

**24<sup>th</sup> ANNUAL AUSTRALIAN POULTRY SCIENCE SYMPOSIUM**

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(University of Sydney)**

**and**

**THE WORLD'S POULTRY SCIENCE ASSOCIATION  
(Australian Branch)**

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2013**

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## THE IMPORTANCE OF THE POULTRY INDUSTRY

HON. J. KERIN

The advent of the personal computer and search engines of varying degrees of sophistication enable computer troglodytes, such as I am, to amass data for re-gurgitation. This enables one to give a quantitative picture of an industry. What is more important are the qualitative aspects of an industry; its contribution to our well being.

So, what are the characteristics of the poultry industry that mark it out as outstanding? Farmers, as with many if not most groupings in our society, resist change. There is always comfort in known certainties and they die hard. The poultry industry is one of adaptation, competition and change.

Industries in Australia live within regulated environments. To gain policy change, to adapt to new technologies and to adjust to the times we are in, nationally and internationally, requires a change in the regulatory map- imposed by either the private or public sector or both.

I lived on and worked on a poultry farm until I was 33, leaving it in 1971. When I left school in 1952 there were 34,000 registered egg producers in NSW. These were registered with the NSW Egg Marketing Board, when the egg producers and major political parties had a belief in 'organised marketing'. To be registered one had to have over 19 laying hens, so one assumes that number of commercial farms was in the mid thousands, say 5-6000. In the village I grew up in, there were 7 commercial farms and some part time producers; today there are none. In NSW today, there would be only a few over 200 commercial farms. All States had similar arrangements and Queensland, the home of rural socialism, at one stage had three egg producing zones. It was prohibited to trade in eggs between the zones. The organic, barn laid etc move has meant that numbers of egg producers is rising again and I understand that ,Australia wide, we now have about 2,000 producers but with the largest three producing over 50% of total egg production. The industry is both scaling up and down! In the post WW2 agricultural commodity exporting boom years, we exported eggs at a lower than the domestic price to England. The British and Europeans were starving. We poultry farmers were buying wheat at a higher price than what it was being sold for on the export market under an International Wheat Agreement, which did not expire until the early 1970s. From the late 1950s and into the 1960s we started to enter a period of over-production and an end to any notion of an export bonanza. There was sense in the overall policy because Australia needed export income so as to afford imports and to enhance our standard of living and to gain foreign exchange for investment. The Commonwealth Government's policy was 'protection all round' until at least 1969.

Although difficult, the egg industry became a model for, near autonomous, structural adjustment. It went through profound change, not the gradual change that occurs in broad-scale farming. It adapted. How did this come about?

The broiler industry commenced as a vertically integrated industry in the 1960s. Grower associations came into being but the dominant hatchery and processing firms were the main drivers of the industry. There are about 800 chicken producers in Australia but Baiada and Inghams produce more than 70% of all production. Scaling up continues; the market at work. Again, the poultry industry became a model for structural adjustment with fierce competition, at all levels. It adapted. How did this come about?

Australians mainly live in cities. We are a highly urbanised society. Diets and lifestyles in our cities have changed. We now do not all go to work 'on an egg', but our consumption is relatively high. We are more likely to eat at a quick food outlet, with over fried chicken, than cook one at home, but our consumption is high. Again the industry has

adapted to changing life-styles. Unlike some industries, we don't think we have to prove to kids that eggs come from chooks. The industry adapts to the new situation. Because we are comparatively rich, a large proportion of our population is able to pay for eggs from hens not raised in the most commercially based conditions. The industry has adapted to the market. The industry adapts.

As a generalisation, farmers readily adopt new technologies but are reticent to embrace institutional change. The time taken to adopt research results is typically long term due to a range of understandable factors but also resistance to new ideas is common. The poultry industry is one which is an 'early adopter'. Vaccines, husbandry types and feed formulation all represent quick adoption within a sophisticated industry. It adopts and adapts. As a lapsed economist I fully understand that one should presume that there will always be oil, phosphates, land, water and all production inputs and that a world population of 10b will present no problems in guaranteeing world food security or in preventing 'food price spikes'. As a lapsed 'bush scientist', I have now been convinced that Global Warming and Climate Change are myths or in any case, if they do exist, they have nothing to do with anthropogenic causes. A former Commonwealth Treasury Secretary and macro-economist has told me so. He would know.

Despite these misgivings, there is a case to propose that the demand for animal protein will rise, that the real price of agricultural inputs will rise and that chicken and eggs will continue to be the cheapest form of animal protein. What evidence is there for this? Can we market poultry products on the basis of being Greenhouse Gas friendly; 'Green Chicken'? We are lectured by all the Pharisees that Australia is now living in the Asian Century and that the "'The Tyranny of Distance' has disappeared. Well, that is pretty good. Another of the great important contributions of the poultry industry may also just be that we are in a position to consider the sale of our services, based on our research and extension into our own industry, and which is applicable to the fast developing world- remember the work by our scientists via ACIAR on Newcastle Disease in Cambodia. Zoonoses are on the rise. We need to continue to adapt.

The importance of the poultry industry goes beyond the stats - but they are important too!

## PHOSPHORUS EVALUATION ASSAYS: SCIENTIFIC APPROACHES AND PRACTICAL IMPLICATIONS

M. RODEHUTSCORD<sup>1</sup> and Y. SHASTAK<sup>1</sup>

### Summary

Phosphorus (P) is an element with special relevance for sustainable food production. All animal species have a specific requirement for P. Excretion of P may negatively affect the environment, and the global raw phosphate stores are limited. Therefore, responsible handling of P sources is necessary along the entire food chain. An optimised use in poultry feeding needs to consider the great differences in P availability between P-containing raw materials and the efficacy of phytase supplements. However, opinions about what P availability is and how it should be measured are different. Approaches that are often used are described in this paper.

The pluralism in the definition of P availability not only causes confusion in communication but it also makes it almost impossible to compare data that originate from different laboratories. Compiling comprehensive feeding tables which are needed by the feed and poultry industries is hampered by this lack in harmonisation. The Working Group No 2 - Nutrition- of the European Federation of Branches of WPSA has identified harmonisation of P evaluation as a major objective and developed a standard protocol for the determination of P availability. Important aspects of this protocol will be addressed in this paper, as well as the impact that it will have and work that still needs to be done.

### I. INTRODUCTION

Phytate is the dominant binding form of phosphorus (P) in plant-based feedstuffs for poultry. Phytate P utilisation by birds is incomplete; in practical feeding, the P requirement of the birds can only be met by inclusion of animal-based proteins, mineral P sources, and enzymes, and combinations of these are often used.

Most of the P used in agriculture is derived from phosphate rock, which is a non-renewable resource, and current global reserves may be depleted in 50-100 years (Cordell et al., 2009). Future generations will face problems in obtaining enough to exist, and further research is needed to avoid problems in the long term (Abelson, 1999). Maintaining P resources in the face of finite global phosphate rock stores has been identified to be one of the greatest challenges for sustainable food production (Gross, 2010; Neset and Cordell, 2012). This implies special challenges for all livestock industries (Rodehutscord, 2008).

In regard to poultry, optimising the supply of available P in the diet by considering differences in the availability of different P sources is one approach to address the problem. The second is to model the requirement of available P of modern genotypes. This paper focuses on the first approach. Knowledge about the availability of P from plant raw materials is important because they contribute most of the dietary P and can largely vary in availability. Phosphorus availability of animal and mineral sources also is important to know; these data are needed to make precise adjustments of P concentrations in diets and because using phosphates contributes to feeding costs.

The term 'availability' will be used to describe the proportion of dietary P that, at a marginal level of P supply, can be utilised to cover the P requirement of an animal

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(Rodehutschord, 2009). It describes the potential of a raw material or diet, and it is not directly related to any binding form of P in the feed.

## II. APPROACHES USED TO DETERMINE PHOSPHORUS AVAILABILITY

Due to the cost of adding P to diets, which has always been relatively high compared to that for other minerals, there has been a lot of research dedicated to determining the necessary dietary levels and quality of P sources in poultry diets (Coon et al., 2002). However, scientists have used different approaches and criteria in the evaluation of P availability. These approaches can be grouped into three categories:

- a) Quantitative approaches;
- b) Qualitative approaches (relative bioavailability);
- c) *In vitro* estimates.

### a) Quantitative Approaches

Assessing the bioavailability of mineral sources by means of digestion and absorption studies seems to be one of the best direct methods, particularly for major minerals (Jongbloed and Kemme, 2002). Moreover, the nutritionist needs an actual quantitative (retention) value for key minerals to assess the true impact of dietary formulations on animal performance and on the elements remaining in animal excreta (Coon et al., 2002). Such quantitative values can be obtained in excreta collection studies, or by measuring the digestibility until the end of ileum (precaecal (**pc**) digestibility), or by comparative whole-body analysis. Radio-labelled isotope techniques can be used to study P absorption and endogenous losses separately, but these techniques will not be considered in this paper.

#### *Retention in balance studies*

The retention of P can be measured either based on complete excreta collection or by using an indigestible marker in the feed and spot sampling of excreta. Different markers were used for evaluating the P availability in poultry, for example, in the studies of Edwards and Gillis (1959), Nwokolo et al. (1976), and Leske and Coon (1999). Van der Klis and Versteegh (1996) used an approach based on quantitative measurements of P intake and excretion, and determined P availabilities of commonly used feedstuffs in 3-week-old male broilers under standardised conditions. Wendt and Rodehutschord (2004) and Rodehutschord and Dieckmann (2005) studied the utilisation of P from different inorganic phosphates in 10-day balance trials with quantitative excreta collection.

#### *Precaecal digestibility*

Hurwitz et al. (1978) already mentioned that the process of P absorption seems to be almost completed in the lower ileum. Thus, pc digestibility, which became highly relevant over the years in the evaluation of proteins and amino acids for poultry, can also be considered as a tool to measure P availability. Grimbergen et al. (1985) and Ketels and De Groote (1988) were the first who reported pc digestibility values for mineral and animal P sources. The P flow at the terminal ileum of broilers appears less sensitive to excess P intake of birds when compared to P retention (Rodehutschord et al., 2012). This can be of advantage in P availability studies because urine is a major pathway of P excretion if P intake is above the requirement. The response in P pc digestibility to increments in dietary P concentration was linear in a wider range of dietary P than the response in P retention was (Rodehutschord et al., 2012). Both retention and pc digestibility measurements provided similar results in the evaluation of mineral P sources when determined at an overall low level of P intake (Shastak et al., 2012a).

*Comparative whole-body analysis*

The total amount of a mineral that is retained by the animal is one of the best response criteria for those minerals with a low mineral turnover such as Ca and P if the animals are fed below their mineral requirement (Jongbloed and Kemme, 2002). However, comparative whole-body analysis is very laborious. A particular challenge is to make a small sample representative for the whole body (Haag, 1939). Thus, studies that employed comparative whole-body P analysis are rare (Dieckmann, 2004; Hemme et al., 2005; Olukosi and Adeola, 2008).

b) Qualitative Approaches (relative bioavailability)

Availability is often calculated as a relative biological value (RBV). A given P source is compared with a standard P source and RBV can be calculated from several biological response criteria, such as animal growth, blood, and bone criteria (Lima et al., 1997). According to Huyghebaert et al. (1980), the calculation of RBV is usually based on the following three techniques if the relationship between the criterion of response and the P level of the diet is linear:

1. The ordinate method (Baruah et al., 1960): at a previously fixed abscissa value, the y-values are compared to each other. The P source whose regression yields the greatest y-value at one particular x-value is considered to be the best biological source.
2. The slope method (Hurwitz, 1964): the slopes of the linear equations are compared to each other. The P source whose regression has the greatest slope presents the highest P availability.
3. The abscissa method (Gillis et al., 1954): comparison of the abscissas corresponding to a previously chosen ordinate. The P source whose regression equation has the lowest x-value at one single y-value is the best biological source.

The second method is the one that was most widely applied in the published literature.

*Bone criteria*

Most of the studies dealing with the investigation of P availability used different bone criteria for the calculation of RBV. Bone mineralisation is a dynamic process that involves adjustment and adaptations to changing physiological conditions (Sapir-Koren and Livshits, 2011). Bone ash and P, bone breaking strength (BBS), or bone densitometry have usually been the criteria of choice in RBV assays.

The tibia bone is one of the most sensitive bones to P deprivation (McLean and Urist, 1961). Thus, tibia ash and P are the most-used criteria (Ammerman et al., 1960; Hurwitz, 1964; Huyghebaert et al., 1980; Nelson et al., 1990; Coon et al., 2007; Shastak et al., 2012b). Femur, toe, and foot ash have also been used as response criteria in P availability studies (Gardiner, 1962; Potter, 1988; Hemme et al., 2005; Garcia et al., 2006; Shastak et al., 2012b).

BBS and bone mineral density were implemented as rapid methods which could replace bone ash in evaluation of bone mineralisation. Rowland et al. (1967), Yoshida and Hoshii (1982) for BBS, and Akpe et al. (1987), Onyango et al. (2003), and Shastak et al. (2012b) for bone densitometry reported a high correlation of these criteria with bone ash.

*Blood criteria*

Decades ago, Gardiner (1962) and Hurwitz (1964) looked into the relationship between dietary P level and inorganic P ( $P_i$ ) concentration of the blood. Summers et al. (1959) used plasma alkaline phosphatase (AP) as the response criterion in P evaluation. More recent investigations tried using blood  $P_i$  or AP (Lima et al., 1997; Hemme et al., 2005; Shastak et al., 2012b) to evaluate dietary P. However, blood  $P_i$

and AP reflect the animal's adaptive reaction and may be influenced by diurnal variation and stress during sampling (Gueguen, 1999). Shastak et al. (2012b) concluded that blood  $P_i$  was not a suitable criterion to study P availability in poultry.

#### *Growth and feed conversion*

Some studies reported that a bioassay based on growth was as good as using bone ash in studying P availability (Summers et al., 1959; Potter, 1988). However, growth occurs as a result of many different processes in the animal (Adeola and Cowieson, 2011), and Jongbloed and Kemme (2002) stated that differences in performance can only be noted at large differences in bioavailability or mineral supply. Nelson and Walker (1964), Huyghebaert et al. (1980), Grimbergen et al. (1985), and Shastak et al. (2012b) concluded that growth and feed conversion were not satisfactory criteria in P evaluation.

#### *Combined response criteria*

Different from the approaches mentioned before, Sullivan (1966) combined three criteria (per cent bone ash, growth, and feed conversion) in a multi-factorial regression equation for the calculation of the RBV (Huyghebaert et al., 1980). Similarly, Soares et al. (1978) combined feed intake, growth, and per cent toe ash to assess P availability. However, these approaches were criticised by Peeler (1972), as the different response criteria provide different RBVs of P from a given source; this reduces the sensitivity of the assay.

#### c) *In vitro* Estimates and Other Approaches

As indicated in the previous chapters, *in vivo* determination of P availability involves complex and laborious procedures. The first attempts to use faster and simpler *in vitro* tests were made in the 1940s (Bird et al., 1945; Gillis et al., 1948). The P solubility in different solutions (water, diluted HCl, citric acid, neutral ammonium citrate) was tested (Day et al., 1973; Sullivan et al., 1992), but it became evident that there was a low correlation with *in vivo* data (Huyghebaert et al., 1980). According to Gueguen (1999), many phosphates that are insoluble in water are actually of high value for animals. Day et al. (1973) also demonstrated that the biological availability of phosphates did not correspond to their solubility *in vitro*. However, some researchers reported some positive relationship between solubility in an acid medium (citric acid) or in neutral ammonium citrate and *in vivo* P bioavailability (Sullivan et al., 1992; Coffey et al., 1994). This discrepancy indicates that it is still worth trying to improve the solubility assays in order to make them more reliable for routine testing of P availability.

### III. THE WPSA AVAILABLE P PROTOCOL

At the 17th European Symposium on Poultry Nutrition, which was held in Edinburgh in August 2009, one session was dedicated to P nutrition. Problems that arise in both science and practical application from the application of the variety of methods in P evaluation were intensively discussed. As an outcome of this discussion, Working Group No 2 -Nutrition- of the European Federation of Branches of WPSA has taken the initiative to suggest a standard for future work on P availability. Experts from both science and the industry collaboratively developed a protocol that includes all technical details of experiments intended to study P availability (WPSA, 2013). A summary of this protocol is given herein.

The protocol has focus on broilers as the main category of the poultry fed. It is known that P availability is different between broilers, turkeys, and ducks (Rodehutsord and Dieckmann, 2005). Differences perhaps also exist between broilers and laying hens. If

availability studies are intended with birds other than broilers, then the new protocol can also be applied with some adjustment in diet composition.

It is recommended to measure the precaecal digestibility of P. Some reasons for this recommendation were already mentioned in the previous chapters. Precaecal digestibility of P of a given feedstuff is tested by a regression approach. This implies that a low-P basal diet is used and a minimum of two levels of the P source under test is supplemented. Using the regression approach implies that a correction for endogenous P losses is not necessary.

A minimum number of six replicated cages per diet is used, and each cage has at least eight birds. Experimental diets are provided for *ad libitum* intake for five days before the content from the lower ileum is collected when birds are between 21 and 28 days old. Digesta is pooled from all birds of one cage. Before the experiment starts, birds are fed a diet that is adequate in all nutrients, including P and Ca.

The experimental diets are adequate in all nutrients except P and Ca, meaning that the raw materials of the diet must be specifically selected. Examples for the composition of the diets are part of the protocol. Diets contain an indigestible marker. The intended level of available P in the basal diet should not exceed 0.15%, which is equivalent to approximately 0.3% total P. The P source under test is supplemented in at least two levels, and the levels are chosen to achieve increments of up to 0.15% of total P originating from the test source. Details depend on whether feedstuffs with a high P concentration (mineral and animal origin) or plant P sources are tested. The ratio of total Ca to total P in the diets can vary between 1.3:1 and 1.4:1. Diets should be pelleted.

The pcdP is calculated for each cage and diet. In a second step, the pcdP of the P source under test is calculated as the slope of a regression line that is calculated for each set of diets that belong together (basal diet and diets supplemented with the respective P source). If two or more P sources are tested in the same experiment, common-intercept multiple linear regression can be used. This allows for the simultaneous determination of pcdP for all tested P sources, including a comparison of slopes. A full example of all calculations is part of the protocol.

The protocol also addresses specific demands in testing the efficacy of supplemental phytase.

This WPSA protocol is seen as the initial step towards harmonisation of P evaluation in poultry and it is open for improvement. Several details had to be set in spite of a lack of experimental evidence and suggestions are made for topics to be clarified in future.

#### IV. PRACTICAL IMPLICATIONS

With a standardised system of P evaluation, the basis for feeding tables and dietary allowances will be formed. State-of-the-art data that are needed by the industries can be provided and can be used in animal feed formulation. This will help to improve the accuracy of feed evaluation and thereby reduce the cost of feeding and improve competitiveness through reduced inclusion of feed phosphates. Once research labs work with the same protocol, it would be easier to collect results and combine them in feeding tables.

Surpluses of phosphates in animal manure can lead to eutrophication and groundwater pollution, especially in areas of intensive animal production. The reduced inclusion of mineral phosphates in animal feeds will decrease P excretion into the environment and contribute to further limiting the environmental burden originating from manure.

Reduced inclusion of feed phosphates will also help to carefully handle P as one of the most limited natural resources. Any tool that will help to improve the utilisation of plant P and to avoid oversupply of feed phosphates contributes to saving the raw phosphate stores for future generations.

## V. NEXT THINGS TO DO

There are two more packages to work on. One is the collection of data that were/are generated according to this protocol and to combine them in feeding tables. Part of it may be taken from published studies that were conducted similar to the new protocol. Another part still needs to be generated in new collaborative research projects. In close linkage with new animal studies, the *in vitro* approaches should be improved and developed into a tool that allows for rapid estimates of P availability under routine conditions. The second major package to work on is to improve the modelling of the available P requirement of different poultry species and categories.

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## CALCIUM TO PHOSPHORUS RATIOS IN BROILERS

R. ANGEL<sup>1</sup>Summary

As we learn more about the negative impacts of calcium (Ca) on the availability of phosphorus (P) and phytate P, as well as on the efficacy of phytase, it highlights how little we know about Ca to P ratios and points to areas where we would benefit from a better understanding of what the broiler actually needs. Historically, the ratio of Ca to P was defined for total Ca and total P in the diet. As the impact of phytate present in seeds and its impact on seed ingredient based P availability began to be understood, a change was made to a total Ca to available P ratio. Unfortunately, available P was defined crudely as the sum of total P in inorganic based sources plus total P in animal based sources and 30% of the P in plant based sources. This definition of available P has been refined and changed over time to reflect a better understanding of phytate P availability but never have we challenged the use of total Ca. Ultimately, for the animal, what is important is the amounts of Ca and P that are available for the animal to be used within the body and these are the values that we need to define accurately. What name is given to these values continues to be debated, but for the purpose of this paper, digestible will be used. The goal would then be to define a range of optimal digestible Ca (dCa) to digestible P (dP) ratios for diet formulation. In order for this to be feasible, we would need to have ingredient dCa and dP values to give a better understanding of the impact of other factors such as phytase, vitamin D and other feed additives, age, and diet ingredient selection on Ca and P digestibilities. We also need to re-explore the impact of dCa on dP and dP on dCa. The focus then of this short paper is to highlight the importance of moving towards a better understanding of Ca and P ratios as well as to highlight how little we know on this subject.

## I. INTRODUCTION.

Historically, there has been a move from the use of a total Ca (tCa) to total P (tP) ratio system (NRC, 1950) to a tCa to inorganic P (iP) ratio (NRC, 1954), to the use of tCa to available P (aP) that appeared in the 1984 NRC. In 1950, the requirements were 1.0% tCa and 0.6% tP (1.66 tCa:tP) (NRC, 1950) and, in the 1954 NRC, the qualification was made giving importance to the availability of P by specifying that, of the 0.6% tP requirement, 0.56% needed to be from an inorganic source. An allowance of 0.3% availability of plant P was also specified. The 1977 NRC still gave requirements in terms of tCa and tP with the proviso that part of the 0.7% tP requirement for P from 0 to 8 weeks be supplied from inorganic sources of P. In the 1984 NRC, requirements for P were given as aP and aP values were also given for ingredients but no change was made to Ca. The Ca:aP ratios recommended were 2.22 to 2.28 throughout growth (hatch to 8 weeks of age) in broilers. By 1994 (NRC, 1994), the term non phytin P (nPP) instead of aP was used but there were minimal if any changes to the values. The tCa to nPP ratios recommended were 2.22 to 2.67 Ca depending on growth stage.

The low digestibility of P in plant sources (Van Der Klis and Versteegh, 1996; Coon and Leske, 1998; Angel et al., 2002; Tamim and Angel, 2003; Tamim et al., 2004) and the variable digestibility of P in animal and inorganic sources (Van Der Klis and Versteegh, 1996; Coon and Leske, 1998) prompted the change in the use of P, from total P to aP, nPP, dP or retainable P that better reflected availability of P in dietary sources. But there has been no move toward a digestible Ca system (dCa). Because of the extensive use of phytases in poultry diets worldwide, the negative effect of Ca on phytate P and on phytase efficacy digestibility (Tamim and Angel, 2003), there has been a push downwards in the use of Ca in poultry diets. Because there are very few data on actual availability or digestibility, as opposed to relative availability, of calcium in

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feed ingredient sources, we assume in general a 100% digestibility for Ca. Clearly this is not correct. Based on reported Ca digestibilities in corn soy diets with no added inorganic Ca or P sources, and the same diet with added limestone, one can calculate an availability of Ca in corn and SBM portion of the diet of 20 to 33% (Tamim and Angel, 2003; Tamim et al., 2004) and for Ca from limestone of between 60 and 70%. The Ca from corn and soy in a corn soy diet represents between 0.17 and 0.21%. We have, up to now, disregarded this low digestibility of this “organic” Ca because it represents a small (20%) percentage in a diet containing 1% Ca, typical of a broiler starter diet. But when we start seeing commercial broiler diets with 0.6% Ca, or even 0.5% Ca in the withdrawal phase, then Ca from the corn and SBM becomes a greater proportion of total Ca.

It is important that we start developing a dCa to dP system if we are to really feed broilers at a requirement concentration; that is, provide diets with concentrations of dCa and dP that meet the needs of the birds. For this, we will need to develop methodologies to determine dCa in ingredients and we will need to review the methodologies used to determine dP, and expected contributions of P and Ca when phytases are used.

## II. VARIABILITY IN REPORTED CALCIUM TO PHOSPHORUS RATIOS

Standard implementation of tCa to dP ratios of 2:1 has occurred commercially for more than 30 years, with the P being called available or digestible. In practical diets, nutritionists try to maintain a 1.8 to a 2.2 tCa to dP ratio. The question is why, and does this constraint lead to diets that do not contain the correct dCa and dP needed by broilers?

Reports abound in the literature where the tCa to dP ratios at “optimal” performance or bone mineralisation are not 2:1. For example, Driver et al., (2005a) reported that a 1:1 tCa to tP ratio maximised body weight gain (BWG) and feed to gain ratios from 0 to 16 d of age in broilers. They fed a corn-SBM diet with graded increases in Ca and P from limestone, monocalcium phosphate and dicalcium phosphate. But they also reported similar BWG with tCa:tP ratios of 0.94 to 1.25. Tibia ash was maximised at ratios of 1.07 to 1.35. Incidence of tibial dyschondroplasia was lowest when diets with a 1.29 to 1.89 tCa to tP ratio were fed but when Ca concentration was between 0.89 and 0.98.

In another study where Ca requirements between 1 and 16 d of age were determined (Driver et al., 2005b), using broken line analysis and 1 concentration of tP (0.63%) and calculated nPP of 0.45%, the requirements reported were at tCa to tP ratios of 0.77, 0.99, 1.14 for BWG, feed to gain ratio and tibia ash, respectively. If one puts these values in terms of tCa to nPP ratios, the ratios would be 1.08, 1.39, and 1.60, respectively. Clearly, the ratios reported by Driver et al., (2005a, b) differ from our standard industry use of 2:1 for tCa to aP ratios and open the door for diets with broader or tighter ratios.

In recent Ca and P requirement work done at the University of Maryland (Jimenez-Moreno et al., unpublished), tCa to nPP ratio for maximal bone ash was found at 1.05% Ca and 0.65% nPP or 1.61 tCa to nPP ratio from hatch to 10 days of age. When retainable Ca and retainable P were measured for this diet, the ratio was 1.64.

Intestinal absorption, or what we are defining as apparent or true (if corrected for endogenous losses) digestibilities or disappearance of Ca or P up to the distal ileum, does not necessarily reflect the bioavailability of these minerals to the whole animal. This is because both Ca and P must be retained and used for metabolism and/or in bone formation and mineralisation. In the case of bone where the bulk of the Ca and P in the body are found, both Ca and P must also be available to the animal and in the body for the production of hydroxyapatite (a complex tricalcium phosphate).

### III. THE CASE FOR THE NEED FOR A DIGESTIBLE CALCIUM SYSTEM IN THE CONTEXT OF CALCIUM TO PHOSPHORUS RATIOS

We have known for some time that the digestibility of P is low in seed based ingredients and variable and not 100% in animal or inorganic based sources (Table1).

**Table 1 - Phosphorus availability from plant and animal sources and feed phosphates (From <sup>a</sup>Van Der Klis and Versteegh, 1996; <sup>b</sup>Coon and Leske, 1998; <sup>c</sup>National Research Council, 1994)**

Ingredient	<sup>a</sup> TP, g/kg	<sup>a</sup> PP, g/kg	<sup>a</sup> AP (% of TP)	<sup>b</sup> Retainable P	<sup>c</sup> nPP, %	<sup>c</sup> nPP, % of TP
Corn	3.0	2.28	29		0.08	28.0
Corn	-	3.96	-	34.9		
SBM	7.1	4.33	61		0.22	35.5
SBM	-	2.39	-	30.8		
Wheat	3.4	2.52	48		0.13	35.1
Wheat	-	3.32	-	30.7		
Wheat Middlings	10.8	7.99	36		0.20	17.4
Wheat Middlings	-	11.85	-	29.1		
Meat and bone meal	60	-	66			
Fish Meal	22	-	74			
Dicalcium Phosphate	181	-	77			
Monocalcium Phosphate	226	-	84			

<sup>a</sup> Availability based on standardised balance trials. <sup>b</sup> Retainable P based on balance trials. Total phosphorus (TP), available phosphorus (aP), phytate phosphorus (PP), non-phytate phosphorus (nPP).

In a study where the digestibility of Ca and P were determined in a corn-SBM starter diet devoid of inorganic Ca or P sources, the digestibility of Ca in the corn and SBM was 33.6% and of P was 67.9% (Tamim et al., 2004). These authors demonstrated the important and large negative impact of Ca on phytate P digestibility when 0.5% Ca from CaCO<sub>3</sub> was added to the corn-SBM diet (analysed Ca in the corn-SBM diet as 0.17% and after adding 0.5% Ca from CaCO<sub>3</sub> was 0.65%). As the Ca in the diet went from 0.17 to 0.65%, the digestibility of phytate P went from 69.2 to 25.4%; this when the only dietary change was the addition of 0.5% Ca from CaCO<sub>3</sub>. Key also from this work was the low digestibility of Ca reported from the corn-soy diet, with no added inorganic sources of Ca or P of 33.6% and by calculation the digestibility of the Ca from the inorganic source, CaCO<sub>3</sub> was 62.7%.

More recent work at the University of Maryland, where true digestibilities of Ca and P from SBM, CaCO<sub>3</sub> and mono calcium phosphate were determined in trials lasting from 8 to 96hr (Proszkowiec-Weglarz et al., unpublished), where purified diets were fed and the specific ingredients were the only source of the P or Ca, true digestibilities of Ca for limestone and mono calcium phosphate were 34.1 and 67.9%, respectively when determined at 40hr post feeding of the ingredient to 25 d old broilers. Of importance is the low Ca digestibility of a feed grade limestone as compared to that in mono calcium phosphate when the absolute Ca concentration was similar.

If, indeed, the digestibility of Ca from mono calcium phosphate is 2 times higher than that of Ca from a limestone, then what are the impacts on Ca to P ratios when phytases are used? When we use phytases in broiler diets, usually inorganic phosphates are decreased or removed

and Ca from limestone is used. If we use an example of a corn-SBM diet with and without phytase, where we give phytase similar matrixes for Ca and P, the amount of mono calcium phosphate in the diet would be reduced by 60% and of limestone by 12%. What are the implications from a digestible ratio perspective? If we assume a dCa to dP ratio in the diet without phytase of 1.6 to 1, then the ratio in the diet with phytase would be close to a 1 to 1 ratio. This change is brought about by the large decrease in the amount of mono calcium phosphate with the much higher dCa.

What Ca to P ratios to use become even more difficult to access in diets with animal Ca and P sources where sometimes, under commercial conditions, we find ourselves adding inorganic P sources to maintain Ca to P ratios. Having ingredient dCa as well as dP values is essential and, to date, little if any dCa ingredient data are available. Requirement work, in the future, should provide data as dCa and dP requirements for optimisation of performance and bone measures. We also must strive to define ranges of dCa to dP that optimise performance and bone mineralisation and minimise welfare issues.

#### IV. CONCLUSION.

It is important to move to a dCa and a dP system that allows nutritionists to formulate diets that meet the needs of the animal. We must accept that dCa in inorganic sources are widely different. Furthermore, when we use phytase, we change Ca source from a calcium phosphate that usually has higher dCa to a limestone that has usually lower dCa and that has a dCa that varies with source.

The current system of tCa to a P value that partially reflects digestibility no longer serves our needs for formulating diets where we minimise P concentrations, reduce costs, use alternate ingredients and feed additives that change both the digestibilities of Ca and P.

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## CALCIUM AND PHOSPHORUS INTERACTIONS IN BROILER NUTRITION: A GEOMETRIC FRAMEWORK APPROACH

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### Summary

Dietary calcium (Ca) and available phosphorus (avP) interactions were investigated using a total of 760 day old Ross 308 male broilers fed from 7-28d in a geometrically designed experiment. . Birds were fed corn-soy diets that were nutritionally similar except for their Ca and avP concentrations. Diets were clustered into low, medium and high Ca:avP densities (12, 13.5 and 15 g/kg, respectively) and at each density, five Ca:avP ratios were fed to generate a geometric nutrient space. Performance data were surface mapped using the Thin Plate Spline procedure of the fields package in R. A significant interaction between Ca and avP was found for feed intake, body weight gain and feed conversion ratio. Generally, performance of broilers was negatively influenced by low avP and high Ca concentrations. Performance was improved as avP concentrations increased up to 5.0 g/kg, however, beyond this level no discernible improvements were found. Increases in both Ca and avP concentrations led to enhanced ( $P < 0.001$ ) P digestibility. An interaction between dietary Ca and avP was found for Ca digestibility ( $P < 0.05$ ) with greater concentrations of Ca improving its digestibility but increased avP having an opposing effect. Nitrogen digestibility was estimated to be maximised within the bounds of feeding diets containing 4.5-5.4 g/kg avP and 8.1-10.0 g/kg Ca. The results of this study emphasise the importance of formulating diets that meet or exceed avP requirements of broilers, particularly when high Ca diets are used.

### I. INTRODUCTION

Calcium (Ca) and phosphorus (P) requirements of poultry have been investigated extensively over the past 50 years. However, the interactions of these two macrominerals are highly complex and are not easily interpreted. The majority of published animal nutritional research is based either on altering a single food property at a time or where multiple factors are changed. While these approaches are valuable in some circumstances, both of these impart a sense of uncertainty where two nutrients interact (Simpson and Raubenheimer, 1999), such as is the case with Ca and P in poultry nutrition.

An alternative to the aforementioned methods is the geometric framework (GF) which graphically models outcomes such as bodyweight and feed intake and maps these in a geometric space that is fashioned by two or more axes. The axes of the GF may be nutritional, environmental or any other input that contributes to the maintenance and growth of the animal. The GF has been used in other species such as insects, rodents, fish and humans to investigate the relationship between the macronutrients; carbohydrate and protein (Simpson and Raubenheimer, 2001; Simpson et al., 2003). In this study the GF was applied to investigate the interaction between varying concentrations and ratios of total Ca and avP on production and nutrient digestibility in broilers.

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## II. MATERIALS AND METHODS

All experimental procedures in this study were conducted in accordance with the University of Sydney Animal Ethics Committee. A total of 720 day old male Ross 308 chicks were obtained from a commercial hatchery (Baiada Poultry Pty Ltd, Marsden Park, NSW) and randomly allocated to cages. Birds were maintained in an environmentally controlled room set at 31 °C for the first five days and this was reduced by 0.5 °C per day until 24 °C (day 21). The photoperiod was 23h:1h (light:dark) for the first five days and then 18h:6h (light:dark) thereafter.

For the first 7 days, all birds were fed a standard commercial broiler starter diet which provided 11.8 MJ/kg AME, 228g/kg CP, 8.8 g/kg Ca and 4.4 g/kg avP (Vella Stock Feeds, Plumpton NSW). On day 7, all birds were individually weighed, wing tagged and randomly allocated to one of 15 dietary treatments in a completely randomised design. Diets were fed as a mash based on corn and soybean meal and were formulated to be nutritionally adequate except for total Ca and avP. Diets were clustered around three total Ca + avP densities of 12, 13.5 and 15 g/kg with five varying ratios of total Ca:avP (4:1, 2.75:1, 2:1 and 1.14:1) within each density. To calculate the apparent ileal digestibility (AID) coefficients for crude protein (CP), energy and minerals, an indigestible marker (Celite 281, Filchem Australia Pty Ltd, Castle Hill, NSW, Australia) was added to diets at a concentration of 20 g/kg. Treatments were replicated five times with eight birds per replicate cage. Feed intake and individual body weight were recorded weekly. Ileal digesta samples were collected from individual birds on day 28 of the study according to the procedures of Ravindran *et al.* (2005). Samples were frozen at -20 °C and freeze dried thereafter.

The gross energy (GE) of diets and excreta were determined using a Parr 1281 adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, USA) that was standardised with benzoic acid. Nitrogen concentration of samples was determined by the Dumas method using a FP-428 nitrogen analyser (LECO<sup>®</sup> Corporation, St. Joseph, MI, USA) as described by Sweeny (1989). Samples were wet acid digested using nitric acid and hydrogen peroxide prior to the determination of mineral concentration by inductively Coupled Plasma-Optical Emission Spectroscopy using a Perkin Elmer OPTIMA 7300 (Perkin Elmer Inc, Waltham, MA, USA) (Peters *et al.*, 2003). The acid insoluble ash component of dried diets and ileal digesta samples were determined according to the method of Siriwan *et al.* (1993).

Performance data were surface mapped using the Thin Plate Spline procedure of the fields package (R Development Core Team, 2011). Treatments are represented as dots overlaid on the contour plots. Interrogation of the data was performed using a quadratic function. Treatment differences were considered significant at  $P < 0.05$ .

## III. RESULTS AND DISCUSSION

The interaction between formulated dietary total Ca and avP on feed intake (FI) for the experimental period are presented in Figure 1. Broilers fed diets with high avP (> 5.0 g/kg) and high Ca (> 10.0 g/kg Ca) had the greatest FI. Feed intake increased as avP increased between 2.4-3.0 g/kg but was less influenced above 4.0 g/kg avP where Ca concentration became dominant. These results are in agreement with Perney *et al.* (1993) who showed that feed intake of broilers was reduced at low avP and was increased as greater avP was provided. Since P is essential for many physiological processes, it may be that when birds are fed low avP diets reduced consumption of high Ca diets may be a response to restore Ca and P homeostasis. However, the mechanisms governing this relationship are yet to be defined.

As indicated by the close proximity of the contour lines (see Figure 2), increased body weight gain response of broilers was more rapid between 2.4-3.0 g/kg avP when compared to increasing avP from 4.0-5.0 g/kg. At low avP concentrations, this relationship was more pronounced when total Ca concentrations were greater than 9.6 g/kg. However, when concentrations of dietary avP were greater than 4.0 g/kg total Ca was more influential to body weight gain (interaction of  $P < 0.01$ ).

Overall FCR was poorest for broilers fed diets with low avP and was less influenced by dietary Ca at these same avP concentrations (Figure 3). Increasing the concentration of avP in diets resulted in an improved FCR and this was optimised when birds were fed the diet formulated with 5.6 g/kg avP and 6.4 g/kg Ca (Ca x avP;  $P < 0.01$ ). The AID coefficients for N, Ca, and P are shown in Figures 4-6, respectively. Maximal AID of N was centred on feeding diets containing 4.5-5.4 g/kg avP and 8.1-10.0 g/kg Ca. Sharp declines in N digestibility were found when birds were fed diets outside of these parameters and suggest that relatively moderate changes in Ca or avP concentrations would result in significant changes to ileal N digestibility.

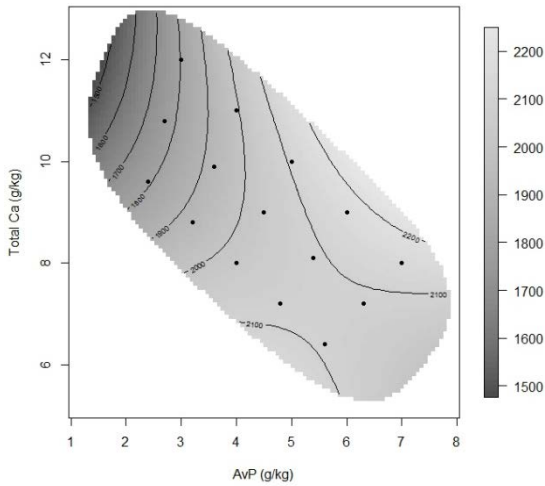


Figure 1 - The effect of total dietary Ca and avP on the feed intake (g/b) of broilers (d7-28).

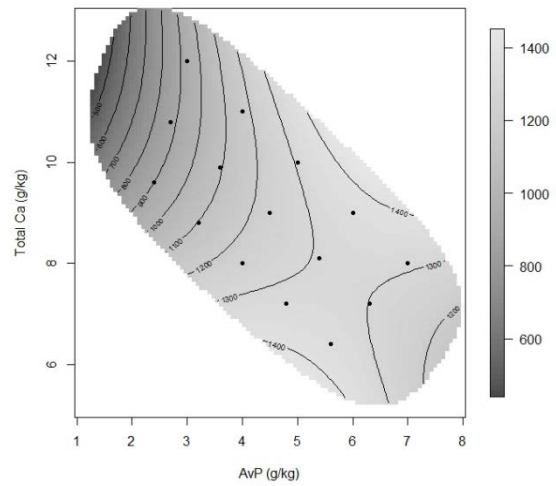


Figure 2 - The effect of total dietary Ca and avP on the body weight gain (g/b) of broilers (d7-28).

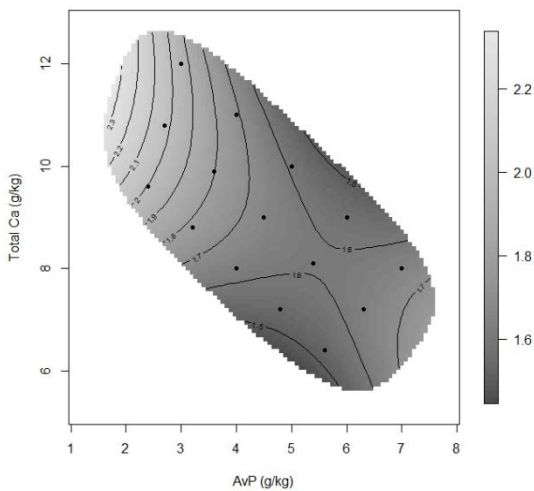


Figure 3- The effect of total dietary Ca and avP on the feed conversion ratio (g:g) of broilers (d7-28).

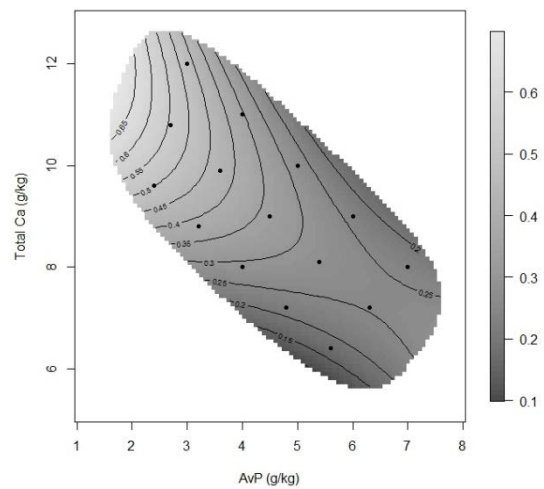


Figure 4 - The effect of total dietary Ca and avP on the apparent ileal digestibility of Ca in broilers (d28).



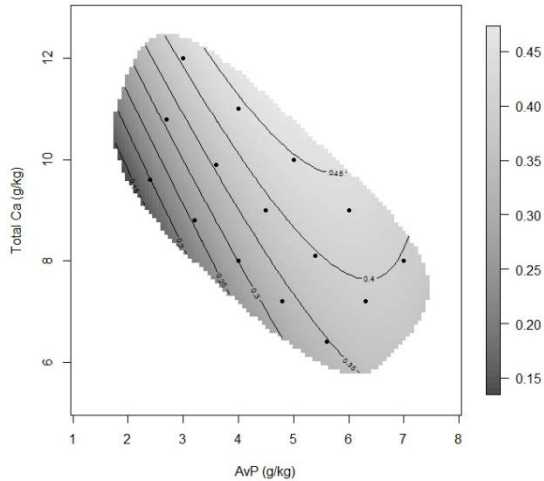


Figure 5 - The effect of total dietary Ca and avP on the apparent ileal digestibility of P in broilers (d28).

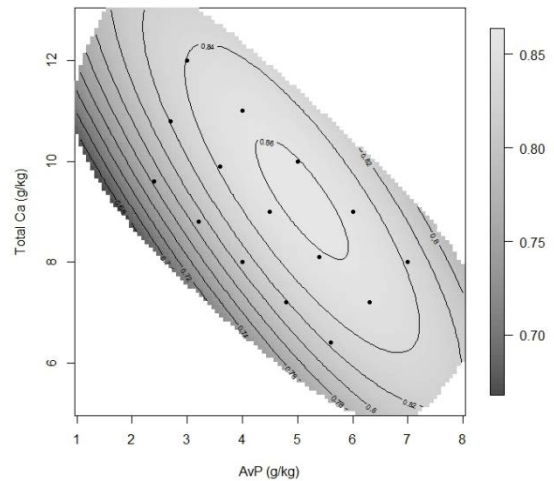


Figure 6 - The effect of total dietary Ca and avP on the apparent ileal digestibility of N in broilers (d28).

Results for the AID of Ca showed there was an interaction ( $P < 0.05$ ) between Ca and avP concentration with birds fed 2.7 g/kg avP and 10.8 g/kg Ca diets having the highest Ca AID in contrast to birds fed the diet containing 5.6 g/kg avP and 6.4 g/kg Ca that had the poorest. The higher ileal Ca digestibility reported in this study is in agreement with previous findings in similar age broilers fed corn-soy based diets (Cowieson et al., 2006). Dietary Ca ( $P < 0.001$ ) and avP ( $P < 0.001$ ) concentrations were both found to influence the AID of P and was greatest when birds were fed diets with 5.0 g/kg avP and 10.0 g/kg Ca and least when birds were fed the 2.4 g/kg avP and 9.6 g/kg Ca diet. In conclusion, within the confines of this study it is apparent that the concentration of avP provided in broiler diets is of greater influence than total Ca on the performance metrics measured. This is made more apparent when diets contain high concentrations of Ca.

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EFFECTS OF LOW CALCIUM AND AVAILABLE PHOSPHORUS DIETS ON PERFORMANCE, SKELETAL CHARACTERISTICS, AND WELFARE PARAMETERS OF ROSS-308 MALE BROILERS

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The aim of this trial was to investigate the effect of lowering dietary calcium (Ca) and available phosphorus (av.P) during Starter (0 to 10d), Grower (11 to 24d) and Finisher (25 to 45d) phases on performance of 4416 Ross-308 male broilers. Diets were wheat-soybean meal, corn gluten meal based, and no phytase added. Birds received one of two dietary Starter treatments: 1.13% Ca; 0.50% av.P or 1.03% Ca; 0.45% av.P. At 10 days, birds were assigned to a Grower/Finisher treatment as part of a central composite design (CCD). The CCD used a combination of one of five dietary Grower treatments (0.64% to 1.04% Ca; 2.2:1 Ca:av.P) with one of five Finisher treatments (0.58% to 0.99% Ca; 2.2:1 Ca:av.P) creating 12 runs (8 treatments + 4 centre points) with 8 replicates per run arranged according to each of the Starter treatments. Daily 23-hrs light was provided up to day 7, and 18-hrs light until depletion. Birds were brooded at 32 °C, with temperature gradually reduced to 20 °C at 27d, and remained at this level until depletion. Liveweight (LW) and mortality adjusted FCR (FCR<sub>adj</sub>) were recorded on days 10, 24, 32, 39 and 45. For lixiscopes (x-ray) bone evaluation, five and four average weight live birds/pen were selected on days 26 and 40, respectively. These birds were also evaluated for any visual leg defects, incidence and severity of hock burn and foot pododermatitis (FPD) at both ages. At 39 days, five average weight birds/pen were processed and carcass characteristics were recorded. At 10 days, there was no significant effect of dietary treatment on LW or livability. The FCR<sub>adj</sub> at day 10 was significantly improved ( $P < 0.001$ ) by lowering dietary Ca (1.020 vs. 1.009). There was a significant linear response to increasing dietary Ca in the Grower phase for 24 day LW (mean 1248.1g  $P < 0.01$ ) and FCR<sub>adj</sub> (mean 1.359;  $P < 0.006$ ). Dietary treatment had no significant effect on lixiscopes scores, incidence of leg defects or FPD at day 26. There was a significant linear response to lowering Ca levels in Grower diets on incidence and severity of hock burn ( $P < 0.01$ ) at 26 days. There was no significant effect of dietary treatment ( $P > 0.05$ ) on 32 day LW (mean 2041.3g). At 32 days FCR<sub>adj</sub> (mean 1.498) was improved linearly by increasing Ca levels during Grower period only ( $P < 0.013$ ). At 39 days, LW (mean 2803.1g) increased linearly by increasing Ca levels in the Finisher period ( $P < 0.004$ ) and there was a positive Grower x Finisher diet interaction ( $P < 0.003$ ). However, FCR<sub>adj</sub> (mean 1.594) was unaffected ( $P > 0.05$ ) at this time point. At 39 days there was a positive linear response in 0.04% of eviscerated carcass yield (mean 70.85%;  $P < 0.02$ ), by increasing dietary Ca in Finisher phase. Neither Grower nor Finisher dietary treatment had a significant effect on lixiscopes analysis or leg defects at 40 days. However, there was a linear response to lowering Ca in Grower diets resulting in a reduction in severity of hock burn ( $P < 0.05$ ). At 45d, LW (mean 3528.5g) and FCR<sub>adj</sub> (mean 1.606) were not affected by the treatments ( $P > 0.05$ ). In conclusion, when using a Starter diet with higher content of calcium it is possible to adopt intermediate dietary levels of Ca in Grower (~0.80%) and Finisher (~0.70%) phases to 45 days without compromising broiler performance or skeletal characteristics.

*Keywords:* broiler, low calcium, low phosphorus, performance, skeleton

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## INTERACTION BETWEEN DIETARY PHYTASE, CALCIUM AND DIGESTIBLE PHOSPHORUS LEVELS ON PERFORMANCE AND TIBIA ASH IN BROILERS

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### Summary

A broiler study was conducted for 36 days with eight treatment groups in a 2 phytase x 2 calcium (Ca) level x 2 digestible phosphorus (dP) level experimental design. Birds exposed to diets low in Ca, and standard in dP performed the best while the high Ca treatments induced negative responses, especially in the first 8 days of experimentation. It was concluded that the level of dP release from the high phytase inclusion was overestimated, while the higher Ca levels included in the study impeded phosphorus (P) digestibility and bone development.

### I. INTRODUCTION

The global availability of inorganic phosphates is limited; phosphorus (P) is the third most expensive ingredient after energy and amino acids. Therefore sustainable animal production requires optimal utilisation of P to reduce the cost of feeding. Over two thirds of P in plant-based feedstuffs is not readily available in poultry as it is bound to phytic acid (PA), which has been commonly thought to be due to the low levels of endogenous phytase (Bedford, 2000; Woyengo and Nyachoti, 2011). However, recent studies have suggested that this is not the case, and in fact chickens possess adequate phytase activity in the intestinal mucosa. The primary issue with phytate digestion is poor substrate solubility in the small intestine due to cation (mainly Ca) interactions (Maenz and Classen, 1998; Cowieson, et al., 2011b). Calcium is known to form insoluble complexes with phytate phosphorus (PP), which hinders phytase activity (Angel, et al., 2002). Therefore, lowering dietary Ca levels can further improve the effect of exogenous phytase on PP degradation. Recent studies have shown that when adding phytase (500 FTU/kg), while at the same time lowering Ca in starter diets from 1% to 0.67% in combination with reduced non-PP (nPP) levels does not impair young bird performance (Létourneau-Montminy, et al., 2010; Powell, et al., 2011).

The use of exogenous phytase to assist the bird in degrading PP has become common practice. In recent years, the use of higher levels of exogenous phytase, referred to as super dosing, has been increasingly promoted as a method to release more PP and to alleviate the antinutritive effects of phytate *per se* (Cowieson, et al., 2011b).

In this study, the interaction between dP, Ca and phytase levels was evaluated with the aim of lowering inclusions of inorganic phosphates by optimising the hydrolysis of PP.

### II. MATERIALS AND METHODS

Three thousand two hundred male Ross 308 broiler chickens were randomly distributed amongst 80 pens, 40 birds per pen, giving 8 treatments with 10 replicates. Each pen, 2.5 x 1.6 m, was fitted with 1 feeder and 4 nipple cup drinkers. Birds had *ad libitum* access to feed and water. From 0-3d, birds had 24 hours of light and from then on 18 hours of light and 6 hours of darkness. At the start of the trial brooding temperature was set to 33°C and then reduced incrementally to 20°C by 29 days. A description of treatments is shown in Table 1. A 3 phase

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feeding program was employed (0-8 d, 8-22 d and 22-36 d). Basal diets were maize/soya with energy increasing from 2850 kcal (11.92 MJ/kg AME) to 2950 kcal (12.34 MJ/kg AME) through phases and crude protein decreasing from 22.5% to 18.5%. The Ca, dP and phytase levels described in Table 1 were achieved by preparing a separate premix of limestone, monocalcium phosphate, phytase and sepiolite, added on top of the basal diet to make up the treatments. Basal diets contained between 2.58 and 2.78 g/kg phytate phosphorous. Birds and feed were weighed on days 0, 8, and 36. At 8 and 36 days of age, 1 animal/replicate, with a body weight (BW) within 5% of the average pen BW was euthanatized, and right tibias were collected, cleaned, and weighed for tibia ash determination. Data was analysed factorially using the general linear models (GLM) procedure of SAS.

**Table 1 - Description of treatments**

Treatment	Phytase level (FTU/kg)	Ca <sup>1</sup> , g/kg. S-G-F <sup>2</sup>	dP <sup>3</sup> , g/kg. S-G-F <sup>2</sup>
1 (control)	500	10.0 – 7.5 – 6.2	4.6 – 3.5 – 3.3
2	1500	10.0 – 7.5 – 6.2	4.6 – 3.5 – 3.3
3	500	10.0 – 7.5 – 6.2	3.7 – 3.1 – 2.9
4	1500	10.0 – 7.5 – 6.2	3.7 – 3.1 – 2.9
5	500	6.7 – 5.0 – 4.1	4.6 – 3.5 – 3.3
6	1500	6.7 – 5.0 – 4.1	4.6 – 3.5 – 3.3
7	500	6.7 – 5.0 – 4.1	3.7 – 3.1 – 2.9
8	1500	6.7 – 5.0 – 4.1	3.7 – 3.1 – 2.9

<sup>1</sup> Total calcium. <sup>2</sup> S-G-F, starter, grower, finisher respectively. <sup>3</sup> According to CVB system (also called retainable P), in formulation, 500 FTU/kg feed was given the nutritive value of 0.96 g dP, and 1500 FTU/kg feed was given the nutritional value of 1.69 g dP. No other nutritional value was given to phytase in formulation.

### III. RESULTS

Increasing phytase dose from 500 to 1500 FTU reduced both feed intake (FI) and feed conversion ratio (FCR), without an effect on BW at 8 days of age (Table 2). Standard Ca levels impeded FI and BW at 8 days of age ( $P < 0.001$ ), in birds receiving diets low in dP resulting in significant Ca\*dP interaction ( $P < 0.001$ ). Low dP levels impaired all performance parameters ( $P < 0.001$ ). A significant interaction between phytase and dP was recorded for d8 BW, with the heaviest chicks observed in treatments with standard dP levels irrespective of enzyme inclusion, while high levels of phytase resulted in a lower bird weight than the lower phytase inclusion concentration, in the low dP treatments ( $P < 0.05$ ).

At d36 a significant interaction between Ca and dP was observed, where standard Ca concentrations resulted in reduced BW and FI only in the birds fed the low dP diets. FCR for the total period was lower in the standard Ca treatments ( $P < 0.05$ ). A tendency for an interaction between Ca and dP was noted, with standard Ca, low dP treatments yielding the most desirable feed conversions ( $P=0.089$ ).

As shown in Table 3, at 8 days of age, tibia ash analysis revealed mineral content of bones from treatments 1, 2 and 7 were the highest ( $P < 0.01$ ), while the lowest were to be found in treatment 4. High levels of dP had a significant improvement on tibia ash % ( $P < 0.05$ ). In addition two way interactions were observed between all factors. At 36 days of age, no effects of treatment were observed on tibia ash %.

### IV. DISCUSSION

The results of this study support previous observations that dietary Ca concentrations are influential in the extent to which birds respond to dP and phytase (Angel, et al., 2002; Tamim, et al., 2004; Cowieson, et al., 2011b). At standard Ca levels, low dP levels had a

negative effect on weight gain at all ages and tibia ash at 8 days, this effect was further compounded by the addition of 1500 FTU/kg of phytase. The observed trend in depressed

**Table 2 - Performance parameters of birds at 8 and 38 days of age**

Phy level	Ca level	dP level	BW8 (g)	DFI8 (g)	FCR8	BW36 (g)	DFI036 (g)	FCR036
Phy x dP								
1500		Standard	215.5 <sup>a</sup>	23.8	1.104	2715	112.7	1.524
1500		Low	204.5 <sup>c</sup>	22.7	1.127	2610	108.4	1.521
500		Standard	214.1 <sup>a</sup>	24.0	1.122	2735	114.2	1.528
500		Low	207.0 <sup>b</sup>	23.2	1.135	2675	111.6	1.526
Ca x dP								
	Standard	Standard	214.4 <sup>a</sup>	23.8 <sup>a</sup>	1.113	2702 <sup>b</sup>	112.6 <sup>b</sup>	1.525 <sup>ab</sup>
	Standard	Low	200.2 <sup>c</sup>	22.3 <sup>b</sup>	1.136	2565 <sup>c</sup>	106.2 <sup>c</sup>	1.516 <sup>b</sup>
	Low	Standard	215.2 <sup>a</sup>	24.0 <sup>a</sup>	1.113	2749 <sup>a</sup>	114.3 <sup>a</sup>	1.527 <sup>a</sup>
	Low	Low	211.3 <sup>b</sup>	23.7 <sup>a</sup>	1.126	2719 <sup>ab</sup>	113.8 <sup>ab</sup>	1.531 <sup>a</sup>
1500			210.0	23.3	1.115	2662	110.6	1.522
500			210.6	23.6	1.128	2705	112.9	1.527
	Standard		207.3	23.1	1.124	2633	109.4	1.520
	low		213.3	23.8	1.120	2734	114.1	1.529
		Standard	214.8	24.0	1.113	2725	113.5	1.526
		low	205.7	23.0	1.131	2642	110.0	1.524
	LSD		3.531	0.50	0.015	73.28	2.600	0.016
	SEM		1.104	0.16	0.005	22.90	0.813	0.005
	Phy		-	**	***	*	***	-
	Ca		***	***	-	***	***	*
	dP		***	***	***	***	***	-
P values	Phy*Ca		-	-	-	-	-	-
	Phy*dP		*	-	-	-	-	-
	Ca*dP		***	***	-	**	***	0.09
	Phy*Ca*dP		-	-	-	-	-	-

a-c = values in a row with unlike superscripts differ significantly where; \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001.

**Table 3 - Tibia ash % at 8 and 36 days of age**

	Phy level	Ca level	dP level	Ash%8	Ash%36
1	500	normal	normal	20.50 <sup>a</sup>	21.27
2	1500	normal	normal	20.47 <sup>a</sup>	21.46
3	500	normal	low	18.91 <sup>bc</sup>	21.38
4	1500	normal	low	17.87 <sup>c</sup>	21.56
5	500	low	normal	19.01 <sup>bc</sup>	21.55
6	1500	low	normal	18.58 <sup>bc</sup>	20.94
7	500	low	low	19.49 <sup>ab</sup>	21.14
8	1500	low	low	19.09 <sup>bc</sup>	20.84
	SEM			0.36	0.21
			dP	*	-
P values			Phy*dP	*	-
			Ca*dP	***	-
			Phy*Ca*dP	**	-

<sup>1</sup>a-c = values in a row with unlike superscripts differ significantly where; \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001. <sup>2</sup>The main affects Phy, Ca, and the two way interaction Phy\*Ca were all insignificant at both ages.

bone development in chicks exposed to high phytase levels in diets with standard Ca and low dP diets suggests that P availability was low. This may be due to an overestimation in P release at the higher phytase inclusion. As a result, diets were deficient in dP, with proportionally higher available Ca levels, capable of further lowering P availability by rendering nPP insoluble due to cation chelation. At low Ca levels, the low dP levels had a less pronounced effect on weight gain and bone ash compared to the high Ca levels. In turn, the high phytase level did not impede young chick bone mineralisation at the lowest dP levels to the same degree as observed in treatments with the same level of phytase and dP at standard Ca levels. Together this indicates a higher P availability at low Ca levels, supporting previous studies (Rama Rao, et al., 1999; Tamim, et al., 2004; Powell, et al., 2011).

Phytase has been shown to enhance energy and protein digestibility, resulting in improvements in BW with reduced FI (Ravindran, et al., 1999; Selle, et al., 2000). In the present study, the significant reductions in BW with 1500 FTU/kg of phytase, in addition to the assumed low P release, may have been due to a shortage of nutrients as a result of the reduced FI and the enzymes failure to compensate by releasing amino acids due to the abundance of Ca cations. At normal dP levels, the low Ca levels resulted in improved growth and litter quality (data not shown) compared to the standard Ca levels.

It can be concluded that reducing dietary Ca concentrations, particularly in the grower, finisher diets, results in improved performance of broilers with no change in bone ash (at slaughter weight) especially when offered diets with marginal dP supply. This strategy may be an effective way to reduce diet costs and improve broiler efficiency under commercial production systems. When increasing phytase dosing from 500 to 1500 FTU in formulation with matrix values of 0.96 and 1.69g dP/kg of feed, the risk of reduced FI should be considered. Therefore, in order to improve the consistency of the beneficial effects of higher doses of microbial phytase, dietary reformulation may be required, with particular attention to amino acid and energy concentrations (Cowieson, et al., 2011a).

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## BIO-EFFICACY OF FEED PROTEASES IN POULTRY AND THEIR INTERACTION WITH OTHER FEED ENZYMES

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### Summary

The most accepted value of the inclusion of exogenous proteases in poultry diets is the improvement of protein digestibility from dietary ingredients. However, effects of exogenous proteases on animal performance do not necessarily reflect the increment in protein digestibility from proteases *in vitro* and a variety of factors determine the bio-efficacy of the application of proteases in chickens. An accurate prediction of amino acid digestibility improvements in response to dietary enzymes is important to maximise the animal performance response and ensure that the cost of including the enzyme is justified. Overestimation of the digestibility effect of protease on individual essential amino acids relative to other amino acids may limit the benefits of increments on the absorption of dietary protein for animal growth. A lack of understanding of what effects proteases alone or in combination have on the digestibility of individual amino acids may further imbalance the amino acid profile provided by the diet. It has been recently demonstrated that improvements in the digestibility of amino acids following exogenous protease application in broiler diets can be accurately predicted as a linear function of undigested amino acids in the diet. The contribution of specific proteases in combination with carbohydrase enzymes in terms of protein digestibility has been confirmed. Nonetheless, effects of protease on the digestibility of other nutrients such as fat and fibre, may play a role in determining the *in vivo* response to protease in chickens. Factors like the age of the birds, the gut health status of the animals, and the profile of gut microbial populations appear to affect the response to proteases in animal performance and require further study.

