corrected FCR (FCRc) (1.390 vs. 1.370, $P = 0.003$). Phytase supplementation significantly ($P = 0.004$) improved final BW (2,247 vs. 2,137 g, for supplemented and unsupplemented bids, respectively) and FCRc was numerically better for supplemented animals (1.375 vs. 1.286). Xylanase addition to basal diets significantly ($P = 0.001$) improved FCRc (1.370 vs. 1.391, for supplemented and unsupplemented bids, respectively).

There was a statistical significant ($P < 0.05$) interaction between phytase and xylanase supplementation on final body weight, which shows the additive/synergistic effect of these enzymes. No further interactions were observed between factors.

Further markers' and digestibility analysis will help understand the exact mechanism through which these improvements were achieved. So far we hypothesise intermittent lighting had an effect on feed retention in the upper gastro-intestinal tract, evidenced by the lower pH found in the crop and gizzard of birds in IL groups (data not shown), and that this enabled better digestibility of nutrients. Also, phytase-activity analysis will hopefully explain why phytase supplementation did not seem to have a positive effect in IL birds further to that achieved with CL.

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LITTER QUALITY: INVESTIGATING THE INTERRELATIONSHIP BETWEEN LITTER MOISTURE CONTENT, pH, WATER ACTIVITY AND ODOUR EMISSIONS

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Wet litter is a recognized issue in commercial poultry production and litter quality has come under great scrutiny with the introduction of farming scheme standards by animal welfare organizations in Australia (RSPCA, 2013). According to RSPCA (2013) meat chicken standards, "litter must be maintained in a dry and friable condition." Litter conditions may affect odour emissions and thus litter properties were studied to correlate with specific odorants.

Two experiments were conducted using Ross 308 male broiler chickens raised in floor pens with fresh pine shavings (10.4 kg/pen) as bedding material. On d 0, the birds were placed in 16 pens with 12 birds (experiment 1) and 10 birds (experiment 2) per pen. Diets contained the same levels of metabolizable energy (ME) (starter/grower/finisher: 12.3/12.8/13 $MJkg^{-1}$) and digestible amino acids but differed in the levels of crude protein, salt and types of feed additives (antibiotic, probiotic and saponin). In experiment 1, approximately 10 g of litter were sampled from each pen on d 29 to measure litter moisture and litter water activity (A_w) using tuneable diode laser water activity meter (AquaLab-TDL, USA). On d 35, a sample of surface litter from under the flux hood was collected from each pen to measure litter moisture and odorants using a selective ion flow tube mass spectrometer (SIFT-MS). Pearson correlation coefficients and associated significance were generated using JMP software to determine the relationship between litter moisture and odorants. The relationship between litter moisture and A_w was determined by exponential regression analysis. In experiment 2, 25 litter samples were collected from the pens from d 25 to d 30 and analyzed for pH and moisture contents. Litter pH was determined by mixing litter and de-ionised water in a ratio of 1:5 and measuring the pH with a pH meter (EcoScan 5/6 pH meter, Eutech Instrument Pte. Ltd., Singapore). The results showed that methyl mercaptan ($r = 0.453$, $P < 0.01$), hydrogen sulfide ($r = 0.482$, $P < 0.01$), dimethyl sulfide ($r = 0.621$, $P < 0.01$), trimethylamine ($r = 0.526$, $P < 0.01$), phenol ($r = 0.409$, $P < 0.05$), indole ($r = 0.503$, $P < 0.01$) and skatole ($r = 0.344$, $P < 0.05$) had significant positive correlations with litter moisture. Dimethyl disulfide tended to be positively correlated with litter moisture ($r = 0.316$, $P = 0.061$) and methylamine ($r = -0.309$, $P = 0.086$), propionic acid ($r = -0.318$, $P = 0.072$) and butanoic acid ($r = -0.318$, $P = 0.072$) tended to be negatively correlated with litter moisture. There was no correlation between litter moisture and odorants belonging to the group of alcohols, aldehydes and ketones. There was an exponential relationship between litter moisture and Aw values on d 29 ($r^2 = 0.938$, $P < 0.01$). Aw increased until it reached a value of 1.0 at a litter moisture of approximately 50%. There was a negative linear relationship between litter moisture and litter pH ($r^2 = 0.54$, $P < 0.01$).

In conclusion, a high litter moisture increased water activity and favoured the emissions of sulfur containing odorants, trimethylamine, phenol, indole and skatole over others. As litter moisture increased, litter pH decreased, which implies that low litter pH associated with high litter moisture may favour the emissions of sulfur containing odorants.

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Notes

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