### I. INTRODUCTION

The use of exogenous proteases in poultry feed has become more prevalent in recent years, following the broader commercial acceptance of other feed enzymes like phytases and xylanases, and increased pressure on the cost of proteinaceous ingredients. Most current commercial proteases for animal feed are alkaline proteases of bacterial origin. Feed cost is reduced with the inclusion of proteases through a reduction of crude protein and first limiting amino acids supplied by dietary ingredients. Nonetheless, the effects of exogenous proteases on animal performance do not necessarily reflect the *in vitro* digestibility of protein from ingredients, but are influenced by a variety of factors that affect their bio-efficacy. For example, serine proteases in broiler chickens do not appear to show a linear dose response on protein digestibility of complete feeds, but an optimum is present (Argüelles-Ramos et al., 2010), after which marginal reductions with increasing protease doses are evident. This suggests that a balance between the hydrolysis of dietary protein and undefined physiological interactions in the intestine may limit further improvements in protein retention due to protease activity. Effects of exogenous proteases are not only confined to the digestion of protein, but extend to the digestion of other nutrients such as fat and starch. Furthermore, the application of proteases cannot be considered in isolation because it often occurs in combination with other feed enzymes, whose mechanisms and subsequent effects on digested nutrients do not appear to be totally independent to those of proteases.

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This paper describes factors that influence the bio-efficacy and application of proteases in broiler diets with emphasis on nutrient digestibility, the interaction of proteases with other dietary enzymes, and nutritional effects of exogenous proteases beside protein digestion.

## II. EFFECTS OF PROTEASES ON AMINO ACID DIGESTIBILITY

A correct estimation of an amino acid matrix to be assigned to exogenous proteases during the formulation of diets is essential to capturing their value in terms of animal performance. That is because an overestimation of the protease effect on the digestibility of essential amino acids, in particular, would create limits to the efficient utilisation of potential improvements on crude protein digestibility. A common mistake in the application of proteases is to assume that a set amount of improvement of protein digestibility, e.g. 3%, can be extrapolated for all amino acids, to calculate an amino acid matrix. In reality, individual dietary amino acids differ widely in their digestibility across different ingredients and diet types. The effect of protease will always be limited by the amount of undigested amino acids present in the small intestine in the absence of the additive. In a recent review, Cowieson (2010) concluded that the inherent digestibility of nutrients in poultry diets prior to enzyme addition is a good indicator of the magnitude of enzyme response.

Romero et al. (2009) conducted a series of studies to determine the relationship between the amount of ileal undigested amino acids and the effects of protease on the ileal digestibility of each amino acid. Four different 21-day digestibility trials were conducted. Each study evaluated the energy and amino acid digestibility of broilers fed corn-soy diets supplemented with a multi-enzyme complex containing xylanase from *T. reesei*, amylase from *B. amyloliquefaciens*, and protease from *B. subtilis* (Avizyme 1502; Danisco Animal Nutrition, DuPont Industrial Biosciences), compared to one containing only xylanase from *T. reesei* and amylase from *B. amyloliquefaciens*, and an un-supplemented control treatment. Diets were corn-soybean meal based in two of the trials, and additionally contained 7-10% corn DDGS in the other two trials.

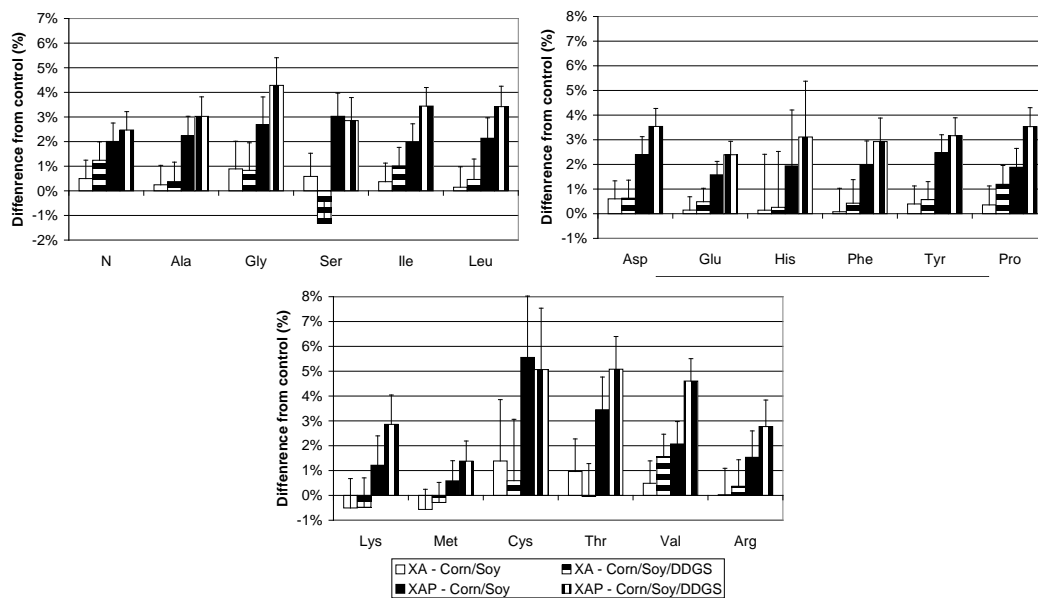


Figure 1 - Percentage change of ileal digestibility of nitrogen and amino acids on a control diet with addition of two different enzyme combinations of carbohydrases with (XAP) or without (XA) a serine protease in broiler chickens.



On average across all four studies, the addition of the xylanase/amylase combination to the un-supplemented control diet increased ileal digestible energy by 78 kcal/kg, whereas the xylanase/amylase/protease combination increased it by 100 kcal/kg. Most notably, the xylanase/amylase/protease treatment resulted in significantly higher digestibility of nitrogen and all amino acids with the exception of methionine, whereas xylanase/amylase did not exhibit significant differences in amino acid digestibility for any of the evaluated amino acids when compared to the control diets. The amino acids with the greatest digestibility response to xylanase/amylase/protease were cysteine (+5.4%), theonine (+4.4%), glycine (+3.6%), and valine (+3.3%), whereas the least responsive amino acids were methionine (+1.0%), glutamine (+2.0%), lysine (+2.0%), and arginine (+2.1%; Figure 1).

To further explore the reasons for the divergence in the digestibility response to xylanase/amylase/protease of different amino acids, the response relative to the undigested fractions of each amino acid in the control diets was analysed (Figure 2). Interestingly, the amount of individual undigested amino acids at the ileal level clearly determined the amino acid digestibility response to proteases on top of carbohydrases.

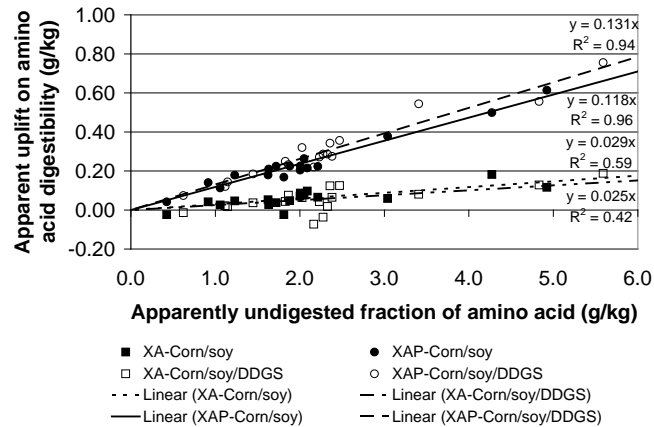


Figure 2 - Uplift of ileal amino acid digestibility of 21-d broiler chickens relative to the amounts of undigested amino acids at the ileal level, in response to carbohydrases or carbohydrases plus a protease in corn/soy or corn/soy/corn DDGS diets. Each point represents measured values for one amino acid in two trials.

Irrespective of the amino acid, a strong linear relationship between the amount of undigested amino acids and the digestibility response to enzymes was evident for xylanase/amylase/protease ( $R^2=0.94$  and  $0.96$ ). These results suggested that protein hydrolysis catalysed by the exogenous protease was responsible for the improvement of apparent ileal digestibility of amino acids. The effect of protease was non-specific to individual amino acids or diet types in this experiment, but it was mostly dependent on the inherent digestibility of amino acids in the diet.

Therefore, the contribution of protease to the digestibility amino acids that are very well digested will be smaller than that for amino acids that are less well digested. It becomes clear that providing greater amounts of highly digestible synthetic amino acids, such as DL-methionine to a diet, must reduce the potential increment in methionine digestibility from a protease. In contrast, amino acids with high concentration or low digestibility, such as glutamic acid, present higher increments on ileal amino acid digestibility from the protease. A conservative approach when recommending matrix values or down-specifications of limiting amino acids for protease supplementation appears to be preferable. Furthermore, digestibility improvement values produced in *in vitro* systems, or *in vivo* systems assessing the digestibility improvements of single ingredients, may overestimate the response of limiting amino acids if the absorption of these amino acids was not properly modelled.

### III. ADDITIVE EFFECTS OF PROTEASE AND OTHER DIETARY ENZYMES

Effects of protease on poultry diets do not appear to be completely limited to protein digestion, but can also affect the digestibility of other nutrients. McAllister (1993), for example, reported increased digestion of corn starch with the use of a serine protease in a rumen *in vitro* model, which the author attributed to the disruption on the protein matrix in starch granules. Similarly, protein digestibility can also be affected by the presence of other dietary enzymes. Effects of carbohydrases and phytases on amino acid digestibility have been demonstrated and appear to involve a reduction of endogenous amino acid losses (Cowieson et al., 2008; Rutherford et al., 2007). It has also been suggested that phytases reduce the association of phytate and protein in the gizzard and proventriculus, increasing protein solubility (Yu et al., 2012). However, as effects of proteases and other dietary enzymes are all dependent on the amount of undigested amino acids present in the digestive tract, increments in amino acid digestibility from different enzymes cannot be additive. Therefore, the nutrient contribution from protease and other dietary enzymes in practical diets should not be determined in isolation.

Romero et al. (2012) conducted a series of studies to better understand the complex interactions of protease with different dietary ingredients and other enzymes. Two studies with 432 21-day or 288 42-day-old Ross-308 broiler males evaluated changes on the ileal energy contribution of substrates in response to xylanase and amylase without, or with protease in four broiler diets. The studies used a 2 x 2 x 3 factorial arrangement of treatments with two base grains (corn-soybean-meal; or wheat-soybean-meal diets); two fibrous protein ingredient levels (with, or without 10% corn-DDGS and 5% canola meal); and three enzyme levels. At 12 d or 32 d, three enzyme levels were applied: a negative control (NC); NC with xylanase from *T. reesei* and amylase from *B. licheniformis*; or NC with xylanase from *T. reesei*, amylase from *B. licheniformis*, and protease from *B. subtilis* (Aextra XAP; Danisco Animal Nutrition, DuPont Industrial Biosciences). At 21 d or 42 d, birds were euthanised; ileal digesta was collected, pooled per cage, and analysed to determine the apparent digestibility of energy, starch, fat, and protein. The increment of ileal energy digestibility of starch, fat, and protein was calculated as the mean change on the coefficient of apparent ileal digestibility of the enzyme treatment compared to the respective control treatment, and multiplied by the measured nutrient content in the diet and the assumed gross energy content of each substrate (starch=4.2 kcal/g; fat=9.4 kcal/g; protein =5.5 kcal/g).

Starch digestibility increased with xylanase/amylase (97.8% at 21 d; 96.6% at 42 d) and xylanase/amylase/protease (97.9% at 21 d; 97.0% at 42 d) compared to the NC (96.3% at 21 d; 93.4% at 42 d) across diets. There were no differences between xylanase/amylase and xylanase/amylase/protease on ileal starch digestion. Xylanase/amylase (84.4%) and xylanase/amylase/protease (85.8%) gradually increased protein digestibility ( $P < 0.05$ ) at 21 d (NC=82.7%); but only xylanase/amylase/protease (85.1%) increased protein digestibility compared to the NC (82.4%) at 42 d. Both xylanase/amylase (83.3%) and xylanase/amylase/protease (84.0%) increased fat digestibility compared to the NC (80.2%) at 21 d. At 42 d, xylanase/amylase (86.6%) increased fat digestibility compared to NC (86.6%); and xylanase/amylase/protease (89.4%) further increased fat digestibility compared to xylanase/amylase.

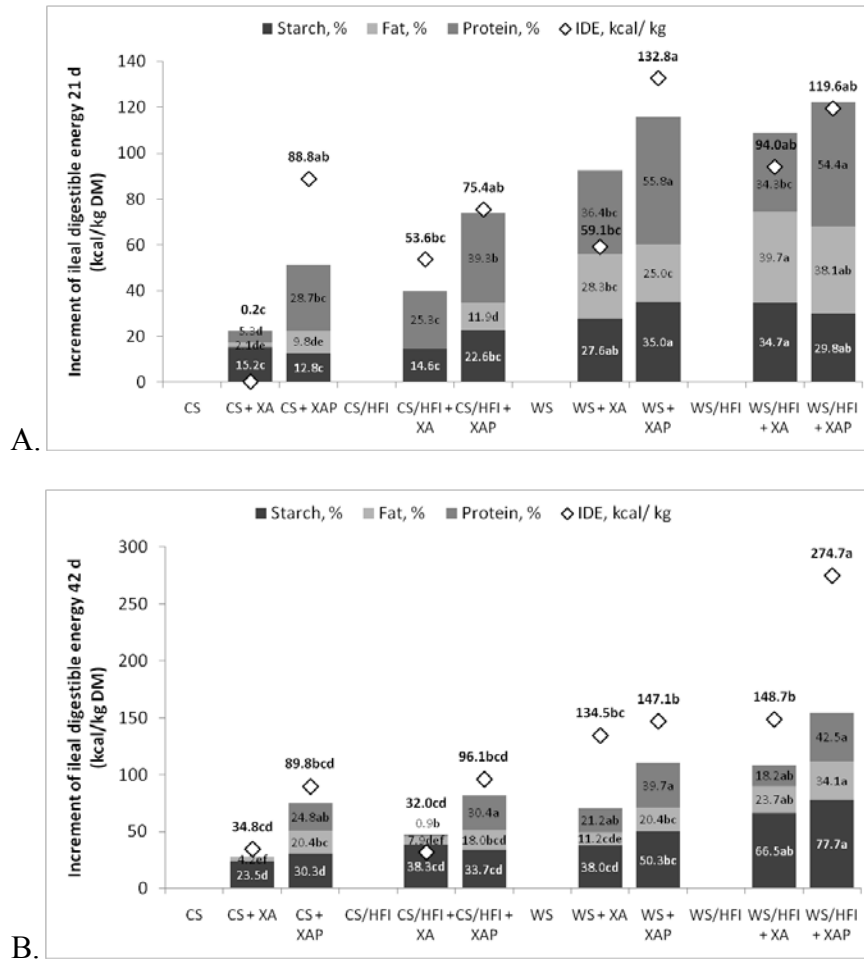


Figure 3 - Improvement in ileal digestible energy due to supplemental enzymes (◊) and calculated ileal energy contribution from starch, fat and protein fractions (bars) in broiler chickens at 21 (A) and 42 (B) days of age. CS=corn/soybean meal-based diet; WS=wheat/soybean meal-based diet; HFI=high-fibre ingredients (corn-DDGS and canola meal); XA=xylanase and amylase; XAP=xylanase, amylase and protease enzymes.

At 21 d (Figure 3), the largest contributor to the increase in apparent ileal digestible energy (AIDE) with xylanase/amylase/protease was the protein fraction, and the protein contribution to digestible energy was greater in wheat than in corn-based diets. For xylanase/amylase, there was a similar trend with the exception of corn-based diets without high fibre ingredients such as corn-DDGs, where the protein contribution to the effect of the enzyme was relatively low. Evidently, fat digestibility had a more prominent role in the total energy effect in response to enzymes in wheat-based diets, whereas the contribution of fat digestibility in corn-based diets was marginal. The extent whereby increments in starch digestibility contributed to the energy contribution of xylanase/amylase or xylanase/amylase/protease was also different between diets, being greater in wheat- than in corn-based diets. At 42 d (Figure 3), the energy contribution from increments in protein digestibility was still relatively high compared to that of starch and fat. Energy contributions from fat were high in 21 d old chickens, but substantially less at 42 d. Only marginal improvements on the energy contribution from starch and fat due to protease were present at 42 days; however, they represented between 9 and 38 kcal/kg depending on the diet type.

Interestingly, the measured energy improvement from enzymes in corn-based diets closely resembled the sum of the contributions from digested starch, fat, and protein at 42 d. However, in wheat-based diets there was a consistent difference between the measured

increase in ileal digestible energy from enzymes and the calculated contributions from starch, fat, and protein. This suggests that the digestibility of other components in the diet (other than fat, starch, and protein) may have contributed to the increased AIDE improvements due to enzymes. Enzyme inclusion may have caused either an increased fermentation of NSPs or the absorption of pentose sugars in the small intestine. The fact that this difference was not present in 21 d, but only in 42 d chickens also suggests interactions with the microbial populations of older birds may have occurred, with a resultant increase in digestion of fibre in the small intestine.

These data demonstrate that, although the main effect of protease was an improvement of protein digestibility, carbohydrases also significantly contributed to protein digestibility at least in some diets and for some growth periods. Similarly, proteases can also contribute to increasing the digestibility of other nutrients and presumably the digestion of NSPs. Other studies have also found overlapping effects of protease and carbohydrases on the digestibility of fat and starch in broiler chickens (Kalmendal and Tauson, 2012). Variability in the digestible nutrient response that is frequently seen with individual enzymes may be ameliorated by an integrated approach to exogenous enzyme utilisation which does not attribute independent additive nutrient contributions to single enzyme activities. It must also be stressed that the interactions of carbohydrases and proteases are likely to be diet-dependent and carbohydrase enzyme effects may differ depending on the nature of the NSPs in different grains and vegetable protein ingredients.

#### IV. PROTEASE EFFECTS ON FIBRE DIGESTION IN CHICKENS

Effects of exogenous proteases on the digestion of fibre in *in vitro* rumen systems have been reported (Colombatto and Beauchemin, 2009). However, specific effects of protease on the digestion of fibre in poultry are not well understood. Olukosi and Romero (2012) recently studied the effects of different protease doses, with or without carbohydrases on the nutrient digestibility and the NSP flow of broiler chickens fed corn/soybean-meal diets with the inclusion of corn-DDGS. A total of 336 1-d old broilers received a standard broiler starter diet until day 14 when they were allocated to seven treatments in a randomised complete block design. Each treatment had 8 replicate cages with 6 birds per replicate cage. Diet 1, the control, contained no enzyme, diets 2 and 3 contained protease from *B. subtilis* at graded levels (protease 1; 5000 or 10000 u/kg), diet 4 contained another bacterial protease (protease 2; 10000 u/kg) whereas diets 5, 6 and 7 contained admixture of xylanase from *T. reesei*, amylase from *B. licheniformis*, and protease from *B. subtilis* (Axta XAP; Danisco Animal Nutrition, DuPont Industrial Biosciences) at graded levels (50%, 100% and 200% the recommended dose for broilers, containing 2500, 5000, or 10000 protease u/kg). The diets were fed for 7 days, excreta samples were collected for the last 3 days of the experiment and ileal digesta samples were collected on the last day of the study.

The total tract flows of arabinose, xylose, galactose, glucose, and glucuronic acid from the NSP fraction (Figure 4) were reduced at the higher doses of protease, and both the intermediate and the high dose of xylanase/amylase/protease. In the specific case of xylose and arabinose, which are the main sugar components of arabinoxylans, the inclusion of xylanase and amylase generally reduced the flow of these sugars compared to protease alone at a comparable dose. These data suggest that fibre degradation may be one of the mechanisms by which proteases increase the digestion of nutrients in chickens. Although the reason of these effects of protease on NSP digestion is not known, studies in ruminant models have suggested that the use of proteases can disrupt cell wall associated proteins, which facilitates microbial colonisation of the substrate (Colombatto and Beauchemin, 2009).

However, effects of exogenous proteases on the gut microbial populations of chickens have not been properly studied.

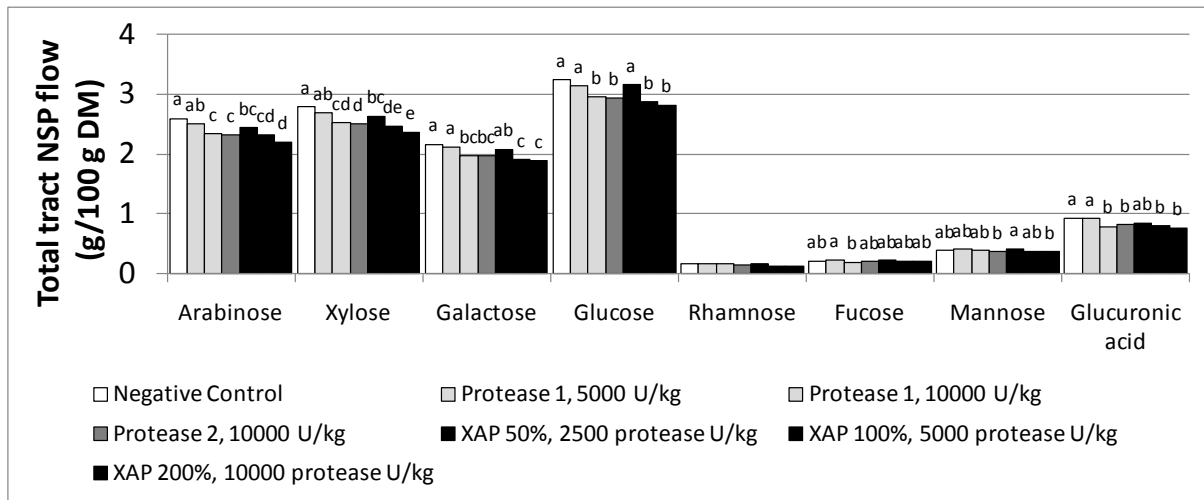


Figure 4 - Total tract flow of total sugars from the non-starch polysaccharides (NSP) fraction in 21-d-old broilers fed corn/soybean meal diets supplemented with different doses of two bacterial proteases or a combination xylanase, amylase, and protease (XAP).

## V. OTHER FACTORS AFFECTING THE BIO-EFFICACY OF PROTEASES

Effects of feed proteases that are not directly related to nutrient digestibility have also been reported in the literature. For instance, Caine et al. (1998) reported a reduction in the level of trypsin inhibitors of soybean meal with the use of a serine protease, which may be present in practice and has not been fully recognised as one of the effects of value for poultry diets. Positive effects of exogenous proteases on the ability of birds to cope with intestinal disease challenges like *Eimeria* infections or necrotic enteritis have also been suggested, although mechanisms are not well understood and the evidence available is not definitive. Peek et al. (2009) found that a protease from *Bacillus licheniformis* increased the body weight gain of broilers challenged with three *Eimeria* species, and suggested that the mechanism was a reduction in the attachment of parasites to the mucus layer. This was supported by an increase in the thickness of the adherent mucus layer of the intestine due to protease. Yan et al. (2011) suggested that a protease avoided the growth of *Clostridium perfringens* in birds challenged with an *Eimeria* vaccine through improved absorption of protein and a reduction in the protein available for bacterial growth. However, other reports have not found clear effects of dietary protease on birds challenged with *Eimeria* vaccines (Walk et al., 2011). Nonetheless, intestinal health appears to be a factor that affects the animal performance responses in the field with the use of proteases, and require further study.

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## EXOGENOUS PROTEASES AND THEIR INTERACTION WITH DIETARY INGREDIENTS

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### Summary

The human population is expected to reach 9 billion by 2038. The rapid urbanization and growing economies of developing countries have increased the disposable income of city dwellers and consequently resulted in an increase in meat consumption. The poultry industry is under severe pressure to feed an increasing population with less cereals and oilseeds available for feeding meat-producing animals. This has been precipitated by competition for corn and cereals by the ethanol industry and the reduced yields of corn and soybean due to droughts. Apart from improved genetics, the development and use of exogenous enzymes, such as proteases, has been a successful strategy to improve feed utilization. A mono-component protease has been demonstrated to significantly improve the digestibility of amino acids in commonly used raw materials such as corn, soybean meal, meat and bone meal and canola meal. Animal trials have also shown that this mono-component protease improved weight gain and feed conversion efficiency of broilers when used either on top of a standard broiler formulation or in low protein diets. However, more work needs to be done to determine the effect of exogenous proteases on other feed ingredients such as sorghum, palm kernel meal and rice bran. Furthermore, we do not fully understand why the various amino acids of tested raw materials respond differently to the effect of a mono-component protease. This information is critical in optimizing the use of raw materials, particularly the alternative protein sources, especially in the face of the rising demand and diminishing supply of soybean meal.

### I. INTRODUCTION

The livestock and poultry industries have made great strides in improving feed efficiency through continuous progress in the areas of genetics, management, disease prevention and nutrition. However, an ever-increasing human population and the needs that come with it, have resulted in competition between human food and energy needs and animals that are farmed for food, leading to an upward spiral in the cost of feed raw materials. According to the US census bureau (2008), within the next 30 years there is going to be 2 billion more people living on earth, raising the human population of the world to 9 billion inhabitants. Although cereal grain production has also been on the rise for the past 40 years, production and consumption have kept pace such that there is almost no food reserve. The situation is further exacerbated by the competition for corn and other cereals by the bio-fuel industry, the increasing meat consumption driven by rapid urbanization in China and India and the adverse effects of global warming on livestock as well as grain production.

This demand and supply mismatch has resulted in a perfect opportunity for feeding strategies that aim to optimize feed utilization by farm animals. In this respect, the development and use of exogenous microbial enzymes to either supplement or complement the animals' own enzymes, with the purpose of enhancing digestion and consequently feed

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utilisation, has proven to be a successful concept in the animal feed industry; it is used on a routine basis to improve the nutritive value of feed ingredients. The most commonly used enzymes are phytases and carbohydrases with high adoption into poultry feeds. With the challenge of high feed prices, as well as limited supplies of feed ingredients, the use of other enzymes to improve feed utilization by the animal has come to the forefront in the last couple of years, with protease being one of them.

## II. WHY USE A PROTEASE IN FEED?

Proteases (or peptidases) are enzymes secreted by many organisms for a number of physiological processes, amongst which is the digestion of feed protein. Animals normally secrete sufficient amounts of enzymes to digest enough of their feed so that they grow and remain healthy under normal conditions. Any increased protein (amino acids) needs associated with the more rapid growth that has been achieved through improved genetics has traditionally been met by adding more protein (or synthetic amino acids) into the feed. This was facilitated by a relatively low cost for most protein-rich ingredients, such as soybean meal (SBM) and synthetic amino acids, such as L-lysine HCL. Thus, an exogenous protease (as a feed supplement) was not considered essential.

Today we face not only the problem of feeding animals of continuously increasing genetic potential (this requires diets increasingly richer in amino acids), but also an unprecedented increase in ingredient prices, leaving very small (if any) margin for profitability. Thus, it has been deemed essential to seek ways to improve the nutritive value of existing ingredients as a means of reducing feed cost. Naturally, protein, being the second most expensive feed component, has received considerable attention worldwide. This has been aided by the negative public image of animal excreta which, if rich in nitrogen (basic component of all proteins), can result in negative environmental impacts.

Here it should be noted that natural feed proteins are never 100% digested by any animal. For broilers, protein in soybean meal is about 82% digestible, whereas protein in maize is around 80% digestible (Ravindran et al., 1998); the rest of the nitrogen in the undigested protein is excreted into the environment. Thus, there is a significant part of feed protein that is clearly being wasted, instead of being used for production of meat, eggs, and milk. The reasons for this inefficiency are varied and include (but are not limited to) the degree of fineness to which feed is ground, the rate of passage of feed through the digestive tract, the age of the animal and its physiological/health condition, the ingredients in the feed as well as their quality (Douglas et al., 2000; Coca-Sinova et al., 2008). All of these variables can be controlled up to a point and at a cost, but supplementing animal feeds with exogenous enzymes such as proteases, if it can be done profitably, is a simpler alternative.

## III. DEVELOPMENT OF A PROTEASE DEDICATED FOR USE IN FEED

Numerous studies of supplemental enzymes in broiler diets have been published over the last 20 years. After publications on phytases, publications on enzyme blends that contain proteases are the next most prevalent. Unfortunately, because the information is for enzyme blends, data do not allow for the evaluation of the effects of any of the individual enzyme activities. Fewer studies have been conducted using single proteases in poultry diets. There is some inconsistency in the published literature as to the impact of proteases. Some authors have reported no positive results or inconsistent results (Simbaya et al., 1996; Marsman et al., 1997; Naveed et al., 1998) while others have shown improvements in broiler performance as well as in energy and nitrogen utilization when proteases were added to broiler diets (Ghazi et al., 2003). In 2002, it was demonstrated by Ghazi et al. (2002) that pretreatment of soybean meal with protease caused an increase in protein solubility and broilers and piglets fed the



pretreated meal as part of a cereal based diet showed increased performance and protein digestibility values. Proteases have also been found to have an impact on the mucus layer thickness in the gastrointestinal tract (Peek et al., 2009). The authors associated this increased thickness with apparent alleviation of the impact of a coccidial infection, resulting in improved body weights. However, until recently, most commercial use of proteases in feed took place via so called enzyme cocktail products where the protease was only present as a side activity. Using such products makes it difficult to determine the *in vivo* effect of the protease as such, as performance improvements can be related to any of the activities present in the products. In 2008, a pure (mono-component) protease dedicated for the feed industry and for the use with any protein source was brought to the market.

The development process of such a product includes several stages. Typically, the process starts with identification of potential enzyme candidates by screening of a large number of microbial strains, pre-selection of these candidates by assessing the *in vitro* effect on different feedstuffs and, finally, *in vivo* digestibility and performance trials to confirm efficacy of the enzyme in the final application. Additionally, registration trials and trials to support global product development need to be conducted. The specific development process of the mono-component protease has been described by Glitsø et al. (2012). Using an *in vitro* digestion model simulating gastric and small intestinal digestion of a corn-soybean meal substrate (Fru-Nji et al., 2011), it was shown that this mono-component protease increased the degree of protein hydrolysis and thereby increased the proportion of soluble low molecular size proteins, hence making the protein better available for uptake by the chicken (Figure 1).

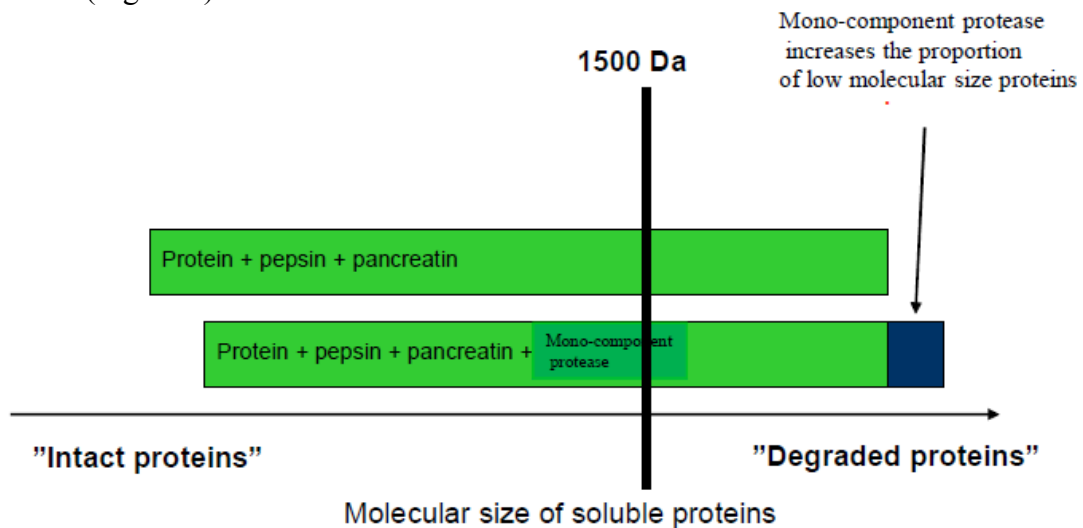


Figure 1 - Schematic presentation of the effect of a mono-component protease on the molecular size distribution of soluble proteins after digestion of a corn-soybean meal blend in an *in vitro* digestion model (described in Fru-Nji et al., 2011).

To validate the *in vitro* results, *in vivo* tests were undertaken to show that the mono-component protease improved feed utilization in broiler diets and improved technical performance of broilers on low protein diets. In Brazil, where a standard industry diet (control) was compared to the same diet with the protease, supplemented at 200 ppm (15000 PROT/kg feed; 1 PROT is the amount of enzyme that releases 1  $\mu\text{mol}$  of p-nitroaniline from 1 mM of Suc-Ala-Ala-Pro-Phe-pNA per minute at pH 9.0 and 37°C), the broilers on the ration with the protease performed significantly better than those in the control group in terms of feed conversion ratio. The cost per kilo live weight of broiler produced was also lower in the treated group.

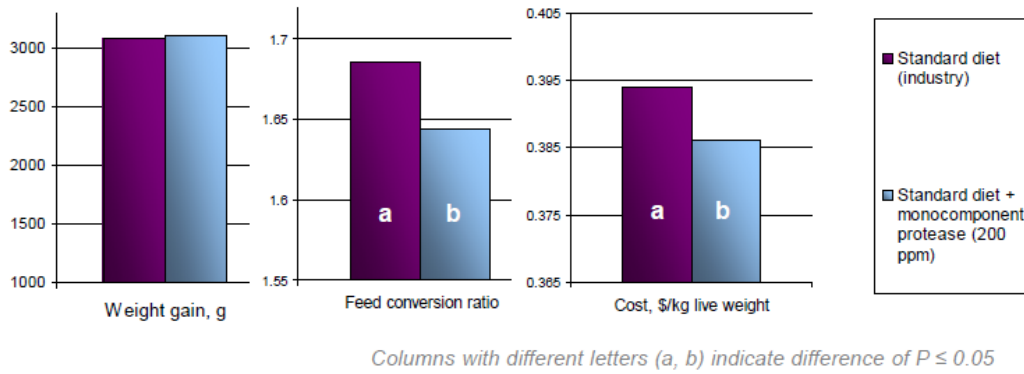


Figure 2 - Effect of a mono-component protease (200 ppm) on broiler performance and production cost (Maiorka et al., 2009)

Numerous trials have since shown that this mono-component protease enables optimization of broiler chicken performance. It improved protein digestibility significantly when added to diets with low crude protein content (Starter: 21% / Grower: 20%) and correspondingly reduced essential amino acid levels (Figure 3). These results translate to significant improvements in weight gain and feed conversion ratio.

The mono-component protease used at concentrations of 200 ppm and greater, increased specific amino acid digestibility, thereby allowing animals to overcome the negative effects on bodyweight gain and feed conversion ratio that resulted from a 10% reduction (by analysis), in diet crude protein, lysine, total sulfur amino acids and threonine and 15% reduction in methionine (Angel et al., 2011).

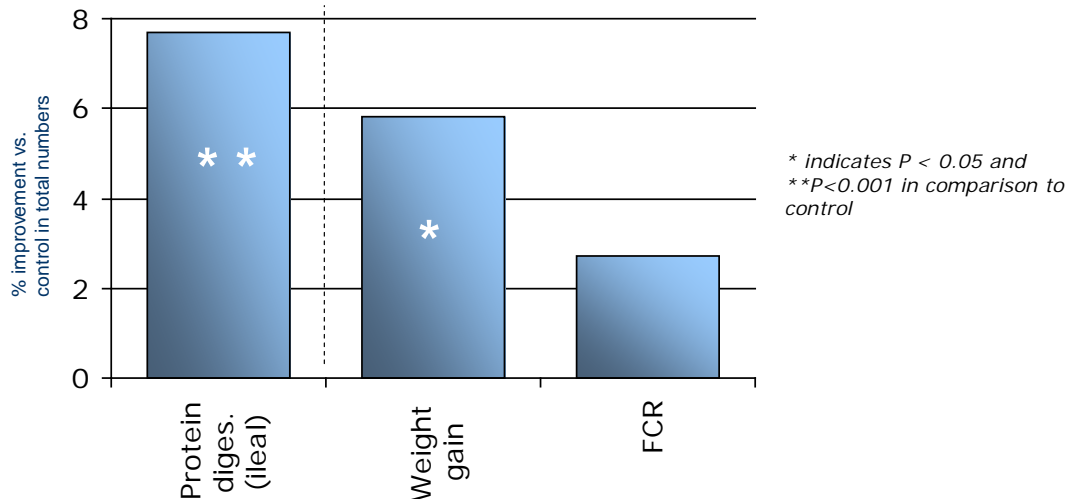


Figure 3 - Effect of a mono-component protease (200 ppm) on apparent ileal protein digestibility, body weight gain and feed conversion ratio (Fru-Nji et al., 2011)

Fru-Nji et al. (2011) further demonstrated that this mono-component protease improved protein and energy digestibility which led to significant improvements in broiler performance.

#### IV. THE EFFECT OF PROTEASE ON DIFFERENT INGREDIENTS

The effect of the mono-component protease in an *in vitro* digestion model was demonstrated by Fischer et al. (2009) using several different feed ingredients from Brazil as substrates. Those results showed that the extent of protein degradation differed for different feed ingredients ranging from 3 to 27% increase in degree of protein hydrolysis (Figure 4).

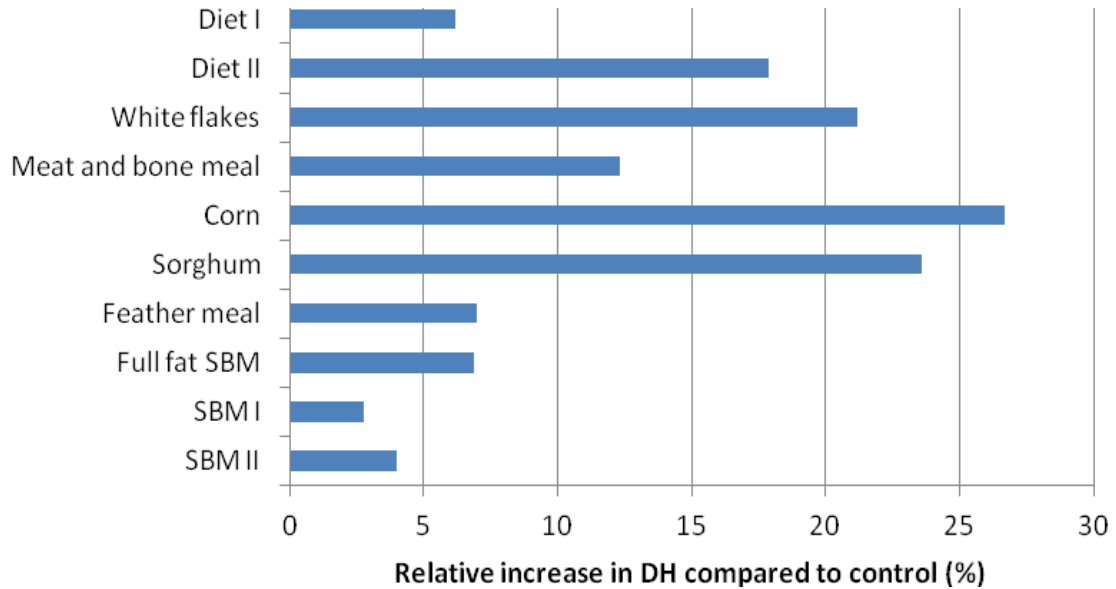


Figure 4 - Relative increase in degree of protein hydrolysis (DH) by an exogenous mono-component protease in an *in vitro* model containing endogenous proteases. Diet I and II represent different commercial broiler diets based on corn, soybean meal and meat and bone meal (Fischer et al., 2009).

Further *in vitro* data have confirmed the effect of the protease on different feed ingredients, cereals as well as oils seeds, and from different regions of the world (Figure 5). The various feed ingredients were incubated in an *in vitro* digestion model utilizing endogenous enzymes (pepsin and pancreatic enzymes) and the protease effect was measured as increase in DH (method published in Fru-Nji et al., 2011) (Unpublished data, Novozymes A/S).

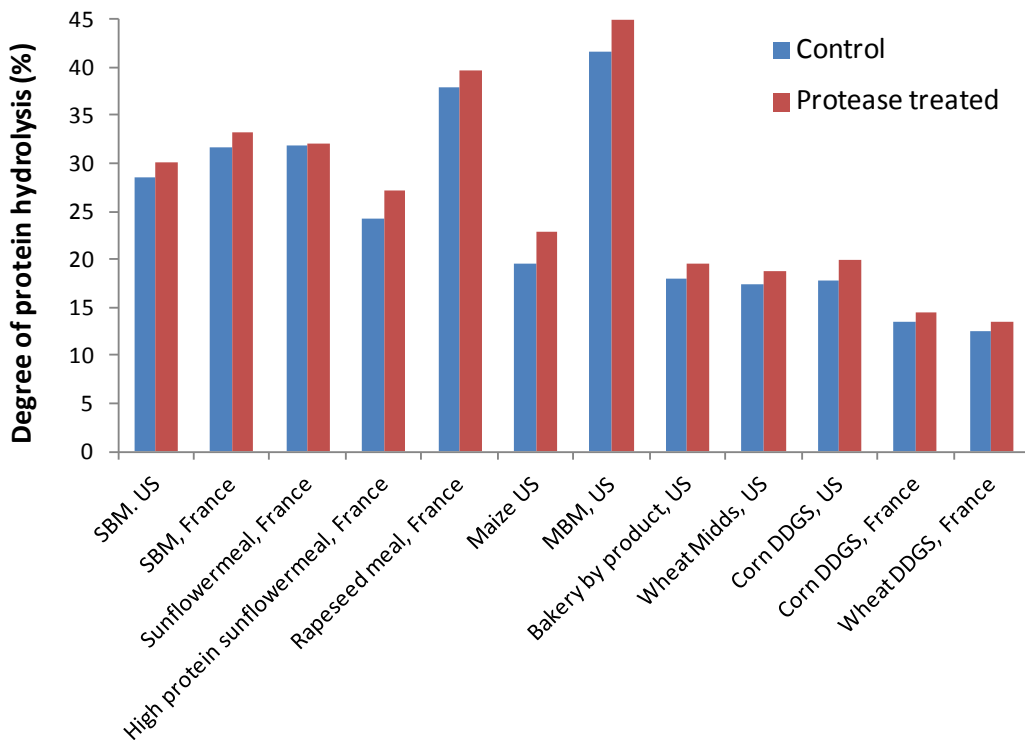


Figure 5 - Increase in degree of protein hydrolysis (DH, %) by a mono-component protease dosed at 100 mg enzyme protein/kg substrate (n=5).

Diversity within feed ingredients is well known, and results from differences in cultivar, country of origin, year to year variation as well as from processing (e.g. oilseed meals). As an example, soybean meals have been shown to vary considerably in nutritional value (De Coca-Sinova *et al*, 2008) and, due to this variation, the effect of a protease can also be expected to vary when testing different batches of soybean meals (Figure 6). The various soybean meal batches were incubated in an *in vitro* digestion system and protease effect on top of endogenous enzymes (pepsin and pancreatic enzymes) was analyzed as increase in DH. Error bars indicate standard deviation and asterisks indicate a significant impact of the protease ( $P < 0.05$ ; Tukey HSD test). (Pettersson and Pontoppidan, 2012)

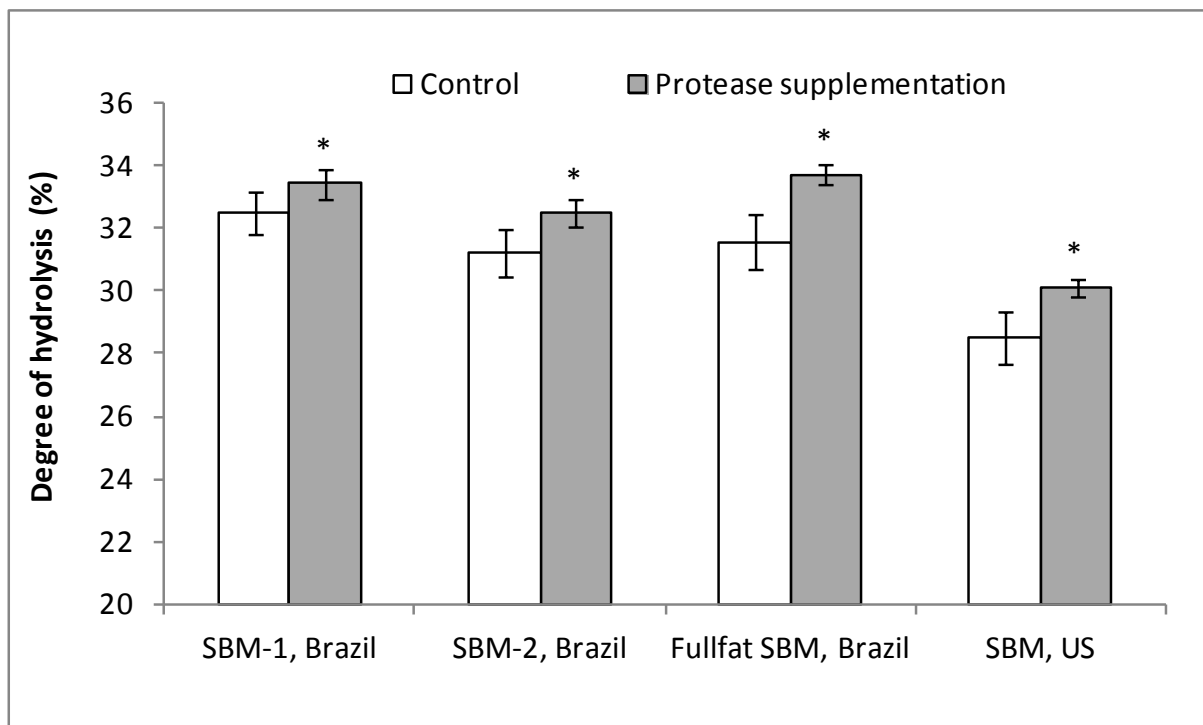


Figure 6 - Increase in degree of protein hydrolysis (DH, %) by a mono-component protease dosed at 100 mg purified enzyme protein per kg of SBM (n = 5).

To verify if the strong protein hydrolyzing effects demonstrated by this protease *in vitro*, could be replicated in chickens, Carvalho *et al.* (2009) and Bertechini *et al.* (2009a, b) carried out a follow up study with a series of *in vivo* trials. They used and adapted a method reported by Matterson *et al.* (1965) in which birds were fed a reference diet and whole portions of this reference diet were replaced by a different feed raw material. Birds were fed diets supplemented with 200 ppm of the protease. Excreta samples were collected and the digestibility of the raw materials was determined.

**Table 1 - Effects of a mono-component protease on the digestibility of amino acids and protein in different feedstuffs**

Amino Acids	Corn		Soybean Meal 46%		Full Fat Soy		Meat and Bone meal 45%	
	Ctrl	Ctrl + ProAct	Ctrl	Ctrl + ProAct	Ctrl	Ctrl + ProAct	Ctrl	Ctrl + ProAct
Lys	87.8	88.0	91.1	94.6**	89.9	92.7**	76.9	82.4**
Met	87.3	87.5	78.5	83.4**	81.0	86.2*	63.7	68.8**
Cys	75.0	79.2**	59.2	65.2**	79.6	83.7*	47.4	51.6*
Thr	72.2	79.3*	78.4	86.8**	80.5	86.7*	66.0	73.1**
Arg	81.5	83.6	79.6	86.6**	81.7	87.4**	48.1	55.0**
Val	81.4	84.7	83.6	87.0*	79.8	86.2**	63.7	67.2**
Asp	84.5	84.6	87.9	90.1	84.9	90.9**	67.7	76.4**
Ser	79.5	83.3**	85.4	89.4**	84.6	88.9*	64.5	70.7**
His	85.5	86.0	77.9	82.8**	81.2	85.4*	70.5	73.5*
Ala	79.4	83.8	71.0	75.1	73.9	81.2**	53.4	57.4*
Pro	82.5	86.0*	77.5	82.0*	79.0	86.4**	53.0	57.7*
Ile	82.2	85.2**	85.7	87.3	82.6	88.1**	60.5	61.6
Leu	88.3	89.5	82.7	84.8	83.6	88.4**	69.9	70.2
Phe	87.1	88.1	86.5	87.9	87.5	90.8**	67.5	69.1*
CP	82.3	84.8*	79.5	83.5*	81.3	86.3*	63.3	67.3*

Adapted from Carvalho et al. (2009 a and b) and Bertechini et al. (2009 a and b)\*P < 0.05, \*\*P < 0.01

Results (Table 1) confirmed that this protease could significantly improve protein digestibility of feedstuffs and that the effect of the enzyme was dependent on the type of raw material used. Other studies were carried out in different regions of the world (Spain and Brazil) using different diets, in which the effects of the protease on the digestibility of amino acids were studied. The results of some of the amino acid digestibility improvements compared to the untreated control are presented in Figure 7. As can be seen, there was an average improvement in the digestibility of amino acids by about 4%. The results also suggest that the digestibility of amino acids in a ration depends on the type of raw material used for the diet. Although the enzyme is supposed to be unspecific to any particular peptide linkage, there were differences recorded for the various amino acids.

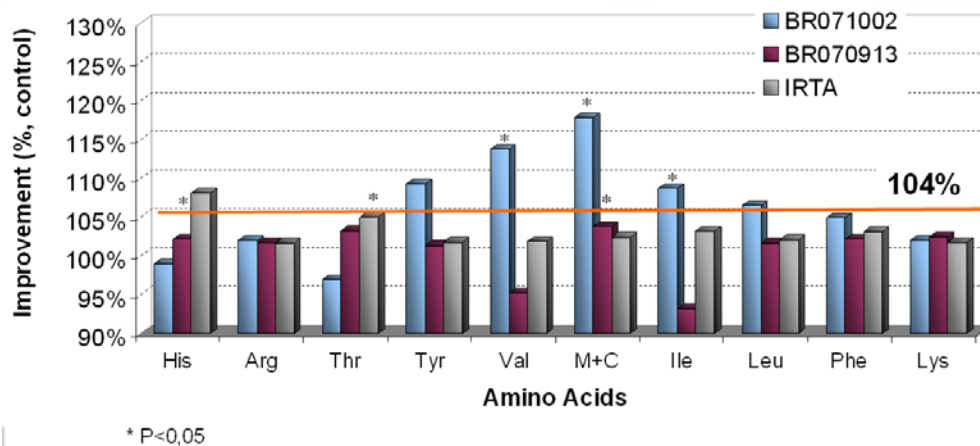


Figure 7 - Improvement in apparent ileal digestibility of amino acids in different diets when broilers were supplemented with a mono-component protease (200 ppm) (adapted from Favero et al., 2009, Maiorka et al., 2009 and Vila et al., 2008).

Another study was carried out by Rosa et al. (2009) in a 3×2 factorial arrangement, with three crude protein and amino acid (CP/AA) levels (21.50, 20.85 and 20.21 % CP, and 1.15, 1.115, and 1.08% Dig Lys; 0.82, 0.795, 0.77% Dig TSAA) with and without protease (200 ppm). Diets were formulated to current industry standards for the starter phase (1-21 days) based on corn, soybean meal and meat and bone meal. Crude protein was then reduced from 21.50% by 3 and 6% respectively to 20.85% and 20.21% to formulate a basal diet that served as a negative control (where negative control refers to all three crude protein levels). Addition of 200 ppm of the protease to the negative control resulted in significant ( $P<0.05$ ) improvements of body weight gain and feed consumption. The birds fed the diet with 6% CP reduction and supplemented with 200 ppm of the protease had similar body weight gain and feed consumption as birds fed the diet with the high CP level.

Iwaniuk et al. (2011) evaluated the effects of inclusion of the mono-component protease on true standardized amino acid digestibility of individual ingredients for broilers. The corn-starch, sucrose and Solka-Flock in the nitrogen free diet were replaced in part by the ingredients being tested such that all the protein in the diet came from the test ingredients. Ingredient inclusion in the final diets was 42% soybean meal (48% CP), 40% meat and bone meal (50% CP), 75% corn DDGS and 96% corn. As shown in Figure 8, the protease improved true ileal digestibility of nitrogen, methionine, cysteine, threonine, valine, isoleucine, arginine, serine, histidine and aspartic acid. The effect of the protease and the magnitude of the effect varied between amino acids and ingredients.

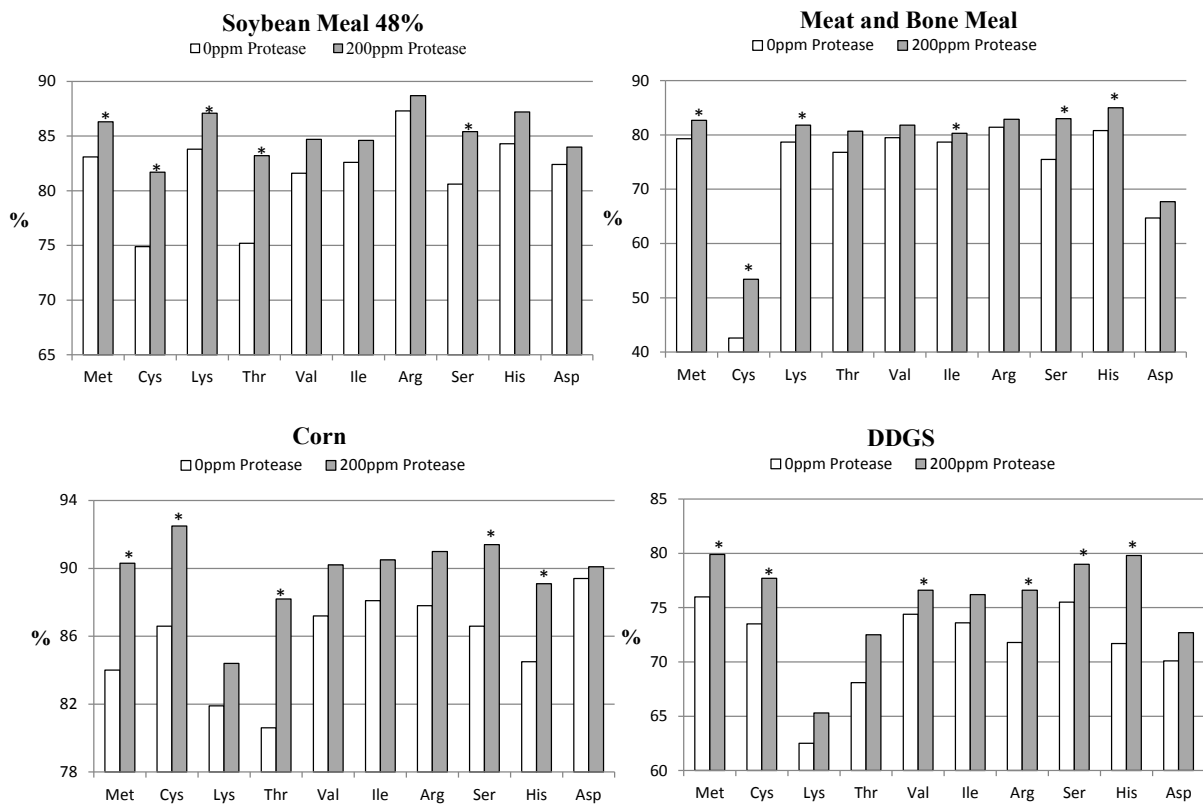


Figure 8 - Improvements in true ileal amino acid digestibility when a mono-component protease was utilized (Iwaniuk et al., 2011). (Means within an ingredient and AA with an \* differ ( $P<0.05$ )).

With the high price of soybean meal, grains and grain by-products, broiler producers are looking for alternative sources of protein. Canola meal in the past was not considered for broiler diets in Asia, but has now become more attractive as a feed ingredient. Gomez et al. (2011) studied the effect of a protease on the digestibility of amino acids and the energy value

of canola meal in starter broiler diets using the substitution method. The results suggested that the availability of amino acids (AID) and the energy values of diets (AME) formulated with canola meal could be enhanced by the addition of a mono-component protease (Figures 9 and 10).

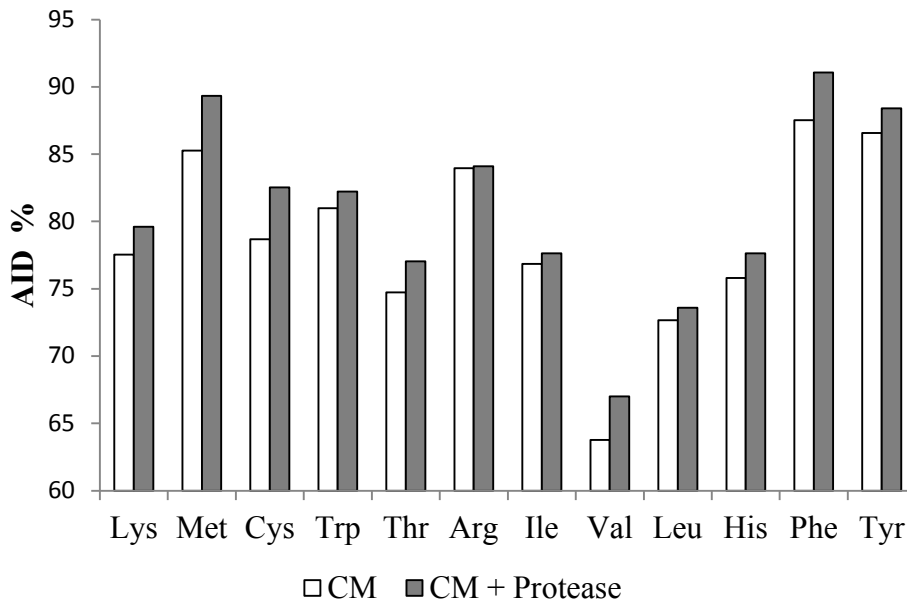


Figure 9 - Apparent ileal digestibility (AID, %) of amino acids in canola meal with and without mono-component protease (Gomez et al., 2011).

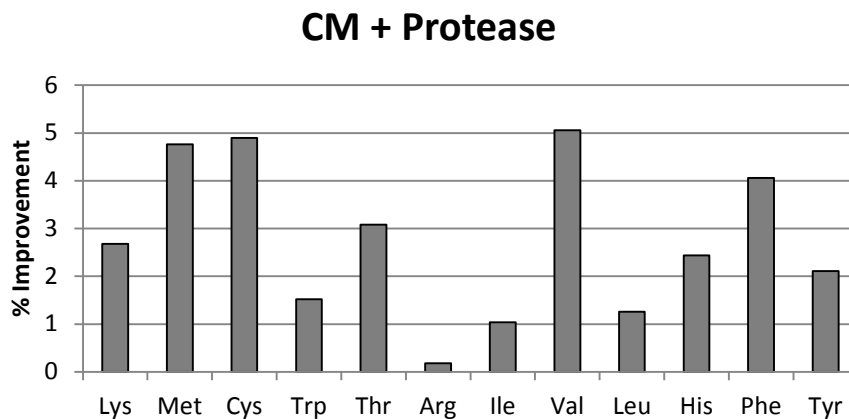


Figure 10 - Improvement (%) of the apparent ileal digestibility of amino acids in canola meal supplemented with a mono-component protease (Gomez et al, 2011).

## V. CONCLUSION

The poultry industry has the enormous task of feeding a rapidly increasing human population. Protein remains one of the most expensive components of the feed. This challenge has led to the development of a mono-component protease (RONOZYME<sup>®</sup> ProAct) that may help the industry to feed broilers more efficiently.

The results presented here demonstrate that this protease is capable of increasing protein digestibility of various feed ingredients and thus provide flexibility in feed formulation. Results from different studies have also demonstrated that it can significantly improve animal performance with different dietary crude protein levels. However, there is

much more work required to determine the effect of a protease on feed ingredients, especially those commonly used in the Asia Pacific region such as sorghum, palm kernel meal and rice bran. Moreover, it is not fully understood why different ingredients respond differently in terms of the improvement in amino acid digestibility. This understanding is critical to optimising the use of raw materials, particularly the alternative protein sources due to the rising demand and diminishing supply of soybean meal.

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PROTEASE SUPPLEMENTATION ENHANCES APPARENT DIGESTIBILITY OF  
AMINO ACIDS AT FOUR SMALL INTESTINAL SITES IN BROILER CHICKENS  
OFFERED SORGHUM-BASED DIETS

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Summary

A feeding study with a 4×2 factorial array of dietary treatments was conducted to determine apparent amino acid digestibilities in the proximal jejunum, distal jejunum, proximal ileum and distal ileum of broilers fed sorghum-based diets without or with exogenous protease. Interactions between intestinal site and protease supplementation were not observed. Intestinal site significantly influenced apparent digestibility coefficients of all amino acids which increased from an average of 0.466 in the proximal jejunum to 0.803 in the distal ileum. Protease improved apparent digestibility of amino acids by an average of 9.16% with significant responses in 14 *ex* 16 amino acids. The more pronounced responses to protease were recorded for proline (14.6%), alanine (12.8%), and leucine (12.7%). The present study indicates that the *Bacillus lichenformis*-derived protease has the capacity to improve amino acid digestibility coefficients in sorghum-based diets.

I. INTRODUCTION

The effects of protease supplementation on nitrogen (N) and starch digestibility in four small intestinal sites of broilers offered sorghum-based diets were determined in an earlier study (Selle et al., 2012). Protease significantly increased N digestibility in the distal jejunum (0.538 *vs.* 0.627), proximal ileum (0.727 *vs.* 0.770) and distal ileum (0.719 *vs.* 0.770). Moreover, protease significantly improved starch digestibility in the distal jejunum (0.678 *vs.* 0.770) and proximal ileum (0.812 *vs.* 0.851). Thus, the intention of the present study was to determine the effect of protease on apparent amino acids digestibility throughout the small intestine given the observed improvement in crude protein (N) digestibility generated by this protease.

II. MATERIALS AND METHODS

The methodology used in this experiment has been previously outlined (Selle et al., 2012). Briefly, a red sorghum (Buster) was hammer-milled (3.2 mm) and incorporated into broiler diets that were steam-pelleted at 80 °C and offered to male Ross 308 chicks (seven replicate cages of six birds per treatment) without and with protease from 7 days to 28 days post-hatch. Dietary composition, nutrient specification and analysed amino acid concentrations of the experimental diets are shown in Table 1. Dependent on treatment, the exogenous protease was incorporated into the sorghum-based diet prior to steam-pelleting at 80°C. The enzyme was a serine endopeptidase from *Bacillus lichenformis* (Cibenza<sup>TM</sup> DP100; Novus International Inc.) with a protease activity of 600,000 units/g and a recommended inclusion rate 300 units/g of feed. The analysed protease activity in the relevant experimental diet was 257 units/g of feed or 86% of the recommended activity-and protease activity was not detected in the corresponding control diet. At day 28, digesta samples were collected in their entirety from the proximal jejunum, distal jejunum, proximal ileum and distal ileum, which were demarcated by the end of the duodenal loop, Meckel's diverticulum and the ileo-caecal

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junction and their mid-points, for apparent amino acids digestibility determination. Amino acids quantitative analyses were completed in duplicate as outlined by Cohen and Michaud (1993). Analyses of variance and tests of significance were performed as a 4×2 factorial array design using JMP® 9.0.0 where significance was determined when  $P < 0.05$ .

**Table 1 - Dietary composition, nutrient specifications and analysed amino acid concentrations in a red sorghum-based broiler diet**

Dietary composition		Nutrient specifications		Amino acid concentrations	
Item	(g/kg)	Item	(g/kg)	Item	(g/kg)
Sorghum	600.0	ME (MJ/kg)	12.84	Arginine	11.11
Soybean meal	224.6	Protein	215.0	Histidine	5.02
Canola meal	60.0	Calcium	9.0	Isoleucine	8.57
Wheat bran	30.0	Total P	7.9	Leucine	19.17
Vegetable oil	21.8	Available P	4.0	Lysine	12.63
Dicalcium phosphate	17.3	Lysine	13.5	Methionine	4.00
Limestone	10.0	Met	6.6	Phenylalanine	10.26
Lysine HCl	4.8	Met + Cys	10.2	Threonine	8.29
Methionine	3.4	Thr	9.0	Valine	9.98
Threonine	1.3	Sodium	1.8	Alanine	10.88
Sodium chloride	0.4	Potassium	8.2	Aspartic acid	17.59
Sodium bicarbonate	4.5	Chloride	2.0	Glutamic acid	38.57
Vitamin-mineral premix	2.0	DEB (meq/kg)	233.0	Glycine	6.82
Celite	20.0			Proline	12.29
				Serine	9.30
				Tyrosine	4.62

### III. RESULTS

The effects of small intestinal site and protease supplementation on apparent amino acids digestibility are shown in Table 2. Interactions between intestinal site and protease inclusion were not observed. Intestinal site significantly influenced apparent digestibility of all amino acids where digestibility in the ileal sites was greater than in the jejunal sites. The average digestibility was 0.466 in the proximal jejunum, 0.643 in the distal jejunum, 0.764 in the proximal ileum and 0.803 in the distal ileum. As a main effect, protease significantly improved the digestibility of proline (14.6%), alanine (12.8%), leucine (12.7%), tyrosine (12.1%), serine (11.5%), phenylalanine (10.5%), valine (10.4%), isoleucine (9.9%) glycine (9.7%), aspartic acid (9.6%), glutamic acid (9.4%), histidine (8.2%), threonine (7.4%), arginine (4.6%). Apparent amino acids digestibility are conventionally determined in the distal ileum where, in this study, protease significantly improved the ileal digestibility of proline (7.9%), alanine (7.1%), leucine (6.7%), serine (6.2%), tyrosine (5.9%), valine (5.5%), glycine (5.5%), phenylalanine (5.4%), aspartic acid (5.3%), isoleucine (5.0%), threonine (4.8%), glutamic acid (4.8%) and histidine (4.5%).

### IV. DISCUSSION

Concentrations of amino acids in sorghum are variable and their ileal digestibility may be highly variable (Bryden et al., 2009); both factors probably contribute to the inconsistent performance of broilers offered sorghum-based diets. The dominant protein fraction in sorghum is kafirin and its proportion relative to glutelin influences both amino acid concentrations and digestibility (Selle et al., 2010). Taylor (2005) suggested that sorghum-

based broiler diets may benefit from the inclusion of protease with the caveat that kafirin may not be readily degraded due to its poor solubility and disulphide linkages, especially following exposure to 'moist heat'. The *Bacillus licheniformis*-derived protease used in the present study can degrade feedstuffs with high cystine content and disulphide linkages and has been reported to enhance broiler performance in maize-based diets (Odetallah et al., 2003, Wang et al., 2008). This is one of the very few studies to investigate the impact of protease on the digestibility of amino acids in sorghum-based broiler diets.

Soybean meal was the largest contributor of amino acids in the experimental diets and amino acid digestibility in soybean meal is greater than in sorghum (Bryden et al., 2009). Kafirin contains relatively high levels of leucine (Selle et al., 2010). Therefore, the 12.6% increase in leucine digestibility observed here suggests that protease addition enhanced amino acid digestibility to a larger extent in sorghum proteins than in 'non-sorghum' proteins (soybean-meal).

Synthetic amino acids in the experimental diets contributed substantial proportions of methionine and lysine. The digestibility of these free amino acids is of a high order and this is consistent with high lysine (0.619) and methionine (0.717) digestibility in proximal jejunum in the sorghum-based diet without protease. This may explain why significant responses to protease inclusion were not observed for these amino acids. Moreover, in the present study, protease did not generate significant improvements in growth performance which also may have been due to the modest improvement in digestibility of these two essential amino acids in response to protease supplementation.

Ileal amino acid digestibility values are widely used in broiler feed formulation. However, amino acids digestibility in four small intestinal sites in the present study were not correlated with previously determined feed intakes, weight gains and feed conversion ratios (Selle et al., 2012). The rate at which amino acids are absorbed from the gut, especially the nutritionally critical amino acids, in relation to starch digestion and glucose absorption may have a greater bearing on growth performance and protein deposition than ileal digestibility coefficients *per se*. Thus, the kinetics of protein digestion and amino acid absorption relative to starch and glucose merits further research.

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**Table 2 - Effect of protease supplementation on apparent digestibility of amino acids in proximal jejunum (PJ), distal jejunum (DJ), proximal ileum (PI) and distal ileum (DI) in broilers at 28 days post-hatch**

Site	Protease	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Ala	Asp	Glu	Gly	Pro	Ser	Tyr
PJ	Nil	0.533	0.427	0.363	0.359	0.606	0.730	0.413	0.405	0.352	0.359	0.416	0.470	0.355	0.368	0.384	0.342
	Plus	0.579	0.498	0.441	0.452	0.633	0.705	0.501	0.457	0.433	0.454	0.498	0.562	0.434	0.487	0.474	0.438
DJ	Nil	0.710	0.612	0.568	0.536	0.758	0.846	0.584	0.580	0.560	0.536	0.585	0.625	0.561	0.531	0.555	0.534
	Plus	0.762	0.686	0.650	0.634	0.791	0.867	0.673	0.643	0.644	0.632	0.667	0.705	0.639	0.633	0.647	0.631
PI	Nil	0.832	0.752	0.726	0.695	0.847	0.916	0.734	0.715	0.715	0.687	0.732	0.755	0.716	0.681	0.715	0.715
	Plus	0.850	0.784	0.766	0.747	0.858	0.931	0.777	0.744	0.757	0.741	0.769	0.795	0.751	0.736	0.761	0.760
DI	Nil	0.862	0.785	0.774	0.748	0.867	0.947	0.781	0.744	0.760	0.733	0.767	0.794	0.752	0.722	0.754	0.763
	Plus	0.881	0.820	0.813	0.798	0.882	0.951	0.823	0.780	0.802	0.785	0.808	0.832	0.793	0.779	0.801	0.808
SEM		0.0233	0.0259	0.0292	0.0287	0.0221	0.0212	0.0253	0.0275	0.0310	0.0285	0.0251	0.0220	0.0287	0.025	0.0258	0.0315
Main effect: Intestinal site																	
PJ		0.556 <sup>c</sup>	0.462 <sup>c</sup>	0.402 <sup>c</sup>	0.405 <sup>c</sup>	0.619 <sup>c</sup>	0.717 <sup>c</sup>	0.457 <sup>c</sup>	0.431 <sup>c</sup>	0.393 <sup>c</sup>	0.407 <sup>c</sup>	0.457 <sup>c</sup>	0.516 <sup>c</sup>	0.394 <sup>c</sup>	0.428 <sup>c</sup>	0.429 <sup>c</sup>	0.390 <sup>c</sup>
DJ		0.736 <sup>b</sup>	0.649 <sup>b</sup>	0.609 <sup>b</sup>	0.585 <sup>b</sup>	0.774 <sup>b</sup>	0.857 <sup>b</sup>	0.628 <sup>b</sup>	0.611 <sup>b</sup>	0.602 <sup>b</sup>	0.584 <sup>b</sup>	0.626 <sup>b</sup>	0.665 <sup>b</sup>	0.600 <sup>b</sup>	0.582 <sup>b</sup>	0.601 <sup>b</sup>	0.582 <sup>b</sup>
PI		0.841 <sup>a</sup>	0.768 <sup>a</sup>	0.746 <sup>a</sup>	0.721 <sup>a</sup>	0.853 <sup>a</sup>	0.924 <sup>a</sup>	0.755 <sup>a</sup>	0.729 <sup>a</sup>	0.736 <sup>a</sup>	0.714 <sup>a</sup>	0.750 <sup>a</sup>	0.775 <sup>a</sup>	0.734 <sup>a</sup>	0.709 <sup>a</sup>	0.738 <sup>a</sup>	0.737 <sup>a</sup>
DI		0.871 <sup>a</sup>	0.803 <sup>a</sup>	0.794 <sup>a</sup>	0.773 <sup>a</sup>	0.875 <sup>a</sup>	0.949 <sup>a</sup>	0.802 <sup>a</sup>	0.762 <sup>a</sup>	0.781 <sup>a</sup>	0.759 <sup>a</sup>	0.787 <sup>a</sup>	0.813 <sup>a</sup>	0.772 <sup>a</sup>	0.750 <sup>a</sup>	0.777 <sup>a</sup>	0.785 <sup>a</sup>
Main effect: Protease																	
Nil		0.734 <sup>b</sup>	0.644 <sup>b</sup>	0.608 <sup>b</sup>	0.584 <sup>b</sup>	0.770	0.860	0.628 <sup>b</sup>	0.611 <sup>b</sup>	0.597 <sup>b</sup>	0.579 <sup>b</sup>	0.625 <sup>b</sup>	0.661 <sup>b</sup>	0.596 <sup>b</sup>	0.575 <sup>b</sup>	0.602 <sup>b</sup>	0.588 <sup>b</sup>
Plus		0.768 <sup>a</sup>	0.697 <sup>a</sup>	0.668 <sup>a</sup>	0.658 <sup>a</sup>	0.791	0.864	0.694 <sup>a</sup>	0.656 <sup>a</sup>	0.659 <sup>a</sup>	0.653 <sup>a</sup>	0.685 <sup>a</sup>	0.723 <sup>a</sup>	0.654 <sup>a</sup>	0.659 <sup>a</sup>	0.671 <sup>a</sup>	0.659 <sup>a</sup>
Significance P =																	
Intestinal site		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Protease		0.046	0.006	0.006	<0.001	0.174	0.782	<0.001	0.024	0.007	0.001	0.001	<0.001	0.006	<0.001	0.001	0.003
Interaction		0.832	0.770	0.806	0.755	0.946	0.715	0.639	0.919	0.839	0.776	0.683	0.518	0.804	0.492	0.691	0.722

<sup>a,b,c</sup>Means within columns not sharing a common superscript are significantly different (P < 0.05).

## MINERAL COMPOSITION OF CALCIUM SOURCES USED BY THE AUSTRALIAN POULTRY FEED INDUSTRY

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### Summary

A mineral compositional survey of 14 limestone sources commonly used by Australian poultry feed manufacturers was performed. Samples were wet acid digested prior to the determination of Ca, P, Mg, Fe, K, Mn, Na, Cu, Sr and Zn concentrations by inductively-coupled plasma-optical emission spectroscopy. The results from this survey show large differences between the highest and lowest determined concentrations in all of the minerals analysed and in particular those of P (241 mg/kg), Na (186 mg/kg), Mn (237 mg/kg), Fe (1397 mg/kg) and K (125 mg/kg) values. Data from this survey indicate that the trace mineral composition of limestone sources is variable and that these values may need to be considered as a part of standard quality control procedures.

### I. INTRODUCTION

Limestone is composed primarily of CaCO<sub>3</sub> and is commonly used as a source of calcium (Ca) in poultry diets. Pure CaCO<sub>3</sub> has a molecular weight of approximately 100g/mole and so is around 400 g/kg Ca. However, CaCO<sub>3</sub> sources used in animal feeding are typically only 370-380 g/kg Ca, sometimes less, due to the presence of other minerals such as Mg or Fe. Furthermore, the bioavailability of Ca to the bird is affected by many factors including the source of Ca, solubility, gut retention time, particle size as well as the presence of other minerals (Oso et al., 2011). A paucity of information detailing the mineral composition of commonly used Ca sources is available to the Australian poultry industry. This paper reports on the determined mineral composition of 15 samples of Ca sources commonly used in the Australian poultry feed manufacturing industry.

### II. MATERIALS AND METHODS

Fourteen samples of limestone (CaCO<sub>3</sub>) in the form of powder (N=13) and grit (N=1) were obtained from various feed mills and distributors throughout Australia. Samples were wet acid digested using nitric acid and hydrogen peroxide (Peters *et al.*, 2003) prior to the determination of Ca, P, Mg, Fe, Cu, K, Mn, Na, Sr and Zn concentration by Inductively Coupled Plasma-Optical Emission Spectroscopy using a Perkin Elmer OPTIMA 7300 (Perkin Elmer Inc, Waltham, MA, USA). All samples were analysed as received in triplicate. Results are sample means presented as g/kg for Ca and Mg and as mg/kg for Fe, K, Mn, Na, Sr and Zn. The concentration of P is reported as mg/kg for all samples except those reported as g/kg.

### III. RESULTS AND DISCUSSION

The results for the determined mineral composition are shown in Table 1. For all samples, the concentration of Cu was below detectable limits and is not reported. All samples contained higher than expected Ca concentrations and when combined with the micro-mineral results this suggests that the samples were not pure CaCO<sub>3</sub>. Calcium concentrations ranged from

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410.8 g/kg to 392.2 g/kg and are broadly in keeping with those found by Reid and Weber (1976).

Sample A (305.3 mg/kg) contained more P than all the other limestone samples and this was approximately 5 times greater than the amount of P contained in sample K (56.5 mg/kg). Concentrations of Fe differed widely between the highest (sample D, 1506 mg/kg) and the lowest (sample N, 109 mg/kg). It is possible that the source of Fe in some of the samples may be attributed to purity of the limestone sample as well as contamination from metal fatigue in mining equipment that was used during the quarrying process.

Greater concentrations of Na and Sr were present in sample C when compared to all of the other samples while increased K was found in samples C and D. Concentrations of Mn varied by 237 mg/kg when comparing sample H (253 mg/kg) to sample O (16 mg/kg). Concentrations of Mg were found to range from approximately 1.0 g/kg to 5 g/kg. Corn-soy diets typically contain 1 to 5g of Mg and are generally below the toxicity limits of birds. However, contamination of mineral sources with Mg and/or the use of dolomitic limestone has been shown to increase diet Mg concentration and result in wet litter in broilers and reduced egg shell quality in layers (Leeson and Summers, 2001). The quality of limestone may become more pertinent for layers where higher concentrations of limestone are used. Zinc concentrations displayed large variation between the lowest (sample D, 0.57 mg/kg) and the highest (sample N, 19 mg/kg).

A further consideration when formulating diets with varying limestone purity is the chelating capacity of phytate and the resultant reduction in phytase efficacy. Phytate is able to chelate mineral ions including Ca, Zn, Fe, Mg, Mn and Cu (Tamim and Angel, 2003). However, the solubility and stability of these phytate-mineral complexes are pH dependent with most being relatively soluble below pH 3.5 while maximal insolubility occurs between pH 4 and 7 (Selle et al., 2000). Importantly, the capacity of these minerals to inhibit phytate-P hydrolysis by phytase varies as  $Zn \gg Fe > Mn > Fe > Ca > Mg$  (Maenz et al., 1999; Angel et al., 2002). *In vitro* work by Tamim and Angel (2003) reported that phytate phosphorus hydrolysis was reduced with the addition of micro-minerals when compared to no minerals being added but no effect was found *in vivo*. The authors postulated that the reason for the discrepancy in results may relate to the concentration of minerals used in the *in vivo* study not being sufficient to elicit a response.

Though the overall concentrations of minerals other than Ca within the limestone sources were relatively low, their presence may be important when formulating diets. For example, a diet formulated with unknown mineral composition of limestone may result in higher concentrations of micro-minerals than expected. This would be more important when samples have higher mineral concentrations than those with lower mineral concentrations. It may also be that the potential effects of the limestone mineral composition may be additive to the mineral composition of the water used on farm and these need to be considered together.

The work reported here suggests that the mineral composition of limestone is highly variable between sources and it is plausible that further variation exists between other sources that have yet to be analysed. Further investigation has been proposed to quantify these differences. Based on the results of this study it may be prudent to conduct regular analysis of limestone samples for mineral composition as a part of regular quality control practices.

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Table 1 - Determined mineral composition of various limestone samples used by the Australian poultry industry

Sample	Origin	Ca (g/kg)	P (mg/kg)	Mg (g/kg)	Fe (mg/kg)	K (mg/kg)	Mn (mg/kg)	Na (mg/kg)	Sr (mg/kg)	Zn (mg/kg)
A	Thailand	405.2	305.3	1.9	207.6	23.6	58.2	14.4	158.0	4.6
B	NSW	398.5	93.8	3.6	1415.5	81.1	241.9	15.8	186.2	2.6
C	QLD	394.1	256.0	4.9	507.8	127.1	62.3	190.3	329.0	2.0
D	SA	395.0	115.3	3.5	1506.4	124.2	210.9	24.8	215.7	0.6
E	Unknown	397.1	204.2	3.0	449.9	45.6	96.6	94.4	237.1	2.7
G	NSW	400.3	124.6	2.9	875.6	100.7	158.5	20.9	182.4	4.9
H	WA	392.2	106.3	2.9	784.9	46.7	253.4	26.7	236.1	10.2
I	NSW	401.2	64.7	1.1	938.8	21.1	149.1	14.7	131.3	2.7
J	QLD	410.8	121.0	1.8	289.9	1.6	138.2	10.6	130.2	11.7
K	NSW	401.6	56.5	1.1	603.3	ND	91.9	11.2	115.7	1.9
L	QLD	402.2	167.9	1.1	719.9	45.1	177.9	15.5	109.5	15.6
M	QLD	407.7	106.7	2.0	244.4	5.5	134.6	4.3	137.1	10.3
N	QLD	409.5	107.1	2.5	108.9	13.9	22.1	9.3	177.4	18.5
O	NSW	403.6	76.8	2.3	124.9	16.4	15.9	6.8	163.2	12.1
	Maximum	410.8	305.3	4.9	1506.4	127.1	253.4	190.3	329.0	18.5
	Minimum	392.2	56.5	1.1	108.9	1.6	15.9	4.3	109.5	0.6



## NON-PHYTATE PHOSPHORUS REQUIREMENT OF BROILERS FED SORGHUM BASED DIETS

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The phosphorus (P) requirement of broilers is still not established despite being the subject of numerous investigations. Estimates of the non-phytate P (NPP) requirements for broilers (Angel et al., 2000a,b) are considerably lower than those published previously (NRC, 1994) and the values currently used by industry. Non-phytate P requirements recommended by NRC (1994) and most recent publications are based on corn-soybean meal diets. The objective of the present study was to investigate NPP requirement with or without phytase supplementation in broiler diets containing sorghum as the only cereal grain component.

Two experiments were conducted with Ross male broilers; Experiment 1 from days 1 to 21 and Experiment 2 from days 22 to 49, post-hatch. The experimental diets based on sorghum, canola meal, soybean meal and meat and bone meal contained graded levels of NPP (2.5 to 5.5 g/kg for starter and 2.0 to 5.0 g/kg for grower and finisher) in increments of 1.0 g/kg. The Ca level was 10.0 g/kg for starter and 9.0 g/kg for grower and finisher and the diets were prepared with or without phytase (Phyzyme XP, 10000 FTU/kg diet) supplementation. Each of the experimental diets was fed to 5 replicate pens, each containing 10 birds; starter, days 1 to 14; grower, days 15 to 21 for experiment 1 and 22 to 28 for experiment 2; finisher, days 29 to 49. Body weight and feed intake were recorded weekly and feed conversion ratio was calculated.

The results of both experiments demonstrate that birds fed diets with the lowest level of NPP (2.5g/kg starter; 2.0g/kg grower/finisher) and without phytase supplementation had significantly ( $P<0.05$ ) lower growth rates that was attributed to the reduced ( $P<0.05$ ) feed intake. Feed conversion ratio by these birds was also inferior ( $P<0.05$ ) to other treatment groups. The other groups all performed to a similar level of growth, feed intake and feed conversion ratio. The addition of phytase to the experimental diets allowed birds fed the lowest level of NPP to perform at a comparable rate to the birds fed the other diets in both experiments. However, no further improvement was observed in birds fed diets with higher levels (4.5 and 5.5g/kg starter, 4.0 and 5.0 g/kg grower/finisher diets) of NPP from day 1 to 21 or from day 22 to 49. The growth performance of birds on diets containing NPP levels of 3.5 g/kg, starter and 3.0 g/kg, grower/finisher gave comparable growth to those fed diets containing higher NPP levels ( $P>0.05$ ).

The findings from the current study suggest that dietary NPP requirement can be substantially reduced to as low as 2.5 and 2.0 g/kg starter and grower/finisher diets, respectively with phytase supplementation; 3.5 and 3.0 g/kg starter and grower/finisher diets, respectively without phytase supplementation. The NPP requirement results obtained in this study refer to one dietary scenario and there is a need to determine bird responses to diets containing different levels of Ca.

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DIETARY CALCIUM LEVELS AND NON-PHYTATE PHOSPHORUS REQUIREMENT OF  
BROILERS FROM DAYS 1 TO 21

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Calcium (Ca) and phosphorus (P) are the major cations required in poultry diets and the importance of maintaining the optimum balance of Ca and P in diets has been known for many years. Our previous study (Li et al., 2013) determined non-phytate P (NPP) requirement using 4 levels of NPP and 1 level of Ca and found that NPP requirement of broilers fed a sorghum based diet was lower than the NRC recommendation (NRC,1994). The objective of the present study was to determine the effect of dietary Ca levels on NPP requirement in broilers fed a sorghum-based diet from days 1 to 21 post-hatch.

Six hundred day-old Ross male broiler chicks were fed experimental diets based on sorghum, canola meal, soybean meal and meat and bone meal. The diets contained 2 levels of NPP (2.5 and 3.5 g/kg starter and 2.0 and 3.0 g/kg grower diets) and 3 levels of Ca (6.5, 10.0 and 12.2g/kg starter and 6.0, 9.0 and 12.0 g/kg grower diets) either with or without phytase (Feedzyme Phytase XP, 1000 FTU/kg diet) supplementation. Each experimental diet was fed to 5 replicate pens with 10 birds per replicate, with the starter diet fed on days 1 to 14 and the grower diet fed on days 15 to 21, post-hatch. All birds had free access to feed and water. Body weight and feed intake were recorded weekly and the feed conversion ratio (FCR) was calculated. Water intake was measured from days 18 to 21. Two birds per replicate were euthanized at the end of experiment for measurement of toe ash.

The results indicate that the dietary Ca level had a significant effect on P requirement (Table 1). Without phytase supplementation, feed intake, body weights were lower as the Ca to P ratio increased ( $P < 0.05$ ). Feed conversion ratio (FCR) was significantly poorer at higher Ca to P ratios. However, with phytase supplementation, the detrimental effect of a high Ca to P ratio was alleviated. Birds on higher dietary NPP had significantly ( $P < 0.05$ ) higher toe ash content compared to birds on lower NPP diets. However, toe ash content decreased as dietary Ca levels increased ( $P < 0.05$ ). Phytase supplementation increased toe ash content ( $P < 0.05$ ). Water intake was lower for birds on higher NPP diets than those on lower NPP diet ( $P < 0.05$ ).

**Table 1 - Broiler body weight at day 21, feed intake and FCR from day 1 to 21**

Starter/grower		Body weight (g/b)		Feed intake (g/b/d)		FCR (g feed/g gain)	
NPP (g/kg)	Ca (g/kg)	-Phytase	+Phytase	-Phytase	+Phytase	-Phytase	+Phytase
2.5/2.0	6.5/6.0	940.4cd	1020.6ab	56.9bc	56.6bc	1.35de	1.28e
	10.0/9.0	729.6f	1008.0ab	51.4e	58.2ab	1.60b	1.29de
	12.2/12.0	585.2g	911.4d	43.4f	54.6cd	1.70a	1.38cd
3.5/3.0	6.5/6.0	1020.0ab	1011.1ab	60.3a	59.1ab	1.31de	1.30de
	10.0/9.0	941.6cd	1041.0a	58.0ab	60.2a	1.37cde	1.29de
	12.2/12.0	833.3e	982.5bc	53.6de	57.9ab	1.46c	1.33de

The values within each parameter with the different letters differ ( $P < 0.05$ )

The results confirm our previous study (Li et al., 2013) that dietary NPP levels can be reduced in broiler diets supplemented with phytase. However, to maximise broiler performance, dietary Ca levels need to be adjusted accordingly.

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## DIETARY NON-PHYTATE PHOSPHORUS LEVELS AND LAYER PERFORMANCE IN THE LAST PHASE OF LAY

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The phosphorus (P) requirement of laying hens is an area of ongoing debate and it is a factor that contributes to hen performance and egg quality, especially late in the laying cycle. Part of the uncertainty regarding P requirements is the basal diet fed in experiments and the variable amounts of phytate P in the diets. For these reasons, industry formulates diets to contain 4.0 to 4.5 g/kg of non-phytate P (NPP). The experiment described below was conducted to evaluate the effects of different levels of non-phytate P (NPP) and phytase on egg production and egg shell quality of hens from 51 to 80 weeks of age.

A total of 480 Hy-Line brown egg laying hens were housed in 6 bird cages in a controlled environmental (22-24°C) shed with 16-hour lighting regimen. There were 8 experimental diets and each diet was fed to 10 replicate cages with measurements recorded from 51 to 80 weeks of age. The experimental diets were based on a sorghum and wheat blend and contained the same levels of calcium (42 g/kg diet), phytate-P (2.6 g/kg diet) with graded levels of NPP (1.5, 2.5, 3.5 and 4.5g/kg diet) with or without phytase (450 FTU/kg). Egg production and defective egg shells were recorded daily. Feed intake, bird body weight, egg weight, and egg shell quality (shell breaking strength, shell weight and shell thickness) were measured every four weeks.

Egg production from 51 to 80 weeks was 79% per hen housed. There was no significant effect of NPP concentration and phytase on egg production. Phytase, however, numerically improved egg production of layers fed the diet containing 1.5 g/kg NPP. There were no significant differences between treatments with regard to egg shell defects. However, birds on the diets containing the higher dietary NPP level (4.5g/kg both with and without phytase supplementation) tended to produce a higher proportion of eggs with defective shells but these differences were not significant. Numerically, the body weights of layers on the diet containing 1.5g/kg NPP were lighter ( $P > 0.05$ ) than the other treatment groups. Dietary NPP levels and phytase had no effect ( $P > 0.05$ ) on feed intake, feed conversion ratio, egg weight or shell weight. Shell breaking strength and shell thickness tended to be lower in layers fed diets containing 4.5g/kg NPP with phytase supplementation compared to birds fed diets with lower NPP levels.

The results indicate that egg production and egg shell quality parameters in layers fed diets containing 1.5 g/kg NPP were comparable to hens fed diets containing higher NPP levels with or without phytase supplementation; and suggest that dietary NPP requirement can be substantially reduced. The NPP requirement results obtained in this study refer to one dietary scenario and there is a need to determine bird responses to diets containing different levels of Ca.

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## CAN WE FEED LAYING HENS WITHOUT SUPPLEMENTAL PHOSPHORUS?

Y.G. LIU<sup>1</sup> and K.Y. ZHANG<sup>2</sup>Summary

The poultry industry generally feeds 350 to 450 mg of non-phytic phosphorus (NPP) per laying hen per day, which is well above what is considered necessary according to recent research. The high NPP supply used is probably due to concerns about inadequate mineralisation of egg shells and skeletal problems. The excess feeding of NPP not only wastes the mineral resource but also excretes extra phosphorus (P) to the environment. This paper presents a brief summary of recent research findings which suggest that laying hens require not more than 200 mg NPP/hen/day, thus an adequate supply would be 220-250 mg/hen/day. A typical formulation without animal protein meal and mineral P source can supply about total P 0.35% or NPP 0.10%, the shortfall 0.12-0.15% can be supplemented with either mineral P (e.g. dicalcium phosphate 5-8 kg/mt) or effective phytase enzyme. This paper describes a series of field studies suggesting that commercial laying hens on corn/soybean meal diets can be fed without using supplemental P, and thus generate appreciable cost savings.

## I. INTRODUCTION

Phosphorus (P) is an essential nutrient in both mammalian and avian species. Its deficiency causes malfunction of multiple organs and tissues. For laying hens, dietary P deficiency leads to decline in feed intake, loss of performance and increased mortality (Liu, 2012). On the other hand, it has proven difficult to define P requirement with precision, due to various complicating factors. Firstly, laying hens are mature birds that can effectively mobilize their body reserve of calcium (Ca) and P for eggshell formation; Secondly, available P cannot be measured directly in diets and is calculated based on table values of individual ingredients that can vary considerably; Thirdly, the bioavailability of various inorganic P sources has not been well determined.

In addition, there is a strong argument to re-evaluate P nutrition of laying hens because P has come to represent the 3<sup>rd</sup> highest cost in formulations after energy and amino acids, and the availability of mineral P sources is becoming restricted.

## II. PHOSPHORUS REQUIREMENTS OF LAYING HENS

In vegetable ingredients, a large portion of P co-exists with phytic acid that is poorly digested by mono-gastric species and its portion of available P (aP) is calculated using a co-efficient estimated from bone mineralization tests on young chickens from 7 to 17 days. As such, the aP contents may vary considerably for the same ingredient. aP and NPP, despite being biologically different, are similar in actual value so we use NPP in most nutrition formulations.

Many studies have been conducted to determine NPP requirement of laying hens, but results are inconclusive. As reviewed by Angel (2011) and Liu (2012), a number of authors suggest NPP requirement in the range between 1.2 to 2.5 g/kg. Karcher et al. (2006) suggest NPP requirement of 1.6 g/kg or 200 mg/hen/d. Tan et al. (2011) confirmed an adequate NPP

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supply was 0.22% for corn based diets. Keshavarz (2003) reported that differences exist among layer strains and ages of birds, whilst Snow *et al.* (2005) found no differences for hens fed on NPP 1.4 vs. 4.5 g/kg and both strains (Hy-Line W-36 and W-98) responded similarly to dietary NPP levels. These findings may reflect the selection methods used by specific breeding firms. Whether there are indeed genetic differences in phosphorus requirements are still worthy investigating across commercial breeds.

The reported NPP requirements of laying hens vary considerably, from 1.2 to 2.5 g/kg or up to 250 mg/hen/d, and the precise requirements will be influenced by the level of dietary Ca, ingredients used, feed intake, age of birds and length of trial. Nonetheless, there is limited research supporting NPP above 2.5 g/kg. At this stage, an NPP supply of 2.0-2.5 g/kg seems a reasonable guideline, with the upper level already providing a comfortable safety margin.

### III. FEEDING HENS WITHOUT USING MINERAL PHOSPHORUS

Apart from revising the NPP requirements, enzyme technology, namely phytase and carbohydrase, can provide an alternative approach. A series of three field trials was conducted to examine feasibility of formulation using enzymes to replace mineral sources of P, and concurrently improve availability of both energy and amino acids.

Exp 1. 324 Lohmann White Leghorn laying hens (Pink-shell) aged from 56 to 76 weeks were allocated to three dietary treatments and each treatment had 6 replicates of 18 birds per replicate. The control group was fed on standard diet containing corn 64.4%, soybean meal 15.5%, rapeseed meal 9.9%, vegetable oil 0.4%, no animal protein meal but 0.75% DCP was formulated to supply P. The negative control contained no DCP, and the treatment diet contained no DCP but a phytase enzyme. The birds' performance is shown in Table 1. As expected, the removal of DCP resulted in reduced feed intake and poor performance, which was fully restored by the addition of phytase (300 FTU/kg) (Lei *et al.*, 2011).

**Table 1 - The effect of DCP and phytase on performance of laying hens**

<b>Diet NPP level, g/kg</b>	<b>2.6</b>	<b>1.4</b>	<b>1.4 + Phytase</b>
NPP intake, mg/hen/day	306	157	157
	1.87 <sup>ab</sup>	1.71 <sup>b</sup>	1.93 <sup>a</sup>
End live wt, kg/bird	84.4	81.0	84.1
Egg production, %	63.1	63.0	62.6
Egg weight, g	117.8 <sup>a</sup>	113.8 <sup>b</sup>	117.3 <sup>a</sup>
Feed intake, g/d	2.18	2.16	2.17
Feed conversion ratio	3.59	3.50	3.62
Eggshell strength, kg	52.4 <sup>a</sup>	47.0 <sup>b</sup>	49.8 <sup>ab</sup>
Tibia ash, %	2.78	9.26	5.56
Mortality, %			

<sup>a</sup>Means not bearing the same superscript differ significantly (P<0.05)

Exp 2. Lohmann White Leghorn hens aged 56 to 76 weeks were fed on a similar corn, soybean meal diet with and without DCP, and the efficacy of an enzyme combination (phytase + carbohydrase) was tested to assess the ability of the enzyme combination to compensate for lower dietary phosphorus, amino acids and ME contents of diets. The trial consisted of 3 treatments: 1) Positive control (PC) (crude protein 15.5%, ME 11.134 MJ/kg, NPP 0.26% from DCP 0.75%); 2) Negative control (NC): no DCP, down-spec in ME 0.209 MJ/kg and amino acids 1.5%; 3) NC + Rovabio<sup>®</sup> Max (containing endo-1,4  $\beta$  xylanase 22,000 visco unit/g, endo 1,3(4)  $\beta$ -glucanase 2,000 AGL unit/g and 6-phytase 10,000 FTU/g, 50 g/mt). Each

treatment had 6 replicates of 18 birds. The trial lasted for 20 weeks and results are shown in Table 2.

**Table 2 - Laying performance after DCP removal and enzyme addition\***

	<b>P. Control</b>	<b>N. Control</b>	<b>N.C. + Rov. Max</b>
Initial wt, kg/hen	1.74 ± 0.10	1.72 ± 0.19	1.84 ± 0.12
Final wt, kg/hen	1.87 ± 0.13 <sup>a</sup>	1.68 ± 0.24 <sup>b</sup>	1.93 ± 0.17 <sup>a</sup>
Mortality, %	2.78 ± 3.05 <sup>b</sup>	13.89 ± 5.83 <sup>a</sup>	2.78 ± 3.05 <sup>b</sup>
Laying rate, %	84.4 ± 5.2 <sup>a</sup>	77.6 ± 7.0 <sup>b</sup>	84.8 ± 1.5 <sup>a</sup>
Feed intake, g/d/hen	117.8 ± 3.05 <sup>a</sup>	113.8 ± 3.34 <sup>b</sup>	116.5 ± 1.43 <sup>ab</sup>
Egg weight, g	63.1 ± 0.98	62.5 ± 0.90	62.3 ± 0.37
Feed conversion	2.18 ± 0.04	2.28 ± 0.23	2.18 ± 0.03
Shell thickness, mm	0.36 ± 0.03	0.34 ± 0.03	0.35 ± 0.02
Shell strength, kg	3.43 ± 0.69	4.05 ± 0.92	3.68 ± 0.90

\*Means not bearing the same superscript in the same row differ significantly ( $P < 0.05$ )

The birds on the NC diet showed lower feed intake and laying rate, and increased mortality. The enzyme addition fully restored those parameters. The results demonstrated that the enzyme not only compensated for NPP deficiency but possibly for lower metabolizable energy and amino acids contents of diets.

Exp 3. Hy-Line Brown birds aged from 20-43 weeks were fed on corn/soybean meal diets with 5 dietary treatments. Each treatment consisted of 9 replicates of 15 birds. The 3 control diets consisted of: 1) Positive control (PC) was standard diet with NPP 2.3 g/kg, crude protein 16.0%, ME 11.506 MJ/kg and Ca 3.6%; 2) Negative control 1 (NC1): no DCP, down-spec ME 0.209 MJ/kg, dig. AA 1.5%; 3) Negative control 2 (NC2): no DCP, down-spec ME 0.355 MJ/kg, dig. amino acids by 3.0% (Table 3a). The combined enzyme (Rovabio<sup>®</sup> Max) was tested on NC1 and NC2 to make up 5 dietary treatments.

**Table 3a - Diet formulations using combined enzyme to supply NPP, ME and DAA**

<b>Ingredients (%)</b>	<b>PC</b>	<b>NC1</b>	<b>NC2</b>	<b>Composition</b>	<b>PC</b>	<b>NC1</b>	<b>NC2</b>
Maize	60.01	62.4	63.8	Crude protein (%)	16.0	15.6	15.0
Soybean meal 48%	18.8	17.9	17.2	ME (kcal/kg)	2750	2700	2665
Soybean oil	2.71	1.62	0.92	ME (MJ/kg)	11.5	11.30	11.15
Sunflower meal 34%	8.00	8.00	8.00	Dig. lys (%)	0.74	0.73	0.72
Calcium carbonate	8.60	9.18	9.17	Dig. met (%)	0.37	0.35	0.35
Dicalcium phosphate	0.89	-	-	Dig. met + cys (%)	0.59	0.58	0.58
Sodium chloride	0.30	0.30	0.30	Dig. thr (%)	0.53	0.52	0.51
DL methionine 99	0.10	0.09	0.09	Calcium (%)	3.60	3.60	3.60
HCl-lysine 98	0.10	0.11	0.12	Total P (%)	0.52	0.36	0.36
Vit./mineral premix	0.40	0.40	0.40	Avail. P (%)	0.25	0.10	0.10

Results (Table 3b) showed that the removal of DCP plus down-spec of ME and DAA decreased feed intake, laying rate, feed efficiency, weight gain and increased mortality. The enzyme addition restored these performance parameters. The hens fed on NC diets showed lower contents in tibia ash, P and Ca, regardless of enzyme addition. The enzyme tended to increase tibia P content. The results suggest that 1) NPP 1.0 g/kg (100 mg/hen/d) is too low to sustain expected performance, which is consistent with the findings of Francesch et al. (2005) and Lei et al. (2011); and 2) confirm the enzyme combination (Rovabio<sup>®</sup> Max) was able to

compensate for lower ME of 0.21-0.35 MJ/kg, NPP 0.15% and digestible amino acids 1.5 - 3.0%.

**Table 3b - Effect of combined enzyme on layer performance from 20 to 43 weeks**

	<b>P. Control</b>	<b>NC 1</b>	<b>NC1+ Enz</b>	<b>NC2</b>	<b>NC2+ Enz</b>
Initial wt, g/hen	1655	1633	1643	1632	1639
Final wt, g/hen	1827 <sup>a</sup>	1656 <sup>b</sup>	1778 <sup>b</sup>	1687 <sup>a</sup>	1777 <sup>b</sup>
Mortality (%)	3.7 <sup>b</sup>	8.9 <sup>a</sup>	2.2 <sup>b</sup>	11.9 <sup>a</sup>	3.0 <sup>b</sup>
Laying rate, %	88.7 <sup>a</sup>	77.2 <sup>b</sup>	88.6 <sup>a</sup>	72.7 <sup>c</sup>	86.8 <sup>a</sup>
Feed intake, g/d	109.6 <sup>a</sup>	100.9 <sup>b</sup>	110.1 <sup>a</sup>	99.2 <sup>b</sup>	108.9 <sup>a</sup>
Egg weight, g	59.7	58.8	59.6	58.4	59.3
Feed conversion	2.073 <sup>c</sup>	2.224 <sup>b</sup>	2.087 <sup>c</sup>	2.344 <sup>a</sup>	2.116 <sup>c</sup>
Tibia ash (% DM)	49.3 <sup>a</sup>	46.6 <sup>b</sup>	46.4 <sup>b</sup>	46.4 <sup>b</sup>	46.2 <sup>b</sup>
Tibia Ca (% DM)	17.2 <sup>a</sup>	16.2 <sup>b</sup>	16.3 <sup>b</sup>	16.3 <sup>b</sup>	16.0 <sup>b</sup>
Tibia P (% DM)	7.70 <sup>a</sup>	7.04 <sup>b</sup>	7.28 <sup>ab</sup>	6.93 <sup>b</sup>	7.27 <sup>ab</sup>

Means within a row not bearing common superscript differ significantly (P< 0.05)

In conclusion, the egg industry may have scope to lower NPP requirements for Lohmann White and HyLine birds on corn /soybean meal diets without animal protein. The data in this paper suggest we can feed laying hens without supplemental P on corn and soybean meal diets provided appropriate phytase enzyme is applied. The use of endo-1,4  $\beta$  xylanase 22,000 visco unit and endo 1,3(4)  $\beta$ -glucanase 2,000 AGL unit/g appears to have beneficial impacts on improving energy uptake and amino acid utilisation.

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## MEASUREMENT OF PHOSPHORUS DIGESTIBILITY IN MAIZE AND CANOLA MEAL FOR BROILER CHICKENS

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### Summary

The present study was conducted to determine phosphorus (P) digestibility in maize and canola meal for broiler chickens. Four semi-purified diets were formulated from each of the ingredients to obtain graded concentrations of non-phytate P. The linear regression method was used to calculate the true P digestibility and true P retention coefficients. The apparent ileal digestibility of P in maize was affected (quadratic,  $P < 0.05$ ) by increasing dietary non-phytate P concentrations, while P retention was unaffected ( $P > 0.05$ ). The apparent ileal P digestibility of canola meal was similar ( $P > 0.05$ ) at different P concentrations. Phosphorus retention in broilers fed diets based on canola meal linearly ( $P < 0.01$ ) decreased with increasing P concentrations. True ileal P digestibility and true P retention coefficients of maize were determined to be 0.676 and 0.632, respectively. The corresponding values for canola meal were 0.453 and 0.475, respectively. No differences ( $P > 0.05$ ) were observed between ileal digestibility and retention coefficients determined for maize and canola meal.

### I. INTRODUCTION

A well-defined criterion for P availability for poultry is necessary to ensure greater efficiency of utilisation of dietary P and reduce the excretion of P into the environment. Three terminologies, namely available P, non-phytate P and retainable P, are used, often synonymously, to describe the available P in feed ingredients, which introduces considerable confusion for feed formulation. Of the various possibilities, however, measurement of digestible P may be the preferable method to assess P availability for poultry (Rodehutsord, 2009). Published data on apparent or true digestibility values of P in common feed ingredients for pigs are available (Fan et al., 2001; Peterson and Stein, 2006). In these studies, regression analysis and direct methods have been used to estimate P digestibility. Corresponding data for poultry are scant. Dilger and Adeola (2006) estimated the true P digestibility of soybean meal for broilers using the regression analysis technique where soybean meal was used as the only dietary source for calcium (Ca) and P.

Retainable P refers to the P that is retained in the body and this term is used in The Netherlands as a measure of P availability in feed ingredients. It is, however, known that increasing dietary concentrations of non-phytate P result in increased levels of plasma inorganic P and, once a physiological threshold is reached, the excess P is eliminated via the urine. Studies by Manangi and Coon (2006) suggest that, for broilers, the critical threshold range for dietary non-phytate P appears to be between 2 to 3 g/kg. Digestibility values of P for pigs are usually determined over the total tract and this approach is workable because faecal samples can be collected without urine contamination (Fan et al., 2001). In poultry, however, total tract measurements will yield misleading data if the dietary non-phytate P concentrations are above the physiological threshold. Thus, the aim of the present study was to determine the true P digestibility and to compare digestible P and retainable P contents of maize and canola meal for broilers.

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## II. MATERIALS AND METHODS

The experiment was arranged as a randomised complete block design with four blocks of eight cages each. Four semi-purified diets were formulated based on maize (236.5, 473, 709.5 and 946 g/kg diet) to contain graded concentrations of total P (0.66, 1.32, 1.99 and 2.65 g/kg; corresponding to 0.19, 0.38, 0.57 and 0.76 g/kg non-phytate P, respectively). Similarly, four semi-purified diets were formulated based on canola meal (135, 270, 405 and 540 g/kg diet) to contain graded concentrations of total P (1.58, 3.16, 4.74 and 6.32 g/kg; corresponding to 0.41, 0.81, 1.22 and 1.62 g/kg non-phytate P, respectively). In each set of diets, maize and canola meal were used as the only dietary source for P. Calcium: non-phytate P ratio was maintained in all diets at 2:1 by the addition of limestone. In addition to the test ingredient, the diets consisted of dextrose, soybean oil, sodium bicarbonate and vitamin-trace mineral premix, and contained 3 g/kg titanium dioxide as an indigestible marker.

Male broilers (Ross 308), 21-day old, were individually weighed and a total of 192 birds were assigned to four blocks based on body weight. Each block had eight cages (six birds per cage) and the eight test diets were randomly assigned to a cage within each block. The diets, in mash form, were offered *ad libitum* and the birds had free access to water. A lighting schedule of 20 h lighting per day was provided. Group body weights and feed intake were recorded on days 21 and 27. Between days 24 and 27, feed intake and excreta output were measured quantitatively per cage for three consecutive days. The daily collections were pooled within a cage, representative samples were taken, freeze-dried and ground to pass through a 0.5 mm sieve and stored in airtight plastic containers pending analysis. On day 28, birds were euthanised by intra-cardial injection of sodium pentobarbitone solution and the digesta from the lower ileum were collected, freeze-dried and prepared for analysis of dry matter, P and titanium. Dry matter was determined by drying samples at 105 °C for 16 hours in a pre-weighed dried crucible in a convection oven (AOAC, 2005; method no: 930.15). Samples were ashed and P was determined colorimetrically at 680 nm (AOAC, 2005; method no: 968.08D). Titanium oxide was determined by the colorimetric method as described by Short et al. (1996). Apparent ileal digestibility (Dilger and Adeola, 2006) and retention (van der Klis and Versteegh, 1996) coefficients of P in the test diets were calculated. Total P output in digesta and excreta at each level of inclusion was regressed against dietary P content to estimate true P digestibility, true P retention and endogenous P losses (Dilger and Adeola, 2006). Data were analysed using the GLM procedure of SAS (2004).

## III. RESULTS AND DISCUSSION

The maize and canola meal samples evaluated in the present study were analysed to contain 2.54 and 9.70 g/kg total P, respectively. No mortality or leg problems were observed during the six day experimental period. Birds responded linearly in weight gain and feed intake when fed maize and canola meal-based diets containing increasing concentrations of non-phytate P (Table 1). However, weight loss was observed when the birds were fed with the lowest dietary inclusion (236.5 g/kg) of maize. Weight gain was highest in birds fed the highest inclusion of canola meal which contained the highest contents of P and crude protein.

Phosphorus intake, P output, apparent ileal P digestibility and P retention coefficient for maize and canola meal-based diets are presented in Table 1. Increasing concentrations of dietary P linearly increased ( $P < 0.0001$ ) both the ileal and excreta P outputs in birds fed maize and canola meal diets. The apparent P digestibility coefficients of birds fed maize-based diets ranged from 0.605-0.704 at the ileal level and 0.451-0.675 over the total tract, respectively. The apparent ileal digestibility of maize was affected (quadratic,  $P < 0.05$ ) by increasing dietary P concentration, while P retention was unaffected ( $P > 0.05$ ). In contrast, the apparent P digestibility of canola meal was similar ( $P > 0.05$ ) at different dietary P

concentrations. Phosphorus retention in broilers fed canola meal diets linearly ( $P < 0.01$ ) decreased with increasing P concentrations.

Strong linear relationships were observed between digesta and excreta P outputs and dietary P intake for both maize and canola meal, which is a primary requirement for the application of regression technique. Existence of such a relationship allows a diet-independent theoretical estimate of endogenous P losses (g/kg dry matter intake) and simultaneous measurement of true P digestibility of a particular feed ingredient in birds. True P digestibility of maize and canola meal, and the estimated endogenous P losses are presented in Table 2. True ileal P digestibility and true P retention coefficients of maize were determined to be 0.676 and 0.632, respectively. The corresponding values for canola meal were 0.453 and 0.475, respectively. No differences ( $P > 0.05$ ) were observed between ileal digestibility and retention coefficients determined for maize and canola meal (Table 2). True ileal P digestibility in maize has not been previously reported, but the ileal digestibility of P in canola meal determined in the present study was considerably lower than the value of 0.66 reported by Adeola and Applegate (2010).

Endogenous P losses estimated for maize at the ileal and excreta levels were 0.0196 and 0.0767 g/kg dry matter intake, respectively. In the birds fed canola meal based diets, the endogenous P losses were determined to be negative at both ileal and excreta levels, with values of -0.335 and -0.344 g/kg dry matter intake, respectively. Reasons for the negative endogenous P losses determined for canola meal in this study are unclear. It is likely that this may be an anomaly reflecting an inherent limitation of the regression method, where the first or last data point could cause the intercept and slope to change. These negative values resulted in calculated true digestibility estimates being lower than apparent values.

In conclusion, the present data demonstrated that the regression method can be successfully used to measure true P digestibility of low and high P ingredients. Under the conditions of the present study, true digestible P values were similar to true retainable P values in maize and canola meal. Total P, true digestible P and true retainable P contents of maize were determined to be 2.5, 1.7 and 1.6 g/kg (as-received), respectively. The corresponding values for canola meal were 9.7, 4.4 and 4.6 g/kg (as received), respectively.

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**Table 1 - Growth performance, apparent ileal digestibility coefficient (AIDC) and total tract retention coefficient (TTRC) of phosphorus in birds fed diets containing graded concentrations of P from maize and canola meal for broilers**

Measurement	Maize-based diets						Canola meal-based diet							
	Diet 1	Diet 2	Diet 3	Diet 4	SEM	Probability L <sup>1</sup> Q <sup>1</sup>	Diet 1	Diet 2	Diet 3	Diet 4	SEM	Probability L <sup>1</sup> Q <sup>1</sup>		
BWG <sup>2</sup> , g/b/d	-5.46	3.69	18.65	34.77	2.28	< 0.0001	0.1525	6.11	29.74	45.33	53.92	1.84	< 0.0001	0.0015
FI <sup>3</sup> , g/b/d	72.68	82.67	97.05	107.32	5.25	0.0003	0.9790	86.55	105.13	111.96	108.73	3.38	0.0004	0.0073
P <sub>i</sub> <sup>3</sup> , g/kg DM	0.69	1.49	1.93	2.75	-	< 0.0001	< 0.0001	1.53	2.59	3.29	5.89	-	< 0.0001	< 0.0001
P <sub>D</sub> <sup>4</sup> , g/kg DMI	0.27	0.44	0.66	0.92	0.04	< 0.0001	0.2465	0.54	0.94	1.59	2.88	0.19	< 0.0001	0.0433
P <sub>E</sub> <sup>5</sup> , g/kg DMI	0.38	0.48	0.88	1.09	0.07	< 0.0001	0.4704	0.46	0.87	1.55	2.72	0.11	< 0.0001	0.0042
AIDC	0.605	0.704	0.656	0.664	0.017	0.1179	0.0227	0.648	0.639	0.516	0.512	0.062	0.0801	0.9689
TTRC	0.451	0.675	0.545	0.605	0.051	0.1756	0.1352	0.697	0.663	0.527	0.539	0.037	0.0032	0.5397

<sup>1</sup>L = linear effect; Q = quadratic effect.<sup>2</sup>BWG = body weight gain.<sup>3</sup>FI = feed intake; P<sub>i</sub> = Dietary P content; DMI = Dry matter intake.<sup>4</sup>P<sub>D</sub> = Ileal P output.<sup>5</sup>P<sub>E</sub> = Excreta P output.**Table 2 - Linear relationship between ileal or excreta P outputs (g/kg DMI) vs. dietary P content (g/kg DM) of maize and canola meal fed to broilers**

	Regression equation <sup>1</sup>	SE of the slope <sup>2</sup>	SE of the intercept <sup>2</sup>	R <sup>2</sup>	Endogenous P loss (g/kg DMI) <sup>3</sup>	Digestibility/ retention coefficient <sup>4</sup>
<b>Maize</b>						
True ileal P digestibility	Y=0.3242X+0.0196	0.03	0.05	0.91	0.0196	0.676
True P retention	Y=0.3676X+0.0767	0.05	0.10	0.76	0.0767	0.632
<b>Canola meal</b>						
True ileal P digestibility	Y=0.5474X-0.3349	0.06	0.22	0.86	-0.3349	0.453
True P retention	Y=0.5246X-0.3436	0.04	0.13	0.94	-0.3436	0.475

<sup>1</sup>Regression of ileal digesta or excreta P output (g/kg DMI) against dietary P content (g/kg DM) as determined by feeding broilers with diets containing graded levels of either maize or canola meal. The slope represents true P indigestibility and the intercept represents the endogenous P loss (g/kg DMI).<sup>2</sup>Standard errors of regression criteria.<sup>3</sup>Calculated as described in Dilger and Adeola (2006).<sup>4</sup>Within each ingredient, digestibility and retention coefficients were similar (P > 0.05).

## USING THE GEOMETRIC FRAMEWORK TO EXPLORE CALCIUM AND PHOSPHORUS INTERACTIONS AND THE EFFECT ON BROILER WELFARE

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### Summary

Calcium (Ca) and phosphorus (P) are the two most abundant minerals in bone and poultry diets; however, they have a complex multifactorial relationship. To explore the interactive effects of Ca and available phosphorus (av.P) on broiler performance and skeletal health, 600 Ross 308 day old male broilers were fed one of 15 dietary treatments. Diets were formulated to one of three total densities of Ca: av. P, with a spread of five different ratios (4, 2.75, 2.1, 1.5 and 1.14:1) at each density. Dietary av.P was more influential on broiler performance than dietary Ca. Feed intake increased with higher av.P levels, but was inhibited at low levels of av.P. Body weight gain increased rapidly with increasing dietary av.P. Latency to lie results showed that birds that were fed diets with a 2.1:1 ratio of Ca: av. P had the longest standing times, indicating better leg health.

### I. INTRODUCTION

The relationship between calcium (Ca) and phosphorus (P) in broiler nutrition is one that is complex and multifactorial. The digestion and absorption of these minerals is influenced by other dietary vitamins and enzymes as well as endogenous hormones. The modern broiler has a high requirement for Ca and P for energy metabolism and skeletal development. Deficiencies in Ca, P or both nutrients can have negative effects on bird performance as well as skeletal health. Due to the selection for rapid growth in broilers, some indirect welfare consequences have arisen; of particular importance are the adverse effects on skeletal health (Shim *et al.*, 2012). Current research shows that due to the high porosity of the cortical bone it is prone to bone deformities, which can impair broiler mobility, which is considered a welfare concern.

To better understand the complex relationship between Ca and P in poultry nutrition, a novel graphical approach termed the geometric framework was used. The geometric framework allows for a graphical representation of two or more nutrients, and explores the interactive effects of these nutrients. This study aims to investigate the optimum dietary Ca and available P (av.P) concentrations for broiler performance and skeletal health using the geometric framework.

### II. MATERIALS AND METHODS

All experimental procedures conducted had approval from The University of Sydney Animal Ethics Committee. A total of 600 Ross 308 day-old male broiler chicks were obtained from a commercial hatchery. Chicks were randomly allocated across 75 cages (750mm x 750mm x 510mm) with eight birds per cage and five replicate cages per treatment. The cages were housed in a temperature controlled room; temperature of 31 °C for the first five days and reduced by 0.5 °C per day until 24 °C (d21). The

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lighting regime was 23h:1h (light:dark) for the first five days and then 18h:6h (light:dark) for the remainder of the study (to d28). Chicks were fed a commercial starter diet for the first seven days before commencing on dietary treatments. Diets were corn-soy based and fed as a mash. Diets were formulated to one of three densities of total Ca:av.P ; high (15 g/kg), medium (13.5 g/kg) and low (12 g/kg). At each density there were five different ratios of Ca:av.P (4, 2.75, 2.1, 1.5 and 1.14:1). Birds had *ad libitum* access to feed and water. At day 27 the skeletal health of five birds per cage was evaluated using the latency to lie (LTL) procedure; whereby individual birds were placed into a plastic tub containing a few centimeters of tepid water and were timed until the bird made an attempt to sit down (Berg and Sanotra, 2003). On day 28 birds were euthanized via injection of a lethal dose (1ml/2kg) of sodium pentobarbitone into the jugular vein. Individual body weights were recorded and the right leg removed for tibia ash analysis. Tibia ash analysis, commonly used to assess bone mineralisation was determined as outlined by Garcia and Dale (2006).

All data were analysed using R version 2.15 (R Development Core Team, 2012). All performance data were analysed using a fixed effect model including blocking and a quadratic response surface model was fitted using the mixed model lme() function. Behavioural LTL times were analysed using Cox's proportional hazard survival analysis.

### III. RESULTS AND DISCUSSION

Bird performance is presented as treatment means over the experimental period of day 7 to 28. The effect of dietary Ca and av.P on feed intake is presented in Figure 1. Feed intake increased with increasing dietary av.P ( $P < 0.0001$ ). Higher feed intakes were associated with high av.P and high total Ca. Feed intake was strongly inhibited by diets with low av.P, and further impaired with the addition of high levels of Ca. Body weight gain (Figure 2) was observed to follow a similar pattern to feed intake. Body weight gain increased rapidly from 1.5 g/kg to 3.0 g/kg of av.P with total Ca having little effect. Concentrations of av.P above 4.5 g/kg had little influence on body weight gain. Total Ca inclusion had a more influential effect on body weight gain at av.P levels higher than 4.5 g/kg resulting in an interaction between Ca and av.P ( $P < 0.01$ ). Previous studies using low av.P ( $< 0.2\%$ ) and high Ca concentrations ( $> 0.9\%$ ) have reported a reduction in broiler growth performance (Rousseau *et al.*, 2012), consistent with the results observed. However, the current results contradict other studies, Dhandu and Angel (2003) and Yan *et al.*, (2001). Both studies found no significant effect on growth performance by varying P concentrations with a fixed Ca concentration. However, the study conducted by Dhandu and Angel (2003) focused on broilers from 32-42 days, a developmental stage where broilers are less sensitive to av.P (Nelson *et al.*, 1990). FCR from day 7-28 was shown to be poorest at low dietary av.P, with rapid improvement as av.P concentrations increased (Figure 3). The effect of dietary Ca and av.P on tibia ash is presented in Figure 4. A significant interaction between of Ca\*av.P was observed ( $P < 0.001$ ).

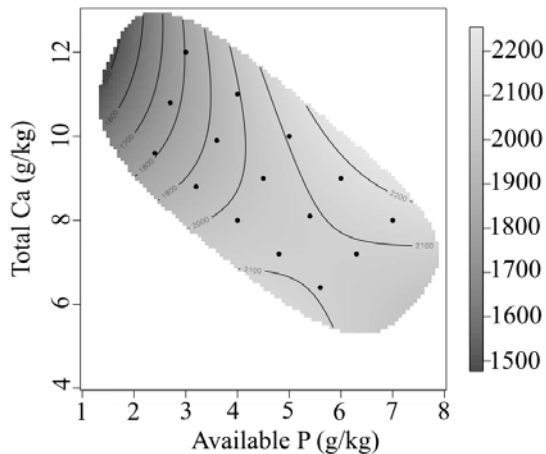


Figure 1 - Graphical representation of the interactive effect of dietary Ca and av.P on feed intake (g/b) from day 7 to 28. Av.P P < 0.0001 Ca P = 0.1167 Ca\*av.P P = 0.0311

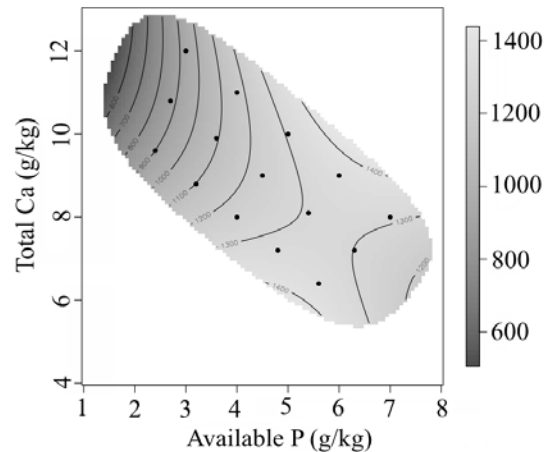


Figure 2 - Graphical representation of the interactive effect of dietary Ca and av.P on body weight gain (g/b) from day 7 to 28. Av.P P < 0.0001 Ca P = 0.0037 Ca\*av.P P = 0.0016

Tibia ash increased with increasing av.P inclusion to around 4.5 g/kg beyond which there was little change in tibia ash. Total dietary Ca was observed to positively influence tibia ash above 4.5 g/kg av.P, resulting in a significant Ca\*av.P interaction. Latency to lie data showed that birds fed diets with an approximate 2:1 ratio of Ca:av.P were more likely to stand for longer (Figure 5). When comparing tibia ash and LTL graphs, birds with poorer tibia ash were also observed to have shorter LTL standing times. These data suggest that the birds receiving these diets have poorer bone mineralisation, which is reflected by impaired mobility and locomotion.

Interestingly, birds with higher tibia ash percentage also had shorter LTL standing times. This indicates that these birds also had impaired mobility, however, this was not associated with reduced bone mineralisation. The intense genetic selection that the broiler has undergone for increased muscle yield has also changed the conformation in the broiler stance and stride. Due to the heavier breast muscle the birds' centre of gravity is further forward, which is supported by short legs with large thigh muscles. These physiological changes have led broilers to walk more slowly, taking short wider steps and increasing contact time with the ground (Mench 2004). This abnormal gait of broilers has been associated with chronic pain (Mc Geown *et al.*, 1999; Danbury *et al.*, 2000). These anatomical changes to broiler locomotion, and the associated chronic pain may help to explain why birds may suffer impaired mobility, yet have high tibia ash percentage. Further investigation is needed to determine the cause of impaired broiler mobility with high av.P diets.

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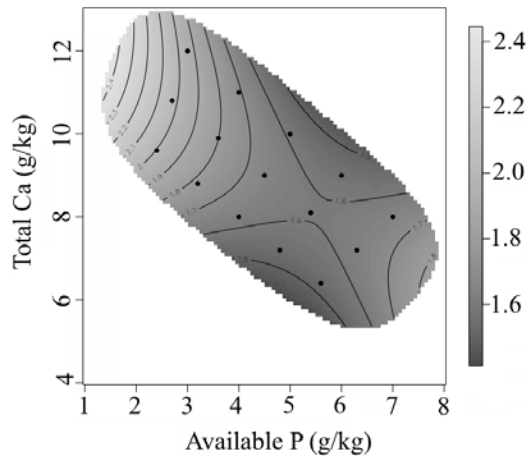


Figure 3 - Graphical representation of the interactive effects of av.P and Ca on FCR from day 7 to 28.  
Av.P P < 0.0001, Ca P = 0.0215,  
Ca\*av.P P = 0.0026

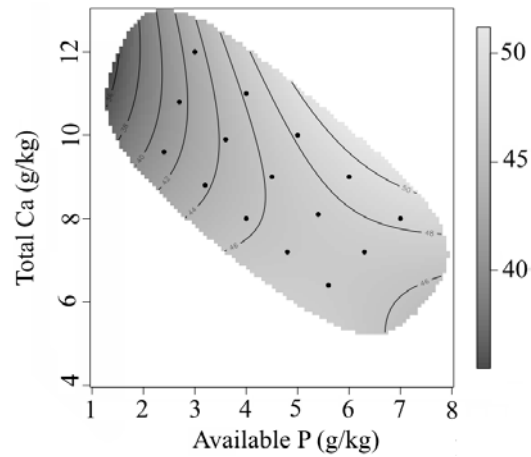


Figure 4 - Graphical representation of the interactive effects of av.P and Ca on Tibia ash (DM%) at day 28.  
Av.P P < 0.0001, Ca P = 0.0031,  
Ca\*av.P P = 0.0009

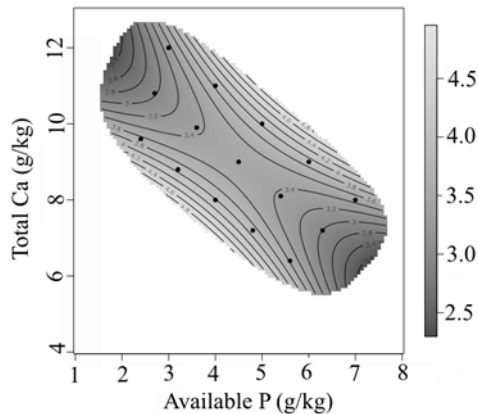


Figure 5 - Graphical representation of the interactive effects of av.P and Ca on Latency to lie time (in minutes) at day 27.  
Av.P P < 0.3531, Ca P = 0.5620,  
Ca\*av.P P = 0.004

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## CALCIUM / AVAILABLE PHOSPHORUS RATIO AND CARBOHYDRASE / PHYTASE AFFECT BROILER PERFORMANCE

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### Summary

The objective of this work was to study the impact of several calcium/available phosphorus ratios (Ca/aP) on broiler performance with or without addition of a carbohydrases/phytase complex (Rovabio<sup>®</sup> Max LC). Four thousand eight hundred 1-day old chicks (Ross PM3) were allocated into 6 treatments: 3 control diets with low levels of aP (0.25% for starter and 0.20% for grower) and different Ca/aP ratios from high (T1: 3.8 for starter and 4.2 for grower), medium (T2: 3.3 and 3.7) and low (T3: 2.8 and 3.2) obtained by changing calcium levels. Each control diet was supplemented with Rovabio<sup>®</sup> Max LC, 200 ml/t (T4 to T6). Each treatment was composed of 8 pens of 100 males. Feed consumption, weight gain and litter quality were measured at 21 and 34d. Results showed that lowering Ca/aP ratio reduced the negative impact of aP deficiency by increasing significantly live weight (+153 and 276g for the medium and low ratios, respectively, in comparison to the high ratio) and feed intake (+247 and 439g), with no effect on feed conversion ratio (FCR). At 21d this reduction in the Ca/aP ratio significantly improved litter score, but this effect faded away during the grower phase on day 34. The addition of the enzyme significantly improved growth performance parameters, excepted for FCR. The best performances were obtained with the diets with the lowest Ca/aP ratio and supplemented with the enzyme. Overall, the enzyme addition showed no impact on litter quality, except that the high Ca/aP ratio diet(T4(T1+E)) produced a significant degradation in litter quality compared with all other diets. In conclusion, high calcium levels accentuated the impact of low available phosphorus diets on broiler performance. The enzyme combination significantly improved growth performance of birds fed on low phosphorus diets and reduced the differences due to Ca/aP ratios. The results suggest that the formulation for a low level of dietary phosphorus requires a simultaneous reduction of dietary calcium level.

### I. INTRODUCTION

Optimisation of phosphorus (P) supply in animal feed remains a major environmental and economical challenge. In this context, nutritional strategies have been developed to limit P overfeeding and excretion, relying on a better adjustment of P supply to meet requirement and improving P availability through introduction of exogenous phytase (Selle and Ravindran, 2007). Nevertheless, low P diets can severely impact growth performance and percentage bone ash in young broilers (Waldroup et al., 1963; Nelson et al., 1965). Overall, P utilisation is governed by digestive and metabolic efficiency, in which several dietary components such as calcium (Ca) and vitamin D play important roles. Increasing the level of dietary Ca beyond dietary requirements or widening the ratio of dietary Ca to P is often associated with a decrease in broiler performance, mineral retention and phytate disappearance (Edwards, 1982). Vitamin D effectively improves the retention of dietary Ca and P of broiler chick diets. As a result, balance among this element might affect animal performance (Létourneau Montminy et al., 2008). However, dietary P reduction can be

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achieved without deleterious effects on performance and bone mineralization if Ca is reduced simultaneously (Driver et al., 2005; Rama Rao et al., 2006). Therefore, it is very important to take into account the Ca/P ratio, particularly when phytase is added to the diet. Driver et al. (2005) clearly demonstrated that animals perform better when phytase is added to low P diets with Ca/total P ratio of 1.1 to 1.4 rather than 1.7 to 2.0, whereas Letourneau-Montminy et al. (2008) observed the highest growth rate when broilers were fed a diet with a Ca/total P ratio of 0.9. Although many studies suggest reductions of dietary Ca level in phytase supplemented broiler diets may be warranted, appropriate dietary Ca level and Ca/P ratio still require more precise definitions. The present study was carried out to assess the effect of several Ca/aP on broiler performance in chicks given wheat-based diets with or without a carbohydrases/phytase complex.

## II. MATERIALS AND METHODS

Four thousand eight hundred 1-day old chicks (Ross PM3) were allocated into 6 treatments: 3 control wheat-based diets with low levels of aP (0.25% for starter and 0.20% for grower, respectively) and different Ca/aP ratios: high (T1: 3.8 for starter and 4.2 for grower), medium (T2: 3.3 and 3.7) and low (T3: 2.8 and 3.2) obtained by changing calcium levels. Each control diet was supplemented with Rovabio<sup>®</sup> Max (Adisseo, Antony, France) (T4(T1+E), T5(T2+E), T6(T3+E)). Each treatment was composed of 8 pens of 100 males. The main characteristics of diets are presented in Table 1. The enzyme was sprayed on diets after pelleting (200 ml/mt). The enzyme activities of diets were controlled by laboratory analysis (Adisseo). Feed consumption, live weight gain and litter quality were measured at days 21 and 34. Litter score were ranged from 1 to 5 corresponding to bad and good litter quality, respectively. Mortality rate was recorded.

Results were tested with a variance analysis using “t” test to compare the different diets, with and without enzyme supplementation. Least square means and standard errors of the means are reported.

## III. RESULTS AND DISCUSSION

All xylanase and phytase activities measured in supplemented diets were in line with the standard recommendation (xylanase: 1,100 visco units/kg and phytase: 500 FTU units/kg). The average mortality rates were 3.3, 3.4, 3.4, 2.0, 3.1 and 3.1% for treatments T1, T2, T3, T4(T1+E), T5(T2+E) and T6(T3+E), respectively. There were no significant differences among treatments and no birds were eliminated due to leg problems (data not shown).

Growth performance is presented in Table 2. For the complete growth period (0-34d) of the three control diets, lowering Ca/aP ratio linearly reduced the negative impact of the low aP diets in that body weight increased by 153 and 276g for the medium and low ratios, respectively. In comparison to the high ratio, feed intake increased 247 and 439g, with no effect on feed conversion ratio. Performances achieved were below genetic provider average for all parameters (Aviagen, 2012). Average (min-max) relative difference among control diets and standard performance were 20 (14 - 21), 58 (55 - 61) and 10 (10 - 11) for final body weight, daily weight gain and feed conversion ratio, respectively. This effect might be partly explained by phosphorus depletion in experimental diets and ratio Ca/aP above the recommendation.

The enzyme supplementation of T1, T2 and T3 diets significantly improved the zootechnical parameters and resulted in higher body weight, weight gain and feed intake than those of the respective control diets ( $P \leq 0.001$ ). However, no effect was observed on feed conversion following the enzyme addition. No interaction was observed between enzyme effect and dietary Ca/aP ( $P > 0.05$ ). Moreover, it is important to note that the best

performance was obtained with the diets of the lowest Ca/aP ratio T6 (T3+E) and supplemented with the enzyme, thus confirming that dietary Ca level could interact with enzyme efficacy. Bone ash content averaged 12.8 and 14.8 % without and with enzyme ( $P < 0.001$ ), respectively. In the opposite, no significant effect of Ca depletion was observed among treatment. Both observations thus suggest aP deficiency for an optimal bone mineralization.

Concerning litter quality evaluation, on day 21, the Ca reduction significantly improved litter score, but this effect faded away afterwards and vanished on day 34. Overall, the enzyme addition had no impact on litter quality, with the exception of the high Ca/aP ratio diet (T4; T1+E) for which there was a significant deterioration in litter quality compared to all other diets.

#### IV. CONCLUSION

The present experiment showed that the effect of phosphorus reformulation on growth performance depends on Ca/aP ratio. Indeed, the benefit of P reformulation can be optimized by a simultaneous reduction of both aP and Ca. The best performance was obtained following enzyme supplementation. The multi-enzyme complex containing NSP-enzymes and phytase brings about more benefits at low Ca/aP ratio (2.8 for starter and 3.2 for grower).

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**Table 1 - Main characteristic of the control diets.**

	1-21 days			22-35 days		
	T1	T2	T3	T1	T2	T3
<b>Composition, %</b>						
Wheat	54.60	55.30	56.00	60.80	61.40	62.00
Corn	10.00	10.00	10.00	10.00	10.00	10.00
Soybean meal	29.90	29.70	29.50	24.10	24.00	23.80
Vegetable oil	2.00	1.80	1.60	2.00	1.80	1.70
DL-methionine	0.20	0.20	0.20	0.20	0.20	0.20
HCl-lysine	0.10	0.10	0.10	0.10	0.10	0.10
L-threonine	0.10	0.10	0.10	0.01	0.01	0.01
Calcium carbonate	1.50	1.20	0.80	1.40	1.10	0.90
Dicalcium phosphate	0.80	0.80	0.80	0.50	0.50	0.50
Choline choride 75%	0.10	0.10	0.10	0.10	0.10	0.10
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Vit/mineral premix <sup>1</sup>	0.40	0.40	0.40	0.40	0.40	0.40
<b>Nutrient content, %</b>						
Crude protein		21			19	
Crude fat		4.2			4.2	
ME, Kcal/kg		2 900			2 950	
Digestible lysine, %		1.17			1.10	
Avail.P, %		0.25			0.20	
Ca/avail. P ratio	3.8	3.3	2.8	4.2	3.7	3.2

<sup>1</sup>Premix provided per kg of feed : vitamin A: 12,000 IU; vitamin D3: 4000 IU; vitamin E: 80mg; vitamin k3: 4mg; vitamin B1: 4mg; Mn: 64 mg; Zn: 72 mg; Fe: 40 mg; Cu: 16 mg; Monensin: 5 g.

**Table 2 - Effect of supplementing diets with a multi-enzyme complex (Rovabio<sup>®</sup> Max) of growth performance of male Ross PM3 broiler (means).**

Groups	T1	T2	T3	T4(T1+E)	T5(T2+E)	T6(T3+E)	CV (%)	Group effect
<b>Ca/avail.P ratio</b>	<b>High</b>	<b>Medium</b>	<b>Low</b>	<b>High</b>	<b>Medium</b>	<b>Low</b>		
<b>Period 1-21d</b>								
Body weight, g	748 a	808 b	859 c	890 d	907 d	958 e	2.3	0.001
Weight gain, g/d	33.5 a	36.3 b	38.8 c	40.2 d	41.1 d	43.5 e	2.4	0.001
Feed intake, g/d	46.0 a	49.5 b	53.0 c	53.9 c	55.4 d	59.3 e	2.7	0.001
Feed conversion	1.37 c	1.36 bc	1.37 bc	1.34 a	1.34 ab	1.35 abc	1.0	0.001
<b>Period 22-34d</b>								
Body weight, g	813 a	907 b	978 cd	926 bc	1020 d	1064 d	4.2	0.001
Weight gain, g/d	62.5 a	69.7 b	75.3 cd	71.2 bc	78.5 d	81.8 d	4.2	0.001
Feed intake, g/d	126.1 a	139.5 b	148.5 c	151.0 c	155.5 d	162.3 e	2.4	0.001
Feed conversion	2.02 ab	1.99 a	1.97 a	2.11 b	1.96 a	1.99 ab	3.0	0.001
<b>Period 0-34d</b>								
Body weight, g	1561 a	1714 b	1837 c	1816 c	1919 d	2032 d	2.8	0.001
Weight gain, g/d	44.6 a	49.1 b	52.7 c	52.1 c	55.1 d	58.4 d	2.9	0.001
Feed intake, g/d	76.6 a	83.9 b	89.6 c	91.1 cd	93.7 d	98.7 e	2.3	0.001
Feed conversion	1.72 bc	1.71 abc	1.70 ab	1.74 c	1.68 a	1.69 ab	1.7	0.01
<b>Litter score<sup>1</sup></b>								
at 21d	2.0 b	2.7 c	3.5 d	1.3 a	2.9 cd	3.2 cd	21.1	0.001
at 34d	2.6 b	2.8 b	3.4 b	1.5 a	2.5 b	2.8 b	27.1	0.01

<sup>1</sup> corresponds to bad litter quality and 5 represents good litter quality  
a, b, c, d, e: significant differences between groups with  $P < 0.05$

## ENZYME COMPLEX IMPROVES PERFORMANCE OF BROILERS FED WHEAT- AND SOYBEAN-BASED DIETS WITH GRADED DENSITY: OPTIMUM DEFINITION

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### Summary

An experiment was carried out to measure regulation of feed intake by broiler chickens fed on graded density diets and possible interaction with enzyme supplementation. Male broilers were randomly assigned to four wheat and soybean meal-based diets providing 10.03, 11.15, 12.26 and 13.38 MJ AMEn/kg with or without NSP enzyme (Rovabio Excel). Each treatment was tested with 12 replicates of 15 birds grown to 35 d of age, to evaluate growth parameters: daily feed intake (DFI), daily weight gain (DWG) and feed conversion ratio (FCR). The results showed that increasing dietary energy density without enzyme addition significantly improved performance. Final body weight and FCR were positively correlated to dietary AMEn content according to a quadratic plateau ( $R^2 = 0.97$ ,  $0.99$  and  $RSD = 43\text{g}$  and  $0.03$ , respectively). Feed intake was significantly correlated to AMEn content for a standardized body weight ( $R^2 = 0.83$ ,  $RSD = 80\text{g}$ ). Over the entire period, the addition of the NSP enzyme improved DWG and FCR by 3% on average. The enzyme effect on FCR was clearly related to dietary energy density ( $P < 0.01$ ), with improvement ranging from +1.4 % for the 13.38 MJ AMEn/kg diet to -6.8 % for the 11.15 MJ AMEn/kg diet. In contrast, no consistent enzyme effect on DFI was observed ( $P = 0.79$ ), in relation to body weight. It is concluded that broilers still possess the ability to control DFI based on desire to regulate energy intake, implying a need for precise formulation tools. The enzyme effect was highly related to dietary density but this relationship was not linear.

### I. INTRODUCTION

Feed normally accounts for up to 70% of the total broiler production cost. Chickens have been traditionally fed with relatively high-energy diets, because, in addition to better feed utilization, it is also assumed that this type of diet maximizes growth rate (Leeson and Summers, 1991). As energy remains the most expensive nutrient, it is now suggested that diet density may be reduced while striving to maintain overall performance. The use of carbohydrases, hydrolyzing insoluble as well as soluble non-starch polysaccharides (NSP), is widely and successfully implemented when viscous cereals (wheat, barley, rye, oats, or triticale) are used (Bedford and Classen, 1992). By breaking down the cell wall matrix, enzymes may facilitate release of nutrients encapsulated in the cell walls, resulting in an easier access of digestive enzymes to their target nutrients (Cowieson *et al.*, 2006) thus improving the nutritional value of the diet. The aim of the present study was to investigate the effect of carbohydrases and diet density on growth performance.

### II. MATERIALS AND METHODS

The study was conducted in a  $4 \times 2$  factorial arrangement of treatments with 4 levels of AMEn (10.03, 11.15, 12.26 and 13.38 MJ AMEn/kg), with or without carbohydrase (Rovabio<sup>®</sup> Excel, Adisseo France SAS, Antony, France) supplementation. All nutrient

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contents remained constant relative to diet AMEn content. Diet nutrient levels were calculated to meet the minimum requirement ratios relative to energy according to the Ross 308 Nutrition Guide (Aviagen, Scotland, 2007). The feeding program consisted of 2 diets, a grower feed supplied from 0 to 21 d and finisher feed from 22 to 35 d. Two extreme diets, low and high density, were formulated as presented in Table 1 and mixed in ratio 2/1 (medium 1) or 1/2 (medium 2) in order to achieve 2 intermediates densities and 4 diets in total distributed in pelleted form. For this experiment, 1440 one-day old Ross 308 male broilers (body weight  $40 \pm 1.4$ g) were allocated to 96 pens, with 15 chickens per pen. Pens were set in a factorial and completely randomized block design with 12 pens per treatment. Performance data ( $n = 96$ ) were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, 2001). The model included diet density ( $n=4$ ), enzyme dose ( $n=2$ ) and interaction ( $n=8$ ) as fixed effects and block as a random effect. Least square means are reported.

### III. RESULTS

Experimental results are summarized in Table 2. As expected, broilers fed the low density diets showed higher ( $P < 0.001$ ) FCR than those fed the higher density diets during growing, finishing and entire rearing periods. Average FCR was 1.82 over the entire period, ranging from 1.48 to 2.20. When standardizing FCR to a 2 kg final body weight (BW), average FCR was reduced to 1.78, but with a wider range (1.39-2.25). The influence was primarily related to BW and BW gain (BWG) changes among treatments. Final BW ranged from 1774 to 2397g, i.e. 26 % increase from low to high density diets. Both parameters were related to AMEn level according to a quadratic relationship ( $R^2 = 0.99$  and  $0.99$ ;  $RSD = 0.02$  and  $36$  for FCR and final BW, respectively). Secondly, these results reflected a negative relationship between DFI and diet density for a standardized BW. Indeed, DFI decreased over the entire rearing period from 118 to 100g/d and AMEn ranging from 11.50 to 13.38 MJ/kg.

Enzyme effects on BW, BWG and FCR were +59g, +2.5g/d and -0.06, respectively for the entire life cycle ( $P < 0.01$ ). The highest effect was observed for the growing period, an average -3.0 % of FCR improvement from enzymes over the entire period was partitioned differently for two growth stages with -3.8 and -2.0 % for growing and finishing periods, respectively. The differing effect was probably associated with a combined effect over the growing period on the DFI and DWG whereas DWG only was affected by enzyme for the finishing period. In addition, enzyme/diet interaction was highly significant for FCR ( $P < 0.001$ ). The greatest FCR improvement with enzyme addition was observed for medium 1 diet (-6.8%;  $P < 0.001$ ), whereas the lowest was observed on the highest energy diet (+1.4 %;  $P = 0.41$ ). Intermediate values were observed for low and medium 2 diets with -2.7 and -3.1 %, respectively.

### IV. DISCUSSION

The pattern of energy intake observed in the present experiment suggests that broilers are able to adjust their feed intake, in response to their energy needs, with the same precision as previously demonstrated (Leeson *et al.*, 1996). Results obtained with pigs (from 20 to 50 kg BW and diet with net energy content ranging from 8.10 to 11.1 MJ/kg) suggested a similar negative relationship between intake and dietary net energy content up to a plateau for the lowest energy value (Quiniou *et al.*, 2012). Quadratic models relating dietary AMEn and animal performance suggest decreased supplementary MJ efficiency with enzyme supplementation, as diet density increases. In other words, 1 MJ reduction in low density diets had a more negative effect than in high density diet. In addition, enzymes could highly shift dietary energy optimum as previously defined for lower density diets. In the present

experiment, enzyme supplementation of reformulated wheat- and soybean meal-based diets was able to compensate for growth depression. These results are in agreement with previous results (Bedford and Classen, 1992). In addition, the present study emphasizes the impact of nutrient density on enzyme efficiency and the requirement for precise feed formulation values. Surprisingly, the enzyme effect was not linear. In other words, an increase in diet nutritional value through enzyme addition will not result in similar performance improvement. A similar study designed for evaluation of enzyme on graded density diets (Bedford and Classen, 1992), also showed that response to enzyme supplementation differed depending on the energy level in wheat and rye diets. Indeed, Bedford and Classen (1992) obtained a maximum FCR improvement for a low density diet (11.80 MJ ME/kg) compared with high density diet (12.85 MJ ME/kg) with about 31.9 and 9.6 % FCR improvement relative to control from 0 to 19 d of age, respectively. In contrast to our work, they observed a continuous improvement in FCR when enzyme was added with decreasing energy level of the diet (- 1.05 MJ ME/kg). The large improvement observed in this study was probably related to the formulation strategy, using a substitution of wheat by rye instead of least cost formulation, resulting in lower range of energy variation than in our study (1.05 MJ ME vs. 3.35 MJ AMEn).

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Table 1 - Composition and calculated nutrient content of basal diets

	Grower		Finisher	
	Low	High	Low	High
<b>Composition, g/kg</b>				
Wheat	402	414	365	472
Wheat bran	470	-	566	-
Soybean meal	83	400	30	350
Vegetable oil	-	144	-	138
DL-methionine	2	4	1	1
HCl-lysine	5	2	3	5
L-threonine	2	1	1	-
Calcium carbonate	11	9	11	9
Dicalcium phosphate	15	15	13	14
Salt	5	5	5	5
Premix <sup>1</sup>	6	6	6	6
<b>Nutrients content, g/kg</b>				
Crude protein	173.9	231.9	159.8	205.2
AMEn <sup>2</sup> , MJ/kg	10.03	13.38	10.03	13.38
Digestible lysine	9.5	12.7	7.3	9.7

<sup>1</sup> Providing per kg of feed: vitamin A = 12 000 IU, vitamin D<sub>3</sub> = 3 000 IU, vitamin E = 100 IU, vitamin K<sub>3</sub> = 3 mg, vitamin B<sub>1</sub> = 2 mg, vitamin B<sub>2</sub> = 8 mg, vitamin B<sub>6</sub> = 3 mg, vitamin B<sub>12</sub> = 0.02 mg, folic acid = 1 mg, biotin = 0.2 mg, pantothenic acid = 15 mg, nicotinic acid = 40 mg, Mn = 80 mg, Zn = 60 mg, I = 1 mg, Fe = 80 mg, Cu = 15 mg, Co = 0.4 mg, Se = 0.2 mg, Ethoxyquin = 0.5 mg, BHA = 0.5 mg and Narasin/nicarbazine = 80 mg.

<sup>2</sup> Apparent metabolisable energy standardized for zero nitrogen retention (AMEn) were calculated from raw material value measurements without enzyme.

**Table 2 - Effect of dietary nutrient density and enzyme supplementation on the performance of broilers (0 to 35 d)**

	Low		Medium 1		Medium 2		High		Statistic <sup>1</sup>				
	-	+	-	+	-	+	-	+	diet	enzyme	inter	R <sup>2</sup>	RSD
<b>Body weight, g</b>													
Initial	40	40	40	40	40	40	40	40	1.000	1.000	1.000	0.08	2
21 day	716 <sup>d</sup>	728 <sup>d</sup>	840 <sup>c</sup>	841 <sup>c</sup>	896 <sup>b</sup>	908 <sup>b</sup>	907 <sup>b</sup>	948 <sup>a</sup>	<0.001	0.020	0.180	0.85	33
Final	1774 <sup>e</sup>	1854 <sup>d</sup>	2173 <sup>c</sup>	2227 <sup>c</sup>	2326 <sup>b</sup>	2360 <sup>b</sup>	2397 <sup>b</sup>	2469 <sup>a</sup>	<0.001	<0.001	0.759	0.90	81
<b>Feed intake, g/day</b>													
Growing	63.0 <sup>b</sup>	60.7 <sup>bc</sup>	65.1 <sup>a</sup>	62.8 <sup>b</sup>	62.1 <sup>b</sup>	61.4 <sup>b</sup>	58.3 <sup>d</sup>	59.0 <sup>cd</sup>	<0.001	0.010	0.047	0.47	2.2
Finishing	178.2 <sup>bc</sup>	186.1 <sup>b</sup>	197.5 <sup>a</sup>	187.5 <sup>b</sup>	177.9 <sup>bc</sup>	174.6 <sup>c</sup>	162.2 <sup>d</sup>	172.4 <sup>c</sup>	<0.001	0.563	0.002	0.50	9.9
Overall	109.1 <sup>bcd</sup>	110.9 <sup>bc</sup>	118.1 <sup>a</sup>	112.7 <sup>b</sup>	108.4 <sup>bcd</sup>	106.7 <sup>cd</sup>	99.8 <sup>e</sup>	104.2 <sup>d</sup>	<0.001	0.793	0.003	0.56	4.6
<b>Daily gain, g/day</b>													
Growing	32.2 <sup>d</sup>	32.7 <sup>d</sup>	38.1 <sup>c</sup>	38.1 <sup>c</sup>	40.8 <sup>b</sup>	41.3 <sup>b</sup>	41.3 <sup>b</sup>	43.2 <sup>a</sup>	<0.001	0.009	0.106	0.90	1.4
Finishing	75.6 <sup>f</sup>	80.5 <sup>e</sup>	95.2 <sup>d</sup>	99.0 <sup>c</sup>	102.1 <sup>bc</sup>	103.7 <sup>b</sup>	106.4 <sup>ab</sup>	108.6 <sup>a</sup>	<0.001	0.003	0.582	0.88	4.7
Overall	49.6 <sup>e</sup>	51.8 <sup>d</sup>	60.9 <sup>c</sup>	62.5 <sup>c</sup>	65.2 <sup>b</sup>	66.3 <sup>b</sup>	67.3 <sup>ab</sup>	69.3 <sup>a</sup>	<0.001	<0.001	0.769	0.92	2.2
<b>Feed conversion ratio</b>													
Growing	1.956 <sup>a</sup>	1.854 <sup>b</sup>	1.711 <sup>c</sup>	1.649 <sup>d</sup>	1.526 <sup>e</sup>	1.487 <sup>f</sup>	1.412 <sup>g</sup>	1.365 <sup>h</sup>	<0.001	<0.001	0.025	0.96	0.038
Finishing	2.358 <sup>a</sup>	2.315 <sup>a</sup>	2.075 <sup>b</sup>	1.900 <sup>c</sup>	1.743 <sup>d</sup>	1.683 <sup>d</sup>	1.524 <sup>e</sup>	1.588 <sup>e</sup>	<0.001	<0.001	0.009	0.91	0.095
Overall	2.199 <sup>a</sup>	2.140 <sup>b</sup>	1.938 <sup>c</sup>	1.806 <sup>d</sup>	1.662 <sup>e</sup>	1.610 <sup>f</sup>	1.483 <sup>g</sup>	1.504 <sup>g</sup>	<0.001	<0.001	0.001	0.95	0.061
Overall AMEn <sup>2</sup>	22.05 <sup>ab</sup>	22.35 <sup>a</sup>	21.61 <sup>b</sup>	20.80 <sup>c</sup>	20.37 <sup>cd</sup>	20.27 <sup>cd</sup>	19.84 <sup>d</sup>	20.56 <sup>cd</sup>	<0.001	0.862	0.004	0.59	0.70
Overall std <sup>3</sup>	2.255 <sup>a</sup>	2.177 <sup>b</sup>	1.895 <sup>c</sup>	1.749 <sup>d</sup>	1.580 <sup>e</sup>	1.519 <sup>f</sup>	1.387 <sup>g</sup>	1.383 <sup>g</sup>	<0.001	<0.001	0.007	0.95	0.071

<sup>1</sup> Variance analysis were realized on complete dataset (n=96) with fixed effect diet (n=4), enzyme (n=2) and interaction

<sup>2</sup> Overall AMEn feed conversion ratio in kJ/g

<sup>3</sup> Overall feed conversion ratio standardized to 2kg final bodyweight

EFFECTS OF ORGANIC SELENIUM SUPPLEMENTATION ON GROWTH  
PERFORMANCE, NUTRIENT UTILISATION AND SELENIUM TISSUE  
CONCENTRATIONS IN BROILER CHICKENS

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Summary

Two organic Se supplements SH (selenohomolanthionine) and SM (selenomethionine), individually and in combination, were included in broiler diets that did not contain any additional Se. Their effects on growth performance, nutrient utilisation and Se tissue concentrations were assessed. Organic Se supplementation numerically increased feed intake and weight gain but there were significant treatment effects on N retention and N-corrected AME. SM improved N retention by 3.2 percentage units (68.4 versus 65.2%;  $P < 0.001$ ); however, SH supported a significantly higher N-corrected AME value than SM by 0.51 MJ (14.15 versus 13.64 MJ/kg;  $P < 0.05$ ). Both SH and SM generated significantly higher Se tissue concentrations in breast muscle and liver in birds relative to the negative control. On average, the two supplements significantly increased Se concentrations in breast muscle (0.270 versus 0.123 mg/kg) and hepatic tissue (0.714 versus 0.382 mg/kg). However, SH generated higher Se concentrations in muscle tissue than SM (0.283 versus 0.257 mg/kg;  $P < 0.05$ ).

I. INTRODUCTION

Selenium (Se) is an essential trace mineral in poultry nutrition as Se has important functions in growth and immunocompetence (Surai, 2002a,b) and as a constituent of selenoproteins, Se impacts on the antioxidant status of poultry (Sevcikova *et al.*, 2006). Inorganic Se is usually incorporated into broiler diets as a constituent of vitamin-trace mineral premixes although evidence is emerging that organic Se may be superior to inorganic Se sources (Wang *et al.*, 2011a,b). However, the importance of the form of organic Se in poultry physiology is not well defined. For this reason two organic Se sources, with putatively different functionality, individually and in combination, were compared in the present study in relation to growth performance, nutrient utilisation and Se tissue concentrations in breast muscle and liver. Also, the effects of organic Se supplementation on oxidative stress of broiler chickens are assessed in a companion paper (Celi *et al.*, 2013).

II. MATERIALS AND METHODS

The feeding study consisted of four dietary treatments in mash form based on wheat, soybean meal and canola meal. Starter diets were offered to birds from 1-21 days and finisher diets from 22-42 days post-hatch and their composition and nutrient specifications are shown in Table 1. The control diet contained a custom vitamin-trace mineral premix formulated to Aviagen recommendations for Ross 308 birds except that it did not contain any selenium. The treatment diets contained 0.3 mg organic Se per kg of diet provided by either SH (BiOnyc® Tor-Sel; 4000 mg/kg Se - primarily composed of selenohomolanthionine), SM (Alltech Sel Plex™; 2000 mg/kg Se - primarily composed of selenomethionine) or an appropriate “Se-equal” blend of the two supplements. Samples of both Se supplements were submitted to the Mark Wainwright Analytical Centre (UNSW) where their composition was determined by inductively coupled plasma mass spectrometry (ICP-MS). SH contained 3902

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mg/kg Se and SM contained 1853 mg/kg Se. Samples of the starter and finisher diets were also analysed and their mean Se concentrations were 0.350 mg/kg in the control diets, 0.855 mg/kg in the SH diets, and 0.930 mg/kg in both the blend and SM diets. Thus the supplements provided an additional 0.555 mg/kg Se on average to the relevant diets as opposed to the usual recommendation of 0.300 mg/kg.

Each of the four dietary treatments were offered to 12 replicates of 6 birds per cage (total: 432 birds) on an *ad libitum* basis from 1-42 days post-hatch as starter and finisher diets. The variables assessed included growth performance (weight gains, feed intakes, feed conversion ratios, mortality rates), nutrient utilisation (N retention and N-corrected AME) and Se tissue concentrations in breast muscle and liver determined by ICP-MS (12 samples per treatment), which provides a high precision analysis of trace minerals. Growth performance and nutrient utilisation data were derived from standard procedures as outlined by Selle *et al.* (2003). Experimental data were analysed by univariate analyses of variance using the IBM®, SPSS® Statistics 20 program. The feeding study complied with the specific guidelines [N00/10-2011/2/5612] stipulated by the Animal Ethics Committee of Sydney University.

**Table 1 - Dietary composition and nutrient specifications for the starter and finisher phases**

Item (g/kg)	Starter diet (1-14 days post-hatch)	Finisher diet (15-42 days post-hatch)
Wheat	590.00	694.00
Soybean meal	295.00	170.00
Canola meal	30.00	45.00
Vegetable oil	35.00	45.00
Limestone	2.50	3.70
Dicalcium phosphate	30.00	25.00
Salt	1.50	1.30
Sodium bicarbonate	5.25	4.60
Lysine HCl	2.50	3.10
Methionine	2.75	2.30
Threonine	0.50	1.00
TMV premix	5.00	5.00
Metabolisable energy (MJ/kg)	12.44	12.99
Protein	222.00	185.00
Calcium	8.90	8.00
Total phosphorus	9.50	8.31
Available phosphorus	4.70	4.00

### III. RESULTS AND DISCUSSION

The effects of organic selenium supplementation of broiler diets on 1-42 days post-hatch growth performance, nitrogen (N) retention, N-corrected apparent metabolisable energy (AMEn) and selenium tissue concentrations in breast muscle and liver are shown in Table 2. The overall

**Table 2 - The effects of organic selenium supplementation of broiler diets on 1-42 days post-hatch growth performance, nitrogen (N) retention, N-corrected apparent metabolisable energy (AMEn) and selenium tissue concentrations in breast muscle and liver**

Treatment	Growth performance				Nutrient utilisation		Selenium tissue concentrations	
	Weight gain (g/bird)	Feed intake (g/bird)	FCR (g/g)	Mortality (%)	N retention (%)	AMEn (MJ/kg DM)	Muscle (mg/kg)	Liver (mg/kg)
Control	2504	4110	1.647	1.39	65.2 <sup>a</sup>	14.14 <sup>b</sup>	0.123 <sup>a</sup>	0.382 <sup>a</sup>
SH	2635	4339	1.648	2.78	64.5 <sup>a</sup>	14.15 <sup>b</sup>	0.283 <sup>c</sup>	0.693 <sup>b</sup>
Blend	2548	4242	1.666	2.78	68.6 <sup>b</sup>	14.03 <sup>ab</sup>	-	-
SM	2540	4217	1.661	2.78	68.4 <sup>b</sup>	13.64 <sup>a</sup>	0.257 <sup>b</sup>	0.734 <sup>b</sup>
SEM	58.71	90.35	0.021	0.718	0.614	0.139	0.006	0.026
Significance (P =)	0.464	0.366	0.896	0.867	< 0.001	0.042	< 0.001	< 0.001
LSD (P < 0.05)	-	-	-	-	1.76	0.397	0.0178	0.0754

<sup>abc</sup>Within columns mean values that do not share a common superscript are significantly different at the 5% level of probability

mortality rate of 2.18% was acceptable and unrelated ( $P > 0.85$ ) to treatment. The overall growth performance of 2575 g/bird weight gain and 4247 g/bird feed intake with an FCR of 1.656 was satisfactory for mash diets and there were no treatment effects ( $P > 0.35$ ) on growth performance. Similarly, there were no significant treatment effects on growth performance from

1-14, 14-28, 29-42 and 1-28 days post-hatch (data not shown). However, over the entire feeding period there were numerical trends in favour of SH birds for feed intake (5.6%) and weight gain (5.2%) relative to the control diet and for feed efficiency (0.9%) relative to the mean of the two other Se-supplemented treatments.

The SM and the blend of the two Se supplements supported significantly greater N retention than the SH and control treatment groups. Thus SM improved N retention by 3.2 percentage units (68.4 versus 65.2%;  $P < 0.001$ ); whereas SH did not influence N retention. SH and the blend did not influence N-corrected AME; however, SH supported a significantly higher value than SM by 0.51 MJ (14.15 versus 13.64 MJ/kg;  $P < 0.05$ ).

Both SH and SM generated significantly higher Se tissue concentrations in breast muscle and liver in birds relative to the negative control, while the blend was not assessed. On average the two supplements approximately doubled Se concentrations by from 0.123 to 0.270 mg/kg in breast muscle and from 0.382 to 0.714 mg/kg in hepatic tissue. However, while there was no statistical difference between SH and SM in hepatic tissue, SH generated higher Se concentrations in muscle tissue than SM by 10.1% (0.283 versus 0.257 mg/kg;  $P < 0.05$ ).

The results of this study suggest that Se may be necessary to optimise the performance of broilers and that the form of organic Se may be of importance. In the present experiment, SH (composed largely of selenohomolanthionine) appeared to be more readily deposited in muscle and have a greater beneficial effect on energy metabolism than SM (composed largely of selenomethionine). On the other hand, SM enhanced N retention to a greater degree than SH. These differences suggest that the form of organic Se may alter the functionality of the molecule *in vivo* as all diets (other than the control) contained quite similar total Se concentrations.

Selenohomolanthionine is a relatively recently discovered selenoamino acid which was first identified in Japanese pungent radish (Ogra et al., 2007). Unlike SM, SH appears

not to accumulate in the pancreas, thus avoiding the pancreatic damage that higher dietary SM concentrations can cause (Tsuji et al., 2010) whilst having comparable efficiency of utilisation. Furthermore, the metabolic pathway of SH is simpler than that of SM as SH is utilised only in the trans-selenation pathway for selenoprotein synthesis. SM, on the other hand, is involved in complex metabolic pathways including displacing methionine in peptide synthesis, mechanisms that do not contribute to Se utilization. Tsuji et al. (2010) compared SM and SH in rats and found that SH is retained for longer in the blood and deposited more slowly in the liver than SM, results that support the results of the present study (Table 2).

It can be concluded that the delivery of organic Se to broilers is beneficial for tissue Se concentrations and nutrient recovery. Furthermore, the form of organic Se may alter the functionality of the product and change the fate of Se in the animal. These mechanisms require further exploration to determine the appropriate amino acid ligand for Se to optimize various biochemical processes and to explore the potential of SH as a microingredient for farmed livestock.

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## THE EFFECTS OF DIETARY SUPPLEMENTATION WITH DIFFERENT ORGANIC SELENIUM SOURCES ON OXIDATIVE STRESS IN BROILERS

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### Summary

The effect of supplementation of diets for broiler chickens with two organic selenium sources on oxidative status was evaluated in 288 Ross birds. The birds were fed either a control diet (no selenium) or experimental diets that contained either selenohomolanthionine (SH) or seleno-methionine (SM) individually, or in combinations of the two, so as to provide 0.3 mg organic Se per kg of diet across all diets. At 42 days post-hatch, one bird from each cage was bled. Plasma was assayed for reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and advanced oxidative protein products (AOPP). Breast muscle and liver tissues were collected and assayed for total antioxidant capacity (TAC), GSH-Px and SOD. Neither of the Se sources influenced plasma concentrations of ROMs, BAP and AOPP; however they increased ( $P > 0.01$ ) GSH-Px and SOD concentrations in plasma, breast muscle and liver. The GSH-Px increases in plasma and breast muscle generated by SH were numerically greater than those generated by SM. In summary, both SH and SM improved the antioxidant status of broilers by elevating activities of antioxidant enzymes in plasma and tissues.

### I. INTRODUCTION

The role of Se in poultry nutrition has been reviewed extensively (Surai, 2002a,b). Selenium is involved in the control of several physiological functions such as growth and immunocompetence. Se, as a constituent of selenoproteins, has structural and enzymatic roles, which impact on the antioxidant status and thyroid secretion of the bird (Sevcikova et al., 2006). Also, Se is a component of the cell enzyme glutathione peroxidase which has a protective function in relation to tissue damage from oxidative stress. Usually, inorganic Se is incorporated into broiler diets as a constituent of the vitamin-trace mineral premix. However, evidence is emerging that organic Se may be superior to inorganic Se sources (Rutz et al., 2003; Wang et al., 2011a,b). For this reason two organic Se sources were compared in the present study. Furthermore, the form of the organic Se was evaluated in order to explore possible functional differences *in vivo*.

### II. MATERIAL AND METHODS

The feeding study consisted of four dietary treatments based on wheat, soybean meal and canola meal. Starter diets were offered to birds from 1-21 days-post-hatch and finisher diets from 22-42 days post-hatch. The composition and the nutrient specifications of the diets are reported in the companion paper (Selle et al., 2013). The control diet contained a custom vitamin-trace mineral premix prepared by International Animal Health Products (Huntingwood, NSW) to Aviagen recommendations for Ross 308 birds. The premix did not contain any selenium. The experimental diets contained either BiOnyc® Tor-Sel (primarily composed of selenohomolanthionine; SH) or Alltech Sel Plex™ (primarily composed of selenomethionine; SM) individually, or in combinations of the two (50% SC:50% SM), so as to provide at total of 0.3 mg organic Se per kg of diet. Each of the four dietary treatments were offered to 12 replicates of 6 birds per cage from 1-42 days post-hatch as “starter” (1-14

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days) and “finisher” diets (15-42 days). The parameters assessed included growth performance, nutrient utilisation and oxidative stress. Growth performance and nutrient utilisation data were compiled by following standard procedures as outlined by Selle et al. (2003) and are presented in the companion paper (Selle et al., 2013). At 42 days post-hatch, one bird from each cage was bled. Blood samples were collected from the jugular vein and were centrifuged ( $1000 \times g$  for 15 minutes) and plasma was stored at  $-80^{\circ}\text{C}$  until assayed for reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and advanced oxidative protein products (AOPP). After blood was collected, birds were euthanised by cervical dislocation and breast muscle and liver tissues were collected, placed on liquid nitrogen and stored at ( $-80^{\circ}\text{C}$ ) in order to determine a range of oxidative stress parameters until assayed for total antioxidant capacity (TAC), GSH-Px and SOD. ROMs and BAP were determined by commercial kits (Diacron, Grosseto, Italy) by FRAS4 (H & D, Parma, Italy). The results of the d-ROMs test were expressed in arbitrary units called Carratelli units' (U. Carr.), where 1 U. Carr. corresponds to 0.08 mg/100 mL. The results of the BAP test are expressed in  $\mu\text{mol/L}$  of reduced iron. The degree of oxidative stress was estimated by the ratio of ROMs/BAP (U. Carr./ $\mu\text{mol/L}$ ) \* 100 = Oxidative Stress Index, given that the relationship between the level of oxidative stress and pathology is higher when ROMs and BAP measurements are so combined (Celi, 2011). Advanced oxidation protein products (AOPP) are novel markers of protein oxidation that were first described and characterised in plasma of uremic patients (Witko-Sarsat et al., 1996). Commercial kits (Cayman) were used for the determination of GSH-Px and SOD activities and GSH and TAC concentrations.

Experimental data were analysed by univariate analyses of variance using the general linear models procedure of the IBM®, SPSS® Statistics 20 program. A probability level of less than 5% was deemed to be statistically significant. The feeding study was conducted so as to comply fully with the specific guidelines for the “evaluation of dietary feed ingredients for broiler chickens” [N00/10-2011/2/5612] as stipulated by the Animal Ethics Committee of Sydney University.

### III. RESULTS

The effects of Se supplementation on parameters of oxidative status in plasma are shown in Table 1. Se supplementation did not influence ( $P > 0.05$ ) reactive oxygen metabolites (ROMs), biological antioxidant potential (BAP), oxidative stress index (OSI) or advanced oxidative protein products (AOPP).

**Table 1 - Effect of selenium supplementation on broiler plasma oxidative status**

Treatment	ROMs (U.Carr)	BAP ( $\mu\text{mol/L}$ )	OSI (arbitrary units)	AOPP ( $\mu\text{mol/L}$ )	GSH ( $\mu\text{M}$ )	GSH-Px (nmol/min .mL)	SOD (U/ml)
Control	29.8	2901	1.05	36.34	20.95 <sup>b</sup>	57 <sup>b</sup>	8.96 <sup>b</sup>
SH	29.5	3016	0.99	41.05	26.01 <sup>a</sup>	191 <sup>a</sup>	12.29 <sup>a</sup>
50-50	25.8	3088	0.84	41.07	26.30 <sup>a</sup>	199 <sup>a</sup>	11.82 <sup>a</sup>
SM	30.1	2987	1.00	47.92	25.86 <sup>a</sup>	164 <sup>a</sup>	11.38 <sup>a</sup>
SEM	2.66	108.7	0.10	7.22	1.35	23.15	0.77
P	0.680	0.666	0.656	0.880	0.016	0.001	0.007

ROMs: Reactive Oxygen Metabolites; BAP: Biological Antioxidant Potential; OSI: Oxidative Stress Index (ROMs/BAP \* 100); AOPP: Advanced Oxidative Protein Products; GSH: Total Glutathione; GSH-Px: Glutathione Peroxidase; SOD: Superoxide Dismutase. Means within a row with different letters are significantly different ( $P < 0.05$ ).

However, there were significant responses to Se supplementation observed for total glutathione (GSH), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD)

plasma concentrations. Relative to the negative control, plasma levels of GSH in birds offered Se-supplemented diets were, on average, increased by 25.1% (26.21 versus 20.95  $\mu$ M;  $P < 0.02$ ). SH increased GSH by 24.2%; whereas, SM increased GSH by 23.4%. Similarly, relative to the negative control, plasma levels of GSH-Px in birds offered Se-supplemented diets were, on average, increased by 230% (188 versus 57 nmol/min.mL;  $P < 0.005$ ). SH increased GSH-Px by 235%; whereas, SM increased GSH-Px by 188%. Again, relative to the negative control, plasma levels of SOD in birds offered Se-supplemented diets were, on average, increased by 35.2% (12.11 versus 8.96 U/ml;  $P < 0.005$ ). SH increased SOD by 37.2%; whereas, SM increased SOD by 27.1%.

**Table 2 - Effect of selenium supplementation on broiler breast muscle oxidative status**

Treatment	TAC ( $\mu$ M Trolox/mg protein)	GSH-Px (nmol/min.mg protein)	SOD (U/mg protein)
Control	0.52	15.30 <sup>B</sup>	5.25 <sup>b</sup>
SH	0.49	31.80 <sup>A</sup>	8.54 <sup>a</sup>
50-50	0.54	27.37 <sup>A</sup>	8.12 <sup>a</sup>
SM	0.50	29.48 <sup>A</sup>	8.90 <sup>a</sup>
SEM	0.05	2.23	1.48

TAC: Total Antioxidant Capacity; GSH: Total Glutathione; GSH-Px: Glutathione Peroxidase; SOD: Superoxide Dismutase. Means within a row with different small and capital letters are significantly different,  $P < 0.05$  and  $P < 0.01$ , respectively.

The effects of Se supplementation on parameters of oxidative status in the liver are shown in Table 3. Se supplementation did not influence ( $P > 0.10$ ) the total antioxidant capacity (TAC) in the liver. However, relative to the negative control, average hepatic levels of glutathione peroxidase in birds offered Se-supplemented diets were significantly higher by 33% (55.14 versus 41.38 nmol/min.mg protein;  $P < 0.05$ ). SH increased GSH-Px by 21%; whereas, SM increased GSH-Px by 37%. Also, relative to the negative control, average hepatic levels of superoxide dismutase in birds offered Se-supplemented diets were significantly higher by 52% (9.9 versus 6.5 U/mg protein;  $P < 0.03$ ). SH increased SOD by 65%; whereas, SM increased SOD by 83%.

**Table 3 - Effect of selenium supplementation on broiler liver oxidative status**

Treatment	TAC ( $\mu$ M Trolox/mg protein)	GSH-Px (nmol/min.mg protein)	SOD (U/mg protein)
Control	0.34	41.38 <sup>b</sup>	6.5 <sup>b</sup>
SH	0.35	49.93 <sup>a</sup>	10.7 <sup>a</sup>
50-50	0.39	54.99 <sup>a</sup>	7.9 <sup>ab</sup>
SM	0.35	56.84 <sup>a</sup>	11.9 <sup>a</sup>
SEM	0.04	4.16	1.26

TAC: Total Antioxidant Capacity; GSH: Total Glutathione; GSH-Px: Glutathione Peroxidase; SOD: Superoxide Dismutase. Means within a row with different letters are significantly different ( $P < 0.05$ ).

The effects of Se supplementation on parameters of oxidative status in breast muscle are shown in Table 2. Se supplementation did not influence ( $P > 0.40$ ) the total antioxidant capacity (TAC) in breast muscle. However, relative to the negative control, average breast muscle levels of GSH-Px in birds offered Se-supplemented diets were significantly higher by 101% (30.76 versus 15.30 nmol/min.mg protein;  $P < 0.005$ ). SH increased GSH-Px by 108%; whereas, SM increased GSH-Px by 93%. Also, relative to the negative control, average breast muscle levels of SOD in birds offered Se-supplemented diets tended to be significantly higher by 80% (9.47 versus 5.25 U/mg protein;  $P < 0.10$ ). SH increased SOD by 57%; whereas, SM increased SOD by 70%.

## IV. DISCUSSION

The observed range of ROMs in this study is lower than those reported in ruminants (Celi et al., 2012). The BAP values reported in this study are consistent with the ranges reported in dairy cattle (Celi and Raadsma, 2010; Celi et al., 2012). In a study conducted in heat stressed sheep, supra-physiological levels of selenium were able to reduce ROMs levels by 10% and increase BAP levels by 5% (Chauhan et al. 2012); therefore the lack of changes in ROMs and BAP after selenium supplementation observed in this study, may be indicative that the level of selenium supplementation was not adequate to induce changes in these biomarkers. There was a trend toward an increase in AOPP concentrations in the majority of treatment groups however this was not significant. The lack of effects on AOPP may reflect the gut health was not compromised in this study. AOPP are pro-inflammatory mediators associated with a number of inflammatory conditions, are considered an indicator of oxidative stress and are associated with embryonic losses in dairy cows (Celi et al., 2011). GSH-Px activity is considered an indicator of oxidative stress and is also related to plasma lipid peroxide content (Di Trana et al., 2006). It is noteworthy that SH significantly increased GSH-Px concentrations in plasma, breast muscle and liver by 235, 108 and 21%, respectively. Alternatively, SM increased GSH-Px concentrations in plasma, breast muscle and liver by 188, 93 and 37%, respectively. The results gathered in this study suggest that Se improved the antioxidant property of broilers by elevating activities of antioxidant enzymes in plasma and tissues and there may be functional differences between SH and SM in broilers.

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## THE EFFECT OF DIETARY ALKALINE PROTEASE SUPPLEMENTATION ON THE PERFORMANCE OF BROILERS AND LAYERS

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### Summary

Feed enzymes are used to improve the digestibility of feed ingredients and reduce the detrimental effects of antinutritional factors. The use of feed enzymes is a common practice in wheat and barley based poultry diets worldwide. However, enzyme producers have found difficulty in developing efficacious and cost effective products for corn-soybean meal and sorghum-soybean meal based diets. Proteases are used in the food industry but protease preparations are now being utilized for animal feed.

Two feeding trials were conducted to determine the effect of dietary protease supplementation on the performance of broilers and layers. In the broiler study, 320 day-old broiler chicks were randomly assigned to four treatments with eight replications of 10 birds. In the layer study, 120 individually caged 20-week old pullets were randomly distributed to four treatments following a completely randomized design. Each treatment was replicated 30 times with one individually caged pullet per replicate. The dietary treatments for broilers and layers were: Treatment 1, basal diet; Treatment 2, reduced crude protein and amino acid diet without protease supplementation; Treatment 3, treatment 2 diet with 125 ppm protease A; Treatment 4, treatment 2 diet with 200 ppm protease B. The broiler study duration was 42 days while the layer study duration was 12 weeks. Results indicated that a 10% reduction in crude protein and amino acid content of the diet adversely affected the performance of broilers but not of layers. Supplementation with protease A or B did not compensate for the reduction in nutrient content of the broiler diets. Supplementation with protease A or B had no effect on layer performance.

### I. INTRODUCTION

Proteolytic enzymes are involved in a great variety of physiological processes and their action can be divided into two different categories. Firstly, limited proteolysis, in which a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formerly inactive protein. Secondly, unlimited proteolysis, in which proteins are degraded into their amino acid constituents.

Since 1958, most of the research efforts in the enzyme field were dedicated to improving the digestibility of some cereals that could replace corn in animal feeds (Fry et al., 1958). Basically the results of this work showed that the addition of the enzyme glucanase improved performance of growing birds when fed barley-based diets while the inclusion of pentosanases to either wheat or rye-based diets also resulted in better poultry performance (Leslie, 1995). The presence of non-starch polysaccharide (NSP) compounds in feed grains causes an increase in digesta viscosity in the intestine. This increase in viscosity reduces nutrient digestibility and absorption with a resulting negative impact on performance of non-ruminant animals (Bedford et al., 1991). As previously mentioned, the main approach to enzyme use in animal feeds has been to improve cereal utilization. However, recently it has been shown that there is great potential in improving the digestibility of other components of the diet, in particular soybean meal. Work by Schang et al. (1997) showed that the proportion

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of gross energy that is metabolized in corn is larger than in soybean meal (i.e. 89- 91% versus 60-75%). Research has indicated that addition of protease increased the true metabolizable energy (nitrogen corrected) values of several plant protein sources by 4 to 14% (Charlton, 1996).

## II. MATERIALS AND METHODS

The enzyme preparations used in the study were provided by two commercial feed additive manufacturers, i.e. from Canada (Protease A) and from Switzerland (Protease B). Protease A is a protease complex of the alkaline serine protease group. Protease B is a preparation of serine protease produced by a genetically modified strain of *Bacillus licheniformis*.

A total of 320 straight-run Cobb one-day old broiler chicks were purchased and randomly distributed to 32 cages with 10 chicks per cage. Four treatments were assigned to the 32 cages of broilers following a completely randomized design (CRD). Each treatment was replicated eight times with a cage of 10 chicks per replicate. The trial duration was 42 days.

For the layer study, a total of 120 20-week old Hy-line pullets were randomly assigned to four treatments following a CRD. Each treatment was replicated 30 times with one pullet per replicate. The trial duration was 12 weeks.

The following treatments were applied to the broiler and layer diets with nutrient specifications shown in table 1:

Treatment 1	Basal diets
Treatment 2	Reduced Crude Protein (CP) and amino acids diets
Treatment 3	Reduced CP and amino acids diets with 125g/tonne protease A
Treatment 4	Reduced CP and amino acids diets with 200g/tonne protease B

**Table 1 - Nutrient specifications of broiler and layer diets.**

Diet	ME, MJ/kg	% CP	% Lys	% Meth	%M+C	%Thr	% Try
B. Starter	13.20	22.50	1.70	0.62	0.99	0.89	0.27
B. Grower	13.40	20.00	1.20	0.60	0.94	0.82	0.23
B. Finisher	13.60	19.00	1.10	0.57	0.90	0.75	0.22
Layer	11.50	17.50	0.91	0.41	0.72	0.70	0.20

## III. RESULTS AND DISCUSSIONS

Table 2 shows the results for broilers. The 10% reduction in CP and amino acid content of the diets resulted in a significant depression in body weight gain and feed conversion efficiency of broilers. However, feed consumption was not influenced with the reduction of CP and amino acids of the diets.

Supplementation with protease A or B did not improve the body weight gain and feed conversion efficiency of broilers fed diets with reduced CP and amino acid content. Likewise, feed consumption was not affected by the addition of protease A or B to the protein and amino acid reduced diets.

**Table 2 - Average performance of broilers fed basal or reduced CP and amino acids diets supplemented with two preparations of alkaline proteases from 1-42 days of age.**

Parameters	T1	T2	T3	T4	CV,%
Body weight gain, kg	2.17 <sup>a</sup>	2.00 <sup>b</sup>	1.96 <sup>b</sup>	1.98 <sup>b</sup>	4.15
Feed consumption, kg	3.85	3.83	3.85	3.83	3.51
Feed efficiency	1.78 <sup>b</sup>	1.91 <sup>a</sup>	1.97 <sup>a</sup>	1.94 <sup>a</sup>	4.38

P &lt; 0.01

The 10% reduction in dietary CP and amino acid content may have been too high for the broilers such that supplementation with either protease A or B could not compensate for the deficiency in nutrients. Feed conversion efficiency responses differ with the level of reduction in CP and amino acids and level of protease activity. Supplementation of exogenous enzymes can positively influence endogenous nutrient losses and the efficiency of the production of endogenous enzymes (Cowieson et al., 2005; Jiang et al., 2008 and Liu et al., 2008). The reduction in protein and amino acids in the diets by 10% could have induced a deficiency in available amino acids for the synthesis of endogenous enzymes. Variability in feed conversion efficiency response suggests the importance of evaluating the availability and levels of substrates for enzyme in feeds and matching them with specific types of enzyme activities to achieve the optimal response (Hruby, 2009).

Table 3 shows the results for layers which indicate that the 10% reduction in CP and amino acid content of the diet did not adversely affect egg production rate, egg weight, feed consumption and feed conversion efficiency. Supplementing the reduced CP and amino acid diet with the proteases did not influence egg production rate, feed consumption or feed efficiency. Eggs laid by hens fed the CP and amino acid reduced diet supplemented with protease B had significantly lower weight than the control treatment.

**Table 3 - Average performance of layers fed basal or reduced CP and amino acids diets supplemented with two preparations of alkaline proteases from 20-32 weeks of age.**

Parameters	T1	T2	T3	T4	CV,%
Egg production, %	92.68	90.43	91.71	92.10	7.38
Egg weight, g	59.56 <sup>a</sup>	58.30 <sup>ab</sup>	58.06 <sup>ab</sup>	57.86 <sup>b</sup>	4.44
Feed consumption, g	109.87	109.75	109.91	109.78	0.34
Feed efficiency	2.01	2.11	2.06	2.06	9.00

P &lt; 0.01

Aklilu (2003), Seguerria (2003) and Bunan (2008) reported significant depression in egg production rate of layers fed diets with reduced CP and amino acid content. The different result seen in this study could be due to different layer strains and different CP and amino acid levels in the diets.

Jackson et al. (1999), Elliot (2002) and Ru (2009) reported positive effects of protease supplementation on egg production from layers. The variability of results obtained from different studies on protease supplementation may be due to differences in activity and concentration of protease preparations including the use of enzyme blends compared with purified protease as well as the microbial source of the enzymes.

## IV. CONCLUSION

Reduction in protein and amino acid content of the diets significantly depressed the body weight gain of broilers. Supplementation with proteases did not affect body weight gain, feed consumption or feed conversion efficiency.

Egg production, egg weight, feed consumption, feed conversion efficiency of layers were not affected by the reduction in CP and amino acid content of the diet or by supplementation with proteases.

Under the conditions of this study, it can be concluded that a reduction of 10% crude protein and amino acid content of the diets adversely affected the performance of broilers but not of the layers. Supplementation of the reduced CP and amino acid diets with protease A or B did not compensate for the reduction in nutrient content of the broiler diets. Layer performance was not significantly affected by protease supplementation of the diet.

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## EFFECTS OF FEEDING AGE AND INCLUSION LEVEL OF WHOLE WHEAT ON PERFORMANCE, CARCASS CHARACTERISTICS AND ECONOMIC BENEFITS OF BROILER CHICKENS

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### Summary

In the last few years, some poultry companies in Australia and Europe have added whole wheat to poultry rations to increase the use of locally grown grains and reduce transport and processing costs (Bennett et al., 2002). A 2x3 factorial design was used to investigate the effect of substituting a pelleted broiler diet with different levels of whole wheat (0, 15% and 30%) at different ages (15d or 22d) on broiler performance and carcass characteristics. The 6 treatment diets were isonitrogenous and isocaloric. Performance was improved by adding 30% whole wheat to the broiler ration, while feeding age had no significant effect on broiler performance. Feeding birds diets containing 30% whole wheat from 22 days of age could obtain better performance and profit.

### I. INTRODUCTION

In Australia, Canada and some European countries, poultry producers have sought to reduce feed ingredient and handling costs by adding whole wheat to poultry rations on farm. Research trials with broiler chickens have demonstrated no significant decrease in weight gain when the feed is diluted with up to 30% whole wheat (Covasa et al, 1994). Some broiler producers have added up to 25% whole wheat in the finisher ration. So, many studies have been done on whole wheat dilution in broiler rations, but a suitable substitution level for whole wheat is still uncertain due to differences in mode of feeding and research conditions. Moreover, the best age to start feeding whole wheat in the diet is rarely studied. This experiment was conducted to investigate the effect of feeding age and inclusion level of whole wheat in diets on growth performance and carcass characteristics of broiler chickens.

### II. MATERIALS AND METHODS

The experiment was carried out using a 2 × 3 factorial arrangement of treatments with two whole wheat feeding ages (15 d or 22 d) and three inclusion levels of whole wheat (0, 15 and 30%) to substitute ground wheat in these diets. Basal diets were formulated to meet nutrient requirements estimated by the NRC (1994). The ingredients and chemical compositions of experimental diets are shown in Table 1. Whole wheat diets were provided from 15 d or 22 d.

A total of 1 260 one-day-old Ross 308 mixed-sex birds (1:1 ratio of males and females) were randomly divided into six treatments, with seven replicates per treatment, 30 birds per replicate. Feed and water were provided ad libitum during the trial. At 21, 35 and 42 d, birds were weighed and feed intake was recorded. At 42 d, 12 birds per treatment were randomly selected and weighed before being slaughtered for carcass characteristics. The wings, breast,

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abdominal fat and gizzard were harvested and weighed.

Data from the growth and carcass characteristics assays were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, 1990). The factors were 2 addition ages and 3 levels of whole wheat. If a significant interaction existed between the main effects, the data were reanalyzed by One-way ANOVA. Statements of probability are based on  $P < 0.05$ .

**Table 1 - Ingredient profile and calculated analysis of starter, grower and finisher diets**

Item	Starter (1 to 21 d)	Grower (22 to 35 d)	Finisher (36 to 42 d)
Ingredients (%)			
Corn	22.09	18.48	14.85
Wheat	40.00	50.00	60.00
Soybean meal	26.30	18.15	11.32
Wheat Enzyme	0.04	0.04	0.04
Premix	0.50	0.50	0.50
Nutrients levels			
Crude protein, g/kg	215.7	193.3	178.7
ME, MJ/kg	11.56	11.97	12.39
Calcium, %	0.80	0.75	0.59
Phosphorus, %	0.57	0.46	0.42

### III. RESULTS

Whole wheat addition level had a significant effect on broiler FCR ( $P < 0.05$ ) (Table 2). FCR with 30% whole wheat was significantly ( $P < 0.05$ ) lower than that of diets without added whole wheat (1.641 VS 1.667). Birds fed 30% whole wheat diets from 22 days of age had the lowest FCR (1.637). The feeding age and level of addition of whole wheat had no significant effects on body weight, average feed intake and survival rate ( $P > 0.05$ ) (Table 2).

Adding whole wheat from 22 days of age significantly increased the relative wing weight ( $P < 0.05$ ) (Table 3). Whole wheat addition level had a significant effect on broiler relative gizzard weight ( $P < 0.05$ ). Birds fed with diets inclusion with 30% whole wheat had heavier relative gizzard weight than birds fed diets with 0 and 15% whole wheat added ( $P < 0.05$ ).

Adding whole wheat from 15 or 22 days of age had the same economic benefit. Adding 15% and 30% whole wheat in diets decreased feed costs and increased profits (Table 4). Overall, adding 30% whole wheat in diets resulted in the lowest feed cost and best economic profit.

### IV. DISCUSSION

Results from field trials indicated that poultry diets can be diluted with whole grain with little or no loss in performance, which encouraged the use of whole wheat in poultry rations. Our results showed that there was no decline in growth performance when broilers were fed diets containing whole wheat. Bennett (2002) reported that whole grain feeding does not affect performance or carcass parameters, but can provide considerable reduction in feed cost for body gain. Engberg (2004) found that feeding a diet supplemented with 23% whole wheat

improved feed utilization. These studies are in accordance with our results.

A higher gizzard weight was observed with the use of whole grains in several studies (Cumming, 1992; Engberg et al., 2004). It has been clearly established that whole grain increased gizzard development (Gabriel et al., 2003a). Similarly, adding 15% and 30% whole wheat in broiler diets increased the development of the gizzard in present study. The mechanism of this effect is not clear, but research has demonstrated that a bird's gizzard can grind whole grain more effectively than a hammer mill (Svihus, 2001), and the digestibility of wheat fed as whole grain is greater than for ground wheat, perhaps because whole wheat stimulates the development of the gizzard. The higher development of gizzard functionality may remove the need for further physiological adaptation of the lower part of the digestive tract (Svihus et al., 2004). This may contribute to an improved feed conversion ratio in the groups fed whole wheat diets in the present study.

Overall, it is possible to add 30% whole wheat to broiler diets earlier to reduce feed cost and receive a health benefit for the chicks.

**Table 2 - Performance of mixed sex chicks at 42 days of age, relative to age of introduction of diets containing whole wheat**

Whole wheat		Body weight, g	Feed intake, g/d/bird (1 to 42 d)	Feed conversion ratio, g/g	Survival rate, %
Feeding age, d	Addition level, %				
15	0	2884	115.6	1.669 <sup>a</sup>	96.3
15	15	2891	114.4	1.646 <sup>ab</sup>	94.9
15	30	2878	113.8	1.645 <sup>ab</sup>	97.7
22	0	2893	115.8	1.665 <sup>a</sup>	97.1
22	15	2839	113.0	1.657 <sup>ab</sup>	96.7
22	30	2919	114.8	1.637 <sup>b</sup>	95.7
SEM		14.46	0.47	0.004	0.50
Main effects					
Feeding age, d	15	2884	114.6	1.654	96.3
	22	2884	114.5	1.653	96.5
Addition level, %	0	2889	115.7	1.667 <sup>a</sup>	96.7
	15	2865	113.7	1.651 <sup>ab</sup>	95.8
	30	2899	114.3	1.641 <sup>b</sup>	96.7
Probabilities					
Feeding age		0.99	0.97	0.94	0.80
Addition level		0.65	0.22	0.008	0.69
Feeding age × addition level		0.44	0.57	0.45	0.30

<sup>a,b,c</sup> Means within a given column with no common superscript are different ( $P < 0.05$ ).

**Table 3 - Carcass characteristics of mixed sex chicks at 42 days of age, relative to age of introduction of diets containing whole wheat**

Whole wheat		Carcass yield, %	Relative Breast weight, %	Relative wings weight, %	Abdominal fat ratio, %	Relative gizzard weight, %
Feeding age, d	Addition level, %					
15	0	74.53	20.52	7.41 <sup>bc</sup>	2.03	0.93 <sup>c</sup>
15	15	75.37	20.50	7.43 <sup>bc</sup>	1.71	1.10 <sup>b</sup>
15	30	75.19	20.36	7.28 <sup>c</sup>	1.85	1.23 <sup>a</sup>
22	0	75.33	20.52	7.86 <sup>a</sup>	1.82	0.93 <sup>c</sup>
22	15	74.93	20.12	7.60 <sup>abc</sup>	1.95	1.10 <sup>b</sup>
22	30	74.87	20.37	7.68 <sup>ab</sup>	1.61	1.18 <sup>ab</sup>
SEM		0.179	0.987	0.05	0.052	0.205
Main effects						
Feeding age, d	15	75.03	20.46	7.37 <sup>b</sup>	1.86	1.09
	22	75.04	20.34	7.71 <sup>a</sup>	1.79	1.07
Addition level, %	0	74.93	20.52	7.63	1.92	0.93 <sup>c</sup>
	15	75.15	20.31	7.52	1.83	1.10 <sup>b</sup>
	30	75.03	20.37	7.48	1.73	1.20 <sup>a</sup>
Probabilities						
Feeding age		0.969	0.739	0.0008	0.504	0.534
Addition level		0.881	0.889	0.394	0.321	<0.0001
Feeding age × addition level		0.317	0.882	0.458	0.104	0.791

<sup>a,b,c</sup> Means within a given column with no common superscript are different (P < 0.05).

**Table 4 - Feed cost and profit of mixed sex chicks at 42 days of age, relative to age of introduction of diets containing whole wheat**

Whole wheat	Feeding age, d	15 15 15 22 22 22						Feeding age, d		Addition level, %		
		0 15 30 0 15 30						15	22	0	15	30
1 to 42 d	Feed cost (Yuan/kg BW)	5.0	4.9	4.9	5.0	4.9	4.9	4.9	4.9	5.0	4.9	4.9
	Profits (Yuan /bird)	4.2	4.4	4.4	4.3	4.1	4.6	4.3	4.3	4.2	4.3	4.5

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## EFFECT OF $\alpha$ -AMYLASE ON PERFORMANCE AND NUTRIENT UTILIZATION OF BROILERS FED DIETS BASED ON CASSAVA AND SOYBEAN MEAL

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### Summary

The effect of a commercial  $\alpha$ -amylase was assessed in a commercial broiler operation in China.  $\alpha$ -amylase was supplemented to a nutritionally marginal diet based on cassava and soybean meal at 1500, 2000 and 2500 U/kg feed, respectively. The growth performance of the birds was recorded over a 21 d period and apparent metabolisable energy (AME), the digestibility coefficients of N, DM and total starch were measured. It can be concluded that the use of  $\alpha$ -amylase in a cassava/soybean-based diet is effective in improving nutrient digestibility and performance of broilers fed nutritionally marginal diets. The optimum supplementation dose of  $\alpha$ -amylase under these trial conditions is between 2000 - 2500 U/kg feed.

### I . INTRODUCTION

Cassava is rich in starch and can be used as an energy source in poultry feed. Starch supplies more than 50% of the metabolisable energy in practical broiler diets (Enting et al., 2005; Wiseman et al., 2000). Noy and Sklan (1995) reported that daily net secretion of amylase into the duodenum was low at day 4 and steadily increased up to day 21. Therefore, an exogenous supply of amylase might be needed to match the requirement of birds and improve starch digestion and therefore performance early in life.

Earlier studies have shown beneficial effects of amylase preparation and  $\alpha$ -amylase-producing bacterial culture on growth and feed efficiency of chicks when supplemented into corn-soybean meal diets.  $\alpha$ -amylase supplementation to corn-soybean meal diets significantly improved daily gain by 9.4% and feed conversion by 4.2% in Cobb male chicks and birds ate more and grew faster than the control (Ritz et al., 1995). Jiang et al. (2007) reported that body weight gain, FCR and feed intake were improved when  $\alpha$ -amylase was supplemented into broiler diets based on corn and soybean meal, and a linear relationship was found between amylase dose rate from 1000 ~ 9000 U/kg feed and body weight gain of birds. Mahagna et al. (1995), however, found no effective response of broilers aged 1 to 14 days when fed a sorghum-soybean meal diet with amylase and protease supplementation. So far, one area that has received little attention in the literature is whether performance profiles of broilers can be improved by addition of an  $\alpha$ -amylase preparation to broiler diets based on cassava meal and soybean meal. The objective of this experiment was to evaluate the effect of  $\alpha$ -amylase supplementation on performance and nutrient utilization of broilers fed cassava-soybean based diets.

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## II. MATERIALS AND METHODS

Four hundred 1-day-old Cobb broiler chickens (Half males) obtained from a local commercial hatchery were randomly assigned into 5 treatment groups consisting of 8 replicates of 10 birds for each. Birds were kept in a fully-closed 3 tier battery cages. Lighting was for 16 h per day. The birds were fed *ad libitum* and water was available all the time. A cassava-soybean based negative control (NC) diet was formulated to be nutritionally marginal in terms of energy only and provided 11.51 MJ of ME/kg. This diet was supplemented with pure  $\alpha$ -amylase preparation (LTAAL-3000AF, Danisco Animal Nutrition, China) at 1500, 2000 and 2500 U/kg feed, respectively. A positive control (PC) diet, which was formulated to be nutritionally adequate (ME, 12.34 MJ/kg), was fed for comparison. Therefore, the treatments used were PC, NC, NC+ 1500 U of amylase/kg, NC+2000 U of amylase/kg and NC+2500 U of amylase/kg. Titanium dioxide (TiO<sub>2</sub>, 0.5%) was added as an indigestible marker. Diets were pelleted at 70°C and an effective thickness of 32 mm and holes 3 mm in diameter. Swabs of excreta were collected daily between day 18 and 21 for the determination of total tract digestibility of nutrients. On day 22, six birds per cage were killed by cervical dislocation for collecting ileal digesta.

## III. RESULTS

Birds fed the NC diet had poorer BWG, feed intake, and FCR compared with those fed the PC diet (Table 1). Supplementation of the NC diet with  $\alpha$ -amylase improved BWG and feed intake. The BWG and feed intake of the broilers fed diet containing 2500 U of  $\alpha$ -amylase/kg were significantly higher than those fed the NC diet ( $P < 0.05$ ), with an improvement of 6.6 and 4.9%, respectively (Table 1).

**Table 1 - The effect of  $\alpha$ -amylase supplementation on performance of broilers**

Treatment	Feed intake (g/bird)	BWG (g/bird)	FCR
Positive (PC)	1182.5 <sup>ab</sup>	825.8 <sup>a</sup>	1.442 <sup>b</sup>
Negative (NC)	1157.2 <sup>b</sup>	771.2 <sup>b</sup>	1.511 <sup>a</sup>
NC+1500	1171.8 <sup>b</sup>	779.5 <sup>b</sup>	1.505 <sup>a</sup>
NC+2000	1192.3 <sup>ab</sup>	806.4 <sup>ab</sup>	1.479 <sup>ab</sup>
NC+2500	1214.5 <sup>a</sup>	822.7 <sup>a</sup>	1.476 <sup>ab</sup>
S.E.M	0.42	0.24	0.01
P-value	*	*	*

Note: Mean values without common superscript within columns differ significantly, NS, not significant,  $P > 0.05$ ; \*  $P \leq 0.05$

AME was approximately 0.65 MJ/kg lower in the NC diet compared with the PC diet (Table 3). Similarly, the digestibility coefficients of N, DM, and total starch were all lower ( $P < 0.05$ ) in the NC diet compared with the PC diet (Table 2). The effect of supplementation  $\alpha$ -amylase to diets on nutrient digestibility coefficients was in line with expectations;  $\alpha$ -amylase supplementation improved AME of NC diet (Table 3). The ileal digestibility coefficients of CP and N retention rate were also improved by  $\alpha$ -amylase addition.

**Table 2 - The effect of  $\alpha$ -amylase supplementation on ileal nutrients digestibility of broilers**

Treatment	Dry matter (g/kg)	Crude protein (g/kg)	Total starch (g/kg)
Positive (PC)	781.2	725.6 <sup>a</sup>	964.3
Negative (NC)	774.3	691.3 <sup>b</sup>	961.2
NC+1500	775.8	714.2 <sup>ab</sup>	960.6
NC+2000	777.2	708.3 <sup>ab</sup>	973.4
NC+2500	784.6	712.3 <sup>ab</sup>	978.3
S.E.M	6.8	11.5	4.9
P-value	NS	*	NS

Note: Mean values without common superscript within columns differ significantly, NS, not significant,  $P > 0.05$ ; \*  $P \leq 0.05$ .

**Table 3 - The effect of  $\alpha$ -amylase supplementation on digestion and metabolite profiles**

Treatment	DM (g/kg)	Total starch (g/kg)	AME (MJ/kg)	N retention rate (g/kg)
Positive (PC)	792.3	969.7	13.41 <sup>a</sup>	692.1 <sup>a</sup>
Negative (NC)	787.5	963.4	12.76 <sup>b</sup>	669.4 <sup>b</sup>
NC+1500	798.3	959.2	12.92 <sup>b</sup>	681.2 <sup>ab</sup>
NC+2000	799.2	973.6	12.94 <sup>b</sup>	678.5 <sup>ab</sup>
NC+2500	801.1	975.8	12.96 <sup>b</sup>	682.6 <sup>ab</sup>
S.E.M	9.1	3.8	0.09	11.5
P-value	NS	NS	*	*

Note: Mean values without common superscript within columns differ significantly, NS, not significant,  $P > 0.05$ ; \*  $P \leq 0.05$ .

#### IV. DISCUSSION

In this experiment, the results that  $\alpha$ -amylase supplementation at 2500 U/kg can significantly improve tBWG and feed intake compared with the NC was consistent with previous reports. Ritz et al. (1995) observed improvements of 3% in daily gain and 4% in feed consumption in 21-d-old broilers fed a corn-soybean meal diet supplemented with an enzyme complex containing predominantly amylase. Gracia et al. (2003) found that  $\alpha$ -amylase supplementation can result in improvements of 8.5% in BWG and 4.2% in FCR, and also significantly increase the feed intake of birds. Jiang et al. (2007) reported that amylase supplementation at doses of 1000 ~ 9000 U/kg can result in improvements of 0 ~ 4.6% in BWG and of 0.1 ~ 3.7% in feed intake of birds, respectively.

In this study,  $\alpha$ -amylase supplementation improved the performance of broilers fed diets containing cassava at day 1 to 21. This result agreed with Ritz et al. (1995) who used corn soybean diets, but not with the findings of Gracia et al. (2003) for corn soybean meal diets. This may be related to amylose, amylopectin and resistant starch content of diet, starch digestion rate and the dose rate of enzyme preparation. Maize starch has approximately 28% amylose and 72% amylopectin while cassava starch has approximately 17% amylose and 83% amylopectin (Gomes et al., 2005). In general, amylose is thought to be less digestible than amylopectin (Annison and Choct, 1991). *In vitro* tests indicated the starch digestion rate of

cassava, wheat and corn were 5.31, 1.38 and 1.38 h<sup>-1</sup>, respectively (Weurding et al., 2001). So there may be a limitation to further improving cassava starch digestion by adding exogenous amylase, because of the higher amylopectin content contained in cassava diets. Supplementation of  $\alpha$ -amylase to nutritionally marginal diets improved the AME of diets, suggesting there may be some benefit to add  $\alpha$ -amylase in the cassava based diet for broilers.

The secretion of amylase per gram of feed intake was low on day 4, steadily increased up to day 7, and then stabilized (Uni et al., 1995; Noy and Sklan, 1995). Therefore, supplemented with  $\alpha$ -amylase may indirectly improve the AME of diets. Douglas et al. (2000) reported improvements in ileal utilization of energy at 21 d of age when amylase, protease, and xylanase were used in a corn-soybean meal diet for broilers.

## V. CONCLUSION

The results of this study indicate that  $\alpha$ -amylase supplementation can improve the productive traits of broilers fed nutritionally marginal diets. The optimum supplementation dose of  $\alpha$ -amylase is between 2000 - 2500 U/kg feed.

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## THE EFFECT OF $\beta$ -MANNANASE ON BROILER PERFORMANCE AND UNIFORMITY

M.E. JACKSON<sup>1</sup>

### Summary

Two broiler experiments were conducted to evaluate the effects of  $\beta$ -mannanase on live performance and uniformity at market age. Uniformity was evaluated by determining individual live weights of all birds in each pen and calculating the % coefficient of variation (CV) for each pen population. Treatments in both experiments included a positive control and a reduced energy negative control treatments with the enzyme added to the negative control. In Experiment 1,  $\beta$ -mannanase added at 200 and 300 g/tonne significantly increased 42 day weight by approximately 7% and improved feed conversion by 12 to 14 points. Uniformity was significantly improved with decreases in the % CV by 2.9 to 3.8 units. In Experiment 2,  $\beta$ -mannanase added at 225 and 400 g/tonne significantly increased 43 day weight by 4.6 and 8.7 % and improved feed conversion by 0 to 16 points, respectively. Forty-two-day uniformity was significantly improved with decreases in the % CV by 1.4 to 2.7 units. The studies demonstrate that  $\beta$ -mannanase improves live performance and uniformity in broilers.

### I. INTRODUCTION

Mannans occur in the forms of glucomannan, galactomannan, glucogalactomannan, and glucorono-mannans in non-starch polysaccharides contained in plants. Mannans are a part of the hemicellulose fraction of plant cell walls in all leguminous plants (Reid, 1985). According to this definition, hemicelluloses include mannan, xylan, galactan and arabinan.  $\beta$ -mannan, also referred to as  $\beta$ -galactomannan is a polysaccharide with repeating units of mannose with galactose and/or glucose attached to the  $\beta$ -mannan backbone (Carpita and McCann, 2000).

$\beta$ -mannans are most prevalent in a wide variety of feed ingredients including soybean meal, palm kernel meal, copra meal, and sesame meal. Since soybean meal is a major protein source in feeds produced around the world,  $\beta$ -mannan is present in most feeds. Other common ingredients such as corn distillers dried grains and canola meal also contribute to the  $\beta$ -mannan content of many diets for monogastric animals. The  $\beta$ -mannan content of a large number of soybean meal samples from various parts of the world has been reported and shown to be reasonable consistent (Hsiao et al., 2006).

The mode of action of  $\beta$ -mannanase in monogastric animals is complex. It is well accepted that  $\beta$ -galacto-mannan inhibits insulin secretion in swine (Sambrook and Rainbird, 1985; Leeds et al., 1980) suggesting a deleterious effect on energy metabolism. This is supported by studies showing a reduced glucose and water absorption in swine (Rainbird et al, 1984). Beneficial effects of  $\beta$ -mannanase on energy metabolism may be associated with an increased stimulation of insulin secretion and a blocking of the adverse effect of  $\beta$ -galacto-mannan on glucose absorption (Jackson et al., 1999).

The mechanism may also be associated with the enzyme's effect on viscosity in the gut.  $\beta$ -galacto-mannan is a viscous polysaccharide, which may contribute to hyperplasia of digestive organs resulting in an increased secretion of pancreatic fluid (Ikegami et al., 1990), thus increasing the energy demand of the intestine.

Experiments have clearly demonstrated that  $\beta$ -galacto-mannans are potent stimulators of the innate immune system. They have been shown to increase the proliferation of

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monocytes and macrophages resulting in secretion of cytokines (Ross et al., 2002; Peng et al., 1991). Aloe vera leaf containing mannan has been used as a natural remedy for accelerating the healing process for minor injuries in humans. The monitoring of acute phase proteins can assess stimulation of the innate immune system. Acute phase proteins are an aspect of the innate immune system, which are known to accumulate in blood to high levels in response to various forms of stress. One acute phase protein known as  $\alpha$ -1-acid glycoproteins (AGP) was monitored in a series of cage and pen trials with poultry (Anderson et al., 2006). These experiments revealed that the AGP level was significantly elevated in broilers after infection with three *Eimeria* species, and also by the exclusion of an antibiotic from the diet, thus establishing a relationship between AGP level and disease-related stress. The addition of  $\beta$ -mannanase to the diets significantly reduced the blood AGP in all trials demonstrating that a reduction in the  $\beta$ -galacto-mannan level in the diet can directly reduce immune stimulation. A reduction in the stimulation of the innate immune system with  $\beta$ -mannanase may result in a reduced expenditure of energy for non-productive purposes.

$\beta$ -mannanase has been shown to improve live performance in broilers. A dose response trial was reported where graded levels of  $\beta$ -mannanase were added to corn-soybean meal-type diets in a 42-day broiler pen trial with results shown on Table 1. Data showed a curvilinear improvement in growth and feed conversion leveling off at the 80-110 MU/tonne inclusion level. In addition, the data suggests a possible benefit in mortality at the highest inclusion level. The enzyme, at its highest level of inclusion, improved growth and feed conversion approximately 4.4 and 3.7%, respectively (P <0.05).

**Table 1 - Effect of varying levels of  $\beta$ -mannanase on 0 to 42-day broiler performance**

Parameter	$\beta$ -mannanase addition rate (MU/ton) <sup>1</sup>			
	0	50	80	110
Weight Gain (g)	2,547 <sup>b</sup>	2,529 <sup>b</sup>	2,651 <sup>a</sup>	2,660 <sup>a</sup>
FCR <sup>2</sup> (g/g)	1.970 <sup>b</sup>	1.965 <sup>b</sup>	1.924 <sup>a</sup>	1.899 <sup>a</sup>
Mortality (%)	5.00 <sup>ab</sup>	6.33 <sup>a</sup>	4.50 <sup>ab</sup>	2.83 <sup>b</sup>

Jackson et al., 2004; <sup>a-c</sup> Means without a common superscript differ significantly (P <0.05)

<sup>1</sup> 1 MU=10<sup>6</sup> enzyme activity units; <sup>2</sup> Feed conversion values corrected for mortality.

A degree of variability in live weights exists in all broiler populations. This variability is caused by inherent stresses from local disease challenges and other factors. The efficiency of modern-day processing operations may be improved by a reduction in the variability of live weights. This can result in an increased throughput as automated equipment is typically adjusted for the average body weight of one or more flocks entering the plant. In addition, the consistency of carcasses or parts can result in a higher value in the marketplace.

Live weight uniformity can be determined in pen trials by weighing all birds at various ages. Although this is a labor-intensive process, it can provide useful information as to the benefits of a feed additive. The percent coefficient of variation (CV) can be determined for each individual pen and data may be statistically analyzed.

Two broiler experiments were conducted to determine the effect of  $\beta$ -mannanase on broiler performance and uniformity.

## II. METHODS

Two pen trials tested male CobbxCobb-500 broilers with corn-soybean meal-animal by-product based practical diets using feeding programs consisting of starter (0-21 d), grower (31-35 d), and finisher (35-42 or 43 d) diets. Phytase was used in all diets and nutrient levels were consistent with practical diets used in the USA. All feeds were pelleted at approximately 80°C and were verified by proximate composition and enzyme analysis and

were formulated on a digestible basis. Diets across treatments varied in metabolisable energy (ME) only by varying levels of fat and other ingredients. Individual live weights of all birds in all pens were determined at marketing age.

In Experiment 1, birds were grown to 42 days with 4 treatments and 8 replications with 50 birds placed per pen. Treatments consisted of a 1. positive control, 2. negative control (minus 0.502 MJ/kg ME), 3. As 2 + 200 g/tonne Hemicell-HT<sup>2</sup>, and 4. As 2 + 300 g/tonne Hemicell-HT. The positive control diets ranged from 13.48 to 12.85 MJ/kg and 22.3 to 18.25% CP.

In Experiment 2, birds were grown to 43 days with 5 treatments and 8 replications with 50 birds placed per pen. Treatments consisted of a 1. positive control, 2. As 1 minus 0.175 MJ/kg ME, 3. As 1 minus 0.323 MJ/kg ME), 4. As 2 + 225 g/tonne Hemicell-HT<sup>2</sup>, and 5. As 3 + 400 g/tonne Hemicell-HT. The positive control diets ranged from 13.47 to 12.92 MJ/kg and 21.75 to 18.00 % CP.

### III. RESULTS

**Table 2 - Results at 42 days, Experiment 1:**

Treatment	BW (g)	FCR	WAFC*	% Mort.	% CV
1. Positive Control	2415 <sup>a</sup>	1.728 <sup>c</sup>	1.685 <sup>c</sup>	5.75 <sup>a</sup>	12.10 <sup>b</sup>
2. As 1 minus 0.502 MJ/kg ME	2135 <sup>c</sup>	1.874 <sup>a</sup>	1.892 <sup>a</sup>	5.50 <sup>a</sup>	15.46 <sup>a</sup>
3. as 2 + Hemicell-HT 200g/t	2287 <sup>b</sup>	1.754 <sup>b</sup>	1.751 <sup>b</sup>	4.00 <sup>a</sup>	11.70 <sup>b</sup>
4. as 2 + Hemicell-HT 300g/t	2283 <sup>b</sup>	1.735 <sup>bc</sup>	1.733 <sup>b</sup>	4.50 <sup>a</sup>	12.57 <sup>b</sup>
Effect of Hemicell-HT 200g/t	7.1%	12.0 pts	14.1 pts	NS	-3.76%
Effect of Hemicell-HT 300g/t	6.9%	13.9 pts	15.9 pts	NS	-2.89%

**Table 3 - Results at 43 days, Experiment 2\***

Treatment	BW (g)	FCR	WAFC*	% Mort.	% CV**
1. Positive Control	2046 <sup>a</sup>	1.897 <sup>bc</sup>	1.874 <sup>d</sup>	4.81 <sup>a</sup>	9.98 <sup>ab</sup>
2. As 1 minus 0.175 MJ/kg ME	1969 <sup>bc</sup>	1.886 <sup>b</sup>	1.892 <sup>d</sup>	4.81 <sup>a</sup>	11.17 <sup>bc</sup>
3. As 1 minus 0.323 MJ/kg ME	1923 <sup>c</sup>	1.966 <sup>d</sup>	1.988 <sup>b</sup>	3.61 <sup>a</sup>	12.19 <sup>c</sup>
4. As 2 + Hemicell-HT 225g/t	2059 <sup>a</sup>	1.894 <sup>bc</sup>	1.867 <sup>d</sup>	3.85 <sup>a</sup>	9.81 <sup>a</sup>
Effect of Hemicell-HT 225g/tt	4.6%	-0.8 pts	2.5 pts	NS	-1.36%
5. As 3 + Hemicell-HT 400g/t	2091 <sup>a</sup>	1.807 <sup>a</sup>	1.768 <sup>c</sup>	4.57 <sup>a</sup>	9.47 <sup>a</sup>
Effect of Hemicell-HT 400g/t	8.7%	15.9 pts	22.0 pts	NS	-2.72%

<sup>a-c</sup> P < 0.05. \* Each 23 g in weight is converted to 0.01 of feed conversion.

\*\* individual weights determined at 42 days

<sup>2</sup> Chemgen Corp, minimum 160 Million units/kg

### IV. CONCLUSIONS

In experiment 1,  $\beta$ -mannanase at both levels significantly improved weights, FCR, and uniformity at 42 days.  $\beta$ -mannanase added at 200 and 300 g/tonne significantly increased 42

day weight by approximately 7% and improved feed conversion by 12 to 14 points. Uniformity was significantly improved with decreases in the % CV by 2.9 to 3.8 units. The additional benefit of increasing  $\beta$ -mannanase from 200 to 300 g/ton was small.

In experiment 2,  $\beta$ -mannanase when added at 225 g/tonne significantly improved weight gain and live weight uniformity. The live performance benefit was superior to that of the positive control which contained an additional 0.175 MJ/kg ME.  $\beta$ -mannanase when added at 400 g/tonne significantly improved final weight, FCR, and uniformity when compared to treatment 3. The live performance benefit was superior to that of the positive control which contained an additional 0.323 MJ/kg ME.  $\beta$ -mannanase significantly improved live weight uniformity by lowering the % CV approximately 2 percentage points.

The studies demonstrate that  $\beta$ -mannanase improved (*or 'can improve'*) live performance and uniformity in broilers.

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## USING NUTRITIONAL GEOMETRY IN FOOD ANIMAL PRODUCTION

S.J. SIMPSON<sup>1</sup> and D. RAUBENHEIMER<sup>2</sup>Summary

Over the past two decades we have developed a unifying framework for nutrition, called the Geometric Framework (GF), within which multiple food components and animal attributes can be distinguished, and the relationships among components and attributes disentangled and then linked to individual performance, ecological outcomes and evolutionary consequences. Geometric framework models have been used to describe how a diversity of animals across multiple taxa regulate their intake of multiple nutrients when experimentally challenged with perturbations to their nutritional environment or nutritional state. This framework has also proved useful to address problems in applied nutrition, for instance to improve diets for domestic animals, characterise the nutritional ecology of endangered species, and explore ways to combat human obesity. Here we set out the basic models and give an example of their use in a food animal production system.

## I. THE GEOMETRY OF NUTRITION

In GF models, individual animals, foods and their interactions are represented graphically in a geometric space defined by two or more food components (for a comprehensive review see Simpson and Raubenheimer, 2012). Foods are represented as vectors through nutrient space at angles determined by the balance of the component nutrients they contain (nutritional rails), and the animal's nutritional state as a point that changes over time. As the animal eats, its nutritional state changes along the vector of the rail for the chosen food. The challenge for animals is to select foods and eat them in appropriate amounts to direct them to their optimal nutritional state (the intake target). Knowing the position in nutrient space of an individual's intake target provides a basis for making predictions about its physiological, behavioural and performance responses to the nutritional environment. An animal can reach its intake target by eating a single nutritionally balanced food, or by mixing its intake from two or more nutritionally complementary foods. If the individual is restricted to a nutritionally imbalanced food, however, it must reach a compromise between over-ingesting some food components and under-ingesting others (termed its rule of compromise), and bear the associated performance consequences.

## II. APPLIED NUTRITION

Much of our research has concerned fundamental biology, but a wide range of applied nutritional problems demand scientifically designed management solutions. There are many examples, but among the most important is the need to feed production animals (Raubenheimer and Simpson 2007).

a) Domestication

The intake targets of animals and rules of compromise they adopt when constrained from reaching their intake target are sculpted over evolutionary time to produce favourable outcomes within the particular ecological circumstances of the animal. In the process of domestication, humans have altered both the characteristics of the animal (through artificial

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selection) and the environment, thereby disrupting the ancestrally evolved match between the nutritional biology of the animal and its nutritional environment. An important challenge of managing domesticated animals is to ensure that they are provided with foods that enable them to meet their nutritional needs: but how to decide what the optimal requirements of a domesticated animal are?

A major complication concerns the question of which factors we wish the diet to optimise. For domesticated animals, the concept of 'evolutionary fitness' in the normal sense no longer applies. Other criteria that come into play when designing diets for food animals include the health and welfare of the animal, economics, logistics, environmental and ethical concerns – and in many cases these conflict. The fact that multiple, conflicting optimisation criteria need to be taken into account is not unique to domestic animals, however. The geometric framework can be used to model these processes.

b) Feeding our food: diet optimisation for animal production

An experiment that illustrates the above issues, and could serve as a reference for experiments in the poultry industry was performed by Ruohonen *et al.* (2007) on a salmonid fish species, the European whitefish (*Coregonus lavaretus*). The aim of this work was to use the GF to define the optimal macronutrient composition in the diet of the whitefish, to quantify the behavioural responses of fish to diets that depart from this composition, and to optimize multiple performance criteria, taking account of economic, environmental and animal welfare concerns.

The protocol for the analysis involved four main stages. The first step was to plot the pair-wise relationships between the three macronutrients separately (fat vs. protein, carbohydrate vs. protein and fat vs. carbohydrate). This showed that, as long as carbohydrate levels were below a damaging threshold (whitefish suffered when carbohydrate was above a relatively low level), the fish regarded lipid and carbohydrate as interchangeable sources of energy. Nutrients that the animal regards as interchangeable can for practical purposes be regarded as a single resource, enabling us to collapse two axes into one (non-protein energy) and plot it against protein. The second step was to construct nutrient intake and growth arrays, according to the usual procedures in the GF. Relative to an estimate for the intake target (derived from self-selection studies in other salmonids), whitefish overconsumed non-protein energy to gain limiting protein when restricted to diets low in % protein. They did this to a greater extent than they overconsumed protein to gain limiting non-protein energy on diets containing a high % protein, probably reflecting the toxic limits to voiding excess protein as ammonium via the gills. Protein growth was more tightly regulated than lipid growth. Thus, fattiness of the flesh was a result of overeating carbohydrate and lipid to gain limiting protein on diets containing a low % protein, while diets high in % protein resulted in low levels of body fat as a result of fish refusing substantially to overconsume protein.

In the third step, we superimposed measures of performance and other response variables onto the intake array. Wet weight growth of fish was consistent across the intake array, but this growth was composed of differing amounts of protein and fat, with flesh protein content rising as dietary % protein increased. Feed efficiency and energy retention efficiency showed little change across the intake array, but nitrogen waste rose with % protein in the food. Commonly-used welfare indicators, such as plasma glucose, plasma cortisol, liver glycogen, and liver somatic index, fell as % dietary protein rose, although the interpretation of these 'welfare measures' is problematic.

The final step was to choose a set of performance responses to be considered in diet optimisation, and to normalise and scale these relative to each other. This involved making *a priori* judgements about which variables were relevant, what each was 'worth' and, for each variable, whether high or low values were the more desirable. Fish and fish feed have a

market price, and there is a price premium for high quality flesh. Environmental and welfare costs are harder to measure, but can be the target for taxes and licensing restrictions which have a measurable cost. We did not conduct a full economic analysis, but for illustration chose four scenarios, in which production costs, flesh quality, environmental impact and animal welfare were prioritised (Ruohonen *et al.* 2007).

### III. DISCUSSION

This study illustrates a major advantage of using the Geometric Framework in applied nutrition: that it places not just foods, but the interaction of the animal with those foods, at the centre of diet optimisation decisions. Taking account of the pre- and post-ingestive regulatory responses of the animal simplifies the problem of identifying optimal diet formulations, while multi-criterion optimisation is a matter of deciding how to select and weight normalised response surfaces and then summing these to arrive at the decision function.

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## PREDICTING FOOD INTAKE IN BROILERS AND LAYING HENS

R.M. GOUS<sup>1</sup>Summary

Birds attempt to consume sufficient of a given food to enable them to meet their nutrient requirements for maintenance, growth and/or egg production, but may fail to do so when constrained by a bulky feed or the inability to lose sufficient heat to the environment. Under constrained conditions maintenance requirements are the highest priority, with potential growth or egg production being reduced in proportion to the constrained intake. Thus, food intake will be governed by the potential performance of the bird, the limiting nutrient in the feed, and any constraints that may reduce the desired intake. The accurate prediction of food intake by a given animal on a given food when housed in a given environment makes it possible to define the optimum economic levels of energy and essential nutrients in feeds for broilers and laying hens thereby improving the method of choosing nutrient specifications to be used in least cost feed formulation. Such systems thinking and modelling, when applied to the problem of feed formulation, leads to a more rational approach in which nutritional decisions are made entirely in terms of the objectives of the business. This advanced approach cannot be achieved without being able to predict voluntary food intake accurately under the conditions encountered in commercial poultry operations.

## I. INTRODUCTION

Being able to predict the food intake of a flock of broilers or laying hens can be likened to discovering the Holy Grail. With such information the optimum economic composition of feeds and feeding programmes may be achieved, thereby moving away from the outdated paradigm of formulating at least cost using tables of nutrient requirements. In a more business orientated approach nutritional decisions are made entirely in terms of the objectives of the business. Under such circumstances dietary nutrient specifications may be chosen that will maximise profitability subject to the constraints which act on the business. This is not the same as feeding to meet a 'requirement'. Economic circumstances will change from time to time, and different nutritional strategies will be needed to maximise margins. For example, using broiler feeds with nutrient contents high enough to maximise breast meat yield is far more expensive than feeding to produce a bird for the farm gate market, yet the returns may be substantially greater as a result of feeding the higher-cost feed. Making accurate decisions and manipulations at this level are not possible without being able to predict food intake.

Birds have evolved to survive: they are capable of choosing from an array of foods such that they will grow and reproduce successfully if the necessary nutrients are available. But they must also be able to deal with shortages and excesses of the various nutrients that they need and consume. If the food available is unbalanced, for example with very low protein content and a high energy content, then in order to consume sufficient protein the bird will have to consume excessive amounts of energy and in the process deposit excess lipid. The Meishan pig is the best example of a domestic animal that has evolved to deal with feeds very low in protein and relatively high in carbohydrate and lipid. When such pigs are given feeds high in protein they do not deposit excessive amounts of fat (Kyriazakis & Emmans, 1995) demonstrating that the excessive fatness usually seen on these animals is a consequence of the way they are fed, and not necessarily the way they would like to be. This

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has been demonstrated in broilers also (Gous *et al.*, 1990). As selection for leanness and improved feed conversion efficiency continues, the resultant genotypes will be unable to deal with poor quality feeds and will be constrained to live on high quality food. We need to be aware of the consequences of such selection.

Complex interactions in the bird must exist to deal with imbalances, unbalanced feeds, toxic compounds etc. and it is therefore highly unlikely that food intake can be successfully predicted using simple (or even rather complex) empirical equations.

## II. USING ENERGY AS THE PREDICTOR OF FOOD INTAKE

The need to predict food intake was realised long ago, and prediction equations such as that of Byerly (1941), below, were the first attempt at doing so. There is a long history of empirical equations that were published in an attempt to predict food intake more accurately, and as many arguments about the best power function to use to describe the maintenance requirements of the bird (see Byerly, 1979 and Byerly *et al.*, 1980).

$$\text{Food intake (g/hen d)} = 0.523 W^{0.653} + 1.135 E + 1.126 dW$$

where  $W$  is body weight (g),  $E$  is egg output (g/d) and  $dW$  is the change in body weight (g/d).

Progress in this regard was made by adding a term for the effect of temperature on the maintenance energy requirement, such as that by Emmans (1974), the equation taking account also of the strain and feathering of the birds:

$$ME_{in}(\text{kJ/bird d}) = W (a + bT) + 8.4 E + 20.9 dW$$

where  $W$ ,  $E$  and  $dW$  are as above, and where  $a$  and  $b$  are constants that depend on strain, temperature and feathering.

In laying hens, by taking account of feathering condition, it was demonstrated that the maintenance energy requirement dropped three-fold (from 8.4 to 26.4 kJ/kg.d.<sup>0</sup>C) (O'Neill *et al.*, 1971), and Sakomura (2004), in a more recent application of such work, showed that the lower critical temperature below which the hen had to consume more energy to maintain energy balance, increased from 19 to 22 and to 24<sup>0</sup>C when the feather cover on the bird was reduced from 100 to 50 and then to 0 %.

By dividing the ME required as calculated by such equations by the ME content of the feed the predicted food intake could be calculated. Unfortunately energy has remained the paradigm for predicting food intake for too long, resulting in the naïve proposal that 'birds eat to satisfy their energy requirement'. This has been reinforced by the misinterpretation of experiments such as that by Leeson *et al.* (1996) in which food intake is shown to increase linearly as a basal food is progressively diluted with more and more of an inert filler. The increased intake has been seen as a desire by the bird to eat more energy, but it is as necessary for the bird to eat more to meet the requirement for all the essential amino acids as well as the micro- and macro minerals and vitamins, but this has largely been ignored when interpreting the results of such trials.

There are many examples to demonstrate that birds eat more food when the content of a limiting nutrient in the feed is reduced further, but these examples have been ignored in favour of the naïve hypothesis that birds eat to satisfy their energy requirements.

## III. WHY DO BROILERS GET FAT?

If birds ate to satisfy their energy requirement, why would birds of the same genotype become fat on some feeds and not on others? Clearly they should all be aiming for the same

degree of fatness, this being part of their requirement for energy. Morris (1968) showed that as the nutrient density of a feed was increased, laying hens ate progressively more energy, and this over consumption was related to the size of the hen (or its characteristic intake of energy when given a feed containing a given ME content). So, although food intake declines as nutrient density (ND) increases, the reduction is not as great as it should be if the birds were eating to satisfy their energy requirement. Fisher and Wilson (1974) found that growing chickens also over-consumed energy as nutrient density was increased, leading to increased levels of fatness on high density feeds. They were unable to reconcile the over consumption of energy with the energy system used, as birds also over-consumed energy when the Net Energy (NE) scale was used to describe the feed energy content.

That chickens do react to feeds of low protein: energy ratio by increasing the lipid content of their gain was first reported by Fraps (1943) and this has subsequently been repeatedly confirmed (Seaton *et al.*, 1978; Jackson *et al.*, 1982 among others). Gous *et al.* (1990) demonstrated that birds only get fat as a consequence of the way they are fed, and that, when given a choice between a high and a low protein feed they choose the combination that ensures a low lipid content in the gain.

When broilers and laying hens are given feeds limiting in an amino acid, using a dilution technique as opposed to a graded supplementation experiment, food intake increases as the dietary amino acid (and protein) content is reduced, and then declines sharply as the deficiency worsens (Gous and Morris, 1985; Gous *et al.*, 1987; Burnham *et al.*, 1992). In spite of the lower outputs in growth and eggs, respectively, body lipid content increases as the amino acid content is reduced indicating that dietary energy content or energy balance is not the factor controlling food intake. Even broiler breeders, regarded as being birds that have a voracious appetite, when confronted with feeds low in protein, will reduce food intake substantially (Bowmaker and Gous, 1991; Fisher *et al.*, 2001). Unfortunately very few researchers analyse carcasses for protein and lipid when conducting response experiments, so the resultant changes in body lipid content are mostly overlooked.

To demonstrate the complexity of the process of food intake regulation, birds that are fat will always attempt to reduce the excessive amounts of lipid by making use of these as an energy source when this is possible (Gous *et al.*, 2012). They do this because they always attempt to maintain an inherent ratio between body lipid and body protein: they fail to maintain this ratio when unbalanced feeds are offered, but can correct the ratio if given the opportunity to do so. It appears that strain differences exist in the extent to which broilers will utilise their body lipid reserves as an energy source, but this can be accounted for when simulating food intake and growth by assuming a higher desired lipid to protein ratio in genetically fat strains.

Further convincing evidence that birds and animals attempt to maintain a genetically determined level of fatness, and that they achieve this by utilising body lipid reserves as an energy source when appropriate, is provided by Kyriazakis and Emmans (1991), who showed that when pigs, made fat by feeding them a low protein food, are given a choice between foods of low and high protein contents, they will select a diet of a composition such that the effects of previous mis-feeding are corrected. Similarly Nonis (2007) demonstrated that broiler breeder hens are capable of maintaining their egg production for up to two weeks on an energy intake of only 80 kJ/d provided sufficient dietary protein is provided daily and provided the birds had excessive body lipid reserves prior to food intake being severely restricted.

It is clear that chickens attempt to control their feed intake so that they achieve a particular fatness and that this level of fatness, which is generally lower than observed on many commercial operations, differs between strains, sexes and degrees of maturity (Gous *et al.* 1990). That this goal is not often achieved provides adequate proof that birds do not 'eat

to satisfy an energy requirement', and that food intake regulation is more complex than this statement suggests.

#### IV. IS THERE A BETTER THEORY TO USE FOR PREDICTING FOOD INTAKE?

There is ample evidence that birds are capable of evaluating a food and adjusting intake accordingly. For example, White Leghorn chicks offered nine ingredients free choice, with the position of the hoppers containing these diverse ingredients being changed twice daily, chose a combination that conformed closely to that of the mixed feed formulated by the researcher, and growth, bone development and feathering were the same as on the compounded feed (Funk, 1932). Also, the results of trials by Gous and Swatson (2000) support the hypothesis that broiler chickens, when provided with two or three foodstuffs containing just one protein source on a free choice basis, which in some proportion will meet their requirements, effectively select a combination which maximises their biological performance. In these trials, combinations of three protein sources were fed to broiler chickens either as mixtures or as a choice, and the performance resulting from the choices made coincided closely with the combination resulting in highest growth rate and food conversion efficiency.

It is not difficult to believe that a bird would attempt to consume sufficient of a given food to enable it to grow or to reproduce. But in order to have a theory to predict food intake it must be assumed that the bird has a goal, not simply to grow, but to grow to its genetic potential so that it can reach sexual maturity and pass on its genes to the next generation (Emmans, 1984; Dawkins, 1990). To breed successfully it also needs to minimise excessive lipid deposition. It can be assumed therefore that each bird has a genetically determined amount of body lipid that it seeks to maintain and that any lipid that is deposited in excess of this would be the consequence of having to over consume energy in an attempt to consume sufficient of the limiting nutrient in the feed(s) on offer. To determine the amount of each nutrient that would be required by each bird each day therefore requires a description of the potential growth of body protein and lipid in the case of a growing bird, or the potential egg output of a laying hen.

Bear in mind that a flock of birds does not have a requirement for a nutrient. An individual can be seen as having a requirement for each of the essential nutrients, for maintenance and for growth or reproduction, and this is usually assumed to be the minimum amount of that nutrient that will meet these requirements. But a flock is made up of individuals all varying in body protein and lipid content, and in their potential to grow protein and lipid, or produce eggs, again varying in age at maturity and the number, size and composition of the eggs that each can produce over a laying period. To suggest that such a flock has a requirement is somewhat ludicrous, but this is, unfortunately, almost universally accepted. A flock responds to a given dietary nutrient content; it does not have a requirement for such. To predict how the flock will respond requires an integration of individual responses in food intake to nutrient supply, where the mean, variance and co-variance of each of the parameters involved in describing the potential of each individual are used to simulate the sample population (the so-called Monte Carlo Method) before simulating the response of each of these individuals to the feed(s).

The potential rate of protein growth of a broiler can be sufficiently described using the Gompertz growth curve, with different parameters being required for body and feather growth. The reason for describing these separately is that these components grow at different rates, and more importantly from a nutritional point of view, they differ in amino acid composition. Strains and individuals within a strain differ in their desired lipid contents, but this, as with all other chemical and physical components of the broiler, may be described in

terms of the body protein weight, using allometry. The potential rate of growth of all body components may thus be determined from relatively few parameters and the amount of each of the essential amino acids and energy required to achieve this potential may be calculated for each day, or even each hour, of the growing period. By comparing the requirement for each nutrient with the amount contained in the feed on offer, the desired food intake for each nutrient may be calculated, with that resulting in the highest desired food intake being the limiting nutrient. To this point the calculations are relatively straightforward: what is more difficult is to determine whether the bird would be capable of consuming the amount of food required to maximise growth.

Constraints would be the bulkiness of the food, the amount of heat that would need to be lost to the environment, and the maximum amount of lipid that the bird could deposit in the gain. Limitations in digesta passage rate associated with rheology or hydration rate resulting from differences in the functional characteristics of the feed (Lentle, 2005) may be accommodated by adjusting the digestibility of essential nutrients in the feed to be formulated, until such time as these effects can be mechanistically modelled.

For laying hens and broiler breeders the procedure is the same as for a growing bird, but although the calculation for maintenance is the same for broilers and laying hens, the potential output in this case is not growth of the body but the deposition of protein and lipid in the egg. This is more complex than predicting growth because there are more factors to consider: the age at sexual maturity influences subsequent egg number and size for a hen; seven parameters are involved in describing the rate of ovulation, more are involved in describing the rate of change in ovulation rate with time; and egg weight must be described in terms of yolk size which varies with time, and with allometry describing the weights of albumen and shell, the sum of which makes up the egg. All these parameters are allocated a mean and standard deviation to generate individuals whose potential egg outputs are thus determined, and the nutrients required per bird each day are then calculated. The desired intake of a given food is thus calculated as for the broiler, and the challenge is to determine whether the bird can consume the amount required, the constraints being the same as for the growing broiler.

## V. UNITS TO BE USED FOR CALCULATING DESIRED FOOD INTAKE

The nutrient content or nutritional value of a feed, for the purpose of calculating the desired food intake, is defined by the amounts of energy and nutrients that it yields, and the units used to describe these must be the same as those describing the nutrient requirements of the individual. In addition, the efficiency with which each of these resources (energy or any nutrient) is utilised for one unit of the various functions (maintenance, body and feather protein growth, body lipid growth etc.) needs to be known, and is assumed to be constant across genotypes, feeds and environments (Emmans and Fisher, 1986). There is some debate about the efficiency with which amino acids are utilised for protein growth, but this probably lies between 0.75 and 0.85.

The digestible amino acid content of a feed is probably universally accepted as being a more accurate descriptor of the amount of amino acid available to the bird than is the total amino acid content, but there is less consensus when describing the energy content of a feed. Metabolisable energy (ME) is the most commonly used scale for describing the amount of energy yielded to a given bird or animal by a given feed, but it does not account for the heat increment of feeding. At high temperatures food intake is often constrained to a level below the desired intake because the bird must remain in thermal balance, and when predicting voluntary food intake it is therefore essential to be able to calculate the amount of heat being generated by the bird in performing the functions of maintenance, growth and production.

This is possible only with the NE system, which provides a way of stating both how much energy is yielded by a given feed and how much of this energy a given animal needs to sustain some stated level of performance (Gous, 2010).

An easily implemental form of NE, the effective energy (EE) system, has been proposed by Emmans (1994). In this system, which applies to both single-stomached and ruminant animals, the heat increment of feeding is considered to be linearly related to five measurable quantities associated with the animal, namely, excretion, fermentation (in ruminants), defaecation and the deposition of protein and lipid. The EE yielded by a feed or feed ingredient can be estimated from the ME, the faecal organic matter content and the amounts of digestible crude protein and lipid. According to Emmans (1994), as the EE values 'can be tabulated for ingredients, and are additive to the extent that ME values are additive, they can be used to formulate diets using linear programming'. For these purposes, and for describing animal performance, the effective energy scale is equivalent to a net energy scale and has proved useful in predicting both the desired and constrained food intakes of broilers offered feeds varying in energy and nutrient content and housed in different environmental conditions (EFG Software, 2012).

## VI. THE CONSEQUENCES OF CONSUMING FOOD

It is very unlikely that a bird would ever be supplied with a food perfectly balanced in respect of all nutrients. It is far more likely that the bird would have to deal with excessive intakes of most nutrients in order to obtain sufficient of the limiting nutrient. In many cases there will be an excessive intake of many amino acids, which would need to be deaminated and the amount not given off as heat would be converted to energy and stored as body lipid. Similarly, excess lipid and carbohydrate intake would result in the deposition of body lipid. Heat output would increase in these cases because of the chemical reactions that take place and the excess heat would need to be lost to the environment. As the environmental temperature increases so the dissipation of heat becomes more difficult for the bird and at some point food intake would be constrained because otherwise body temperature would rise and the bird would die. Similarly, on very low protein feeds the amount of lipid that would need to be deposited in the gain would be more than the bird could be capable of, and again the desired intake would be constrained. Feed bulk would similarly constrain intake in some cases although this is unlikely in broilers fed diets based on maize or wheat and soybean.

There are instances where the bird senses that the food on offer may be harmful, and as a result food intake is reduced. For example, when a second-limiting amino acid is added to a diet marginally deficient in one or more indispensable amino acids an imbalance occurs and the primary response is a decrease in food intake (Harper *et al.*, 1970; Park, 2006). Similarly, the presence of toxins in the feed may result in the bird reducing food intake (Bryden, 2012). Imbalances may be modelled, with difficulty, but the constraints resulting from the presence of toxins are difficult, if not impossible, to predict in a simulation model.

The extent to which food intake is constrained under the above circumstances needs to be solved either by iteration or by the use of simultaneous equations. Once the actual intake has been determined, the consequence of the lower-than-desired intake of the limiting nutrient on growth or egg production can be modelled. Considering the process described above to predict actual food intake and performance it should be clear that it is impossible to model actual performance using any form of empirical equation: the potential performance can certainly be described using empirical equations, but too many interacting factors contribute to the actual performance to allow this to be predicted in the same way.

There are instances reported in the literature where food intake appears to have been enhanced rather than constrained, and this phenomenon is usually explained as an



improvement in palatability. Introducing the concept of palatability into a model to predict food intake is dangerous. The term is often applied loosely to a number of ingredients and additives that may appear to enhance food consumption, whilst the reason for the apparent increase in intake resulting from its inclusion remains unclear. For example, both Alenier and Combs (1981) and Cantor and Johnson (1983) demonstrated that when birds are given, in addition to a compounded feed, a range of separate ingredients such as fishmeal, bone meal and fermentation products, some of which apparently contained ‘unidentified growth factors’, they would choose to consume these ingredients in addition to the compounded feed because they were ‘more palatable’. It is more likely that the birds identified an essential nutrient in these ingredients that was absent from the compounded feed and actively chose to obtain this nutrient from the ingredients offered as a choice. This would not constitute proof that the ingredients chosen were more palatable. However, some ingredients, such as oils and fats, reduce dustiness thereby improving the palatability of a food, as does the pelleting process in some instances (Jensen et al. 1962; Rinehart, 1981). Such changes in intake are difficult to model because of the difficulty in describing the physical characteristics of a feed, and the preferences by the bird for such.

Food intake needs to be an output from a simulation model, not an input, if the model is to be of any real value in helping to define the optimum economic levels of energy and essential nutrients to be included in feeds for broilers and laying hens. Because the state of a broiler changes continually, as does the laying performance of a flock of hens, the optimum feed composition on any one day is unlikely to remain optimal throughout the production cycle, and because food intake can be predicted as the state and/or reproductive performance changes, it is possible to optimise the feeding programme to be used during the production cycle. This would involve changing the feed composition at different stages of production, with the optimum economic feeding programme and feed composition within each phase of the programme being determined mathematically. Such optimisation routines are available (EFG Software, 2012) which rely entirely on being able to predict the intake of a given food by an individual under given environmental conditions as the production cycle progresses. Poultry nutritionists need no longer rely on tables of nutrient requirements when formulating feeds; more sensible and advanced alternatives are available which will enable nutritional decisions to be made in terms of the objectives of the business.

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## RESPONSE OF BROILER CHICKENS TO DIETARY ENERGY AND ITS RELATIONSHIP TO AMINO ACID NUTRITION

H.L. CLASSEN<sup>1</sup>

### Summary

The relationship between dietary energy and amino acids is fundamental to the formulation of broiler diets. A historical perspective on this relationship is that broilers alter their feed intake to match dietary energy and therefore amino acid supplementation should be adjusted in accordance. However, control of feed intake is complex and even if this perspective is correct, multiple factors can prevent the appropriate adjustment in feed intake. Furthermore, the validity of broilers altering feed intake in response to dietary energy has been questioned and our research supports this view. Dietary energy did not affect feed intake when factors that confound broiler response were minimized, and reduced performance in low energy diets suggested that energy was insufficient to support the utilization of dietary amino acids. The results support approaches to feed formulation that ensure adequate dietary energy to maximize the protein accretion potential of the diet.

### I. INTRODUCTION

Dietary energy and amino acids are the largest components of poultry diets and also the most expensive. Therefore understanding the relationship between these dietary components is of major significance. An important part of this relationship is the importance of these nutrients in controlling feed intake. Nutrition is one of many factors that control feed intake and it is beyond the scope of this paper to discuss this complex topic in its entirety (Ferket and Gernat, 2006; Applegate, 2012). Instead, the focus of the paper will be on the nature of diet energy effects on feed intake with specific reference to factors that may affect or camouflage the effects of energy per se.

### II. HISTORIC PERSPECTIVES

The response of growing chickens to dietary energy has been the subject of much research and early research has been thoroughly reviewed (e.g. Fisher and Wilson, 1974; Pesti and Smith, 1984; Leclercq, 1986). These reviews provide a historical perspective and undoubtedly have shaped nutritional practice. A commonly accepted role of diet energy is shown in the following quote written in the beginning of the review by Fisher and Wilson (1974):

“The primary response to dietary energy concentration is seen in feed consumption and in productive efficiency rather than in production level”

This statement implies that within a reasonable range of dietary energy, feed intake responds to diet energy to maintain a constant nutrient intake, which in turn affects production efficiency. Fischer and Wilson (1974) outlined this response by examining much of the existing literature (160 comparisons) based on publications where ingredients were known and of a practical nature, data were available on growth and feed consumption, and the number of energy levels permitted the use of regression analysis. In experiments meeting these requirements, feed intake was shown to go down with increasing energy, but statistical

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analysis revealed that diet energy only accounted for 28% of the variation in feed intake. This finding can be interpreted as a further indication of the multitude of factors that impact feed intake. Interestingly dietary energy was more successful in predicting growth rate (52% of variation) and feed efficiency (83% of variation). This summary further demonstrated that bird gender, age and breed influenced the response of growing chickens to dietary energy, and also highlighted other factors that may impact the comparison of energy levels such as feed density and the nature of feed ingredients. These factors, in fact, confound interpretation of the specific effect of dietary energy. Of importance, quality of individual experiments was not a criterion for inclusion or exclusion in their summary. This was similarly not included as a criterion by others (e.g. Pesti and Smith, 1984). Failure to critically evaluate experiments raises questions about the value of research summaries, and the lack of recognition or definition of confounding factors, further reduces their predictive ability. In the following sections, the author will attempt to address the factors that may affect or confound results in experiments designed to determine the effect of dietary energy on broiler performance and more specifically feed intake.

### III. FACTORS AFFECTING RESPONSE OF BROILERS TO DIETARY ENERGY

#### a) Broiler genotype

Selection of broiler genotypes has been extensive with obvious phenotypic changes such as growth, feed efficiency, increased meat yield, reduced carcass fat, and improved bird health. Many of these changes were scientifically demonstrated by Havenstein et al. (2003), and it is recognized that broiler chickens continue to evolve in response to selection pressures, whose specific nature is also changing. Physiological changes have occurred as a result of selection and these are the cornerstones of obvious phenotypic traits, but there are also changes that either are not obvious or have not been clearly identified such as mechanisms of feed intake control. The less clear understanding of the physiological mechanisms on feed intake in avian in comparison to mammalian species is partially responsible for this lack of clarity in changes occurring with increasing growth rate (Richards et al., 2010). A positive correlation between feed intake and growth is expected and scientifically confirmed (Siegel and Wisman, 1966; Pym and Nichols, 1979). It is also clear that feed intake has a heritable basis and will change in association with selection for correlated traits or as a result of direct selection (Pym, 2005). That there may be differences in feed intake control mechanisms in broiler strains has been recognized for some time (Burkhart et al., 1983, Lacy et al., 1986), as have differences in how rapidly growing breeds respond to dietary energy content with some breeds demonstrating no or little effect (Pym and Nicholls, 1979; Pym, 2005). Therefore, breed differences are an important confounding factor in the study of feed intake responses to dietary energy. Furthermore, the continuing selection and sometimes rapid changes within commercial broiler stock show that understanding breed responses to dietary energy is a moving target and requires periodic re-evaluation.

#### b) Diet composition and digestible nutrient content

When comparing the response of broiler chickens to dietary energy, one of two approaches can be taken: 1) formulation of one diet and then diluting it with materials with little nutritional value or effect, or 2) formulation of diets using practical feed ingredients. Both approaches can be criticized for potentially influencing the response of birds to dietary energy. In the case of diet dilution, the assumption that diluting materials have little or no nutritional influence other than reducing diet energy content can be questioned. Use of practical ingredients has the advantage of using feedstuffs that might be used in commercial applications, but this can also be a disadvantage because of variable nutritional qualities. As a consequence, there is considerable potential for broiler response to be related to the nature of

ingredients that are either not found in all diets or are incorporated at vastly different levels. Most research on the effect of dietary energy has taken the practical feed ingredient approach. Some of the ingredient factors that may influence broiler response are nutrient or anti-nutrient levels, nutrient digestibility and post-digestion metabolic effects.

Formulation of diets relies on the use of dietary specifications derived from a variety of sources, which are usually based on averages of results from digestibility research. In research designed to estimate the impact of dietary energy on broilers, this has the potential to reduce accuracy since specific samples of ingredients can vary considerably from these averages. In research on nutritional requirements, it is well accepted that the level of the nutrients in diets should be measured to confirm experimental assumptions. Yet this has not been the case for the vast majority of studies on dietary energy. Further reducing accuracy in these studies is the failure to recognize known broiler age differences in energy retention. It appears logical that ingredients or diets used in this research should be assessed for energy retention in an age appropriate manner to reduce the potential for inaccurate nutrient profiles.

A key aspect of diet formulation is the level, balance, and digestibility of amino acids. Diets from early research on dietary energy were formulated based on total amino acid content and a limited number of amino acids (sulphur amino acids, lysine). Lower density, higher fibre ingredients frequently have lower amino acid digestibility and this has the potential to impact growth and other response criteria. Failure to take into account amino acids beyond sulphur amino acids and lysine may result in the diet being limited by another amino acid, thereby affecting growth potential. Finally, amino acid balance and the ideal protein concept (Emmert and Baker, 1997) can also affect broiler response to dietary energy. Amino acids in excess of that required for protein synthesis and other aspects of body metabolism are catabolised, and this process incurs an energy cost (Sklan and Plavnik, 2002) and is metabolically stressful. It is only in most recent years that some or all of these issues related to amino acids have been taken into account in dietary energy research. The relationship of amino acids to energy will be discussed in more detail later in this report.

The nature and amount of fibre in diets can also affect broiler response and fibre is a key method of reducing diet energy. That soluble fibre can be an anti-nutritional factor has long been recognized (Choct et al., 1992) and therefore alleviation of its effects via exogenous dietary enzymes is required for proper dietary energy comparisons. Initial research on dietary energy in my lab utilized barley as a key ingredient in low energy diets. This research occurred before the commercialization of exogenous enzymes so the soluble  $\beta$ -glucan content in barley affected not only the digestible nutrient content of the barley, but also the remainder of the diet. Insoluble fibre, often considered to have little nutritional impact other than nutrient dilution, can also affect feed intake and digestive function (Svihus and Hetland, 2001, Svihus, 2011).

Fat is the most common ingredient used to increase dietary energy and possibly has the most potential to be confounding in dietary energy studies. Fat is recognized as providing more nutritional benefit than can be attributed to its metabolizable energy (ME), the measure of energy most used in previous research (Pesti and Smith, 1984). This "extra-caloric effect" can be partially attributed to the proportionally lower heat increment of fat. To derive this beneficial effect, fat must be digested and absorbed and this is not only affected by fat source but also by bird age; younger birds do not utilize fats as well as older birds and the degree of difference is related to fatty acid chain length and saturation. However, fat effects on digestive function also increase utilization of other dietary nutrients and this effect is complicated by level and nature of fat in the diet (Mateos and Sell, 1980; 1982). Further, fat can affect diet palatability, thereby affecting research results and in particular feed intake. Use of net energy (or equivalent) should enhance the accuracy of energy delivery in diets varying in fat content, but other beneficial effects of fat are more difficult to estimate. With

few exceptions research on broiler response to dietary energy has failed to account for the important influence of dietary fat.

c) Feed form and feed processing

Feed form is well recognized to influence broiler performance with birds fed pelleted diets growing more rapidly and more efficiently (McKinney and Teeter, 2004; Skinner-Noble et al., 2005; Brickett et al., 2007). A primary reason for the increase in productivity is thought to relate to bird behaviour, more specifically, reduced energy use due to less time eating and more time resting (McKinney and Teeter, 2004). However, other mechanisms may also play a role. Although generally regarded as minimal, pelleting may affect nutrient digestibility, with the nature of the response ingredient dependent. Diet presentation can also impact both the ability of birds to consume feed as well as its palatability for consumption (Latshaw, 2008). Volumetric density is reduced in mash feeding and this can impact the ability to consume sufficient nutrients for maximum production, particularly when diets are low in nutrient density. Particle size in mash diets can further impact diet palatability and this effect is modified by the level of dietary fat. Therefore, the characteristics of a mash diet can confound broiler response to dietary energy. Regardless of the mechanism, pelleting diets affects the effective caloric value of feed (McKinney and Teeter, 2004). Pellet quality is also impacted by diet ingredients, so experiments should be designed to ensure comparable quality across energy treatments. It would seem rational that research related to the response of birds to diet energy should employ the processing technique that it is being used to predict.

d) Bird age

The response of broilers to dietary energy has been shown to be affected by bird age, with younger birds seemingly less able to respond appropriately because of physical limits in digestive tract capacity (Brickett et al. 2007). This could also relate to the reduced digestive ability (Sklan, 2001) and the nature of the diet ingredients and form.

e) Energy to protein ratio

The relationship of energy to protein in experimental diets examining the effect of diet energy has important implications on broiler response. Experiments have generally followed one of two approaches, providing digestible or total amino acids above bird requirement or balancing amino acids to the level of dietary energy. The latter approach is often based on the assumption that birds have the ability to maintain energy intake by adjusting feed intake to match the energy in the diet. If this is incorrect, as discussed above, or the response is unpredictable because of other factors, this approach can produce invalid results because lower energy diets would be amino acid deficient. Broiler response is affected by the most limiting factor in the diet and therefore diets containing insufficient levels of indispensable amino acids or energy will have lower response potential. The partitioning of energy and protein for maintenance and production purposes will certainly be affected by dietary balance. The protein (amino acid) sparing effects of dietary energy is a good example of partitioning change as less protein is used to meet the energy requirements of the bird and more is directed towards growth and lean tissue accretion.

The use of calorie to protein ratio for describing the relationship between these two important diet fractions is outdated other than to discuss this relationship in the most general of terms. More relevant is to describe the relationship of energy to the level of ideal digestible amino acids (most limiting amino acid) and minimum levels of protein to supply nitrogen for non-essential amino acid synthesis.

f) Environment and disease

Numerous non-nutritive factors can affect bird performance and hence have the potential to affect the feed intake response of broilers to dietary energy (Applegate, 2012). It is beyond the scope of this report to examine them in detail, but environmental factors of significance include temperature, environmental contaminants such as ammonia, housing density, water and feed availability and lighting programs. Disease challenge and immunological stress are also important in defining bird response. The nature of these effects is complex but among the mechanisms of action, partitioning of dietary energy as a result of increased heat production and confounding effects on feed intake merit mentioning.

## IV. RECENT RESEARCH

With the above factors in mind, research was undertaken to investigate the impact of dietary energy, while attempting to minimize confounding factors (Cho, 2011). Diets were formulated to contain 11.30, 11.86, 12.42 and 12.97 MJ/kg of ME using practical ingredients (main ingredients – corn, wheat, barley, soybean meal, canola meal and canola oil), and fed to Ross x Ross 308 mixed sex broilers housed in litter floor pens. The following aspects of the experimental design were implemented to reduce confounding influences on broiler response.

- Ingredient AMEn and apparent ileal amino acid digestibility were determined at 5-6 and 21 d of age in broiler chickens and diets were formulated using age appropriate values.
- Diets were formulated on a digestible amino acid basis with ideal protein balance.
- Diets were formulated to maintain a constant ratio between the ether extract content and AMEn of the diets.
- Diets were pelleted and fed in crumble or pellet form. Crumble and pellet quality, as assessed by sieving (particle size) and pellet quality index procedures, demonstrated minor differences among diets. To reduce the impact of ingredients on pellet quality, pellet binders were added to the diets.
- Exogenous enzymes were used to negate the impact of soluble fibre found in wheat and barley.
- Environmental conditions (temperature, ventilation) were optimized or specified (e.g. space allocation).
- Anti-coccidial agents and growth promotants were used to minimize subclinical and clinical disease.

In the first experiment, all diets were formulated to contain the digestible amino acid requirements outlined by Aviagen (2007). The four levels of energy were fed from 0, 10 or 26 d of age until trial end at 35 d. Body weight and feed intake were monitored every 5 d for the length of the trial. With minor exceptions, dietary energy did not affect feed intake regardless of when treatments were initiated. Body weight gain increased and feed to gain ratio (mortality corrected) decreased with dietary energy with significant effects most often found during the starter and grower phases. The results demonstrate that this strain of broilers did not increase feed intake to maintain the same energy intake. Effects of growth rate and feed efficiency were similar to previous research but not to the same degree.

Because the levels of digestible amino acids in the first trial were relatively high, it was of interest to examine the interaction of dietary energy with lower levels of dietary amino acids. The second trial used the same ingredients and energy treatment levels, but these were fed in combination with three levels of amino acid supplementation that approximated 90, 80 and 70% of Aviagen (2007) recommendations. Significant interactions were found for most response criteria assessed. At the highest level of dietary amino acids, body weight and

carcass and breast yield increased, and feed to gain ratio decreased with dietary energy, but feed intake was not affected. With the intermediate level of amino acids, dietary energy did not affect response criteria. With the low levels of dietary amino acids, weight gain, feed intake, and carcass and meat yield decreased with diet energy content. The interactions between diet energy and amino acids for feed intake and body weight are shown in Figures 1 and 2.

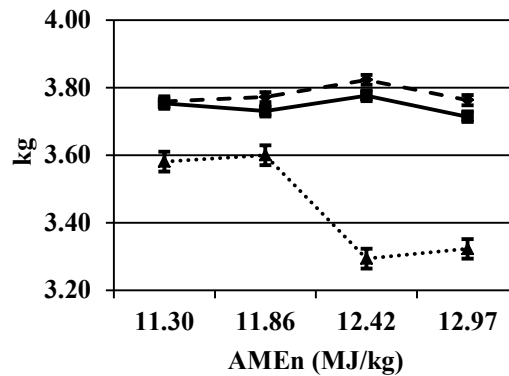


Figure 1 - Effect of dietary energy (MJ/kg) on feed intake of broilers fed 90 (◆), 80 (■) and 70% (▲) of Aviagen (2007) amino acid specifications.

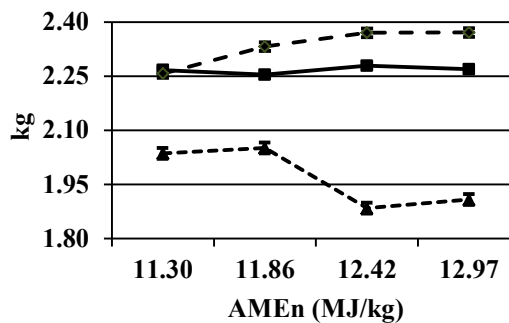


Figure 2 - Effect of dietary energy (MJ/kg) on body weight of broilers fed 90 (◆), 80 (■) and 70% (▲) of Aviagen (2007) amino acid specifications.

These data confirm the lack of energy effect on feed intake in diets containing moderate to high levels of dietary amino acids. Performance data from the 90% amino acid level suggest that the low levels of energy were inadequate to provide for bird maintenance and maximum growth. It supports the concept that increased energy spares amino acids from catabolism and permits their use in protein synthesis. The carcass and breast meat yield responses were similar to that of body weight, which supports this conclusion. No obvious increase in fat content was noted. Therefore, not all increases in body weight associated with increasing diet energy are due to increased fat deposition. At the 80% level of amino acids, production characteristics were unaffected suggesting that all energy levels were adequate to match the growth potential provided by the low levels of amino acids. The reduction in feed intake, growth and carcass characteristics with increasing energy in the 70% amino acid diets may have occurred because of the large imbalance between energy and amino acids in the diet. Of note, feed to gain ratio was unaffected by diet energy for this amino acid level.

Are these results in agreement with previous research? The results from more recent research continues to provide variable results with some research supporting the eat to meet energy requirements (Leeson et al., 1996; Dozier et al., 2006; Lemme et al., 2005) while at least some other research has failed to see an effect of dietary energy on feed intake



(Plumstead et al., 2007; Latshaw, 2008; Delezie et al., 2010; Li et al., 2011). Although more recent papers are more likely to account for the confounding papers described above, at least some of these factors remain unaccounted for in most research.

## V. WHERE TO FROM HERE?

Energy and protein are the most expensive components of broiler diets with energy emerging in prominence due to increased competition for starch- and oil-enriched feedstuffs. This reality is likely not to change in the near future and as the poultry industry responds to world demand for high quality animal protein, the efficient use of ingredients will become paramount. In particular, generating a comprehensive understanding of the amount of energy required for maintenance and production, and the balance between energy and ideal amino acid content will increase in importance. It is the author's opinion that research to provide this understanding must be based on current genotypes and industry practice, and avoid confounding factors that reduce the value of research. Perhaps an international collaboration with cross pollination of ideas and a common experimental design will lead provide us with this important nutritional understanding.

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STARCH AND NITROGEN DIGESTION KINETICS INFLUENCE GROWTH  
PERFORMANCE AND NITROGEN RETENTION  
IN RED SORGHUM-BASED BROILER DIETS

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Summary

A study was conducted to examine the effect of starch and nitrogen (N) digestion kinetics on performance of broilers using sorghum-based diets as a model. Starch and nitrogen digestion kinetics were determined by using an exponential mathematical model to relate digestion coefficients at proximal jejunum, proximal ileum and distal ileum with mean retention times in each segment and these digestion kinetics were compared with previously determined performance parameters. Starch digestion rate was positively correlated with weight gain and relative gizzard weight was correlated with nitrogen digestion kinetics. N retention was negatively correlated with starch digestion rates but not correlated with nitrogen digestion rates. From predicted glycaemic indices, the rate of glucose absorption was highly correlated with enhanced feed efficiency. Thus this study demonstrates that even under *ad libitum* feeding regimes digestion kinetics of starch and nitrogen modified feed efficiency, weight gain and N retention in broiler chickens.

I. INTRODUCTION

The present study is an extension of an earlier experiment (Selle et al., 2012) which investigated the shortcomings of the grain sorghum as a feedstuff for poultry. One reason for the inferiority of sorghum relative to wheat proposed by Black et al. (2005) was a lack of synchrony in starch and protein digestion. The gastrointestinal tract has a substantial energy demand (Cant et al., 1996), which is largely met by the oxidation of luminal non-essential amino acids (Reeds et al., 1999); whereas, glucose is required for the stimulation of protein accretion in breast muscle (Yaman et al., 2000). Thus, the relative availability of glucose or amino acids, arising from different starch and protein digestion rates, may influence both nutrient absorption and protein deposition. In practice, broilers have unrestricted access to feed and *ad libitum* feeding regimes are often thought to accommodate any differences in rates of digestion/absorption of nutrients. However, *ad libitum* feeding does not equate with continuous feeding because birds fed pelleted diets spent less than 5% of illuminated time eating under a '12 hours-on' lighting pattern (Jensen et al., 1962). Thus despite unrestricted access to feed it does appear that consumption patterns still provide scope for asynchronies in digestion and absorption to impact on broiler performance. Therefore, this study investigated the digestion kinetics of starch and nitrogen and related them to 6-27 days post-hatch broiler performance with feed access dictated by an '18 hours-on' lighting regime.

II. MATERIALS AND METHODS

The general methodology used in this experiment has been outlined previously (Selle et al., 2012) and is not repeated herein. Specifically, digesta samples were collected in their entirety from the proximal jejunum, proximal ileum and distal ileum, freeze-dried and weighed to determine mean retention time (MRT) and apparent digestibility of starch and nitrogen (N) using acid insoluble ash (AIA) as the inert dietary marker. The digestion time (t) was

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calculated from the sum of MRT determined in each intestinal segment. Mean retention time was calculated using the following equation:

$$\text{MRT (min)} = (1440 \text{ (min)} \times \text{AIA}_{\text{digesta}} \times \text{W(g)}) / \text{FI}_{24\text{hr}} \times \text{AIA}_{\text{feed}}$$

where  $\text{AIA}_{\text{digesta}}$  is the AIA concentration in the digesta (mg/g),  $\text{W}$  is the weight of dry gut content (g),  $\text{FI}_{24\text{hr}}$  is the feed intake over 24 h before sampling (g),  $\text{AIA}_{\text{feed}}$  is the AIA concentration in the feed (mg/g) and 1440 equals minutes per day. On average, MRT in proximal jejunum, proximal ileum and distal ileum were 16, 35 and 41 min, respectively. Mean retention time in the duodenum and distal jejunum was not determined but were taken as 5 and 41 min, respectively, based on two other sorghum studies completed by the Poultry Research Foundation (unpublished data).

The pattern of fractional digestibility coefficients was described by relating the digestion coefficient at each site with the digestion time ( $t$ ). The curve of digestion was described by the exponential model developed by Orskov and McDonald (1979):

$$D_t = D_\infty (1 - e^{-kt}) \quad (1)$$

where  $D_t$  (g/100g starch or nitrogen) is the percentage of starch/nitrogen digested at time  $t$  (min), the fraction  $D_\infty$  is the amount of potential digestible starch or nitrogen (asymptote) (g/100g starch or nitrogen), digestion rate constant  $k$  (per unit time,  $\text{h}^{-1}$ ) would mean a 100 % starch digestion within 1 hour when it is equal to 1. Starch absorption was assumed not to take place proximal to the small intestine. Nitrogen digestion in this study was determined as apparent N digestibility, which unlike starch, is impacted by endogenous N flows.

The glycaemic indices are the area under the digestogram (AUC), which is obtained by integrating Eq. (1) between digestion times  $t_1$  (usually  $t = 0$ ) and  $t_2$  (Eq. 2):

$$\text{AUC} = \left[ D_\infty t + \frac{D_\infty}{k} (e^{-kt}) \right]_{t_1}^{t_2} \quad (2)$$

This is because correlation between glycaemic index (GI) and hydrolysis index (HI),  $\text{GI} = 39.71 + 0.549\text{HI}$  ( $r=0.894$ ,  $P < 0.05$ ) (Goni et al., 1997), where  $\text{HI} = \frac{\text{AUC}}{\text{AUC}_{\text{reference food}}}$ .  $\text{AUC}_{\text{ref}}$  is a fixed constant in this equation; therefore, AUC can be used as an indicator of glycaemic index. The Microsoft Excel Solver® was used to compute parameters of the modified first-order kinetic (Eq. 1) by minimising the sums of squares of residuals with the constraint that  $D_\infty \leq 100\%$ .

### III. RESULTS

The previously reported growth performance and digestibility coefficients are shown in Table 1 (Selle et al., 2012) and the results of starch and nitrogen digestion kinetics are shown in Table 2. Starch and nitrogen were gradually digested along the small intestine, with starch being digested more rapidly than nitrogen in the jejunum. On average, the starch and nitrogen digestion rate constants were 2.02 and  $1.36 (\times 10^{-2} \text{ h}^{-1})$ , respectively. Birds fed intact pelleted diets had superior weight gain and feed efficiency, which was associated with the highest feed intakes and most rapid starch and nitrogen digestion rates. Pearson correlations between digestion kinetics and reported parameters of broiler performance are shown in Table 3. Starch digestion rate and glycaemic response were positively correlated with weight gain. Birds with heavier relative gizzard weight had higher nitrogen digestion rate and potential digestible nitrogen. The rate of glucose absorption, as per the predicted glycaemic index (AUC), was highly correlated ( $r = -0.710$ ,  $P < 0.002$ ) with enhanced feed efficiency. N retention was negatively correlated with starch digestion rate ( $r = -0.597$ ,  $P < 0.01$ ) and glycaemic response ( $r = -0.550$ ,  $P < 0.02$ ); however, it was not correlated with nitrogen digestion rate ( $P > 0.25$ ).

## IV. DISCUSSION

In this study, sorghum was coarsely-ground (6.0 mm hammer-mill sieve) prior to steam-pelleting and secondary reductions in particle size in the pellet press (4.0 mm) almost certainly influenced digestion rates. The starch digestion rate constant in reground mash was 51 % higher than unprocessed mash ( $2.10$  vs.  $1.39, \times 10^{-2} \text{ h}^{-1}$ ); similarly, the nitrogen digestion rate was 38 % higher in reground mash diets ( $1.31$  vs.  $0.95, \times 10^{-2} \text{ h}^{-1}$ ).

**Table 1 - Effects of dietary treatments on growth performance [weight gain (WG), feed intake (FI), feed conversion ratio (FCR), relative gizzard weights (RGW), N retention], starch and N digestibility coefficients from 6-27 days post-hatch**

	Mash	Intact Pellet	Reground Pellet	SEM	P - value
WG (g/bird)	1235 <sup>b</sup>	1457 <sup>a</sup>	1272 <sup>b</sup>	22.389	<0.001
FI (g/bird)	2179 <sup>b</sup>	2450 <sup>a</sup>	2175 <sup>b</sup>	47.522	0.001
FCR (g/g)	1.765 <sup>a</sup>	1.682 <sup>b</sup>	1.709 <sup>b</sup>	0.0207	0.037
RGW (g/kg)	28.11 <sup>a</sup>	23.65 <sup>b</sup>	22.14 <sup>b</sup>	0.6174	<0.001
N retention (%)	61.23 <sup>a</sup>	55.82 <sup>b</sup>	56.82 <sup>b</sup>	1.2016	0.007
Starch digestibility coefficients					
Jejunum	0.395 <sup>c</sup>	0.681 <sup>a</sup>	0.568 <sup>b</sup>	0.0262	<0.001
Proximal ileum	0.838	0.882	0.877	0.0164	0.159
Distal ileum	0.893	0.901	0.886	0.0103	0.566
Nitrogen digestibility coefficients					
Jejunum	0.374	0.472	0.386	0.0345	0.126
Proximal ileum	0.647 <sup>b</sup>	0.703 <sup>a</sup>	0.714 <sup>a</sup>	0.0138	0.009
Distal ileum	0.724	0.740	0.735	0.0101	0.544

\*Means within rows followed by different letters are significantly different at  $P = 0.05$

**Table 2 - Effects of dietary treatments on digestion kinetics [potential digestible starch (PDS), area under starch digestion curve (AUC), starch digestion rate ( $K_{starch}$ ), potential digestible nitrogen (PDN), nitrogen digestion rate ( $K_{nitrogen}$ )]**

Form	PDS (%)	AUC (g min/g dry starch)	$K_{starch}$ ( $\times 10^{-2} \text{ h}^{-1}$ )	PDN (%)	$K_{nitrogen}$ ( $\times 10^{-2} \text{ h}^{-1}$ )
Mash	99.30	89.36 <sup>b</sup>	1.39 <sup>b</sup>	99.31 <sup>a</sup>	0.95
Intact Pellet	97.56	125.80 <sup>a</sup>	2.57 <sup>a</sup>	83.31 <sup>b</sup>	1.82
Reground Pellet	99.41	118.10 <sup>a</sup>	2.10 <sup>a</sup>	95.36 <sup>a</sup>	1.31
SEM	1.0507	4.3641	0.2108	3.8056	0.2268
P - value	0.399	<0.001	0.004	0.025	0.051

\*Means within columns followed by different letters are significantly different at  $P = 0.05$

**Table 3 - Pearson correlations between digestion kinetics and broiler performance**

Item		WG	FI	FCR	RGW	N retention
PDS	r =	-0.276	-0.272	0.082	0.545	0.344
	P =	0.268	0.275	0.746	0.680	0.163
$K_{starch}$	r =	0.567	0.448	-0.422	-0.526	-0.597
	P =	0.014	0.062	0.081	0.025	0.009
AUC	r =	0.642	0.399	-0.710	-0.783	-0.550
	P =	0.004	0.101	0.001	<0.001	0.018
PDN	r =	-0.496	-0.376	0.359	0.487	0.271
	P =	0.037	0.124	0.144	0.040	0.276
$K_{nitrogen}$	r =	0.416	0.304	-0.328	0.466	-0.252
	P =	0.086	0.220	0.183	0.051	0.314

Feed efficiency is pivotal to sustainable chicken-meat production. Feed conversion ratio was correlated with starch digestibility in the proximal jejunum ( $r = -0.517, P < 0.03$ )

and proximal ileum ( $r = -0.554$ ,  $P < 0.02$ ), but not in distal ileum and FCR was not correlated with N digestibility in the previous study. However, there was a stronger correlation between FCR and glycaemic index than starch digestibility coefficients. The relevant linear regression equations predict an increase in glycaemic index from 89.36 to 125.80 will translate to a 4.5 % improvement in FCR (1.768 vs. 1.688) and an 11.1 % increase in weight gain (1239 vs. 1377 g/bird). Glucose triggers insulin secretion in a concentration-dependent manner via defined pathways (Henquin, 2000) and insulin prompts net protein deposition and moderates protein turnover (Grizard et al., 1999). Importantly, Tomas et al (1998) showed that protein turnover was negatively correlated with feed efficiency.

N retention was significantly correlated with starch but not nitrogen digestion rates; however, starch was more rapidly digested than nitrogen (2.02 vs. 1.36 units). As suggested by Black et al. (2005), asynchronies or imbalances in starch and protein digestion rates may compromise protein deposition and broiler performance. Therefore, rapid starch digestion, in concert with slow protein digestion, may have depressed N retention in this study. Instructively, Weurding et al. (2003) demonstrated that slowly digestible starch significantly improved feed efficiency (1.55 vs. 1.58 units) and weight gain (1426 vs. 1400 g/kg) compared with rapidly digested starch in 9-30 day broilers. Thus the balance of either slowly digestible starch (pea-maize) or rapidly digestible starch (tapioca-maize) in relation to protein (soybean meal) in the Weurding et al. (2003) study impacted on performance and protein utilisation.

Finally, in this study, broiler performance parameters were correlated with kinetics of starch and nitrogen digestion but not with digestibility coefficients of starch and nitrogen in the distal ileum. Therefore, the dynamics of starch and nitrogen digestion are more important determinants of performance than ileal nutrient digestibility coefficients and the latter value is limited because they are static assessments.

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## ADDITIVITY OF ILEAL AMINO ACID VALUES OF SORGHUM WITH DIFFERENT PROTEIN SOURCES

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Sorghum is used widely in Australian poultry diets, primarily as an energy component. It is usually fed in diets that contain wheat but may be the only cereal in diets when wheat prices are high. Feed ingredients with low amino acid concentrations and containing anti-nutritional factors have been reported to mask additivity in a complete diet (Laplace et al. 1989). The presence of anti-nutritive factors (phytate, kafirin and polyphenols) in sorghum may have an impact on protein and amino acid digestibility and additivity. In complete diets, sorghum is fed with a range of protein meals and the objective of this study was to determine if ileal crude protein and amino acid digestibility coefficients of mixed diets containing sorghum and different protein sources can be predicted from the values determined with individual feed ingredients

Five mash assay diets containing sorghum (600g/kg) and one of the following protein meals (279g/kg); sunflower, soybean, canola, cottonseed or meat and bone meal, were prepared (Angkanaporn et al., 1996). Celite (20 g/kg), as a source of acid insoluble ash (AIA), was used as an indigestible marker in all experimental diets. Each diet was fed to 3 replicate cages with 12 broilers per cage from days 14 to 23 post-hatch. On day-23, all birds were euthanised and digesta from the lower ileum were collected and pooled per replicate, frozen and lyophilised. Nitrogen, amino acids and AIA in feed and ileal samples were analysed with standard laboratory protocols. Digestibility coefficients of protein and amino acids of mixed diets were determined and calculated/predicted based on the digestibility value of individual ingredients. The values for individual ingredients were determined in a parallel bioassay using chicks of the same age and methodology that has been described in detail (Bryden et al., 2009). Individual amino acid digestibility coefficients and level contributed by each ingredient were used to calculate and predict the digestibility coefficient of protein and amino acids in the mixed diet. Predicted digestibility coefficients were subtracted from the respective determined values of each diet and tested for significance.

No significant difference was detected in the measured and predicted ileal digestibility coefficients of protein in any of the mixed diets. Determined and predicted ileal digestibility coefficients values of most of the amino acids were insignificant in the sorghum +sunflower meal diet except for lysine and tyrosine that were higher than predicted. In the sorghum +MBM diet the determined values were greater for Met, Ile, Lys, and Tyr and lower for Asp and Gly than their predicted digestibility coefficients. The determined digestibility coefficient of all the amino acids was greater in the sorghum +canola meal diet except for Leu. Interestingly, the determined digestibility coefficients of most amino acids in the sorghum +cottonseed meal diet was lower than predicted values except Thr, Val, His, Ser, Gly and Tyr.

These findings demonstrate that, in most instances, individual amino acid digestibility of sorghum and a range of protein feed ingredients can be used to predict the amino acid digestibility of mixed diets containing these ingredients. However, the precision of additivity differed with different protein sources indicating some interaction of the protein source with sorghum.

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## NET ENERGY VALUE OF BROILER DIETS AS AFFECTED BY HIGH INCLUSION OF DISTILLERS' DRIED GRAINS WITH SOLUBLES

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Inclusion of distillers' dried grains with solubles (DDGS) has been shown to reduce the metabolisable energy (ME) of poultry diets. It is not known what effect this ingredient has on heat production and therefore dietary net energy (NE), especially when included at high levels. In terms of NE, the effectiveness of enzyme addition for such diets is also unknown. The present study was designed to evaluate the impact of DDGS, as a fibrous ingredient, on the energy utilisation of broiler chickens using the indirect calorimetric method. Two levels of sorghum DDGS (0 and 300 g/kg) without or with an enzyme cocktail (xylanase, protease, amylase and glucanase) were used in a 2 x 2 factorial arrangement of treatments involving a total of 32 male broilers (Ross 308). The basal diet comprised maize, wheat, soybean meal and canola oil and diets were isoenergetic and isonitrogenous. Birds were fed experimental grower diets from d 18 to 28. From d 21 to 24, birds were assessed for heat production using 16 closed-circuit calorimetric chambers. Each treatment was replicated four times with two birds per replicate. During the same period, the ME content of the diets was determined by the total collection method and NE was calculated as fasting heat production (as 450 KJ/BW<sup>0.70</sup>) + energy gain (Noblet et al., 2010).

Inclusion of DDGS in the diet impaired ( $P < 0.05$ ) feed conversion ratio (FCR) (Table 1). Despite the negative effect of DDGS inclusion on ME of the diets, ME intake (MEI) of the birds remained unaffected by experimental treatments. However, birds given the diets containing DDGS produced more ( $P < 0.01$ ) heat and had a lower ( $P < 0.05$ ) respiratory quotient (RQ) than the control birds. As a result of the difference in heat production (HP), the NE value of the diet and the efficiency of ME for NE were poorer ( $P < 0.05$ ) on the DDGS-diets. Addition of the enzyme cocktail tended to reduce HP while it had no effect on the ME and NE contents or the NE:ME ratio. Effects of increased dietary fibre (62 %) on gut fermentation and gut development may provide some explanation for the higher HP in the birds fed diets containing DDGS. The differences in NE:ME ratio for the diets without or with DDGS suggests a higher sensitivity of NE compared to ME for the assessment of energy utilisation in broilers when high levels of DDGS are included in the diets.

Table 1 - Heat production and utilisation of energy in broiler chickens

	Diet		Enzyme			Significance <sup>1</sup>	
	Control	+DDGS	-	+	SEM	Diet	Enzyme
FCR (d 21 - 24)	1.46	1.54	1.51	1.49	0.019	*	NS
Mean BW (kg/b)	1.12	1.09	1.09	1.11	0.015	NS	NS
MEI (kJ/kg BW <sup>0.70</sup> )	1413	1420	1401	1432	22.8	NS	NS
HP (kJ/kg BW <sup>0.70</sup> /d)	890	936	928	898	6.2	**	0.07
RQ	1.06	1.02	1.03	1.06	0.006	*	*
ME (kJ/g)	12.98	12.40	12.66	12.72	0.055	***	NS
NE (kJ/g)	9.24	8.46	8.74	8.96	0.007	***	NS
NE:ME (%)	71.2	68.2	69.0	70.4	0.61	*	NS

\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .<sup>1</sup>Diet x enzyme interaction was not significant ( $P > 0.05$ )Noblet J, Van Milgen J, Dubois S (2010) *Proc. Aust. Poult. Sci. Symp.* **21**, 26-35.<sup>1</sup> School of Environmental and Rural Sciences, University of New England, Armidale, NSW 2351, Australia.<sup>2</sup> INRA, UMR1348 Pegase, F-35590, St-Gilles, France.<sup>3</sup> Poultry Cooperative Research Centre, Armidale, NSW 2351, Australia.



## METHODOLOGY TO DETERMINE NET ENERGY IN BROILERS

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The energy cost in broiler diets is likely to continue to increase in the future. Data on heat increment of raw materials used in poultry feed formulations are lacking. An examination of methods to determine net energy is necessary to further develop an accurate net energy assay system. An indirect calorimetry system has been developed at UNE to gather information on respiratory quotient (RQ), heat production, AME and net energy in broilers.

The results of two experiments are described. The first was designed to examine the variation and sensitivity of data collected from indirect calorimetry chambers. Twenty four, 21 d old male broilers (Ross 308) were allocated to 12 closed-circuit chambers. Birds were acclimatised to chambers for four days prior to collecting RQ data and calculation of heat production (HP) using the Brouwer equation from 25 d to 27 d. Apparent metabolisable energy (AME) was determined by total collection of excreta. Birds were fasted for 14 h with fasting HP (FHP) determined on d 28. A second experiment compared AME, HP and NE as measured by indirect calorimetry (IC) or comparative slaughter (CS) in high and low fiber diets. For IC, male broilers (Ross 308; n=32) were used (2 per chamber) to determine AME, HP and NE between 21 d and 24 d as described by Noblet et al. (2010). AME was determined by total collection of excreta. For CS, male broilers (Ross 308; n=240) were used. Birds were slaughtered and analysed at 18 d and 28 d bracketing median growth rate (ADG/BW) of birds in chambers to determine increases in carcass gross energy (GE). Titanium dioxide was used as an indirect marker to determine AME. After taking diet into account in the model, differences in methods were examined.

**Table 1 - Energy determination by IC in broilers fed a wheat-SBM-meat-canola diet (25-27 d)**

	Mean	SEM	CV (%)
BW, g (25 to 27d average)	1286	19.3	6.01
Average daily gain (ADG), g	97.8	11.2	11.5
FCR	1.337	0.031	8.1
ME intake, kJ/kg BW <sup>0.70</sup>	1380	21.8	5.5
HP, kJ/kg BW <sup>0.70</sup>	799	12.7	5.5
RQ	1.02	0.01	4.2
FHP kJ/kg BW <sup>0.70</sup> (28 d)	578	7.2	4.6
ME, kJ/g feed	12.9	0.10	3.2
NE, kJ/g feed	9.92	0.11	3.7
NE:ME	76.9	0.01	3.7

Results from the first experiment (Table 1) showed high variation in ADG between chambers but low variation in determination of AME, HP and NE. Based on this variation, it is expected that four replicates would be sufficient to detect an 8% difference in NE between treatments at the 5% level of significance (Aaron and Hays, 2004). In the second experiment, AME, HP and NE as measured by IC vs CS (12.7 vs 12.6 kJ/g feed; 913 vs 887 kJ/kg BW<sup>0.70</sup>; and 8.82 vs 9.47 kJ/g feed, respectively) were not significantly different ( $P > 0.05$ ). The results indicate that AME, HP, and NE can be accurately determined by IC. Furthermore, FHP can be determined using the same birds in the IC but not in the CS method.

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## EFFECT OF VARYING DIETARY METABOLISABLE ENERGY AND PROTEIN ON PERFORMANCE OF BROILER CHICKENS

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### Summary

The objective of this study was to delineate the effects of varying dietary metabolisable energy (ME) and crude protein (CP) on growth performance and carcass traits of broilers from 0 to 42 days of age. A total of 450 day-old Cobb male broilers were randomly distributed to nine dietary treatments consisting of 3 levels (high, medium, and low) of ME and CP. At 42 days of age, there was a significant interaction ( $P = 0.001$ ) between ME and CP on body weight gain (BWG) and feed intake (FI) but not for feed conversion ratio (FCR). Birds fed low ME had significantly ( $P = 0.002$ ) higher FCR compared with feeding medium and high ME; however, there was no significant effect of reducing dietary CP on FCR. Carcass traits including ready to cook yield (RTC) and abdominal fat (AF) were significantly reduced as dietary ME was reduced, but breast weight (BrW) was not affected. Reducing dietary CP did not affect RTC and BrW but AF increased ( $P = 0.032$ ) in birds fed low CP relative to those fed high CP. In conclusion, dietary energy can be reduced without negatively affecting growth or carcass performance when feeding low CP diets balanced with supplemental amino acids. This reduction in dietary energy ensures a better balance between protein and energy leading to reduced abdominal fat and improved overall carcass quality.

### I. INTRODUCTION

Energy and protein are quantitatively and qualitatively the most important nutrients in a feed. Optimising the dietary concentration of energy and protein (amino acids) is essential to maximise production and to minimise diet costs. Dietary energy in excess of maintenance and production requirements of broilers would lead to increased fat deposition and reduced carcass quality with added production cost. Bertechini (1987) reported improved body weight gain (BWG) and feed conversion ratio (FCR) of broilers (29-56 days of age) fed diets with increasing nitrogen-corrected apparent metabolisable energy (AMEn) from 11.72 to 12.55 MJ/kg however it was not clear that the improvement in the BWG was due to increased protein or fat deposition. Oliveira Neto et al. (1999) showed similar responses when dietary energy was increased from 12.55 to 13.82 MJ AMEn/kg in 42 day old broilers but the optimal protein deposition was observed at 13.00 MJ/kg with no further improvements with increasing AMEn. This study suggests that the dietary energy above what is required for maximum protein accretion would lead to fat deposition.

Animals, including poultry have requirements for specific amino acids rather than protein *per se*. With supplemental amino acids now commercially readily-available, there is more opportunity to reduce the dietary CP levels while maintaining the performance and production. Feeding low protein diets balanced with supplemental amino acids will lead to increased nitrogen utilisation while reducing nitrogen excretion. In addition, these types of diets can reduce the need for dietary energy required to break down dietary intact protein and for excretion of the excesses of amino acids. With the increasing prices of the protein feedstuffs and also the pressure on the environmental sustainability there is a great opportunity to minimise excesses of dietary energy and protein. Therefore, the objective of this experiment was to evaluate the effect of reducing dietary metabolisable energy (ME) and crude protein (CP) levels on performance of commercial broilers.

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## II. MATERIALS AND METHODS

A total of 450 1-day-old Cobb male broiler chicks were randomly distributed to nine treatments. Each treatment was replicated with 10 pens of 5 birds each in wire floored stainless steel battery brooders in an open sided poultry house. The trial was conducted between May and July with minimum and maximum recorded temperature and humidity of 27-39C and 24-65%, respectively. The nine treatments were based mainly on corn-soybean meal and consisted of 3 levels of ME in combination with 3 levels of dietary CP. They were fed to broiler chickens from 0 to 42 days of age. Diets were formulated to contain minimum amino acid concentrations based on ideal protein for starter (0 - 11 days), grower (12 - 28 days) and finisher (29 - 42 days) phases according to AMINOChick<sup>®</sup> 2.0 with the exception of the Ile:Lys ratio, which was reduced marginally by 3% as a test of the recommendation of Ile:Lys ratio. The high CP diet (HCP) was supplemented with DL-Methionine (DL-M), L-Lysine-HCl (L-Lys) and L-Threonine (L-Thr) to reflect current industry practice. Then, the dietary CP level was reduced by 5 and 10% of HCP while maintaining the minimum amino acid concentrations. As a result, the medium CP diet (MCP) was supplemented with DL-M, L-Lys, L-Thr and L-Valine (L-Val), and the low CP diet (LCP) was supplemented with DL-M, L-Lys, L-Thr, L-Val and L-Isoleucine (L-Ile). Dietary ME levels of 12.34, 12.76 and 13.18 MJ/kg were considered as high ME (HME) during starter, grower, and finisher phases, respectively. The ME concentrations were then reduced by 0.42 and 0.84 MJ/kg to create the medium ME (MME) and low ME (LME) diets. Dietary nutrient concentrations are provided in Table 1. Feed and water were provided *ad libitum*.

**Table 1 - Calculated nutrient concentrations of diets, as-is basis**

	ME MJ/kg	CP, g/kg			Minimum dietary standardised ileal digestible (SID) amino acid contents , g/kg							
		HCP	MCP	LCP	Lys	M+C	Thr	Try	Arg	Ile	Leu	Val
Starter	12.34											
0-11 d	11.92 11.51	252	239	226	13.0	9.4	8.3	2.3	13.9	8.6	16.8	10.3
Grower	12.76											
12-28 d	12.34 11.92	210	200	189	10.8	8.1	7.0	1.9	11.3	7.3	14.5	8.6
Finisher	13.18											
29-42 d	12.76 12.34	196	187	177	9.9	7.6	6.5	1.7	10.5	6.8	13.4	8.0

Body weight (BW) and feed intake (FI) were recorded at 11, 21, 28, 35 and 42 d of age, and BWG and FCR were calculated. At 42 d of age, one bird from each pen weighing close to the average body weight ( $\pm 5\%$ ) of the respective pen was selected and removed from feed for 12 h to record BW loss during pre-slaughter holding period. The birds were slaughtered by cervical dislocation to measure carcass traits. Ready to cook yield (RTC; including giblet), weight of breast (BrW) and abdominal fat (AF) were recorded and expressed as g per kg pre-slaughter live BW of the respective bird. Data were subjected to two-way factorial analyses following the completely randomised design (Snedecor and Cochran, 1980) to determine the interaction and independent effects of ME and CP. Treatment means were separated using Duncan's multiple range test (Duncan, 1955). Treatment differences were considered significant when  $P < 0.05$ .

## III. RESULTS AND DISCUSSIONS

The effects of dietary ME and CP on growth performance and carcass traits are given in Table 2. At 42 days of age, interaction between dietary ME and CP significantly influenced BWG and FI. In the birds fed HME, reducing the dietary CP balanced with supplemental amino acids did not influence the BWG. However, BWG in birds fed HCP diets was significantly higher compared with those fed MCP or LCP diets at MME. Among the birds fed LME, highest BWG was observed in groups fed MCP compared with those fed LCP.

**Table 2 - Effects of varying dietary metabolisable energy (ME) and crude protein (CP) on growth performance and carcass traits of broilers at 42 days of age<sup>1</sup>**

ME	CP	BWG, g	FI, g/bird	FCR	RTC, g	BrW, g	AF, g
HME	HCP	1902 <sup>abcd</sup>	3021 <sup>abc</sup>	1.59	803.7	221.2	13.70
	MCP	1920 <sup>abc</sup>	2924 <sup>bc</sup>	1.52	764.5	212.7	15.51
	LCP	1923 <sup>abc</sup>	3027 <sup>abc</sup>	1.57	769.2	215.3	17.26
MME	HCP	2021 <sup>a</sup>	3135 <sup>ab</sup>	1.55	764.8	206.1	9.89
	MCP	1785 <sup>cd</sup>	2798 <sup>c</sup>	1.57	759.0	216.9	13.2
	LCP	1859 <sup>bcd</sup>	2944 <sup>bc</sup>	1.58	760.7	217.6	11.75
LME	HCP	1857 <sup>bcd</sup>	2949 <sup>bc</sup>	1.59	749.0	213.6	8.52
	MCP	1983 <sup>ab</sup>	3199 <sup>a</sup>	1.61	749.5	215.7	6.57
	LCP	1773 <sup>d</sup>	2875 <sup>c</sup>	1.62	756.3	210.7	12.40
<b>Main effects</b>							
ME	HME	1915	2990	1.56 <sup>b</sup>	779.0 <sup>a</sup>	216.4	15.49 <sup>a</sup>
	MME	1889	2959	1.57 <sup>b</sup>	761.5 <sup>b</sup>	213.5	11.62 <sup>b</sup>
	LME	1871	3008	1.61 <sup>a</sup>	751.6 <sup>b</sup>	213.4	9.16 <sup>b</sup>
CP	HCP	1927	3035	1.58	772.5	213.6	10.69 <sup>b</sup>
	MCP	1896	2974	1.57	757.7	215.4	11.77 <sup>ab</sup>
	LCP	1852	2949	1.59	762.1	214.5	13.8 <sup>a</sup>
SEM		14.9	24.3	0.006	2.76	2.04	0.50
<b>P value</b>							
ME		0.483	0.710	0.002	0.001	0.797	0.001
CP		0.124	0.332	0.185	0.097	0.958	0.032
ME x CP		0.001	0.001	0.077	0.082	0.485	0.141

<sup>1</sup>BWG=body weight gain; FI=feed intake; FCR=feed conversion ratio; RTC = ready to cook; BrW = deboned breast weight; and AF = abdominal fat pad HME=high ME; MME=medium ME; LME=low ME; HCP=high CP; MCP=medium CP; LCP=low CP.  
<sup>a,b,c,d</sup> Means having common superscripts in a column do not vary significantly ( $P < 0.05$ ).

There was no main effect of reducing CP on BWG, FI and FCR whereas reducing dietary ME increased FCR in broilers fed LME relative to those fed HME or MME (Table 2). Higher FCR ( $P = 0.002$ ) in the groups fed LME may indicate marginal deficiency of dietary energy to optimise the live weight gain. No response to the variation in dietary CP level on the feed utilisation indicates that CP levels can be reduced without sacrificing performance of broilers by balancing the amino acid profile via use of supplemental amino acids.

The interaction between the levels of ME and CP did not influence ( $P > 0.05$ ) carcass parameters (Table 2). However, weights of RTC and AF were decreased by reducing dietary ME from HME to MME or LME level while no differences ( $P > 0.05$ ) were observed between MME and LME. There was no effect of reducing dietary ME on BrW. Thus a reduction in RTC as a result of reducing the dietary ME was mainly due to a decrease in AF indicating energy provided to birds fed the HME diet was above the requirement for maximum protein deposition. Similarly, Oliveira Neto et al. (1999) showed that live BW was maximised by feeding high energy diets but the protein deposition was maximised at an

intermediate level of dietary energy. Additionally, Jiang et al. (2006) reported no difference in breast meat yield despite an increase in BWG of birds fed diets containing relatively high ME (12.13 to 12.76 MJ/kg) than those fed low ME (11.72 to 12.34 MJ/kg). Previous research from our lab has also shown that 90% of the breeder recommended dietary energy was sufficient for the optimal protein deposition as indicated by higher breast meat yield (Evonik, 2005).

These studies indicate that the higher BWG due to the feeding high dietary ME is mainly attributable to higher fat deposition. Similar to previous studies, the present study demonstrates that the requirement of dietary ME to maximise protein deposition is lower than to maximise live weight gain. This is especially true in broilers fed low protein diets balanced with supplemental amino acids possibly due to a reduced energy requirement to breakdown intact proteins and deamination of amino acids. In the current study, RTC and BrW were not affected ( $P > 0.05$ ) by variations in CP whereas AF was progressively increased ( $P < 0.05$ ). In the LCP diets, which contained supplemental amino acids including DL-M, L-Lys, L-Thr, L-Val and L-Ile, energy that would have otherwise been needed to break down intact protein and to excrete excess amino acids was likely spared and thus ultimately deposited as fat. This study suggests that adoption of low CP diets should be accompanied by an adjustment in dietary energy.

Results of this study suggest that the dietary energy can be reduced without negatively affecting growth or carcass performance while feeding low protein diets balance with supplemental amino acids without sacrificing broiler performance. This reduction in dietary energy ensures a better balance between protein and energy leading to reduced abdominal fat and improved overall carcass quality.

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## DOES PELLETING IMPROVE NUTRIENT DIGESTIBILITY AND ENERGY UTILISATION IN BROILERS?

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### Summary

The true impact of pelleting on the utilisation of nutrients in broiler diets has not been clearly delineated due to the pronounced effect of pelleting on broiler performance. It appears that the better growth responses achieved by pellet feeding, compared to mash diets, are obtained at the expense of utilisation of nutrients and energy. Possible negative effects on nutrient utilisation are compensated by increased feed intake and the resultant high nutrient intake in pellet-fed broilers.

### I. INTRODUCTION

It is well accepted that offering feed to broilers in pellet form enhances the economics of production by enhancing growth performance and feed efficiency. Although it is generally believed that pelleting improves nutrient digestibility, there is little published evidence to support this belief. On the contrary, some reports suggest that pelleting may decrease nutrient utilisation under some conditions. Limited published data available on the effect of pelleting on nutrient utilisation is reviewed in this paper.

### II. EFFECTS ON STARCH DIGESTIBILITY

Since gelatinisation increases the access of starch to enzymatic degradation, starch digestibility may be expected to be increased in pelleted diets. However, the extent of starch gelatinisation that occurs during pelleting is generally small and is probably of modest importance (Svihus et al., 2004; Zimonja et al., 2008). Svihus (2001) reported that high starch digestibility usually coincides with mash feeding, whereas low starch digestibility is associated with feeding cold-pelleted diets. In this study, apparent ileal starch digestibility coefficients of cold-pelleted diets containing high levels of four varieties of wheat was of less than 0.83. It was suggested that starch digestibility of wheat-based diets is negatively correlated to feed intake. Based on a significant increase in starch digestibility when a wheat-based diet was diluted with cellulose, Svihus and Hetland (2001) speculated that an overload of wheat starch in the small intestine, due to feed over-consumption, is the major cause of low starch digestibility. Significant decreases in starch digestibility of wheat-based diets, from 0.959 in mash diets to 0.842 and 0.834 in diets pelleted at 60 and 90 °C, respectively, in broilers due to pelleting have been reported by Abdollahi et al. (2011). Selle et al. (2012) found that pelleting had no effect on the ileal starch digestibility of sorghum-based diets (Table 1). These limited data suggest that pelleting is not beneficial for starch digestibility and may even decrease starch digestibility as a result of high feed intake, at least in wheat-based diets.

### III. EFFECTS ON PROTEIN DIGESTIBILITY

Limited feed-processing experiments have been conducted to assess whether proteins may become more digestible as a consequence of feed processing. Carré et al. (1991) did not find any increase in protein digestibility in broiler chickens as a result of pelleting. Duodu et al.

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(2002) reported that cooking (10 min at approximately 95 °C) reduced *in vitro* protein digestibility of sorghum, but not of maize. The poorer protein digestibility of cooked sorghum was explained by the formation of enzyme-resistant disulphide-bonded oligomeric proteins that occur to a greater extent in sorghum than in maize. It was also suggested that pericarp components, germ, endosperm cell walls, and gelatinised starch could be possible factors limiting protein digestibility in sorghum. Selle et al. (2010) suggested that steam-pelleting of sorghum-based diets at temperatures above 90 °C may provide sufficient ‘moist-heat’ to form disulphide linkages in kafirin and, compromise protein and starch digestibility in sorghum. Abdollahi et al. (2011) reported lower nitrogen digestibility in wheat-based pellet diets (0.836 and 0.822 in diets pelleted at 60 and 90 °C, respectively) compared to mash diets (0.847) (Table 1). Thus, it is reasonable to assume even if pelleting influences protein digestibility, it may not be a positive effect. However, Selle et al. (2012) showed that pelleting of sorghum-based diets had no effect on nitrogen digestibility, but depressed nitrogen retention coefficient in broilers by an average of 9.1% from 0.606 in mash diets to 0.551 in intact pellets (Table 1). The depression in nitrogen retention was partly attributed to the pelleting inducing the formation of disulfide linkages. These researchers suggested further studies to investigate the possibility of the compromising effect of pelleting on the utilisation of protein after digestion and absorption.

#### IV. EFFECTS ON ENERGY UTILISATION

It has been reported by Svihus et al. (2004) that pelleting increased the apparent metabolisable energy (AME) of wheat-based diets from 11.6 to 11.8 MJ/kg. However, the increase in AME was not associated with improvements in starch digestibility. In contrast, Amerah et al. (2007) reported negative effect of pelleting on nitrogen-corrected AME (AMEn) of a wheat-based diet. In their study, pelleting reduced the AMEn of the diet from 12.5 to 11.8 MJ/kg (Table 1). Abdollahi et al. (2011) also reported a decrease in AME of wheat-based diets from 14.10 to 13.75 and 13.42 MJ/kg due to pelleting at 60 and 90 °C, respectively (Table 1).

The positive effect of pelleting on feed efficiency has been consistently observed (Jensen et al., 1962; Latshaw and Moritz, 2009) and is due partly to the reduction in feed energy used for maintenance. McKinney and Teeter (2004) reported that pelleting contributed 0.78 MJ MEn/kg diet at 100% pellets (no fines), with this value decreasing with increasing proportions of fines to pellets, but still contributing 0.32 MJ MEn/kg for 20% pellets. Skinner-Noble et al. (2005) reported a contribution of 0.63 MJ MEn/kg diet from pellets compared to a mash diet. A recent study by Latshaw and Moritz (2009) showed that broilers fed pellets had lower heat increment and utilised feed energy more efficiently for productive purposes than those fed mash.

#### V. NUTRIENT INTAKE OF PELLET-FED BIRDS

A number of factors are considered as motivations behind the pelleting of broiler feeds. Feed intake is the major factor driving body weight gain and an increased feed intake is the primary motivation for pelleting broiler diets (Svihus et al., 2004). Nutrient and energy intakes calculated from some of the previously discussed data on nutrient digestibility and energy utilisation are also presented in Table 1. In general, increased feed intake resulted in higher intakes of digestible protein, digestible starch and AME, and retained more protein in pellet-fed birds compared to mash-fed birds. It is noteworthy that the higher nutrient and energy intakes in pellet diets have been achieved while having lower nutrient digestibility and AME content than mash diets. This highlights the importance of feed intake when broilers are fed pellet diets.

In summary, the importance of pellet feeding to broilers is not questionable. However, if pelleting, while enhancing the growth performance, lowers the nutrient utilisation, then the wastage of feed nutrients will unwittingly increase. Further studies are warranted to investigate how nutrient utilisation is influenced by feed form in broilers. The negative effect of pelleting on starch digestibility in wheat-based diets is documented (Svihus, 2001), but the effects on the digestibility of protein, amino acids, fat, calcium and phosphorus in different grains have not been evaluated and merit further investigation.

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**Table 1 - Nutrient digestibility, energy utilisation and intake in broiler chickens fed mash, pellet and reground pellet diets**

Grain type	Feed form	Nutrient and energy utilisation	Value	Nutrient and energy intake <sup>1</sup>	Value	Reference
Sorghum	Mash	Ileal starch digestibility	0.897	-	-	Selle et al. (2012)
	Pellet		0.887		-	
	Reground pellet		0.890		-	
Sorghum	Mash	Ileal nitrogen digestibility	0.726	Digestible protein intake (g/bird)	333	Selle et al. (2012)
	Pellet		0.738		383	
	Reground pellet		0.737		352	
Sorghum	Mash	Nitrogen retention	0.606	Protein retained (g/bird)	278	Selle et al. (2012)
	Pellet		0.551		286	
	Reground pellet		0.550		263	
Sorghum	Mash	AMEn (MJ/kg DM)	12.53	AMEn intake (MJ/bird)	23.83	Selle et al. (2012)
	Pellet		11.78		24.61	
	Reground pellet		12.61		24.27	
Wheat	Mash	Ileal nitrogen digestibility	0.847	Digestible protein intake (g/bird)	219	Abdollahi et al. (2011)
	Pellet at 60 °C		0.836		259	
	Pellet at 90 °C		0.822		249	
Wheat	Mash	Ileal starch digestibility	0.959	Digestible starch intake (g/bird)	445	Abdollahi et al. (2011)
	Pellet at 60 °C		0.842		468	
	Pellet at 90 °C		0.834		454	
Wheat	Mash	AME (MJ/kg DM)	14.10	AME intake (MJ/bird)	14.17	Abdollahi et al. (2011)
	Pellet at 60 °C		13.75		16.73	
	Pellet at 90 °C		13.42		15.85	
Wheat	Mash	AMEn (MJ/kg DM)	12.50	AMEn intake (MJ/bird)	10.36	Amerah et al. (2007)
	Pellet		11.80		14.89	
Wheat	Cold-pelleted	Ileal starch digestibility	0.790	Digestible starch intake (g/bird)	299	Svihus and Hetland (2001)
	Reground pellet		0.950		273	

<sup>1</sup>Values have been calculated using the data from the references.

AME intake (MJ/bird, DM) = Feed intake (kg, DM) x AMEn content of the feed (MJ/kg, DM).

Digestible nutrient intake (g/bird) = Feed intake (kg) x nutrient content of the feed (g/kg) x digestibility coefficient of nutrient.

## ENZYME RESPONSE IS AFFECTED BY SUBSTRATE TYPE

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The variety of enzyme products available in the marketplace is considerable (PUBCRIS). Individual products differ markedly in enzyme composition and activity for the same target species regardless of ration (substrate)-type. This study demonstrates the effect of substrate-type on reducing sugar production from three enzyme mixtures with potential application in poultry feeds.

Three substrate types (corn:soy (CS), wheat:soy (WS) and low-soy (LS) mixtures) were prepared from grains and protein meals to reflect ingredient compositions common in poultry feeds, c. 18% crude protein and 11.7 MJ/kg AME. CS and WS mixtures contained a minimum grain content of 650g/kg. The LS mixture used (/kg) 600g grain as corn:wheat (1:2), 50g soy bean meal and 150g canola meal. Samples were ground to pass a 0.25mm screen and incubated in triplicate with xylanase alone (X, 2U/g), with a combination of neutral protease and amylase mixture (NPA, 1U/g and 0.2U/g) and with each enzyme combined (X+NPA). Reducing sugar production (RSP) was estimated at hourly intervals for 5 hours as described by Lü and Zhou (2011). Statistical analyses of absorbance values were undertaken using a GLM procedure blocked for substrate type, enzyme inclusion and incubation time (Minitab V14).

RSP as estimated from absorbance values was affected ( $P < 0.001$ ) by both substrate type and enzyme combination (Table 1). Xylanase alone produced only modest improvements in RSP (0.020) compared to NPA (0.195) and X+NPA treatments ( $0.183 \pm 0.005$ ). Response to xylanase alone was greatest for the LS substrate and showed no significant effect ( $P > 0.05$ ) on the CS mixture. In contrast, responses to the NPA treatment were greater on the CS mixture. The X+NPA treatment also showed highest RSP response on the CS mixture.

**Table 1 - Mean Reducing Sugar production is influenced by enzyme and substrate type.**

	Corn:Soy	Non-Soy	Wheat:Soy	Enzyme (main effect)
Xylanase alone	0.000a	0.040b	0.020c	0.020a
Neutral Protease + Amylase	0.265a	0.147b	0.172b	0.195b
Xylanase + Neutral Protease + Amylase	0.214a	0.156b	0.178b	0.183b
Substrate (main effect)	0.160a	0.114b	0.124b	0.005

Pooled SEM: main effects, 0.005; substrate x enzyme terms, 0.008.

Inclusion of neutral protease and amylase increased the degradation of each ration-type over the use of xylanase alone, as estimated by RSP. These results suggest that neutral protease and amylase may be useful additions to enzyme formulations intended to stimulate nutrient release from ingredients commonly used in poultry feeds. However, the RSP technique does not reflect changes in the substrate that impact on nutrient absorption or gut health (Jia et al., 2009) which may underestimate the value of xylanase in formulations for younger birds.

PUBCRIS database; <http://www.apvma.gov.au>

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**CARBOHYDRASES IMPROVE PERFORMANCE OF BROILERS FED BOTH  
NUTRITIONALLY ADEQUATE AND DOWNSPEC WHEAT DIETS**

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Although the main non-starch polysaccharides (NSPs) in wheat are arabinoxylans, considerable amounts of  $\beta$ -glucan and cellulose are also present. In soybean meal (SBM) and canola meal, the major polysaccharides are pectins and cellulose. Since more plant ingredients containing different forms of NSPs are being used for diet formulations, an enzyme combination with additional activities each differing in substrate preference may further enhance nutrient utilization. A study was conducted to investigate the use of Rovabio Excel LC (liquid), an enzyme complex containing 19 carbohydrase activities on broilers fed a nutritionally adequate or downspec diet. The positive control (PC) was a nutrient adequate wheat-SBM based diet with its specifications varying according to starter, grower and finisher stages. Nutrient levels in the negative control (NC) were 0.366 MJ/kg ME and 1.5% digestible amino acids (DAA) lower compared to PC. A total of 1008 day-old Ross broiler chicks were randomly allocated to four treatments with seven replicates of 36 birds each and reared until d 42. It was a 2 x 2 factorial design where PC and NC either contained Rovabio Excel (200ml/tonne) or did not. On d 27 and d 42, ileal digesta samples from each pen were collected for the analysis of nutrient digestibility and viscosity.

Birds fed the downspec diet had 2.3% poorer FCR ( $P < 0.01$ ) at the end of the experiment. Inclusion of Rovabio Excel improved body weight by 3.2% ( $P < 0.05$ ) and FCR by 3.1% ( $P < 0.01$ ) from 0 to 42 days. Ileal viscosity was reduced in birds consuming feed treated with Rovabio Excel on day 42 ( $P < 0.05$ ) with a trend for reduced viscosity from Rovabio Excel on day 27 ( $P = 0.07$ ). From 0 to 27 days, Rovabio Excel significantly improved apparent ileal digestibility of crude protein ( $P < 0.01$ ) regardless of the diet type. The addition of Rovabio Excel possibly improved protein digestion by depolymerizing viscous NSPs thus allowing endogenous proteases to better access the substrate. The breakdown of physical entrapment of protein in plant cell wall architecture may be another essential mechanism through which the enzyme complex exerted its action. Overall, the inclusion of a multi-carbohydrase enzyme complex reduced the ileal digesta viscosity and enhanced the crude protein digestibility, leading to a complete offset of the performance loss of birds fed the downspec diet.

**Table 1 - Performance of broilers fed Rovabio Excel from 0 to 42 days**

Treatment means	Weight kg/bird	Intake kg/bird	FCR	Livability %
PC	3.246	5.227	1.633 <sup>b</sup>	92.6
PC + RB XL	3.343	5.212	1.580 <sup>a</sup>	93.1
NC	3.235	5.320	1.669 <sup>c</sup>	96.6
NC + RBXL	3.345	5.336	1.617 <sup>b</sup>	93.1
Main effects and interactions				
Diet	NS	NS	< 0.01	NS
RBXL	<0.05	NS	< 0.01	NS
Diet * RBXL	NS	NS	NS	NS

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# INTERACTIVE EFFECTS OF EXOGENOUS ENZYMES AND DIRECT FED MICROBIALS ON DIGESTIBILITY, INTESTINAL INTEGRITY, PATHOGEN COLONIZATION AND PERFORMANCE IN FIRST-CYCLE LAYING HENS

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## Summary

A total of 288 Hy-Line W36 laying hens were utilized to understand the interactive effects of an exogenous enzyme (EE) blend containing xylanase, amylase, and protease (XAP) and direct-fed microbial (DFM) supplementation on energy digestibility, gut integrity, pathogen colonization and performance. Corn-soy bean meal-dried distiller's grain with solubles-based diets consisting of a positive control with 12.18MJ/kg (PC), negative control with 11.76MJ/kg (NC), NC + EE, NC + EE + DFM, were fed to 25-40 wk old laying hens. There were no significant differences in feed intake or hen day egg production, hen body weight, egg weight, egg mass, and egg characteristics over the entire experimental period. The combination of EE and DFM increased ( $P < 0.05$ ) nitrogen corrected apparent metabolizable energy compared to NC fed birds at wk 38 and wk 40 of hen age. Hens fed the various diets did not differ in ileal villus height, crypt depth and villus height: crypt depth ratio measured at the completion of the experimental period. D-glucose and L-lysine active transport was significantly increased with EE supplementation and addition of DFM increased ( $P < 0.01$ ) ileal mucin mRNA expression. Apparent endotoxin permeability in the colon ( $P < 0.01$ ) and *Campylobacter spp.* colonization ( $P = 0.05$ ) were significantly reduced with DFM supplementation while trans-epithelial electrical resistance was increased ( $P < 0.01$ ). These results indicate that supplementation of the EE and DFM combination in corn-SBM-DDGS based diets increased energy digestibility and enhanced gut integrity and possibly reduced pathogen load in first-cycle laying hens.

## I. INTRODUCTION

Dietary energy has become an expensive component of poultry feed and thus a major factor in determining the cost of production (Pardue, 2010). As laying hen feed constitutes nearly 75 % of the cost of egg production (Donohue and Cunningham, 2009), efficient and complete utilization of dietary energy is essential. Another concern for poultry producers is the energetic cost involved in maintaining healthy poultry flocks (Klasing, 2007; Applegate et al., 2010). Sub-clinical pathogenic challenges increase energy expenditure towards perpetual activation of acute phase immune response (Applegate et al., 2010). Thus, evaluation of alternative methods to increase productive energy partitioning by means of improving gut integrity and controlling sub-clinical pathogenic challenges is necessary. In the context of releasing dietary energy and increasing gut integrity, exogenous enzymes (EE) and direct-fed microbials (DFM) have been used as viable options. Although the effects of both EE and DFM on poultry species have been researched previously (independent of each other), their interactions have not been characterized to any great extent. An experiment was designed

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with the hypothesis that supplementation with an EE containing xylanase, amylase, and protease and a DFM containing 3 different proprietary *Bacillus* strains in corn-SBM-DDGS-based diet to first-cycle laying hens will increase energy utilization as well as gut integrity. The objective of this experiment was to determine the effects of the combination of EE and DFM on energy digestibility, gut integrity, pathogen colonization and performance in first-cycle laying hens.

## II. MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University before the initiation of the experiment. A total of 288 Hy-Line W36 laying hens (Hy-Line International, Dallas Center, IA) were fed a standard diet from 22 to 24 wk of age to allow the hens to adapt to the experimental facility. The experiment was 16 wk long, from 25 to 40 wk of hen age, with four treatment groups; positive control (PC), negative control (NC), NC with EE (NE), and NC with EE and DFM (NED). Experimental diets were corn-SBM-DDGS-based and the PC diet was formulated to meet or exceed breeder recommendations of 12.18MJ/kg. The NC diets were similar to the PC with the exception of a 0.42MJ/kg reduction in ME to induce a minimal energy stress on the hens. The EE and the DFM were added at a dose rate of 0.0375 % and 0.05 %, respectively and all treatment diets were supplemented with 300 FTU phytase enzyme (Danisco Animal Nutrition, Marlborough, UK). An experimental unit (EU) was defined as three adjacent cages of three hens each (439 cm<sup>2</sup> per hen), resulting in eight EU for each of the four treatment groups. Hens were randomly allocated to EU at the start of experiment.

Feed intake was determined weekly and hen-day egg production (HDEP) was recorded daily. Body weight (BW), egg weight, egg mass, and egg quality such as egg solids (constituting albumen and yolk), shell weight, albumen weight, yolk weight, and Haugh unit were determined every four weeks. Excreta samples were collected for the last 48 hr of wk 36, 38 and 40 of hen age to determine nitrogen corrected apparent metabolizable energy (AMEn). Colon contents were collected from all hens fed NE and NED diets on hen age wk 40 to quantify colonization levels of *Campylobacter spp.* (*C. coli*, *C. jejuni* and *C. lari*). On the last day of the experiment, ileal samples were collected from one hen per EU from all the dietary groups. Ileal morphology, nutrient transport, mucin (MUC2) mRNA expression and trans-epithelial electrical resistance (TER) were determined from the ileal tissue samples. Apparent endotoxin permeability co-efficient (*Papp*) and TER were determined from the colon samples collected from one randomly selected hen per EU from NE and NED groups. Data were analyzed by MIXED procedure of SAS with protected least square means to separate means and the significance was ascertained at  $P \leq 0.05$ .

## III. RESULTS AND DISCUSSION

The feed intake, HDEP, BW, FCR in terms of feed:egg mass, egg weight, egg mass, egg solids and egg characteristics between dietary groups did not differ statistically ( $P > 0.05$ ). The AMEn was significantly different between dietary groups during wk 38 ( $P < 0.01$ ) and wk 40 ( $P < 0.05$ ), while a trend was observed in wk 36 ( $P < 0.10$ ). Hens fed PC, NE and NED diets had higher AMEn while NC fed hens had lower AMEn. The higher AMEn for hens fed diets supplemented with EE and DFM may indicate that the combination increased energy digestibility in a partially additive manner and DFM increased the consistency of response in AMEn to EE. This is in agreement with previous research in which EE increased energy digestibility by degrading dietary fiber (Pirgozliev et al., 2010) while DFM altered intestinal

microbiota to favor an increased activity of intestinal enzymes and energy digestibility (Dierick, 1989).

The ileal villus height, crypt depth and villus height: crypt depth ratio did not differ significantly between the dietary groups ( $P > 0.05$ ). Ileal active transport of D-glucose and L-lysine was increased for hens fed EE supplemented diets compared to hens fed either PC or NC diets ( $P < 0.01$ ). Neither DL-methionine nor L-glutamine transport was significantly different between the dietary groups ( $P > 0.05$ ). Increased glucose uptake has been found to stimulate trans-epithelial sodium which in turn increases the uptake of sodium dependent amino acids like lysine (Lind et al., 1980). Intestinal mucins act as a selective barrier protecting the cell by virtue of their negatively charged filamentous protruding structure, preventing bacterial translocation (Van Klinken et al., 1995; Smirnov et al., 2005). Supplementation with DFM increased the mRNA expression of ileal mucin gene (MUC2) compared to hens fed other dietary groups ( $P < 0.01$ ), indicating the DFM played a role in increasing luminal mucin content.

**Table 1: Effects of dietary supplementation of exogenous enzymes (EE) and direct fed microbials (DFM) on energy digestibility, ileal nutrient transport, and intestinal integrity in first-cycle laying hens fed corn-soy-DDGS based diets from hen age wk 25 to 40.**

Parameters	PC <sup>1</sup>	NC <sup>2</sup>	NE <sup>3</sup>	NED <sup>4</sup>	SEM <sup>5</sup>	<i>P</i> -value
AMEn wk 36 (MJ/kg)	14.10	13.94	14.44	14.40	0.16	0.09
AMEn wk 38 (MJ/kg)	14.69 <sup>a</sup>	13.59 <sup>c</sup>	14.09 <sup>b</sup>	14.17 <sup>b</sup>	0.15	$\leq 0.01$
AMEn wk 40 (MJ/kg)	14.55 <sup>a</sup>	14.04 <sup>c</sup>	14.09 <sup>bc</sup>	14.46 <sup>ab</sup>	0.14	0.03
Glucose Transport ( $\mu\text{amp}/\text{cm}^2$ )	1.78 <sup>b</sup>	1.46 <sup>b</sup>	3.85 <sup>a</sup>	4.77 <sup>a</sup>	1.63	$\leq 0.01$
Lysine Transport ( $\mu\text{amp}/\text{cm}^2$ )	2.25 <sup>b</sup>	2.33 <sup>b</sup>	4.59 <sup>a</sup>	4.29 <sup>a</sup>	0.86	$\leq 0.01$
MUC2 mRNA <sup>6</sup>	1.53 <sup>b</sup>	1.51 <sup>b</sup>	1.46 <sup>b</sup>	2.01 <sup>a</sup>	0.27	$\leq 0.01$
TER <sup>7</sup> ( $\Omega/\text{cm}^2$ )	-	-	818 <sup>b</sup>	956 <sup>a</sup>	28.69	$\leq 0.01$
<i>Papp</i> <sup>8</sup> ( $\mu\text{g}/\text{ml}\cdot\text{min}\cdot\text{cm}^2$ )	-	-	2.70 <sup>a</sup>	1.58 <sup>b</sup>	0.47	$\leq 0.01$
<i>Campylobacter coli</i> ( $\times 10^6\text{cfu}$ )	-	-	6.64 <sup>a</sup>	4.44 <sup>b</sup>	0.62	0.05
<i>Campylobacter jejuni</i> ( $\times 10^6\text{cfu}$ )	-	-	0.53 <sup>a</sup>	0.29 <sup>b</sup>	0.14	0.05

<sup>1</sup>Positive Control; <sup>2</sup>Negative Control; <sup>3</sup>NC + Enzymes; <sup>4</sup>NC + Enzymes + Direct fed microbials

<sup>5</sup>Pooled standard error of mean

<sup>6</sup>Ileal mucin gene (MUC 2) mRNA expression values are reported in arbitrary units relative to 28S gene.

<sup>7</sup>Colon trans-epithelial electrical resistance

<sup>8</sup>Colon apparent permeability co-efficient (*Papp*) for endotoxins

Intestinal tight junctions (TJ) are major paracellular barriers connecting individual intestinal epithelial and endothelial cells to each other (Denker and Nigam, 1998). When ion conductance across the TJ is far greater than across apical and basolateral membranes, a condition known as leaky epithelia (Albin et al., 2007), it reduces the integrity of TJ (Shen et al., 2011) leading to lowered trans-epithelial electrical resistance (TER). In the present experiment, the ileal TER did not differ ( $P > 0.05$ ) between dietary groups, but supplementation of DFM increased ( $P < 0.01$ ) the colon TER. Endotoxins, commonly known as lipopolysaccharides (LPS), are cell wall components of Gram-negative bacteria that increase leaky epithelial conditions, resulting in bacterial translocation across epithelia (Tomita et al., 2004). The apparent permeability co-efficient for *E. coli* LPS transport across colon epithelia was reduced for hens which received DFM supplemented diet ( $P < 0.01$ ). This observation also validates the increased TER for colon epithelium with the DFM fed group, indicating increased TJ barrier function contributing to reduced endotoxin transport (Albin et al., 2007; Shen et al., 2011). Colonization levels of *C.coli* ( $P = 0.05$ ), *C.jejuni* ( $P = 0.05$ ) were significantly decreased with DFM supplementation while there were no detectable

levels of *C. lari* in either NE or NED fed hens. Reduction in the colonization of *Campylobacter spp.* has been linked to reduced luminal pH by DFM organisms (Fooks and Gibson, 2002). In summary, supplementation of EE increased energy digestibility and ileal nutrient transport while DFM increased ileal mucin mRNA expression, colon TER and reduced endotoxin permeability and *Campylobacter spp.* colonization.

#### IV. CONCLUSIONS

Reducing dietary energy by 0.42MJ/kg in NC diet did not alter feed intake, body weight or hen performance parameters. The combination of EE and DFM increased AMEn and DFM appeared to increase the consistency of response in AMEn to EE in laying hen diets. Supplementation of EE increased ileal nutrient absorption, supplementation of DFM increased intestinal epithelial barrier function and integrity while reducing *Campylobacter spp.* colonization compared to hens fed control diets. Addition of EE and DFM in corn-SBM-DDGS based diets will improve gut health and energy utilization of first-cycle laying hens.

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**METABOLIZABLE ENERGY OF FEEDSTUFFS IN MEAT-TYPE GROWING DUCKS  
DETERMINED BY TOTAL OR PARTIAL EXCRETA COLLECTION METHODS**

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Although duck production is increasingly important, diet formulation for ducks normally uses the metabolizable energy values obtained from chickens because of limited information on nutrient requirement for ducks (Adeola et al., 1997). This study aims to determine the apparent metabolizable energy (AME) values of common feed ingredients for meat-type growing ducks using the total or partial excreta collection methods. In this experiment, a total of 275 21-day-old Cherry Valley ducks was used. Experimental design, diets, feeding and total excreta collection were as described previously (Hoai et al., 2011). Celite was added as source of acid insoluble ash (AIA) at the rate of 20 g/kg. AIA measurement and digestibility calculation were conducted as described by Ravindran et al. (1999). The experiment was conducted in 5 replicates (pens) of 5 ducks each per ingredient. The experimental diets were fed for 7 days and the excreta from each pen were collected over the last 4 days for AME determinations.

**Table 1 - Apparent metabolizable energy (AME) of feedstuffs for ducks determined by total or partial excreta collection (MJ/kg DM)**

Ingredients	Total collection	Partial collection	Difference	P
Maize	15.15 ± 0.41	15.45 ± 0.57	0.29	0.379
Broken rice	15.14 ± 0.61	15.51 ± 0.93	0.36	0.483
Rice bran	12.87 ± 0.91	12.13 ± 1.33	-0.74	0.334
Wheat bran	12.74 ± 2.25	12.73 ± 1.28	-0.01	0.991
Cassava meal	13.80 ± 0.83	14.40 ± 1.56	0.59	0.474
Soybean meal with hull	12.28 ± 0.55	11.72 ± 1.09	-0.56	0.333
Fishmeal 550 g CP/kg	13.09 ± 0.42	12.44 ± 0.78	-0.65	0.140
Fishmeal 600 g CP/kg	13.31 ± 0.53	12.26 ± 1.09	-1.05	0.088

*Mean ± SD*

The results in Table 1 show that there were no significant differences in AME values of feed ingredients determined by the total or partial excreta collection methods. The differences in AME values determined by these two methods ranged from 0 (in wheat bran) to 1 MJ/kg (in fishmeal 600 g CP/kg). AME values determined by the partial excreta collection method were higher than those determined by the total collection method in ingredients with low ash content (maize, broken rice and cassava meal). In contrast, for ingredients with high ash content (rice bran, soybean meal and fish meals), AME values were higher when determined by the total excreta collection method.

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RELATIONSHIP BETWEEN THE APPARENT METABOLISABLE ENERGY AND  
GRAIN CHARACTERISTICS OF MAIZE

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Maize is an important energy source in poultry feed formulations in New Zealand. No published data are available on the variability in apparent metabolisable energy (AME) content of maize grown in New Zealand. A total of 24 samples, representing 8 cultivars, were collected from different growing locations in the North Island. The samples were subjected to the measurement of various grain characteristics (bulk density [BD], 50g kernel number [KN], protein content, oil content and stentvert hardness [SH]). The AME was assayed using the rapid method of Farrell (1978) with two modifications. Male broiler chickens (28-day old) were used instead of adult cockerels and assay diets containing 965 g/kg wheat and 35 g/kg vitamin and mineral supplements were used instead of substituting 500 g/kg of the cereal into a sorghum-basal diet. Each assay diet, in mash form, was offered ad libitum to four replicates (6 birds/ replicate) per sample. The correlation coefficients of AME with the grain characteristics were calculated. As summarised in the Table below, the AME of maize samples showed considerable variation, ranging between 11.86 and 14.81 MJ/kg dry matter.

Cultivar	Number of samples	Average AME (MJ/kg dry matter)	Range (MJ/kg dry matter)
1	4	12.97	12.82 – 13.20
2	4	13.58	13.12 – 14.22
3	2	13.51	13.32 – 13.69
4	2	13.70	13.58 – 13.81
5	5	14.10	13.31 – 14.81
6	2	13.61	13.60 – 13.61
7	2	13.59	13.58 – 13.61
8	3	12.91	11.86 – 13.65

The variation determined in grain characteristics were: BD, 52.8 to 80.9 kg/hl; KN, 134 to 258 kernels; protein content, 76 to 113 g/kg; SH, 2.9 to 9.6 kj; and oil content, 25 to 42 g/kg. The AME was not correlated with protein content ( $r=-0.01$ ;  $P=0.98$ ) and poorly correlated with BD ( $r=0.37$ ;  $P=0.11$ ). Significant correlations were observed with KN ( $r=-0.49$ ;  $P<0.05$ ), SH ( $r=0.58$ ;  $P<0.01$ ) and oil content ( $r=0.56$ ;  $P<0.01$ ) and these grain characteristics may be useful predictors of the AME of maize.

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## INFLUENCE OF EXOGENOUS XYLANASE ON PERFORMANCE AND NUTRIENT DIGESTIBILITY OF BROILERS FED WHEAT-BASED DIETS.

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### Summary

Five dietary treatments were used in a 49-day broiler trial to assess the effect of xylanase on performance and nutrient digestibility. Treatments included an industry-standard control diet and four further diets where xylanase was introduced in a diet with or without a metabolisable energy density dilution (0.25MJ/kg) either from day 1 or day 28. The addition of xylanase with no associated energy dilution from day 1 resulted in the greatest benefit on performance, with significant improvements in weight gain compared with the industry standard control diet at day 28 and at day 49. Addition of xylanase from day 28 (with no energy dilution) resulted in a significant improvement in FCR between 1-49 d. Energy dilution with the inclusion of xylanase from day 1 or day 28 resulted in performance comparable to the xylanase-free control diet with no energy dilution. In no case was feed intake affected.

Addition of xylanase improved ileal digestible energy values at 28 days by around 0.35MJ/kg and ileal nitrogen digestibility coefficients by around 3%. At 49 days of age xylanase improved ileal digestible energy values by around 0.9MJ/kg and ileal nitrogen digestibility coefficients by around 4.6%.

It can be concluded that supplemental xylanase is effective in improving performance and in broilers fed wheat-based diets, by improving digestive efficiency. When added to an industry control diet, performance was dramatically improved and when added to an energy reduced diet (-0.25MJ/kg) performance was equal to the standard diet.

### I. INTRODUCTION

Exogenous xylanase has been routinely added to non-ruminant diets for more than 20 years. In diets based on cereals such as wheat and rye, the mechanism of action is likely to centre on viscosity reduction and improved diffusion of nutrients in the intestine. Though this mechanism is almost certainly involved, interactions with fats and a reduction in the presence of bacteria in the small intestine following xylanase addition, may also be important. The effect of exogenous enzymes on the microbial populations in the avian hind gut has been considered for many years. The hydrolysis of structural fibre by exogenous xylanase may provide xylo-oligomers which have been shown to increase bifidobacteria populations in the caeca and FCR improvement (Courtin et al., 2008). These oligosaccharides are partially fermented in the caeca and colon yielding a range of volatile fatty acids resulting in changes in the profile of short-chain fatty acids in the distal GI tract. These fatty acids have some energy value for the host animal but perhaps more importantly reduce pH, restrain the proliferation of putrefactive microorganisms, encourage the proliferation of enterocytes and may directly mediate gastric emptying, perhaps via the same infrastructure involved in the ileal brake mechanism.

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## II. EXPERIMENTAL DETAIL

A total of 1500 Cobb 500 day-old male chicks were obtained from a commercial hatchery and randomly allocated to one of the five dietary treatments in a completely randomised design. Each treatment was replicated twelve times with twenty-five chicks per replicate pen. At 28 and 49 days of age, three birds per pen were euthanized for the purpose of digestibility measurements and the contents of the distal half of the ileum content were collected. Ileal contents were immediately frozen at -18°C prior to being freeze-dried and ground to pass a 0.5mm screen.

Diets were based on wheat and soybean meal and basal diets contained the following in the starter (1-14 days), grower (15-28 days) and finisher (29-49 days) phases; energy 12.80, 13.10, 13.40 MJ/kg; digestible Lysine (dLys) 12, 9.5, 8.6 g/kg; Ca 9.5, 8.5, 8.0 g/kg and digestible P (dP) 4.5, 3.6, 3.3 g/kg. There were five treatments; treatment 1 was a basal diet. Treatment two was identical to treatment 1 except xylanase was included from day 28 only. Treatment three was as treatment two (xylanase fed from day 28) but the diet from day 28 was energy reduced (less 0.25MJ/kg). Treatment four was identical to treatment one except xylanase was included from day 1. Treatment 5 was as treatment four (xylanase fed from day one) except the diet from day one was energy reduced (less 0.25MJ/kg). All diets were steam pelleted at 80 °C in a Palmer pelleter mill with a steam pressure of 150 kDa and a seven second conditioner residence time. Acid insoluble ash (Celite; AIA) was used at 20 g/kg as an indigestible marker. The xylanase enzyme (Econase XT) used in the current study was supplied by AB Vista Feed Ingredients (Marlborough, UK). This xylanase preparation contained 160,000 units/g of endo-1,4-β-xylanase activity (EC 3.2.1.8).

The gross energy (GE) of diets and lyophilized digesta were determined using a Parr 1281 adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, USA). Nitrogen concentration of samples was determined by the Dumas method using a FP-428 nitrogen analyser (LECO® Corporation, St. Joseph, MI, USA). The acid insoluble ash component of dried diets and ileal digesta samples were determined allowing the calculation of apparent ileal digestibility coefficient of N and energy.

All data were exported to JMP v.9.0 (SAS Institute, Cary, NC, USA) and subjected to analysis of variance. Means were separated by Tukeys LSD and were considered significant at P<0.05.

## III. RESULTS

The effect of xylanase addition on broiler performance is presented in Table 1. There was an improvement (P<0.05) in bodyweight gain associated with the addition of xylanase to the control diet (treatment 1 versus 4). The birds that received the energy-dilute diet supplemented with xylanase had performance equivalent to the control group (P>0.05, treatment 1 versus 5). Diet 5 returned an FCR not different from the control group.

During the period 1-28 days, there was a significant improvement in bodyweight gain with the addition of xylanase to the control diet and this was reflected in a significant improvement in FCR (P<0.05). However, the dilution of metabolisable energy by 0.25 MJ/kg (diet 5) returned an intermediate performance that was not different from the control.

Terminal bodyweights were significantly higher for the birds that received xylanase throughout the trial (diet 4) compared with those that received the industry standard control diet. The combination of energy dilution and xylanase addition (diet 3 and diet 5) resulted in performance equivalent (P>0.05) to the industry standard control diet (diet 1). Feed conversion ratio was improved by the addition of xylanase on an 'over-the-top' basis (diet 4 vs. diet 1). The addition of xylanase from d28 with no energy dilution resulted in an improvement in FCR and this tended toward significance compared with diet 1 (P<0.05).

When xylanase was added in combination with a reduction in energy density there was a muted benefit on FCR but performance was equivalent ( $P>0.05$ ) to the control diet (diet 1).

**Table 1 - Influence of xylanase addition to a wheat/soy-based diet on broiler performance**

ID <sup>1</sup>	BWG	FI d1-	FCR	BWG	FI d1-	FCR	BWG	FI d1-	FCR
	d1-14 (g/b)	14 (g/b)	d1-14 (g:g)	d1-28 (g/b)	28 (g/b)	d1-28 (g:g)	d1-49 (g/b)	49 (g/b)	d1-49 (g:g)
1	370.0 <sup>b</sup>	455.5	1.23 <sup>ab</sup>	1344 <sup>b</sup>	2047	1.52 <sup>a</sup>	3870 <sup>b</sup>	6343	1.64 <sup>a</sup>
2	373.0 <sup>ab</sup>	466.9	1.25 <sup>b</sup>	1359 <sup>b</sup>	2075	1.53 <sup>a</sup>	3981 <sup>ab</sup>	6185	1.55 <sup>b</sup>
3	370.4 <sup>ab</sup>	465.3	1.26 <sup>b</sup>	1361 <sup>b</sup>	2070	1.52 <sup>a</sup>	3935 <sup>ab</sup>	6284	1.60 <sup>ab</sup>
4	395.6 <sup>a</sup>	464.4	1.18 <sup>a</sup>	1449 <sup>a</sup>	2062	1.42 <sup>b</sup>	4071 <sup>a</sup>	6224	1.53 <sup>b</sup>
5	367.1 <sup>b</sup>	450	1.23 <sup>ab</sup>	1391 <sup>ab</sup>	2066	1.49 <sup>a</sup>	3986 <sup>ab</sup>	6269	1.57 <sup>ab</sup>
Pooled	6.4	8.07	0.02	16.9	41.7	0.027	53.6	84.9	0.022
SEM									
P	<0.05	NS	<0.05	<0.001	NS	<0.05	0.09	NS	<0.05

<sup>1</sup>See description above. 1 was "PC" throughout. 2 was "PC" to d28 and then "PC" plus xylanase from d28-49. 3 was "PC" to d28 and then "NC" plus xylanase from d28-49. 4 fed "PC" plus xylanase throughout. 5 was "NC" plus xylanase throughout.

The effect of xylanase addition with and without energy dilution on the ileal digestibility of energy, dry matter and nitrogen is presented in Table 2. On d28, the addition of xylanase, irrespective of whether a corresponding energy dilution was applied (treatments 4 and 5), resulted in an increase in ileal digestible energy of around 0.3-0.4MJ/kg. This increase was significant when treatments 1-3 (which were identical to d28) were contrasted to either treatment 4 or 5 ( $P<0.05$ , contrast not shown). The dilution of dietary energy (diet 5 vs. diet 4) had no detectable effect on ileal digestible energy at d28 ( $P>0.05$ ). Similar effects were observed with the apparent coefficient of ileal nitrogen digestibility where there was an improvement ( $P<0.05$ ) when diets 1-3 were contrasted against either diet 4 or diet 5. On d49, xylanase addition (either with or without an energy density dilution) improved ileal digestible energy (around 0.9MJ/kg;  $P<0.001$ ) and ileal nitrogen digestibility coefficients (around 4.6%;  $P<0.001$ ) compared with the xylanase-free control diet.

**Table 2 - Influence of xylanase addition on the coefficient of apparent ileal dry matter digestibility (CAIDMD), apparent ileal digestible energy (AID) and the coefficient of apparent ileal nitrogen digestibility (CAIND) at d28 and d49**

Diet ID	Day 28			Day 49		
	CAIDMD	AID (MJ/kg)	CAIND	CAIDMD	AID (MJ/kg)	CAIND
1	0.76 <sup>ab</sup>	13.16 <sup>ab</sup>	0.86 <sup>ab</sup>	0.75 <sup>b</sup>	13.08 <sup>b</sup>	0.86 <sup>c</sup>
2	0.76 <sup>ab</sup>	13.13 <sup>ab</sup>	0.86 <sup>ab</sup>	0.81 <sup>a</sup>	13.92 <sup>a</sup>	0.90 <sup>a</sup>
3	0.75 <sup>b</sup>	12.89 <sup>b</sup>	0.85 <sup>b</sup>	0.79 <sup>a</sup>	13.72 <sup>a</sup>	0.88 <sup>b</sup>
4	0.78 <sup>a</sup>	13.47 <sup>a</sup>	0.88 <sup>a</sup>	0.82 <sup>a</sup>	14.12 <sup>a</sup>	0.90 <sup>a</sup>
5	0.78 <sup>a</sup>	13.45 <sup>a</sup>	0.87 <sup>a</sup>	0.80 <sup>a</sup>	13.76 <sup>a</sup>	0.89 <sup>ab</sup>
Pooled	0.006	0.1	0.005	0.007	0.132	0.004
SEM						
P	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001

## IV. DISCUSSION

A major conclusion from the present experiment is that the timing of addition of exogenous xylanase is critical to the outcome, especially in the young bird as is the effect of fat inclusion in the diet.

Rosen (2002) reported the results of a holo-analysis of 1322 independent peer-reviewed experiments where xylanase was added to broiler diets. In this analysis, a significant 'not day old' term emerged where the overall efficacy of xylanase was muted when xylanase was introduced several days, or weeks, after the experiment began and axiomatically enhanced when xylanase was introduced immediately post-hatch. It is conceivable that the introduction of xylanase immediately post-hatch results in the gradual emergence of a distal gut microbial community that has an advantageous profile for nutrient recovery and GI tract integrity.

The interactive effect of dietary fat and exogenous xylanase has been reported and appears to have been influential in the present study. Cowieson et al. (2010) found that the removal of 20g/kg vegetable oil from a broiler starter diet in order to accommodate the anticipated energy effects of supplemental xylanase resulted in a reduction in ileal amino acid digestibility of around 4%. The authors speculated that the removal of dietary fat altered the rate of passage of feed in the GI tract, perhaps mediated via gastric residency.

Finally, the ileal brake mechanism may play an important role in the efficacy of xylanases. Modern day broilers may have a feed passage rate that is too rapid for optimal digestibility of nutrients and thus some decrease in transit rate may be beneficial. One hormone, amongst many of which may be involved in this process, is Peptide YY (PYY) and has been shown to delay gastric emptying (Savage et al., 1987). In various species, PYY has been shown to be released in response to dietary fibre and fat. Early experiments with broilers have shown that those fed diets containing xylanase have significantly higher levels of plasma PYY (Masey O'Neill et al., 2012). Thus, the displacement of dietary fat with exogenous xylanase, perhaps acutely but not uniquely in the neonate (Cowieson et al., 2010), may inadvertently reduce the overall efficacy of the xylanase

It can be concluded that exogenous xylanase is an effective way to reduce FCR and enhance BWG of broilers. Optimum response was achieved by the addition of xylanase from d1 (immediately posthatch) without dilution of dietary energy. However, inclusion alongside dietary energy reduction of 0.25MJ/kg resulted in performance equivalent to the control.

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STEAM-PELLETING TEMPERATURES, SORGHUM VARIETY AND FEED FORM  
INFLUENCE GROWTH PERFORMANCE, NUTRIENT UTILISATION AND  
NITROGEN DIGESTIBILITY IN BROILER CHICKENS

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Summary

An increase in conditioning temperatures at which diets based on two sorghum varieties were steam-pelleted from 65 to 97°C reduced protein solubility and significantly depressed feed efficiency and the two parameters were correlated ( $r^2 = 0.322$ ). Broilers offered intact pellets had significantly higher feed intakes and weight gains than birds on reground mash but nutrient utilisation was compromised. Sorghum #3-based diets supported significantly better feed efficiency, weight gain and N retention than sorghum #5, which may have been a consequence of a higher kafirin content in the sorghum #5 variety.

I. INTRODUCTION

Sorghum has a relatively high starch gelatinisation temperature and for this reason sorghum-based broiler diets are often steam-pelleted at high conditioning temperatures to achieve acceptable pellet integrity. While compacted, pelleted diets offer a physical feed form advantage over mash diets as they facilitate prehension and increase feed intakes (Jensen et al., 1962), high conditioning temperatures may have adverse effects on the nutritive qualities of pelleted diets (Raastad and Skrede, 2003) and sorghum is especially vulnerable to hydrothermal processes (Selle et al., 2010). To investigate these aspects equivalent diets based on two selected sorghum varieties were steam-pelleted at 65 or 97°C and offered to broiler chicks as intact pellets or reground mash from 7-28 days post-hatch.

II. METHODS AND MATERIALS

This section is abbreviated because the methodology of the present study is essentially similar to a parallel study reported by Selle et al. (2012). Six red sorghum varieties from the 2009 Liverpool Plains harvest were evaluated in a previous feeding study and two were selected for the present experiment. Sorghum #3 contained (g/kg) 112 protein, 712 starch, 8.33 phytate with a Symes' particle size index (PSI) texture of 10 and sorghum #5 contained 126 protein, 700 starch, 8.51 phytate with a PSI texture of 9. With soybean and canola meals, the two sorghums were incorporated into nutritionally equivalent diets with an energy density of 12.8 MJ/kg and 215 g/kg protein and 4.5 g/kg nonphytate P. These diets were steam-pelleted at 65 or 97°C and offered from 7-28 days post-hatch as intact pellets or reground mash as a 2 x 2 x 2 factorial array of dietary treatments. Each of the eight treatments consisted of six replicates of six male Ross 308 chicks per cage. Parameters assessed included growth performance, relative gizzard weights, N retention, N-corrected AME and apparent N digestibility in the distal jejunum and distal ileum. The protein solubilities of sorghum *per se*, unprocessed and steam-pelleted diets were determined by the Promatest method, as described by Odjo et al. (2012), which compares the solubility of the feedstuff or test diet with albumin as the 100% soluble standard.

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### III. RESULTS AND DISCUSSION

Steam-pelleting the #3 sorghum-based diet reduced its Promatest protein solubility from 53.3% to 46.3% at 65°C and 35.5% at 97°C; the corresponding values for sorghum #5 were 50.0, 42.4 and 31.4%. The results of the feeding study are tabulated and the overall 7-28 day weight gain of 1278 g/bird was inferior to the 1553 g/bird performance objective for male Ross 308 chicks. Three significant interactions between main effects were observed and the most notable was the sorghum variety and feed form interaction ( $P < 0.01$ ) for efficiency of feed conversion. Birds on sorghum #5 diets had higher FCR with pelleted diets; whereas, sorghum #3 birds had lower FCR on pelleted diets. As expected, broilers offered diets based on sorghum #3 significantly outperformed their sorghum #5 counterparts in feed efficiency by 4.8% (1.524 versus 1.601;  $P < 0.001$ ), weight gain by 3.7% (1302 versus 1255 g/bird;  $P < 0.01$ ) and N retention by 5.6 percentage units (63.6 versus 58.0%;  $P < 0.05$ ). This was expected because sorghum #3 *per se* held advantages in protein solubility (49.5% versus 41.2%), pepsin digestibility (65.8 versus 61.6%) and probably contained less kafirin. Sorghum #3 had an estimated kafirin content 58.0 g/kg and a 'kafirin index' of 4.3; whereas, sorghum #5 contained an estimated 68.1 g/kg kafirin with an index of 6.8. The kafirin index is calculated from sorghum NIR aminograms [g/kg: leucine – (arginine + histidine + lysine)] on the premise that kafirin is rich in leucine but contains a paucity of basic amino acids (Mosse et al., 1988). Alternatively, phytate levels and grain textures of both sorghums were similar.

Diets fed as intact pellets generated significant increases of 9.8% in feed intake and 9.1% in weight gain relative to reground mash diets. However, N retention was significantly decreased by 5.4 percentage units, AMEn by 0.83 MJ and jejunal and ileal N digestibility by 9.5 and 3.7%, respectively. Thus it appears that the substantially higher feed intake of intact pellets compromised nutrient utilisation presumably as a result of 'overconsumption'.

Elevating conditioning temperatures from 65 to 97°C increased relative gizzard weights by 4.0% (20.79 versus 19.24 g/kg;  $P < 0.001$ ), AMEn by 0.26 MJ (11.74 versus 12.00 MJ/kg;  $P < 0.05$ ) but, importantly, depressed feed efficiency by 2.46% (1.582 versus 1.544;  $P < 0.001$ ). As a main effect, conditioning temperatures did not influence ( $P > 0.50$ ) jejunal N digestibility but when intact pelleted diets are considered separately, there was a quite substantial numerical reduction of 8.7% (0.473 versus 0.518;  $P < 0.07$ ) that approached significance.

Steam-pelleting diets at 97°C reduced average protein solubility from 51.7% in unprocessed diets to 33.5%. However, it was previously determined that steam-pelleting the two sorghums individually at 90°C reduced their average protein solubility from 45.4 to 13.2%. Therefore, it may be deduced that the average reduction in protein solubility of sorghum *per se* was from 45.4 to 13.2%; as opposed to the reduction from 55.0 to 44.1% for the balance of proteins in the diet. Thus steam-pelleting generated a considerably more pronounced reduction in sorghum protein solubility than in soy and canola proteins. Pearson correlations between dietary protein solubilities and the *in vivo* parameters assessed are instructive. Protein solubility was significantly correlated with feed conversion ratios ( $r = -0.567$ ;  $P < 0.001$ ), AMEn ( $r = 0.384$ ;  $P < 0.01$ ) and tended to be correlated with N retention ( $r = 0.266$ ;  $P < 0.10$ ). When only intact pelleted diets are considered, protein solubility was correlated with feed conversion ratios ( $r = -0.557$ ;  $P < 0.01$ ), AMEn ( $r = 0.447$ ;  $P < 0.05$ ), N retention ( $r = 0.447$ ;  $P < 0.05$ ) and jejunal N digestibility ( $r = 0.423$ ;  $P < 0.05$ ).

It was established in a previous study (Selle et al., 2012) that in the order of 30% of the reduction in protein solubility generated by steam-pelleting sorghum-based diets can be attributed to the formation of disulphide bridges. The likelihood is that this mainly occurred between cysteine residues in the  $\gamma$ - and  $\beta$ -kafirin fractions located in the periphery of protein

bodies in sorghum endosperm, which is thought to impede digestion of the central  $\alpha$ -kafirin core (Duodu et al., 2003). Therefore, in diets fed as intact pellets it is relevant that the increase in conditioning temperatures numerically reduced jejunal N digestibility from 0.518 to 0.473 and this reduction was significantly correlated to protein solubility of the diets.

Finally, steam-pelleting two sorghum-based diets at 65°C and 97°C reduced protein solubility and the increase in conditioning temperatures significantly depressed feed efficiency by 2.5% (1.582 versus 1.544). Protein solubilities were significantly correlated with feed conversion ratios ( $r^2 = 0.322$ ), which indicates that the Promatest method may be a valuable indicator. Diets based on sorghum #3 outperformed sorghum #5 by 4.8% in feed efficiency, 3.7% in weight gain and 5.6 percentage units in N retention and in its native state sorghum #3 was higher in both protein solubility and pepsin digestibility. While phytate, starch, protein levels and grain texture of the two sorghums were similar there is the likelihood that sorghum #5 contained more kafirin, which may have contributed to the differences observed in broiler performance.

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**Table 1 - Effects of dietary treatments on growth performance, relative gizzard weights, nutrient utilisation and apparent nitrogen digestibility coefficients in the distal jejunum (AJD) and distal ileum (AID) from 7 to 28 days post-hatch**

Treatment			Weight gain (g/bird)	Feed intake (g/bird)	FCR (g/g)	Rel. gizzard wt. (g/kg)	N retention (%)	AMEn (MJ/kg DM)	Nitrogen AJD	Nitrogen AID
Sorghum	Feed form	Temperature								
#3	Reground	65°C	1236	1873	1.516	18.86	66.5	12.91	0.563	0.777
		97°C	1241	1922	1.549	20.47	63.3	12.48	0.548	0.708
	Intact	65°C	1364	2040	1.497	18.08	63.0	12.17	0.526	0.729
		97°C	1366	2097	1.535	20.53	61.4	11.98	0.483	0.721
#5	Reground	65°C	1224	1910	1.561	20.21	61.0	11.94	0.515	0.753
		97°C	1190	1906	1.602	21.55	63.1	11.81	0.564	0.761
	Intact	65°C	1295	2071	1.600	19.81	55.1	10.98	0.509	0.728
		97°C	1311	2151	1.641	20.60	52.9	10.69	0.463	0.705
SEM			20.936	32.533	0.0129	0.314	1.7441	0.1693	0.0305	0.0186
Main effects										
Sorghum variety #3			1302 <sup>b</sup>	1983	1.524	19.49	63.6	12.38 <sup>a</sup>	0.530	0.734
Sorghum variety #5			1255 <sup>a</sup>	2009	1.601	20.54	58.0	11.35 <sup>b</sup>	0.512	0.736
Feed form										
Reground pellet			1223 <sup>a</sup>	1903 <sup>a</sup>	1.557	20.27 <sup>b</sup>	63.5	12.28 <sup>a</sup>	0.547 <sup>a</sup>	0.749 <sup>a</sup>
Intact pellet			1334 <sup>b</sup>	2090 <sup>b</sup>	1.568	19.76 <sup>a</sup>	58.1	11.45 <sup>b</sup>	0.495 <sup>b</sup>	0.721 <sup>b</sup>
Conditioning temperature										
65°C			1279	1974	1.544 <sup>b</sup>	19.24	61.4	12.00 <sup>a</sup>	0.528	0.747
97°C			1277	2019	1.582 <sup>a</sup>	20.79	60.2	11.74 <sup>b</sup>	0.514	0.724
Significance (P =)										
Sorghum variety (S)			0.003	0.258	< 0.001	< 0.001	< 0.001	< 0.001	0.419	0.841
Feed form (FF)			< 0.001	< 0.001	0.243	0.025	< 0.001	< 0.001	0.020	0.035
Conditioning temperature (CT)			0.872	0.057	< 0.001	< 0.001	0.321	0.034	0.519	0.091
Interactions										
S x FF			0.298	0.486	0.006	0.479	0.036	0.091	0.954	0.378
S x CT			0.671	0.751	0.768	0.035	0.352	0.670	0.481	0.246
FF x CT			0.441	0.319	0.894	0.754	0.558	0.855	0.162	0.577
S x FF x CT			0.383	0.417	0.894	0.122	0.241	0.406	0.430	0.092

## ARE WE TURNING CHICKENS INTO COWS: HOW MUCH GRASS DO FREE RANGE BROILERS EAT?

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### Summary

Fourteen hundred and forty as hatched Cobb 500 broilers were divided equally among four experimental treatments in a 2 x 2 factorial design, involving conventional or free range production systems and diets with and without in feed antibiotics. Alkane concentrations in the litter were measured and compared with alkane profiles of the intake components (grass, diet pellets and woodchip) in order to estimate total grass intake from the range area. Grass consumption was estimated to be 3.72-4.24 % of total “as fed” intake. Considering the number of hours the birds spent on the range and the average feed intake from d21-42, this equates to 1.55-1.78 grams of grass per bird per hour of range access in this study. Taking into account grass consumption, range access resulted in an increase in feed intake by 4.1% ( $P < 0.01$ ) and FCR by 9-11 points ( $P = 0.082$ ). It can be concluded that broilers reared under free-range conditions eat a substantial quantity of grass. Under some circumstances this may be advantageous, reducing the consumption of expensive pelleted feed. However, the nutrient profile of grass is not complementary to the formulated ration and its consumption is likely to lead to an array of nutritional and physiological changes for the bird. Further work is required to explore the nutritional and health consequences of grass consumption for free-range broilers, particularly considering energy, amino acid and mineral balance and the effect on gastrointestinal physiology, immunology and microbiology.

### I. INTRODUCTION

Free-range broilers and layers are less efficient converters of feed into saleable meat and eggs and generally have higher mortality than conventionally-reared poultry. In broilers, the performance gap has been quantified as a 10-12 point increase in FCR and a 2-3% increase in mortality in free-range compared with conventionally-reared birds (Durali et al., 2012). This increase in FCR, however, may still be under represented considering free range birds also have access to supplementary feed sources on range. Although it has been established by observation studies that chickens eat grass while on range (Glatz et al., 2005, Miao et al., 2005), there have been minimal attempts to quantify the amount of grass consumed and its effect on performance and digestibility in birds. Some of the methods that have been used so far are by measuring reduction in sward height (Elbe et al., 2004), or comparing herbage mass in areas grazed by hens to an area from which they have been excluded (Jondreville et al. 2011). Other methods involve invasive procedures such as analysis of crop, gizzard and faecal contents which cannot be repeated for individual birds (Antell and Ciszuk 2006; Jondreville et al. 2011; Milby; 1961; Takahashi et al. 2006). One of the most suitable methods is to use the n-alkanes (Hatt et al. 2001; Ordakowski et al. 2001; Premaratne et al. 2005). So far there have been very few attempts to use this methodology in birds. The first reported study on use of alkanes in chickens was conducted by Hameleers et al (1996), who were able to determine their faecal recovery. In another study, alkane analysis was used to study intake and nutrient digestibility in pigeons (Hatt et al. 2001). Recently, soil and herbage intake was measured for free range layers using this methodology to evaluate the impact of nutrient restriction on ingestion (Jondreville et al. 2011). If grass consumption can be

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quantified accurately, then an important outcome would be to provide birds with feed that would compensate for the nutritional imbalances caused. This study attempts at establishing the use of alkanes as internal markers for estimating the intake of grass.

## II. MATERIALS AND METHODS

A total of 1440 Cobb 500 as hatched broilers were allocated to one of four treatments each with twelve replicates in a 2x2 full factorial design, the factors being conventional or free-range production system and conventional (with in-feed antibiotic growth promoter (AGP+)) or free range (AGP-) diet. Day old chicks received a numbered wing tag at placement and were randomly allocated to 48 pens (30/pen, density of 15 kg/m<sup>2</sup>) with ad libitum access to feed and water in a tunnel ventilated shed. Broilers were kept at a temperature of 31°C for days 1-4 and thereafter this was reduced by 0.5°C/day to 24°C. Although chicks were assigned to treatment diets on day 1, free range access was available to birds only from day 21 onwards. Ten percent of birds in the free range treatment were chosen randomly and manually assigned to the range from day 21-28 for 2 hours. The number was increased to 20% birds from day 28-35 and to 30% from day 35-42 for three hours (all chosen randomly) on range to represent the free range preference by broilers on a commercial production farm (pers. comm., Durali 2012). The range had a homogenous growth of Kikuyu grass (*Pennisetum clandestinum*) as the main herbage and was mowed down to 2.5 inches before assigning the birds. Pen wise body weights and feed intake were recorded at weekly intervals during the 42-day trial, corrected for mortality and FCR calculated.

Litter samples which consisted of woodchip along with excreta were collected on day 42 from each pen using the “coning and quartering” technique to represent an even distribution of the representative excreta. Grass, diet pellets and clean wood chip samples were also collected on day 42, dried to a constant weight in a freeze drier and ground through a 0.5 mm screen Cyclone mill. Alkane concentration in grass, diet pellets, woodchip and litter samples was determined using a modification of Mayes et al. (1986) methodology and gas chromatography. The identity of odd chain alkanes (C25 to C33) was determined from their retention times relative to the known standard. The area under the peak for each alkane was determined using an integrator (Model 3393A, Hewlett Packard), and peak areas were converted to amounts of alkane by reference to the internal standard C32.

Recovery of each alkane was used to calculate the proportion of the ingested ingredient, which was recovered in excreta. Diet proportions were estimated using a non-negative least squares procedure in the software “EatWhat” (Dove and Moore, 1995). In this study, only five odd chained alkanes (C25, C27, C29, C31, and C33) that were found in traceable concentrations were used for diet proportion estimates. The concentration of individual alkanes in excreta was corrected allowing for incomplete recovery based on published values by Hameleers et al. (1986) and number of birds per pen. By subtracting the contribution of woodchip, the intake of pellets and grass was established. Feed intake and FCR were corrected by adding the calculated grass consumption for day 21-42. Performance and alkane data were analyzed using JMP 9.0, 2010. ANOVA was conducted to evaluate the system and diet effects on performance as well as effect of individual alkanes on excreta recovery rates. In all cases, significance was set at  $P < 0.05$ .

## III. RESULTS AND DISCUSSION

Before accounting for grass consumption in the calculations, performance of birds on free range system appeared to be better than those raised on conventional system with a significant decrease seen in feed intake of birds ( 121gm vs. 130 gm/b/day,  $P < 0.001$ ) and a significantly lower FCR for birds with range access ( 2.15 vs. 2.33,  $P < 0.001$ ). However,

birds on range were seen to be eating grass. Alkane analysis was used to quantify grass consumption. The alkane profiles of the diet components showed that the predominant alkanes recovered were the odd chain alkanes C25, C27, C29, C31 and C33. While C31 (27.77 mg/kg DM) and C33 (21.12 mg/kg DM) showed up in higher concentrations than any other alkanes in grass, all alkanes showed up in more abundance in grass as compared to the other diet components. Woodchip showed the lowest amount of alkanes ranging from 0.028 mg/kg DM of C25 to 0.256 mg/kg DM of C29 recovered from it, whereas the two diet pellets showed higher concentrations of C25 (1.013 and 1.069 mg/kg DM), C27 (1.283 and 1.360 mg/kg DM), and C29 (1.260 and 1.407 mg/kg DM).

The n-alkane concentrations in litter for the different production systems and diets are presented in Figure 1. Alkanes recovered from litter samples showed significant rise in C31 and C33 alkanes in birds on a free range system and with access to grass (  $2.17 \pm 0.082$  and  $1.13 \pm 0.041$  mg/kg DM) as compared to the conventional system (  $0.54 \pm 0.082$  and  $0.27 \pm 0.04$  mg/kg DM). No significant difference was seen in alkane recoveries between the two diets.

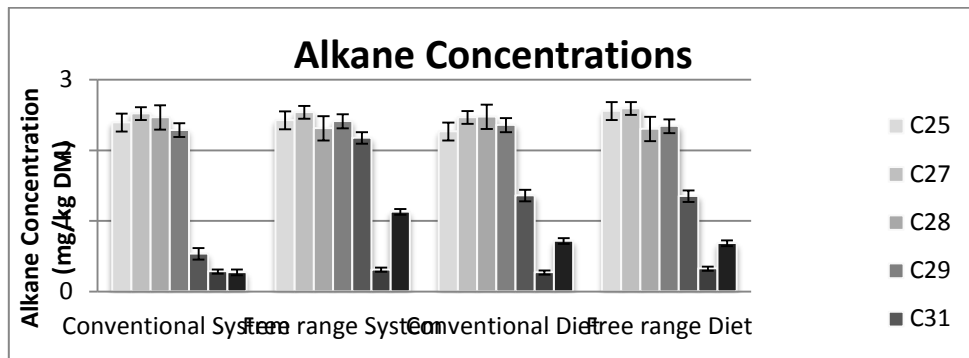


Figure 1 - Alkane recoveries (mg/kg DM) for conventional and free range systems and diets.

No grass was detected in excreta of birds reared under the conventional production system. Figure 2 shows the proportion of ingested ingredients as recovered from excreta of birds that had access to grass and the two diet treatments.

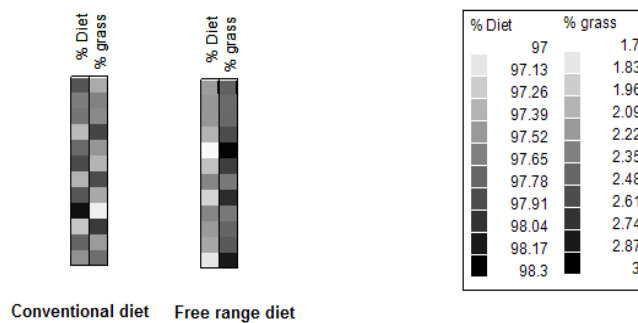


Figure 2 - Amount of grass and pellet diets ingested by birds in a free range production system.

Diets intended for consumption by free-range birds are not routinely formulated to accommodate the modifying effects of grass consumption on digestible nutrient intake. However, the consumption of grass at the levels outlined above would have a substantial diluting effect for dietary energy and would result in a significant increase in dietary potassium concentrations. The implications of these changes in dietary nutrient supply are currently being explored in a subsequent trial where both standard and high energy density broiler diets were systematically diluted with grass. The outcomes of this work will shed light on possible nutritional contributions to the relatively poor performance of free-range broilers

in Australia. Inadvertent changes in either dietary energy density or in dietary electrolyte balance (DEB) may be of importance, especially during summer months where DEB balance becomes critical to control metabolic alkalosis.

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## COMPARISON OF FREE-RANGE AND CONVENTIONAL BROILER PERFORMANCE AND DIGESTIBILITY

T. DURALI<sup>2</sup>, M. SINGH<sup>1</sup>, P. GROVES<sup>1</sup> and A.J. COWIESON<sup>1</sup>

### Summary

Fourteen hundred and forty as hatched Cobb 500 broilers were divided equally among four experimental treatments in a 2 x 2 factorial design involving conventional (no range access) and free range (range access) production systems and diets with and without in-feed antibiotics (AGP). Weekly body weight, feed intake, weight gain, feed conversion rate (FCR) and mortality data were collected and analysed to identify effects of diets, production systems and interaction between them. Diet had no effect on mortality, weekly weight gain and feed intake; however access to range area (day 22 to 42) had significant effects on mortality, weekly weight gain and feed intake. The group that had range access experienced higher mortality, lower feed intake and lower weight gain ( $P < 0.05$ ). There was no interaction between diet and production system for any performance indicators. The Acid Insoluble Ash (AIA) method was used to calculate apparent ileal energy digestibility (AIE) and apparent ileal dry matter (AIDM) digestibility. There was no interaction between range access and diet for the AIE and AIDM of low body weight birds; however there was a significant positive effect of range access for high body weight birds. Range access improved ileal digestibility of energy and dry matter in birds in the non-AGP diet group by 6% and 6.6% respectively ( $P < 0.01$ ) while the AGP+ group experienced a 1.4% and 1.7% decrease in both digestibilities, respectively. It can be concluded that providing range access to broilers may have advantages and disadvantages as it can result in improvement of energy and dry matter digestibility but may be associated with an increase in mortality and a decrease in feed consumption. Further work is required to explore the nutritional and health consequences of range access and grass consumption for free-range broilers, particularly considering energy, amino acid and mineral balance and the effect on gastrointestinal physiology, immunology and microbiology.

### I. INTRODUCTION

Free range broiler production is growing rapidly in Australia. In 2006, free-range broiler production accounted for 4% of total broiler production and today it is around 15% (ACMF, 2011). Commercial free-range broiler production is associated with poorer growth rate, higher feed conversion and higher mortality compared with conventional broiler production. Weeks et al., (1994) demonstrated that free range broilers had significantly lighter body weight ( $4.08 \pm 0.08\text{kg}$ ) than conventionally reared broilers ( $4.49 \pm 0.08\text{kg}$ ) at ten weeks of age. This performance gap has been observed in a long term commercial comparison study as a 2-3% increase in mortality, 10-15 points increase in feed conversion ratio and a retardation in growth rate in that birds took an extra 2.5 days to reach a 2.45 kg target body weight (Durali et al., 2012). This 'performance gap' may be as a result of the absence of in-feed antibiotics (AGP), poorer digestive health, gastrointestinal disease challenge, nutritional inadequacy due to unpredictable consumption of pasture and insects and increased activity. The positive effects of antibiotic supplementation of diets has been well documented since their introduction in the 1950's (Moore et al., 1946, Couch and Reed, 1950, Whitehill et al., 1950, Elam et al., 1951a, Elam et al., 1951b). Most of the in-feed antibiotics have two common features; they are mostly active against Gram positive bacteria and their absorption from the gastrointestinal tract is limited (Feighner and Dashkevicz, 1987). The mechanisms of AGP action vary for different groups of antibiotics (Ferket, 2004). They may interfere with cellular metabolism of microorganism, bacterial cell wall building and maintenance or protein transformation at ribosomal level (Ferket, 2004). As a result, antibiotics appear to limit proliferation of bacteria and thus permit the host to perform more

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closely to their genetic potential. Free range broilers and layers may consume unknown amounts of pasture with unknown nutrient value and this could affect their performance. This study attempts to identify effect of range access and unknown pasture consumption on performance of birds fed diets with and without in-feed antibiotic.

## 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 design, the factors being free-range or conventional production, with or without in-feed antibiotic (Zinc Bacitracin (15%) (30mg/kg feed)). Day old chicks received numbered wing tags at the day of placement and were randomly allocated to 48 pens (30 birds per pen) with *ad libitum* access to feed and water. Each individual pen had one tube feeder and a drinker line with five nipples per pen. Feed was formulated according to Cobb 500 nutrition specifications and produced in the Poultry Research Foundation feed mill. Feed was allocated to pens on the basis of 0.7 kg starter feed, 1.2 kg grower feed and ad libitum finisher feed per bird. Range access was made available to birds from day 21 onwards. Between days 21-28, 10% of total birds in the free range treatment pens were chosen randomly and given access to 3 hrs in a 6m<sup>2</sup> of range pen made of aluminium frame and chicken wire with 3m<sup>2</sup> shade cloth on top. Range pens had no feeders and drinkers. Between 29 and 35 days, 20% of total birds were given range access and 30% were given range access between days 35-42. Three hours range access was selected, based on personal observations of broiler range usage on a commercial free range broiler farm over 12 months. Mortality was recorded daily; body weight and feed consumption were recorded at weekly intervals. Feed conversion ratio (FCR) was calculated weekly. On day 42, three of the lightest and three of the heaviest birds in each pen were selected, euthanised and their ileal digesta was collected and pooled by treatment for ileal energy and dry matter digestibility analysis. Performance, mortality and digestibility data were analysed Standard Least Square ANOVA using JMP 9.0 (2010). Significance was set at P<0.05.

## III. RESULTS AND DISCUSSION

An unexpectedly low weight gain in the AGP + treatment group was observed between 21 to 28 days of age [Table 1]. Body weight of AGP+ and AGP- treatments at day 7 and 14 were slightly better than Cobb 500 broiler performance target (Figure 1). Grower rations were introduced on day 18 and subsequently the growth rate of the AGP+ group fell behind by 53gm on day 28 (Figure 2). All feeds were analysed for crude protein, fat, ash, Ca, Na, Cl, Mn, and fibre content. Cl level in AGP + grower feed was found to be 50% lower than AGP- grower feed. The effects of a relative deficiency of Cl may result in poor growth (Leach and Nesheim, 1963, Klasing and Austic, 2003, Garland and Pritchard, 2008). This feed formulation deficit compromised any ability to compare growth rates across treatments after the starter phase. Diet had no significant effect on body weight, mortality, weight gain or feed intake from 0-21 days. However birds fed with diet AGP + had significantly lower FCR, (0.831) than diet AGP- (0.909) from days 0-7 (P < 0.001) and days 8-14 (P < 0.05). Range access had significant effects on mortality, weekly weight gain and feed intake however this had no effect on weekly body weight and weekly FCR. There was no significant difference in mortality in first 3 weeks among the groups. However range access, regardless of the diet received, was associated with significantly higher mortality between 22-42 days (P < 0.05). There was no significant diet by range access interaction for any of the performance indicators.

Not including AGP in the diet resulted in significantly lower AIE and AIDM digestibility in low body weight birds (P < 0.05) but had no effect on high body weight birds (Table 2). Providing range access after 21 days of age had no significant effect on AIE and AIDM digestibility in either low or high body weight birds. There was no significant interaction between diet and range access for AIE and AIDM digestibility in low body weight birds; however this interaction was significant, with range access having a positive effect, for high body weight birds. Range usage improved AIE and AIDM digestibility in the non-AGP diet group by 6% and 6.6%

respectively ( $P < 0.01$ ); however a decrease of 1.4% and 1.7% respectively was observed in the AGP+ group.

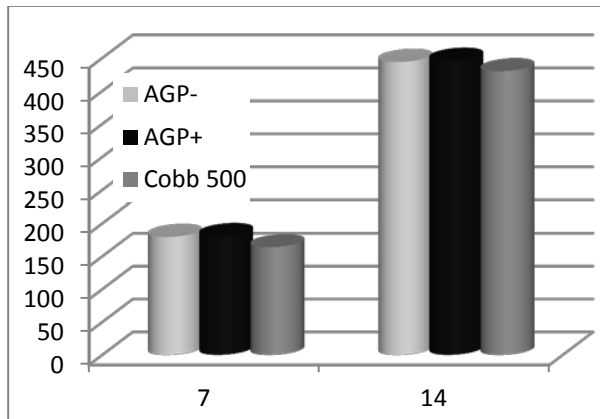


Figure 1 - Body weight on day 7 and 14.

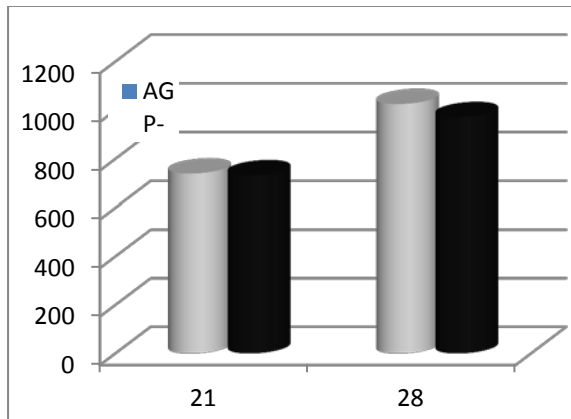


Figure 2 - Body weight on day 21 and 28.

Commercial free range diets are not customarily formulated to accommodate the effects of range access and unknown grass consumption on digestible nutrient intake. The range access improved ileal dry matter and energy digestibility in this study, possibly due to improved physical digestion by the gizzard. However, unknown grass consumption could have had a considerable dilution effect on dietary energy and could result in a significant change in dietary mineral concentrations. The implications of these changes in dietary nutrient supply are currently being explored in a subsequent trial where both standard and high energy density broiler diets were systematically diluted with grass. The outcomes of this work will help to understand poor performance of free-range broilers in Australia.

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**Table 1 - Weekly body weight gain (gram)**

Main effect means	1 <sup>st</sup> week	2 <sup>nd</sup> week	3 <sup>rd</sup> week	4 <sup>th</sup> week	5 <sup>th</sup> week	6 <sup>th</sup> week
AGP+	134	265	287	239 <sup>a</sup>	279	342
AGP-	133	264	295	285 <sup>b</sup>	299	368
Range Access (RA)	-	-	-	253	281	351
No-Range Access (NoRA)	-	-	-	271	297	359
AGP+ RA	-	-	-	225	272	354
AGP+ Non RA	-	-	-	252	287	329
AGP- RA	-	-	-	280	291	348
AGP- Non RA	-	-	-	290	306	388
	0.3909	0.9327	0.1319	0.002	0.1669	0.3725
P Value	-	-	-	0.1192	0.2745	0.7864
	-	-	-	0.4660	0.9909	0.2779

**Table 2 - Effect of diet, range access and their interactions on free range broiler digestibility**

	High Body Weight Apparent Ileal Energy Digestibility (%)	Low Body Weight Apparent Ileal Energy Digestibility (%)	High Body Weight Apparent Ileal Dry Matter Digestibility (%)	Low Body Weight Apparent Ileal Dry Matter Digestibility (%)
AGP-	73.80	70.23 <sup>a</sup>	69.76	66.88 <sup>a</sup>
AGP+	75.32	74.84 <sup>b</sup>	71.75	71.83 <sup>b</sup>
Range Access (RA)	75.72	74.05	71.96	70.92
No-Range Access (NoRA)	73.40	71.02	69.54	67.80
AGP-RA	76.81 <sup>a</sup>	72.60	73.05 <sup>a</sup>	69.15
AGP-NoRA	70.79 <sup>b</sup>	67.86	66.47 <sup>b</sup>	64.62
AGP+RA	74.63 <sup>ab</sup>	75.50	70.87 <sup>ab</sup>	72.69
AGP+NoRA	76.00 <sup>a</sup>	74.18	72.62 <sup>a</sup>	70.98
	0.2560	0.0294	0.1613	0.0227
P Value	0.0845	0.1460	0.0901	0.1432
	0.0074	0.4084	0.0046	0.5055

## COMPARISON OF ANHYDROUS AND HYDROCHLORIDE FORMS OF BETAINE ON BROILER PERFORMANCE

D. CRESWELL<sup>1</sup>

### Summary

A trial was conducted at Bangkok Animal Research Centre (BARC), June-July, 2012, as part of the ongoing program of research on betaine for broilers. This trial demonstrated that both anhydrous betaine and betaine hydrochloride in equi-molar amounts are effective for use in diets reduced in choline, methionine and oil, tending to give improved Live Weight Corrected FCR (LWC FCR). Betaine hydrochloride was shown to be effective when used in drinking water.

### I. INTRODUCTION

Betaine has been extensively reviewed as an additive for poultry diets (Metzler-Zebeli et al., 2009). Positive effects have been shown on carcass yield and breast meat. As a methyl donor, betaine may partially substitute other methyl group donors such as methionine and choline. As an osmolyte, betaine controls movement of ions and water into and out of intestinal cells, at a lower energy cost than use of ion pumps. This reduces the energy cost of maintenance (Campbell et al., 1997). There are two commercial forms of betaine, anhydrous betaine and betaine hydrochloride. Few comparisons have been made between these two forms. Creswell (2012) showed increased carcass yield with the hydrochloride form, while Phillip (2012) demonstrated the osmolyte properties of betaine hydrochloride, as it is a zwitterion at the point of absorption in the small intestine. This study aimed to compare the two forms of betaine on broiler performance, when included in diets reduced in choline, methionine and energy (oil) or when added in drinking water.

### II. MATERIALS AND METHODS

Three hundred newly hatched male broiler chicks of commercial strain (Arbor Acres Plus) were randomly allocated to five treatments with six replications using 10 male birds in a pen as an experimental unit. Diets were wheat-corn-soybean meal-palm oil. A Negative Control (NC) treatment was formulated by removing all choline chloride, 1.25 kg/t DL Methionine and 0.31 MJ/kg ME (17-23 kg/t palm oil) from the Positive Control (PC). The two betaine sources were added to the NC in equi-molar amounts. Details of the treatments are shown in Table 1. In a fifth treatment, the NC was fed, and betaine hydrochloride was added to drinking water at 0.10% (w/v).

The experiment was conducted in a closed house with tunnel ventilation and evaporative cooling system. Birds were raised on solid-concrete-floor pens using rice hulls as bedding material. Experimental diets were fed in pelleted and crumble form to 12 days and in pellet form thereafter until the end of the trial. The average max/min temperature and relative humidity in the experimental house were 36.2/30.8 °C and 39.3% during 0-7 days of age, 31.7/26.7 °C and 76.3% during 7-16 days of age, 30.4/26.5 °C and 80.9% during 16-38 days of age, respectively.

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**Table 1 - Treatment design**

Treatment	1	2	3	4	5
Basal diet	PC	NC	NC	NC	NC
Betaine, kg/t	0	0	2.75 <sup>1</sup>	2.15 <sup>2</sup>	DW <sup>3</sup>
Choline chloride 60%	Yes	No	No	No	No
ME reduction, MJ/kg	No	-0.31	-0.31	-0.31	-0.31
DL Methionine, Starter kg/t	2.41	1.12	1.12	1.12	1.12
DL Methionine, Grower kg/t	2.40	1.12	1.12	1.12	1.12
Digestible M+C, starter %	0.84	0.715	0.715	0.715	0.715
Digestible M+C, grower %	0.803	0.678	0.678	0.678	0.678

<sup>1</sup>Hydrochloride <sup>2</sup>Anhydrous <sup>3</sup>Added in drinking water at 0.10%/w/v

### III. RESULTS AND DISCUSSION

Results presented in Table 2 and Figure 1 show excellent performance, being 2768 grams LW and 1.577 FCR at 38 days. Precision was also good, at 1.75-2.55 percent C.V. for most performance parameters.

Effects of the treatments on LWC FCR are seen in Table 2 and Figure 1. Figure 1 enables these treatment differences to be seen most clearly.

Treatment differences were small and few differences were significantly different. Removing the choline chloride, methionine and oil from the PC tended to give poorer LWC FCR (1.577 vs 1.601). Adding betaine hydrochloride to the NC gave an improved LWC FCR ( $P=0.05$ ) than the NC (1.556<sup>a</sup> vs. 1.601<sup>b</sup>).

Comparison of betaine hydrochloride and anhydrous betaine showed similar results, with a trend of better LWC FCR for betaine hydrochloride (1.556 vs 1.589). Both betaine forms gave better LWC FCR than their control, the NC treatment. In the case of betaine hydrochloride the difference was significant and for anhydrous betaine it was numerically different.

Addition of betaine hydrochloride to drinking water was successful in that there were no problems with slime development in the drinking water lines, and water consumption was normal. LWC FCR for this treatment was equal to the PC (1.574 vs. 1.577) and tended to be better than the NC (1.574 vs. 1.601).

These results suggest that betaine may be used in this way by adding to diets reduced in the nutrients choline, methionine and energy (oil), and as such lower in cost. The betaine containing diets were around USD 10/t lower in cost than the PC at the time of formulation, including the cost of the betaine.

### IV. CONCLUSIONS

This trial indicates that both betaine hydrochloride and anhydrous betaine may be useful in reducing feed costs. When used in diets reduced in added choline, methionine and energy (oil) they will reduce feed costs and give at least equal broiler performance.

Betaine hydrochloride has been shown to be used in drinking water without problems.

**Table 2 - Effect of treatments on Live weight, (LW), weight gain, feed intake (FI), feed conversion ratio (FCR), live weight corrected FCR and liveability at 38 days**

Treatment	Betaine	LW, g	WG, g	Feed, g	FCR	LWC FCR <sup>8</sup>	Liveability (%)
PC	0	2768	2725	4293	1.577	1.577 <sup>ab</sup>	100
NC <sup>1</sup>	0	2739	2697	4299	1.594	1.601 <sup>b</sup>	98.3
NC <sup>1,2</sup>	2.75 <sup>5</sup>	2754	2712	4204	1.553	1.556 <sup>a</sup>	96.7
NC <sup>1,3</sup>	2.15 <sup>6</sup>	2722	2679	4227	1.578	1.589 <sup>ab</sup>	98.3
NC <sup>1,4</sup>	DW <sup>7</sup>	2779	2737	4313	1.577	1.574 <sup>ab</sup>	98.3
P value		0.644	0.636	0.276	0.177	0.051	0.825
Pooled SEM		28.24	28.25	41.20	0.011	0.008	1.93
C.V. %		2.51	2.55	2.37	1.75	1.83	4.81

<sup>1</sup>NC is PC – added choline chloride – 1.25 kg/t dl methionine – 0.31 MJ/kg ME, <sup>2</sup>NC +BtHCL, <sup>3</sup>NC+ BtANH, <sup>4</sup>NC+BtHCLW, <sup>5</sup>Hydrochloride, <sup>6</sup>Anhydrous, <sup>7</sup>Drinking Water, <sup>8</sup>LWC FCR, calculated for a LW of 2768 grams, at 2.4 points per 100 grams  
<sup>ab</sup> (P<0.05)

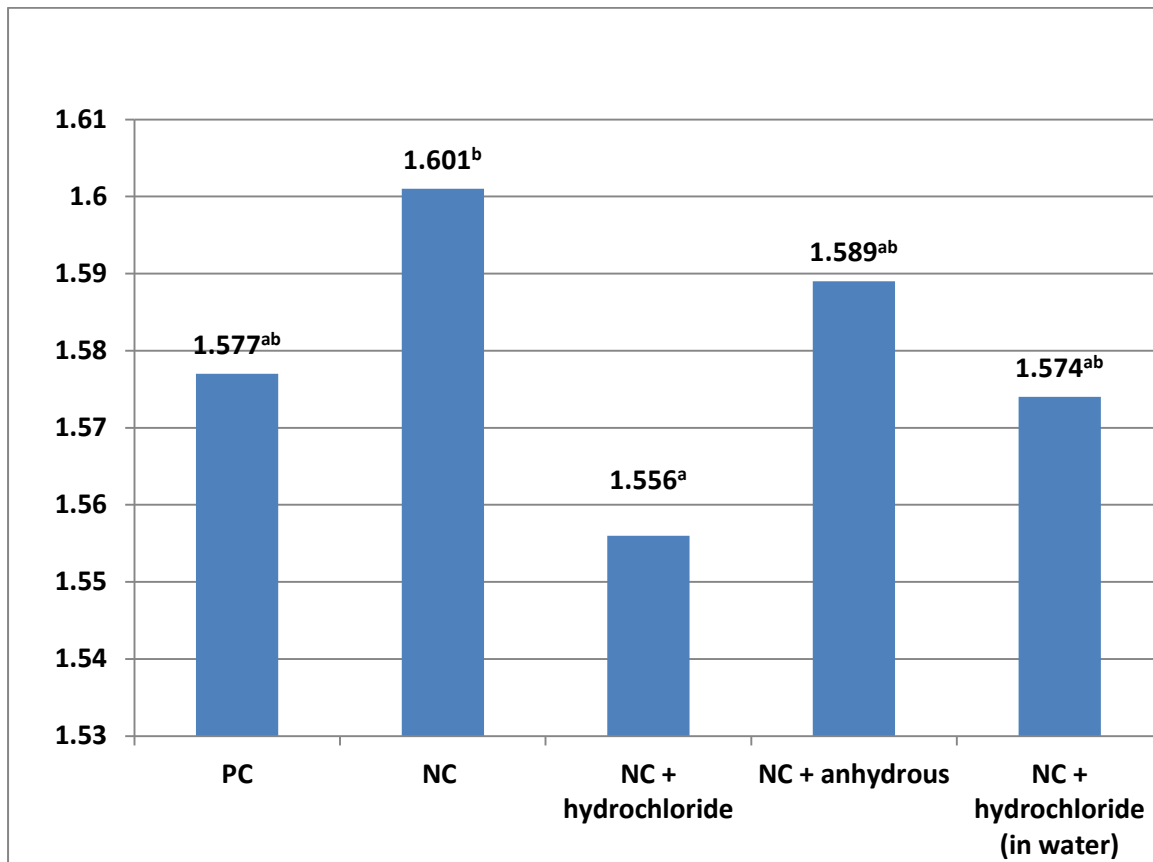


Figure 1 - Effect of betaine treatments on LWC FCR (Liveweight Corrected FCR) at 38 d. <sup>ab</sup> P<0.05

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EVALUATION OF BETAINE AS AN *IN OVO* FEEDING NUTRIENT FOR BROILER CHICKENS

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The incubation period (21 d) represents about 37 % of the entire lifespan of the modern broiler bird. Organ and overall body development during incubation are related to the final market weight. The technology for *in ovo* feeding has been largely developed (Uni and Ferket, 2003) but the uptake rate is slow. This may partly be due to lack of suitable nutrients, which not only promote pre- and post-hatch growth but can support good hatchability. In the present study, 0.5 ml of a solution of betaine was injected into standard size 18 d embryonated broiler eggs at 10, 50, 100 and 200 mg betaine per egg. There were un-injected control eggs and sham control eggs injected with 0.5 ml Milli-Q water. The eggs were re-set to hatch and chicks were maintained in their respective groups and fed on a standard diet (based on sorghum and maize) to 21 d of age.

**Table 1 - Feed intake (g/bird), body weight gain (g) and FCR (g feed/g weight gain) of broiler birds after *in ovo* feeding of betaine on 18 d of embryonic age<sup>1</sup>**

Response	Age (d)	Milli-Q		Betaine level mg/egg				SEM
		Control	water	10	50	100	200	
Hatchability (%)		93.0	60.0	60.0	70.0	85.0	56.7	
Hatching wt (g)		53.9 <sup>b</sup>	39.8 <sup>d</sup>	42.2 <sup>c</sup>	47.9 <sup>c</sup>	56.8 <sup>a</sup>	42.0 <sup>c</sup>	1.09***
Feed intake (g/b)	1-3	42	41	40	41	44	41	0.49
	1-10	296	295	292	295	302	299	2.69
	1-21	1273	1295	1293	1274	1283	1303	15.44
Weight gain (g)	1-3	30 <sup>c</sup>	42 <sup>a</sup>	41 <sup>a</sup>	36 <sup>b</sup>	31 <sup>c</sup>	44 <sup>a</sup>	1.07*
	1-10	259	269	275	276	262	277	2.93
	1-21	994	1013	1054	1009	993	1015	11.55
FCR	1-3	1.42 <sup>a</sup>	0.97 <sup>c</sup>	0.96 <sup>c</sup>	1.15 <sup>b</sup>	1.43 <sup>a</sup>	0.95 <sup>c</sup>	0.038***
	1-10	1.14 <sup>ab</sup>	1.10 <sup>bc</sup>	1.06 <sup>c</sup>	1.07 <sup>c</sup>	1.15 <sup>a</sup>	1.08 <sup>c</sup>	0.009**
	1-21	1.28	1.28	1.23	1.27	1.29	1.29	0.011

<sup>1</sup> Each value represents the mean of 6 replicates for each treatment group; <sup>a, b, c, d</sup> Values with unlike superscripts within each row are significantly different; SEM = Standard error of mean, \*\*\*P < 0.001; \*\*P < 0.005

Of the treated groups, 100 mg/egg gave the best hatchability, 85 %, although this was lower than the control (untreated) group (93 %) (Table 1). Hatching weight was also highest (P < 0.001) in the group that received 100 mg. Feed intake was not affected by treatment. Similarly, body weight gain and feed conversion ratio between hatch and 21 d were not significantly different although the group that received 10 mg/egg was superior in these two variables. These results suggest that administering betaine at 100 mg/egg would not adversely affect hatchability but did not significantly improve subsequent growth and feed efficiency. There is a need for further evaluation of this nutrient, including the use of an appropriate buffer rather than water in the preparation of the test solution. There are on-going analyses of samples for development of mucosal histology, immunity and digestive enzyme activities.

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## IN OVO FEEDING OF GLYCEROL TO BROILER CHICKENS

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### Summary

In recent years, the poultry industry has intensified the search for technologies that provide nutrients to the embryo, especially through *in ovo* feeding (IOF). Recently, many studies have been conducted to determine what kinds of compounds, solutions or nutrients should be introduced into the egg to improve the chicken's performance and health. Therefore, this study evaluated the effects of *in ovo* injection of glycerol on embryo yolk weight and organ weight at hatch and 24 hours post-hatching, and performance of broiler chickens to 21 days of age. Seventy two birds were killed to measure yolk sac weight and organ weight parameters (gizzard, small intestine and liver) and, for the performance study, 300 male chickens were allocated to six treatments with five replicates of ten birds each. Solutions with different levels of glycerol were prepared: 1) distilled water 2) 1.2% of glycerol 3) 2.4% of glycerol 4) 3.6% of glycerol 5) 4.8% of glycerol, and 6) 6.0% of glycerol. There were no effects ( $P > 0.05$ ) of glycerol level on organ weight. There was a quadratic effect on the yolk sac at 24 hours after hatch. Linear and quadratic effects were observed on feed intake and body weight gain, respectively, at seven days of age. Results indicate that injection of glycerol *in ovo* increased the yolk sac weight 24 after hatch and improved the body weight gain at day seven for chickens fed *in ovo* up to 2.4% of glycerol.

### I. INTRODUCTION

The developing chick embryo relies on the nutrients provided by the egg rather than maternal influence. Thus, there has been an intensive search for technologies that provide nutrients to the embryo, other than what is present in the egg. This process is known as *in ovo* feeding (IOF).

Glycerol is a polyalcohol, an oily, colourless, viscous liquid with a sweet flavour, and soluble in water. During metabolism, glycerol can be used as a carbon skeleton for gluconeogenesis, oxidative phosphorylation and skeleton for triglycerides (Lin et al., 1976). During incubation, glucose and glycogen are utilized as energy sources, in preference to lipids and protein, especially during the last few days before hatch (Moran Jr, 2007; Zhai et al., 2011). The embryo is then driven toward anaerobic catabolism of glucose, which is dependent on the amount of glucose held in the glycogen reserves of the liver, kidneys, and muscles and on the glucose generated by gluconeogenesis from amino acids, glycerol, and lactate (Christensen et al., 2001; De Oliveira et al., 2008). The objective of the current study was to examine the effects of *in ovo* injection of glycerol on the embryo yolk weight and organ weight, and performance of broiler chickens until d 21.

### II. MATERIALS AND METHODS

Chicken eggs from a breeder flock (39 wk of age), with four days of storage, were obtained from a commercial hatchery, preheated for 4 hours (27-29°C) and incubated in a single-stage incubator according to standard hatchery practices. At 17.5 d of incubation, 996t eggs containing viable embryos were weighed and randomly divided into 6 treatment groups of

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166 eggs each. On the same day, each egg was candled to identify the location of the amnion, disinfected with ethyl alcohol 70%, a hole was punched using a 21-gauge needle and 1 mL of *in ovo* feeding (IOF) solution was injected into the amnion. The treatments contained different levels of glycerol (99%), as follows: 1) distilled water, 2) 1.2% of glycerol, 3) 2.4% of glycerol, 4) 3.6% of glycerol, 5) 4.8% of glycerol, and 6) 6.0% of glycerol.

Six chicks from each group were killed by cervical dislocation, on the hatch day and 24 hours after hatching for assessment. The chick was weighed followed by excision of gizzard, liver, small intestine and yolk sac, which were also weighed. The allometric weight of the digestive organs and the yolk sac was calculated based on live body weight. Three hundred male chickens were randomly assigned on the hatch day into six treatments, each with five replicate pens of 10 chickens. Birds were housed in a shed equipped with manual drinker and feeder, wood shaving litter, and supplemental incandescent heat. During the performance study, birds and feed were weighed at one, seven, and 21 days of age to determine feed intake (FI), body weight gain (BWG), and feed conversion rate (FCR). Polynomial regression coefficients were obtained by fitting data to a simple linear model, considering the significance level, determination of correlation coefficient, and the biological response of the birds.

### III. RESULTS AND DISCUSSION

The weight of the gizzard, small intestine and liver at hatch and 24h after hatch was not influenced ( $P>0.05$ ) by *in ovo* glycerol injection. However, yolk sac weight presented a quadratic effect at 24 hours after hatch day ( $Y=15.78-2.555x+0.339x^2$   $R^2=0.35$ ). Zhai et al. (2011) observed differences in yolk sac weight and on the rate of yolk absorption of embryos fed with or without carbohydrate solutions *in ovo*.

**Table 1 - Gizzard, small intestine (SI), liver, and yolk sac weights (g/100g live weight) of chicks at hatch and 24 hours after hatch from eggs injected different levels of glycerol.**

	Level of Glycerol (%)						CV%	Probability		
	0	1.2	2.4	3.6	4.8	6.0		L	Q	DQ
Hatch										
Gizzard	5.86	6.87	5.82	6.08	6.61	6.31	18.39	NS	NS	NS
SI	1.79	1.84	1.78	1.71	2.02	1.98	15.79	NS	NS	NS
Liver	2.02	2.34	2.22	2.10	2.29	2.32	16.53	NS	NS	NS
Yolk Sac	17.03	17.04	17.35	16.39	17.08	17.42	14.00	NS	NS	NS
24 hours after hatch										
Gizzard	7.11	6.82	7.03	7.51	6.94	7.59	13.98	NS	NS	NS
SI	3.34	3.53	3.55	3.52	3.82	3.76	14.05	NS	NS	NS
Liver	3.02	2.80	2.64	2.83	2.90	3.05	12.85	NS	NS	NS
Yolk Sac <sup>1</sup>	15.59	13.76	11.50	9.80	12.65	12.18	17.97	*	*	NS

\* $P < 0.01$ ; NS – not significant. L – linear effect; Q – quadratic effect; DQ – quadratic deviation effect

<sup>1</sup> $Y=15.78-2.555x+0.339x^2$   $r^2=0.35$

There were no effect ( $P>0.05$ ) of glycerol on the body weight (BW) at hatch day. The FI ( $P<0.01$ ) was increased by glycerol *in ovo* injection at seven days of age. There was a quadratic effect of glycerol level on BWG of chickens at day 7. However, the FCR was not influenced ( $P>0.05$ ) in response to the glycerol levels *in ovo*. Feed intake showed a linear response ( $Y=0.139-0.05x$   $r^2=0.32$ ) to glycerol levels, increasing in line with rising levels of



glycerol. Body weight gain presented a quadratic effect ( $Y=0.117-0.017x+0.064x^2$   $r^2=0.53$ ) in response to the glycerol levels (Table 2). Although FI and BWG has been affected at seven days, no significant difference was observed at 21 days of age (Table 2). Results from a study of Uni et al. (2005) demonstrated that *in ovo* feeding of carbohydrates and  $\beta$ -hydroxy- $\beta$ -methylbutyrate at 17.5 d of incubation can improve the energy status of embryos and improve early growth, to enhance the genetic potential for late embryonic and early posthatch growth.

**Table 2 - Body weight (BW-g), feed intake (FI-g), body weight gain (BWG-g), and feed conversion rate (FCR) of chickens from eggs injected with different levels of glycerol at hatch day, 7, and 21 days of age.**

	Level of Glycerol (%)						CV%	Probability		
	0	1.2	2.4	3.6	4.8	6.0		L	Q	DQ
Hatch day										
BW	40	41	42	40	41	43	3.10	NS	NS	NS
Day 7										
FI <sup>1</sup>	139	161	170	164	177	167	13.19	*	NS	NS
BWG <sup>2</sup>	117	140	148	133	135	137	10.53	*	*	NS
FCR	1.18	1.14	1.14	1.23	1.22	1.22	10.23	NS	NS	NS
Day 21										
FI	1165	1240	1209	1192	1173	1197	6.57	NS	NS	NS
BWG	764	816	827	771	812	781	7.10	NS	NS	NS
FCR	1.53	1.52	1.46	1.55	1.44	1.53	6.01	NS	NS	NS

\*P < 0.01; NS – not significant. L – linear effect; Q – quadratic effect; DQ – quadratic deviation effect  
<sup>1</sup>Y=0.139-0.05x  $r^2=0.32$ ; <sup>2</sup>Y=0.117-0.017x+0.064x<sup>2</sup>  $r^2=0.53$

In conclusion, the use of solutions with 2.4% of glycerol increased the weight gain of chickens at seven days of age, but this result was not maintained to d 21. Increased levels of glycerol up to 3.6% reduced weight of the yolk sac.

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THE STRUCTURAL BASIS OF EGG SHELL TRANSLUCENCY AND ITS ROLE IN  
FOOD SAFETY OF TABLE EGGS

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Eggshell translucency is the appearance of lighter coloured regions of the shell visible when an egg is viewed over a light source. Some research has been conducted into the causes of translucency in the past but there has been little work recently and the cause of translucency remains uncertain. This study aimed to identify the structural cause of eggshell translucency and determine if translucency correlates with bacterial penetration of eggs.

Experiments confirmed that translucency is caused by a build-up of moisture in the structures of the shell because translucency disappears when shells are dried out but reappears following rehydration of the shells. The presence of the shell membrane does not appear to be integral to the appearance of translucency because removal of shell membrane and rehydration of egg shells showed no difference with or without membrane present.

Observation of the mammillary layer of eggshells under the scanning electron microscope revealed some features that were correlated with a high incidence of translucency such as type B mammillary bodies and cubic cone formations. The size and density of the mammillary bodies did not correlate with translucency score.

A CT scanner was used to provide high resolution details of shell structure. While these images showed several underlying features common to translucent shells, there was no consistent single feature that could be associated with a high incidence of translucency. However, counts of the number of pores per unit area that passed directly through the shell, branched internally or branched externally showed differences among translucency score categories (see Table). The number of straight pores was inversely related to translucency score whereas the number of externally branching pores was directly correlated. However, studies of egg shell conductance and pore density for a range of translucency scores showed no statistically significant differences although shell conductance tended to increase with translucency score whereas pore density tended to decrease.

**Table 1 - Types of pores in shells of different translucency scores (4 = highest incidence)**

Translucency Score	Average number of Pores per sample (0.91 mm <sup>3</sup> )		
	Straight	Internal	External
1	<sup>a</sup> 4.55	0.55	<sup>b</sup> 0.09
3	<sup>b</sup> 5.86	0.57	<sup>b</sup> 0.43
4	<sup>c</sup> 3.08	0.50	<sup>a</sup> 1.92

Whole egg and agar eggs studies were conducted to investigate the ease with which *Salmonella* Agona could penetrate washed and unwashed shells of different translucency scores. For whole eggs, penetration was more likely at 37°C than at 20°C incubation and washed and unwashed eggs were not different. For the agar egg studies, unwashed eggs had a higher penetration rate than washed eggs. There was a tendency for penetration to occur more often in eggs from translucency scores 2 and 3. Egg shell translucency is more likely caused by a combination of factors including pore branching, mammillary layer ultrastructure and perhaps the density of the shell calcite.

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## EFFECT OF HEAT TREATMENT OF RAPESEED MEAL ON PERFORMANCE AND CARCASS CHARACTERISTICS OF BROILER CHICKENS

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### Summary

The objective of this experiment was to study the impact of feeding heat-processed rapeseed meal (RSM) on broiler performance. Rapeseed was procured from the market and subjected to cooking temperature of 100 °C for 160 min to produce a heat damaged (HD) RSM. A total of 640 Ross 308 male broilers were randomly assigned to four dietary treatments with eight replicates and 20 birds per replicate from 10 to 28 days of age in a completely randomized design. Dietary treatments were: 1) good quality RSM; 2) HD RSM; 3) HD RSM adjusted for changes in total amino acid content (HD-total AA); and 4) HD RSM adjusted for changes in standardized ileal digestible (SID) amino acid content due to heat damage (HD-SID AA). There was a reduction (39%) in the amino acid composition of HD RSM compared with good quality RSM. Body weight gain, feed intake, feed conversion ratio, total carcass and breast meat yield were ( $P < 0.05$ ) reduced by replacing the good quality with HD RSM. However, growth performance and carcass yield improved marginally when HD RSM was corrected for its total AA composition. Growth performance and carcass yield further improved when HD RSM was corrected for its SID amino acid composition. It can be concluded that the impact of heat damaged RSM can be mitigated by accounting for differences in the SID amino acid content.

### I. INTRODUCTION

Rapeseed meal is a protein-rich ingredient widely used in animal feeding. During RSM production, it is heat processed and as a result, the quality of RSM can be enhanced and/or diminished depending on the conditions during heat processing. During processing, heat treatment destroys the enzyme, myrosinase, which is responsible for hydrolyse glucosinolates present in rapeseed. Hydrolyzed glucosinolates release sulphur and free goitrogenic substances, such as oxazolidinethione, isothiocyanates and thiocyanates into the rapeseed oil and meal (Larsen, 1981), which decreases the nutritive value of RSM.

The destruction of myrosinase via heat processing is a critical step to improve the nutritional quality of RSM. However, if heat-processing is not properly controlled, it can also have a detrimental effect on the protein quality of RSM. The destruction and loss of amino acids such as lysine, cystine, and arginine in soybean meal due to inappropriate heat treatment has been previously reported by several researchers (Parsons et al., 1992; Fontaine et al., 2007 and Boucher et al., 2009a,b). It has been also shown that excessive heat treatment not only decreased the total amino acid contents but also the digestibility of amino acids was affected (Parsons et al., 1992). Kluth et al. (2010) demonstrated a SID amino acid reduction for soybean meal and distiller's dried grains with solubles as a result of over-processing. Similarly, excess heat treatment applied during rapeseed processing will have a negative effect on the digestibility of amino acids of RSM (Newkirk and Classen, 2002). They reported that feeding toasted canola meal resulted in reduced growth and feed efficiency in broilers compared with those fed non-toasted meal. In addition, Newkirk et al. (2003) demonstrated that the apparent ileal digestibility of amino acids in broilers were higher in

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non-toasted compared with toasted canola meal. The objective of the present study was to further evaluate the impact of feeding HD RSM on growth performance and carcass characteristics of broilers.

## II. MATERIALS AND METHODS

Rapeseed meal used in the present experiment was produced at the pilot plant for oil extraction (CREOL) of the Technical Centre for Oilseed crops in France (CETIOM). Rapeseed from commercial batches of the 2010 harvest was purchased from a local cooperative. The seed batch was processed under standard conditions to obtain the good quality RSM. To achieve the HD RSM, the residence time in the cooker at 100 °C was extended from 90 to 160 min. The good and HD RSM were analyzed to confirm reduction in protein quality. On average, the total amino acid content of the HD quality of RSM was 5% lower than of the good quality (Table 1). The impact of HD on SID amino acid content of RSM was previously determined (Evonik facts and figures no.1599, 2012), and this was used as the basis for estimating changes in SID values for good versus HD RSM. The application of the estimated SID to the RSM for the current trial led to a bigger difference in SID amino acids between the good and the HD RSM (Table 1).

**Table 1 - Total and standardized ileal digestible (SID) amino acid (AA) contents (g/kg) of good quality and heat damaged (HD) rapeseed meal (RSM; standardized to 88 % dry matter).**

	Good quality RSM		Heat damaged RSM		% reduction of SID AA in HD vs. good quality RSM
	Total AA	SID AA	Total AA	SID AA	
Crude protein	336.3		334.4		
Methionine	6.7	5.1	6.6	2.9	43
Cystine	7.7	5.1	7	2.6	49
Met+Cys	14.5	10.2	13.6	5.5	46
Lysine	17.2	12.2	11.1	4.9	60
Threonine	14.8	9.5	14.7	5.6	41
Tryptophan	4.5	3.3	4.4	2.4	27
Arginine	19.5	15.7	15.6	9.4	40
Isoleucine	13.4	9.7	13.4	7.2	26
Leucine	23.2	17.1	23.1	11.2	35
Valine	17.5	12.8	17.4	9.5	26
Histidine	8.4	6.5	7.7	4.1	37
Phenylalanine	13.4	10	13.1	6.5	35

A total of 640 male Ross 308 broilers were used. Each treatment was replicated eight times with 20 birds per replicate from 10 to 28 days of age. For the first nine days, chicks were fed a commercial crumbled starter feed adequate in energy and all nutrients. The birds were individually weighed at day 10 and randomly allocated to pens. Pens measured 2.0 m x 1.5 m each, and were equipped with bell drinkers, feeding troughs, and wood shavings as litter. Room temperature and lighting was in accordance to breeder recommendations.

The experimental diets were formulated to meet nutrient and energy requirements of broilers from 10-28 days of age except for amino acids. The ideal amino acid ratios to SID lysine of all essential amino acids were followed as recommended by Evonik (QuickChick 1.2), however the absolute level of SID lysine was reduced (10 vs. 11 g/kg diet) in order to operate in the sensitive part of the response curve. The dietary treatments were: (1) Good quality RSM with diet formulated on the basis of analyzed amino acid level combined with respective SID coefficient published by Evonik (2006); (2) HD RSM without any amino acid

adjustment hence assuming same nutrient levels as good quality RSM; (3) HD RSM adjusted for total amino acid content based on analysis (HD-total AA); and (4) HD RSM adjusted for SID amino acids accounting for the degree of heat damage (HD-SID AA). The HD RSM diet represented the case in which heat damage of RSM is not identified and the materials are thus used like regular quality. In practice this may occur if each single batch of ingredient is not analyzed. The inclusion level of RSM was similar in all 4 treatments (150 g/kg of the diet), and this was done to avoid a negative influence from anti-nutritive factors like glucosinolate. Diets were pelleted and analysis of compound feed confirmed accurate feed preparation. Nutrient composition of experimental diets is presented in Table 2. Growth performance and carcass characteristics were measured at the end of 35 days of feeding period.

**Table 2 - Nutrient composition (g/kg) of experimental diets**

Nutrient composition	Experimental diets			
	Good	HD	HD-total AA*	HD-SID AA**
ME, kcal/kg	12.91	12.91	12.91	12.91
Crude protein	198.1	197.8	195.7	209.3
SID Lysine	9.72	8.62	9.09	9.72
SID Methionine	3.99	3.66	3.55	4.20
SID Met+Cys	7.19	6.51	6.36	7.19
SID Threonine	6.22	5.64	5.42	6.22
SID Tryptophan	2.06	1.93	1.89	2.07
SID Arginine	10.64	9.71	9.48	10.58
SID Isoleucine	6.71	6.33	6.28	6.71
SID Leucine	12.92	12.05	11.84	12.83
SID Valine	7.78	7.30	7.17	7.78
SID Histidine	4.46	4.11	4.03	4.39
SID Phenylalanine	7.92	7.39	7.26	7.90

\*Analyzed total AA using NIR; presented SID amino acid levels similar to good quality RSM was used without correction for heat damage;

\*\*Analyzed total AA using NIR; SID amino acid levels are corrected to account for heat damage.

### III. RESULTS AND DISCUSSION

On average (of all 10 essential amino acids), the SID amino acid content in HD RSM decreased by 39% compared with good quality RSM (Table 1) indicating that the imposed heat treatment of RSM greatly reduced its amino acid digestibility. The severity of SID reduction for lysine was highest (60%) which is due in part to the Maillard reaction (Mauron, 1981).

Significant differences in performance were observed across dietary treatments indicating the impact of heat treatment and the success of adjusting nutrient specifications of RSM for the heat damage (Table 3). Birds fed the HD RSM diet had a significantly lowest final body weight, body weight gain, and feed intake and worst feed conversion ( $P < 0.05$ ). However, bird performance were gradually improved to the same level or slightly above the level achieved by those fed good quality RSM when the diets contained HD RSM adjusted for total amino acids (HD-total AA) and for SID amino acids (HD-SID AA). These responses indicate that in the case of heat damage RSM, diet adjusted for total amino acid content partially recovers the performance depression observed. But diets additionally adjusted for SID changes provided similar performance as the good quality RSM diet.

Dressing (% of live weight) and breast meat yield (% of carcass) was also decreased in birds fed HD RSM relative to those fed good quality RSM ( $P < 0.05$ ). However, adjusted total amino acid content of RSM numerically increased carcass yield and significantly

increased breast meat yield ( $P < 0.05$ ), followed by a further numerical increase with the additional adjustment for SID.

**Table 3 - Effects of experimental diets on performance of broilers (10-28 days)**

Parameters	Experimental diets			
	Good	HD	HD-total AA	HD-SID AA
Final body weight, g	1479 <sup>bc</sup>	1285 <sup>a</sup>	1446 <sup>b</sup>	1516 <sup>c</sup>
Body weight gain, g/d	58.0 <sup>bc</sup>	48.8 <sup>a</sup>	56.5 <sup>b</sup>	59.8 <sup>c</sup>
Feed intake, g/d	94 <sup>b</sup>	89 <sup>a</sup>	98 <sup>bc</sup>	99 <sup>c</sup>
Feed conversion ratio, g/g	1.62 <sup>a</sup>	1.82 <sup>c</sup>	1.73 <sup>b</sup>	1.65 <sup>a</sup>
Dressing, % of live weight	61.2 <sup>ab</sup>	60.4 <sup>b</sup>	60.6 <sup>ab</sup>	61.7 <sup>a</sup>
Breastmeat, % of carcass weight	32.7 <sup>a</sup>	30.4 <sup>b</sup>	32.3 <sup>a</sup>	32.7 <sup>a</sup>

<sup>a, b, c</sup>Means having common superscripts in a column do not vary significantly ( $P < 0.05$ )

The results from this study indicate that only adjusting the nutritional matrix of heat damaged RSM for total amino acid content does not completely correct bird performance when compared to bird performance using good quality ingredients. Growth performance and carcass yield of broilers fed HD RSM diets corrected for SID amino acid composition was improved and similar to that of birds fed good RSM diets. In conclusion, the impact of heat damaged RSM can be mitigated by accounting for differences in the SID amino acid content.

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## RELATIONSHIP BETWEEN SHANK, KEEL LENGTH AND FERTILITY IN MEAT-TYPE JAPANESE QUAIL

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### Summary

A study was undertaken to assess the relationship between growth and skeletal traits and reproductive performance traits in commercial Japanese quail strains. Two hundred and twenty five, 8 week old Japanese quail, representing five strains, were housed in cages and pair-mated to determine the relationship between morphometric and reproduction traits. Shank and keel length and live weight were recorded in males and females. Cloacal gland area, ejaculate volume, and sperm concentration were also recorded in males. Strains that had shorter keel length, both for males and females, had higher supply of sperm compared to strains with longer keel length ( $P < 0.05$ ). Keel length in females was positively correlated with live weight ( $r = 0.394$ ;  $P < 0.01$ ), but negatively correlated ( $r = -0.383$ ;  $P < 0.01$ ) with sperm supply. Keel length in males was positively correlated with ejaculate volume ( $r = 0.226$ ;  $P < 0.05$ ) and sperm concentration in an ejaculate ( $r = 0.291$ ;  $P < 0.01$ ), but negatively correlated ( $r = -0.355$ ;  $P < 0.01$ ) with the cloacal gland area. This study suggests the desirability of monitoring morphometric traits in Japanese quail to avoid undesirable correlated changes in reproductive performance traits.

### I. INTRODUCTION

Skeletal development is an important factor in the attainment of optimum live weight and uniformity for meat type poultry. A number of conformational traits are known to be good indicators of body growth (Edward, 2000). In general, shank and keel length are positively correlated with live weight. Selection for longer keel and shank length is regarded as desirable because a longer keel allows more space for breast muscle deposition (Wolanski et al., 2004), while increased shank length and width help dissipation of heat and are positively correlated with mating success (Mahrous and Radwan, 2011). Changes in skeletal conformation and leg dimensions may, however, impede semen transfer (Soller et al., 1965).

Japanese quail occupy an important position in commercial poultry production because of their desirable meat and egg characteristics. Over the last three to four decades quail have been selected in commercial breeding programs for increased growth rate and meat yield. Consequently, the mature weight of current commercial quails is considerably greater than that of 40 years ago. Similarly to meat chickens, changes in body weight and frame size, shank and keel length may possibly be linked with reproductive ability of breeding flocks (McGary et al., 2003; El-Sahn, 2007; Galal et al., 2002). Keeping in mind this phenomenon, this study investigated the relationship between body weight, shank and keel length, and male and female reproductive traits in Japanese quail. We used sperm supply to eggs (the number of sperm present in the outer perivitelline layer and holes made by sperm in the inner perivitelline layer of the ovum) as a measure of reproductive performance of quail (Wishart and Staines, 1995; Farooq et al., 2012).

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## II. MATERIAL AND METHODS

The breeder quail used in the study were provided by the Game Farm Pty Ltd. (Galston NSW, Australia) and were housed in the Native Animal Facility of the University of Western Australia. The house environment was maintained at 22-26°C temperature, 14/10-h light/dark cycle per day, adequate ventilation and *ad-libitum* feed and water supply. The quails were given quail breeder diet containing 20.0% CP and 11.5 MJ/kg ME.

The study was conducted with 8 and 16-week old quails representing five strains, maintained as primary breeding lines to produce meat type breeders (9 males x 36 females x 5 strains, n=225). All quails were housed individually in quail cages. Pair mating was carried out and approximately 30 eggs per pair were collected, marked and opened to estimate sperm supply to the ova by counting the number of sperm reaching the outer perivitelline layer (PVL), and the holes made by sperm in the inner perivitelline layer of the ovum (Wishart and Staines, 1995; Staines et al., 1998; Farooq et al., 2012).

A piece (1.5 x 1.5 cm) of PVL was collected, washed and stained with Hoescht dye 0.01 mM solution (Sigma-Aldrich Co. Castle Hill, NSW, Australia) to mark sperm nuclei. The sperm nuclei were visualized with a fluorescence microscope (Olympus BX60-FL, Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) using a 'U' filter cube with 372 nm excitation and 456 nm emission wavelengths. For counting sperm holes, Schiff's reagent (Sigma-Aldrich Co. Castle Hill, NSW, Australia) was used for staining the IPVL section as described by Bramwell et al., (1995). Counting was carried out in six fields (5.7 mm<sup>2</sup> total area) along the horizontal axis passing through the germinal disc (GD) region.

Shank length (SL) was measured on the left leg as the distance from the middle of the foot-pad to the hock joint (Robinson et al., 1996). Keel length (KL) was measured from the point of fusion in the clavicle to the ventral portion of the sternum. The cloacal gland area (CGA) was measured using the method proposed by Siopes and Wilson (1975). Semen was collected by the teaser method as described by Chelmonska et al., (2008). Ejaculate volume (EV) was recorded using an automatic pipette. Sperm concentration (SC) was estimated using a spectrophotometer and a previously established standard curve for Japanese quail sperm concentration.

We analysed data for main effects of age, strain and the interactions using the General Linear Models procedure of PASW Statistics 18, Release Version 18.0.0 (=D3 SPSS, Inc., 2009, Chicago, IL, www.spss.com). Means were compared by LSD (Least Significant Difference test). Pearson correlation coefficients of PASW Statistics 18 were generated to compare the sperm supply, live weight (LW), cloacal gland area, ejaculate volume, sperm concentration and male and female shank and keel lengths.

## III. RESULTS

The mean values for shank and keel length, body weight and sperm supply are given in Table 1. Strain 1, 2 and 5 had significantly ( $P<0.05$ ) longer keel lengths than strains 3 and 4. However, shank length was not significantly different between the strains. Females from strain 3 and 4 had significantly ( $P<0.05$ ) lower body weights than strains 2 and 5 while males from strains 3, 4 and 5 were significantly ( $P<0.05$ ) heavier than males from strain 1. Strain 3 had higher ( $P<0.05$ ) sperm supply than strains 1, 2 and 5 while strain 4 had higher ( $P<0.05$ ) sperm supply than strains 1 and 2.

Data presented in Table 2 shows rank correlations between the investigated traits. There was a significant positive correlation in females between keel length and live weight and a significant negative correlation between live weight and sperm supply. Male shank and keel length was positively correlated with ejaculate volume and sperm concentration and there was a significant positive correlation in males between cloacal gland area and sperm



supply. There were significant negative correlations in males for sperm supply with ejaculate volume and sperm concentration.

**Table 1 - Mean values for shank and keel length, live weight and sperm supply index for male and female Japanese quails of 5 different strains.**

Strain	Male				Females			
	Shank length (cm)	Keel length (cm)	Live weight (g)	Sperm supply (n)	Shank length (cm)	Keel length (cm)	Live weight (g)	Sperm supply (n)
1	3.34±0.01	8.00±0.04 <sup>a</sup>	265±3.7 <sup>b</sup>	34±4.0 <sup>b</sup>	3.35±0.01	7.92±0.04 <sup>b</sup>	320±3.5 <sup>cd</sup>	33±3.8 <sup>b</sup>
2	3.35±0.01	7.98±0.04 <sup>a</sup>	269±3.7 <sup>ab</sup>	39±3.9 <sup>b</sup>	3.34±0.01	7.91±0.04 <sup>b</sup>	327±3.5 <sup>bd</sup>	37±3.8 <sup>b</sup>
3	3.34±0.01	7.72±0.04 <sup>c</sup>	273±3.8 <sup>a</sup>	51±4.0 <sup>a</sup>	3.33±0.01	7.76±0.04 <sup>a</sup>	315±3.7 <sup>ac</sup>	50±4.0 <sup>a</sup>
4	3.34±0.01	7.85±0.04 <sup>b</sup>	276±3.8 <sup>a</sup>	45±4.1 <sup>ac</sup>	3.34±0.01	7.69±0.04 <sup>a</sup>	319±3.6 <sup>c</sup>	43±3.9 <sup>ac</sup>
5	3.34±0.01	8.0±0.04 <sup>a</sup>	273±3.9 <sup>a</sup>	41±4.2 <sup>bc</sup>	3.34±0.01	7.87±0.04 <sup>b</sup>	330±3.8 <sup>bd</sup>	40±4.1 <sup>bc</sup>

<sup>a-c</sup> Means with different superscript in columns differ significantly ( $P < 0.05$ )

**Table 2 - Pearson correlation coefficients for SL, KL, LW, CGA and SS for males and females**

Males	KL	SL	LW	CGA	EV	SC
Shank length (SL)	0.226*					
Live weight (LW)	0.055	-0.025				
Cloacal gland area (CGA)	-0.355**	-0.022	-0.123			
Ejaculate volume (EV)	0.226*	0.194	-0.095	0.037		
Sperm concentration (SC)	0.291**	0.183	-0.111	-0.029	0.457**	
Sperm supply (SS)	-0.198	-0.123	-0.134	0.293**	-0.293**	-0.260*
Females	KL	SL	LW			
Shank length (SL)	0.109					
Live weight (LW)	0.394**		0.034			
Sperm supply (SS)	-0.383**		-0.175		-0.452**	

\*  $P < 0.05$ ; \*\*  $P < 0.01$

#### IV. DISCUSSION

The positive correlation between female live weight and keel length, revealed by this study, supports the previously reported (Wolanski et al., 2004) strong association between keel length and muscle deposition. Whether this association can be reflected in selection for improvement in live weight remains to be demonstrated. In males, no relationship was found between body weight, and shank and keel length. Heavier males recorded better fertility compared to those with lower body weight. This finding was contrary to our expectation that heavy weight males would have lower relative reproductive performance due to reduced mating ability as was previously reported for broiler breeders (Siegel and Dunnington, 1985). Rather, this finding is in accordance with McGary et al., (2003) who similarly reported no effect of male body weight on fertility in broilers.

The significant positive correlation between male keel length, ejaculate volume, and sperm concentration are in agreement with the reports of El-Sahn (2007) who found significant positive correlation between keel length, ejaculate volume and sperm concentration in Bandarah chicken cocks. Similarly Galal et al., (2002) demonstrated a positive phenotypic correlation between shank length and ejaculate volume, percentages of abnormal sperm, and percentages of dead sperm in Norfa chicken cocks. However, the

negative correlation between keel length and cloacal gland area, and sperm supply revealed by this study, suggests that for quail these measures of fertility may be negatively affected by selection for improved meat yield.

In conclusion keel length in male and female quails appears to be associated with measures of fertility, including sperm supply. More comprehensive gender and strain specific studies, however, are required to fully evaluate these relationships, thus ensuring that reproductive traits are not adversely affected by selection for economic traits.

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## DEVELOPMENT OF METHODS FOR RECOVERY AND QUANTITATION OF VIRAL NUCLEIC ACIDS FROM BROILER LITTER

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### Summary

We investigated the development of standardised methods of extraction and quantitation of viral nucleic acids from broiler litter. To detect and quantify virus we used fully quantitative Taqman® qPCR assays with plasmid-based standard curves to quantify Marek's disease virus (MDV, dsDNA), infectious laryngotracheitis virus (ILT, dsDNA), Fowl adenovirus (FAdV, dsDNA), chicken anaemia virus (CAV, ssDNA) and infectious bursal disease virus (IBDV, dsRNA). A series of experiments examined the effects of litter washing, blending, bead beating, and removal of inhibitors using polyvinylpyrrolidone (PVPP). To evaluate the qPCR assays and the DNA extraction techniques we monitored the recovery of fixed amounts of virus added to litter samples. The three litter types used were hardwood shavings, softwood (pine) shavings and rice hulls. Hardwood shavings were shown to contain high levels of PCR inhibitors but these could be neutralised by PVPP. Detectable virus recovery was good for IBDV and CAV, but low for the dsDNA viruses ILT and MDV. The third dsDNA virus, FAdV, was unable to be detected. The four detectable viruses were detectable in all fractions of material (retentate after filtration, and in both the pellet and supernatant fractions following centrifugation of the filtrate) with highest concentrations in the pellet. The results indicate that a method based on washing samples with buffer containing 0.15% Tween-80 followed by bead beating and PVPP treatment would enable detection of most DNA and RNA viruses from litter with the greatest concentration of virus found in the pellet fraction after centrifugation. Work is ongoing to resolve the low recovery rate of dsDNA viruses and to simplify the litter processing and DNA extraction further.

### I. INTRODUCTION

The Australian broiler industry has a well-recognised problem with both the supply of litter material in some regions, and with the disposal of spent litter. This is driving a trend towards increased reuse of litter to raise successive chicken batches. One of the risks associated with this practice is carryover of litter-borne viral and bacterial pathogens between successive batches of chickens. This risk has been confirmed and quantified for some viral pathogens, using a chick bioassay developed for this purpose (Islam et al., 2012). The chick bioassay has also been used successfully to determine the efficacy of litter treatments such as pasteurising by heaping or windrowing between batches of chickens in reducing virus transmission between batches in used litter (Walkden-Brown et al., 2010). While the bioassay has proved a useful research tool, in practice it is complex and expensive to implement and it would be ideal if a direct test of viral load in litter could be developed. Quantitation of viral load using quantitative real-time PCR (qPCR) is an attractive option (eg. Guan et al., 2008) but to be effective requires resolving of three issues: i) Ensuring adequate representation of the original environmental material in the final nucleic acid sample assayed; ii) Ensuring that the PCR reaction is not compromised by inhibitors; iii) Ensuring that the reaction is measuring infectious virus, not inactivated virus with intact nucleic acids.

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As part of a project to investigate these issues and determine the feasibility and utility of direct assay of viral pathogens in poultry litter using qPCR we needed first to develop suitable qPCR tests for 5 viruses and develop effective methods extraction and quantitation of viral nucleic acids from broiler litter. This paper reports our findings in this regard.

## II. MATERIALS AND METHODS

Fully quantitative Taqman® qPCR assays with plasmid-based standard curves were used to quantify Marek's disease virus (MDV), infectious laryngotracheitis virus (ILTV), chicken anaemia virus (CAV), Fowl adenovirus (FAdV) and infectious bursal disease (IBDV). MDV, ILTV and FAdV are double stranded DNA viruses, CAV is a single stranded DNA virus and IBDV is a double stranded RNA virus. Methods were either adapted from published assays or developed by the project as follows: MDV (Islam et al., 2006); CAV (Zhang 2009); FAdV, modified HEX-S (Steer et al., 2009); ILTV (Callison et al., 2007), IBDV developed by project from GenBank sequence data of the VP2 gene (Ignjatovic and Sapats 2002).

Specific pathogen free (SPF) litters (LT11-C-LP1) based on pine shavings, hardwood shavings and rice hulls were generated by placing SPF chickens on these litters for 42 days then storing at -20°C and following drying for 7 days at 37°C until required. Infective litter was generated by raising broilers vaccinated with MDV, ILTV, IBDV, CAV and FAdV live vaccines on pine shavings for 42 days (LT11-C-LP2). Stored infective pine shavings from the study of (Islam et al., 2012) were also used (Samples LT09-C-CB9).

Study 1 comprised a number of small-scale tests to demonstrate isolation of a DNA (MDV) and an RNA virus (IBDV) from naturally contaminated litter and testing of various preparation steps prior to nucleic acid extraction. These treatments included washing the litter with MilliQ water, or TE buffer containing 0.15% Tween-80 to release the virus from the litter (shaking or blending); washing the litter with 10% beef extract then precipitation of virus with 8% PEG 6000 (Guan et al 2008).

Study 2 comprised a formal factorial experiment testing the effects of litter washing method (TE buffer plus 0.15% Tween-80 or 10% beef extract), wash times (2 hr and 16 hr at 4°C), blending (blend, no blend) and bead beating (bead beat, no bead beat) on detection of MDV and IBDV in DNA/RNA extracted from unstrained slurry, or the retentate or filtrate following straining of the slurry.

Study 3 tested for the presence of PCR inhibitors in litter of the different types by addition of fixed amounts of viral DNA to serial dilutions of extracted litter material and then investigated the effects of polyvinylpyrrolidone (PVPP) in overcoming inhibition.

Study 4 was a large factorial experiment in which fixed amounts of commercially available vaccine for 4 of the viruses and infective dust for MDV were added to 100 g of SPF litters of different types and recovery in different fractions determined by qPCR following a range of preparatory steps. Samples of the pine shavings, hardwood shavings and rice hulls litters were incubated in TE buffer plus 0.15% Tween-80 on ice with shaking for two hours (blending twice during this time) then filtered through a fine nylon mesh (approximately 1mm<sup>2</sup>). The retentate (R) was retained and the filtrate centrifuged at 17,500g for 30mins at 4°C, to provide a supernatant (S) and pellet (P). The R, S and P fractions were then subjected to the following treatments in a complete factorial design: Bead beating (BB) for 5 minutes or not; and treatment with PVPP or not.

Total nucleic acids were extracted using magnetic bead technology (MagMax™ Total RNA isolation kit Ambion) manually or in the automated Kingfisher Flex 96 (Thermo Fischer Scientific) although the Qiagen QIAmp DNA stool extraction kit was also tested in

Study 1. The final eluate was diluted 1:10 and used as the template in the qPCR reaction for all 5 viral assays. Assay results were generally expressed as viral copies (VC) per unit.

Where relevant the significance of fixed effects and their interactions in the various studies were determined by ANOVA with means separation by protected Student's T test, or in the case of ratios, by contingency table analysis. Analyses were performed with JMP 9 (SAS Institute, NC, and USA). Means are presented  $\pm$  SEM.

### III. RESULTS

Study 1: As this comprised a series of small tests, formal statistical analysis was not attempted. Nucleic acid targets of both MDV (DNA virus) and IBDV (RNA virus) were successfully recovered from pine shaving litter material naturally contaminated by infected chickens. Significant amounts of both were detected in the retentate, filtrate and pellet fractions following litter preparation. Use of Tween-80 to wash the litter provided better overall results for both viruses than water or the beef extract. Water failed to release IBDV from the litter sample with the majority of the viral RNA detected in the initial litter strainings, however it was successful in releasing MDV. Conversely IBDV was satisfactorily detected using beef extract whilst MDV was not. Blending and shaking samples in Tween 80 produced high recoveries for both viral targets so this was explored further in Study 2.

Study 2: MDV, but not IBDV target sequence amplified successfully in this experiment. For MDV the overall mean value for viral load was  $8417 \pm 1813$  VC/g litter for all preparation methods and fractions. Increasing wash time from 2 hr to 16 hr at 4°C significantly reduced the number of positive samples from 96% to 67% ( $P = 0.006$ ). Washing with Tween buffer resulted in significantly higher MDV recovery than washing in beef extract buffer ( $11,585$  v  $5,245$  VCN/g litter,  $P = 0.05$ ). More viral DNA was recovered in the filtrate ( $15,161$  VC/g litter) than the solid retentate after straining ( $1,275$  VC/g litter) ( $P = 0.004$ ) with unstrained slurry intermediate ( $8,815$  VC/g litter). There were no other significant main effects although there were trends towards improved recovery with blending, and reduced recovery with bead beating.

Study 3: Initial spiking tests using a 7 x 10-fold serial dilution of DNA extracted from pine shavings, rice hulls and hardwood shavings indicated that the qPCR reaction for MDV was inhibited in undiluted samples for all litter types, with amplification of the target first occurring at dilutions of 1:10 for pine shavings and rice hulls and 1:1000 in hardwood shavings. When DNA was extracted from infective litter dried at 37°C rather than stored at -20°C, similar results were obtained except that amplification occurred at a dilution of 1:100 in the hardwood shavings. The spike test was repeated using a 6 x 2-fold serial dilution of DNA extracted from hardwood shavings with 6.6% w/v PVPP added to the extracted DNA sample and mixed for 5 minutes, 1 hour or overnight at room temperature. Amplification of the target product occurred at dilutions of 1:4, 1:2 and neat respectively. In all of the tests, once amplification occurred, there was no effect of further dilution on the amount of target recovered when adjusted for the dilution factor.

Study 4: The dataset comprised 144 qPCR results. CAV, IBDV, ILTV, and MDV, but not FAdV were readily detected in all three litter types with overall percentages of positive qPCR results of 69%, 43%, 22% and 26% respectively ( $P < 0.001$ ). When no PVPP was used the percentage of positive samples was significantly lower from hardwood (6%) than rice hulls (50%) or softwood, (47%) ( $P < 0.0001$ ) but this effect was completely removed by inclusion of PVPP with values of 48%, 48% and 42% respectively, indicating a selective effect of PVPP in overcoming the PCR inhibition observed with hardwood shavings. The effect of PVPP was evident for all viruses but was greatest for CAV and least for IBDV. For the three DNA viruses no amplification occurred at all on hardwood shavings in the absence

of PVPP. The pellet had the highest ratio of positive samples (47%) followed by the supernatant (41%), then the retentate (33%) ( $P = 0.07$ ). PVPP increased the percentage of positive samples in the pellet ( $P = 0.008$ ). Bead beating increased the proportion of positive samples from 35% to 45% ( $P = 0.09$ ). This was observed for all viruses except ILTV for which there was no difference with or without bead beating. The mean measured recovery rates of added virus were 18.0, 10.4, 0.065 and 0.052 % for IBDV, CAV, ILTV and MDV respectively ( $P < 0.001$ ). The virus recovery rate was not significantly affected by any of the other factors in the experiment.

#### IV. DISCUSSION AND CONCLUSIONS

This work confirms other published work (eg. Guan et al., 2008) demonstrating that viral nucleic acids can be reliably amplified from poultry litter. Clearly the type of litter is important, with hardwood based litter material containing PCR inhibitors. Fortunately these were able to be neutralised with PVPP. For all viruses significant amounts were found in the retentate after filtration, and in both the pellet and supernatant fractions following centrifugation of the filtrate. The results indicate that a method based on washing samples with TE containing 0.15% Tween-80 followed by bead beating and PVPP treatment would enable detection of both DNA and RNA viruses from litter with the greatest concentration of virus found in the pellet fraction after centrifugation. Detectable virus recovery was good for IBDV and CAV, but low for the dsDNA viruses ILTV and MDV. The third dsDNA virus, FAdV, was unable to be recovered at all. Work is ongoing to resolve the low recovery rate of dsDNA viruses and to simplify the litter processing and DNA extraction further.

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## EFFECTS OF VARIOUS ADDITIVES TO REUSED BROILER LITTER ON LITTER AMMONIA PRODUCTION, CHICKEN WELFARE AND PERFORMANCE

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### Summary

We investigated the effects of adding alum, sodium bisulphate, bentonite, zeolite and NaturClean CSM® at 3.2, 3.2, 13, 13 and 0.9 % by weight respectively to reused litter on moisture content, pH, ammonia production, bird liveweight and conditions linked to welfare including scores for footpad dermatitis, hock burn, breast burn and breast feathering. Treatments, including no amendment, were applied to four pens each 2.25 m<sup>2</sup> with half the pens having 20% additional water added. Nineteen broiler chicks were reared in each pen up to day 42 with sampling of the litter and birds at various intervals. Litter moisture content of 31% on day 0, reduced to approximately 21% on days 7 and 14 before increasing to a value of 53% on day 42. All litter amendments except NaturClean CSM® reduced ammonia production with the greatest reductions seen with alum and sodium bisulphite on days 7 and 14. Amendments worked similarly in litter with or without water addition. There were no significant effects of litter amendment on bird weights or measures of welfare with welfare measures generally worsening with time and with initial addition of water. Ranking of reused litter treatments from 1 to 6 (best to worst) for each welfare measure, followed by the averaging of those rankings, provided mean rankings of 1.75, 2.25, 3, 3.75, 4.75 and 5.5 respectively for reused litter without amendment, bentonite, alum, sodium bisulphite, NaturClean CSM® and zeolite treatments. Both acidifying and adsorbent litter additives have potential to significantly reduce ammonia concentrations on reused litter. Further work is required to determine optimum inclusion rates and more clearly identify effects on welfare and performance.

### I. INTRODUCTION

Multiple batch litter reuse reduces spent litter production and has potential to improve the fertiliser value of spent litter. Australian producers have been reluctant to reuse litter for multiple broiler batches because of concerns about pathogen transfer between batches, increased ammonia production from litter and increased incidence of footpad dermatitis and other skin infections leading to compromised productivity. However the scarcity and high prices of bedding materials and reductions in value of spent litter are making litter reuse increasingly attractive to producers. Some of the potential problems associated with litter reuse are ameliorated if brooding occurs on new litter, followed by grow out on used litter. However if chicks are to be brooded on used litter, amelioration of ammonia production is desirable, particularly in winter months when ventilation rates are low.

Chemical amendment of litter to reduce ammonia release from reused litter is widely used internationally but is only starting to be practiced in Australia. There are two main types of litter amendments that claim to reduce ammonia: acidifiers and adsorbents. Application of amendments aims to improve air quality, reduce moisture and add value to spent litter by retaining nitrogen and phosphorus. This study investigated the effects of applying five locally

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available litter amendments to reused litter on litter ammonia production, chicken weights and a range of welfare measures.

## II. MATERIALS AND METHODS

A  $6 \times 2$  factorial experiment was conducted with five litter amendments (as well as no amendment) applied to end-of-batch, single-use pine shavings litter at two moisture levels. Litter had been heaped for 7 days prior to spreading to a depth of 6–8 cm. Alum, sodium bisulphate, bentonite, zeolite and NaturClean CSM® bedding conditioner (a proprietary mixture of mineral and plant extracts) were applied at 3.2, 3.2, 13, 13 and 0.9 % by weight or 0.425, 0.425, 1.56, 1.56 and 0.1 kg/m<sup>2</sup> respectively to the surface of the spread litter in four pens each 1.5 x 1.5 m, prior to chick placement with Ross broilers. Water was added to two of the four pens for each amendment to increase initial litter moisture content by an estimated 20% by weight. Two external control pens had fresh pine shavings without extra moisture. Nineteen chickens per pen were reared to 42 days.

Ammonia production from litter samples collected on days 7, 14, 21, 28 and 42 was measured in duplicate at 30°C using the chamber acid trap method of Miles *et al.* (2008). Briefly, 660 mL subsamples of litter (49–427 g) were placed in sealed 1 litre containers with air pumped through them at 215 mL/min. After equilibration at 30°C overnight, total ammonia production was determined for 6 hours for each sample chamber. Airflow exiting each sample chamber was allowed to bubble through two successive flasks of boric acid solution with the added indicators bromocresol green & methyl red. After titrating with hydrochloric acid to a red colour endpoint, ammonia production was calculated as milligrams of N lost during the sample exposure to regulated airflow in the system, as follows: mg of N = (mL of HCl) × (HCl molarity) × MW<sub>N</sub>. Following the initial run, 20% water by weight of litter was added and allowed to equilibrate overnight before ammonia production was measured again. Litter dry matter (oven drying for 48 hr at 105°C) and pH (pH meter, 1:20 litter solution) were assessed, the former on all collection dates, the latter on days 28 and 42.

Ten chickens randomly selected from each pen on days 7, 14, 28 and 42 were individually weighed and assessed for footpad dermatitis (FPD) by scoring size (% area of the foot) and severity (discoloration, erosion, and necrosis) of lesions on a 10-point (0 to 9) scale of increasing severity (Allain *et al.* 2009). On days 28 and 42, hock burn (HB), breast burn (BB) and breast feather cover (BF) were also measured. HB and BB were scored 0 to 2 on an ascending scale of severity while BF was scored using a 4-point scale (0 to 3) of ascending quartiles of breast feather coverage.

Animal measurements were averaged for each pen, and these, together with all litter and ammonia measurements, were subjected to two repeated measures analyses using mixed REML models with pen fitted as a random variable. Analysis 1 included only the reused litter treatments and tested the fixed effects of day, litter treatment and water addition (20% water added or not). Analysis 2 included the new litter control but excluded all reused litter treatments with added water and tested the fixed effects of day and litter treatment. Data were analysed using JMP 9 (SAS Systems, USA). Least squares means and SEM are presented.

## III. RESULTS

Analysis 1 of litter moisture content revealed significant overall effects of day of sampling ( $P < 0.0001$ ) and water addition ( $P = 0.035$ ) (Figure 1a) with a trend towards a significant effect of treatment ( $P = 0.06$ ; Table 1). Analysis 2 showed a significant interaction between the effects of day and treatment with the new litter treatment showing the greatest increase over the course of the experiment (8.9% at day 0 to 55.7% at day 42). Litter pH at days 28 and 42 was significantly affected by day ( $P < 0.0001$ ) and treatment ( $P = 0.0003$ , Table 1). Litter pH



at day 28 ( $8.7 \pm 0.05$ ) was significantly higher than at day 42 ( $8.03 \pm 0.05$ ) with most of the decline occurring in the treatments without acidifiers.

Analysis 1 of ammonia production revealed significant effects of litter treatment ( $P < 0.0001$ ), day ( $P < 0.0001$ ), initial moisture ( $P = 0.02$ ) with significant interaction between the effects of day number and treatment ( $P < 0.0001$ ) and day and initial moisture ( $P = 0.02$ ). Ammonia production was higher on day 7 than days 14 and 21, before increasing to new highs on days 28 and 42. The acidifying treatments had a greater effect on reducing ammonia than the adsorbents, with NaturClean CSM® having no significant effect (Table 1). The widest differences were observed prior at days 7 and 14, particularly for the acidifiers (Figure 1b). Analysis 2 also showed a significant overall effect of litter treatment ( $P = 0.006$ ) with the new litter treatment having significantly lower ammonia production than used untreated litter, but significantly higher levels than reused litter treated with alum or sodium bisulphite.

**Table 1 - Least squares means<sup>1</sup> for the main effect of litter treatment<sup>2</sup>. All sampling days are included. Means not sharing a common letter within columns differ significantly ( $P < 0.05$ )**

Analy-sis	Litter treat.	Litter moisture	Litter pH	Ammonia	LW (g)	FPD	Hock burn	Breast burn	Breast feather
1	Reused	29.3	8.76 <sup>a</sup>	2.08 <sup>a</sup>	959	2.52	0.30	0.74	2.8
	R+B	26.4	8.57 <sup>ab</sup>	1.86 <sup>c</sup>	977	2.87	0.45	0.69	2.5
	R+Z	33.1	8.10 <sup>cd</sup>	1.91 <sup>bc</sup>	939	3.22	0.65	0.91	2.2
	R+A	36.8	8.07 <sup>d</sup>	1.74 <sup>d</sup>	896	3.24	0.45	0.72	2.5
	R+SB	30.6	8.33 <sup>bc</sup>	1.73 <sup>d</sup>	923	3.08	0.56	0.86	2.5
	R+NC	39.5	8.53 <sup>ab</sup>	2.02 <sup>ab</sup>	945	3.39	0.59	0.74	2.4
SEM		2.86	0.082	0.035	20	0.41	0.10	0.09	0.10
P value		0.063	0.0003	0.0001	0.166	0.704	0.223	0.503	0.063
2	New	29.3±	8.38±	1.84±	889±	3.02±	0.30±	0.63±	2.6±
		4.35	0.20	0.040	31	0.53	0.12	0.19	0.14

<sup>1</sup>LM – litter moisture (%), Ammonia production [Log<sub>10</sub> (mg N/10L litter/hr)]

<sup>2</sup>R = Reused, B = Bentonite, Z = Zeolite, A = alum, SB = sodium bisulphite, NC= NaturClean CSM®

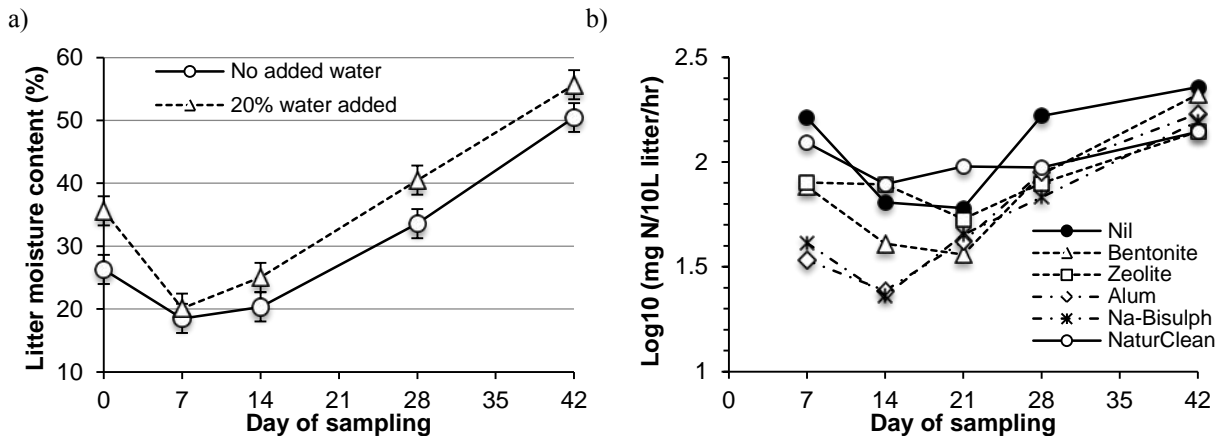


Figure 1 - a) Least squares means for moisture content of litter by day and water addition; and for b) ammonia production Log<sub>10</sub> (mg N/10L litter/hr) by treatment and day (Analysis 1).

Analysis 1 of liveweight revealed a significant overall effect of day (127, 345, 1085, 2201 g at days 7, 14, 28 and 42 respectively  $P < 0.0001$ ) with no significant other main effects or interactions including the effect of treatment ( $P = 0.17$ , Table 1). Analysis 2 produced a similar result with a non-significant effect of treatment ( $P = 0.12$ ). Interestingly chicks on unamended used litter had the highest value for mean liveweight ( $1004 \pm 31$  g) and those on new litter had the lowest value ( $889 \pm 31$  g).

Analysis 1 of FPD score revealed a significant overall effect of day (1.44, 2.59, 3.95 and 4.25 at days 7, 14, 28 and 42 respectively  $P < 0.0001$ ) and a significant interaction between the effect of water addition and day ( $P = 0.013$ ) with the negative effect of water addition increasing with time. There was no effect of litter treatment on FPD or other measures linked to welfare (Table 1). Hock scores were higher ( $P < 0.0001$ ) on day 42 (0.75) than day 28 (0.25), and higher on litter to which water had been added (0.6 v 0.4,  $P = 0.0282$ ). Breast burn scores were also higher ( $P < 0.0002$ ) on day 42 (0.98) than day 28 (0.57) but no other effects were significant. Breast feathering scores were worse on litter to which water had been added (2.36 v 2.62,  $P = 0.01$ ) with a significant interaction ( $P = 0.01$ ) between this and litter treatment with the adverse effects of moisture only observed on the zeolite, sodium bisulphite and NaturClean CSM® treatments.

#### IV. DISCUSSION AND CONCLUSIONS

All litter amendments with the exception of NaturClean reduced ammonia production from litter with the greatest reductions of 0.5-0.7  $\text{Log}_{10}(\text{mg N}/10\text{L litter/hr})$  occurring with the acidifying additives alum and sodium bisulphite. The effects were greatest on days 7 and 14, during the brooding period, although some effect was observable throughout the experiment, consistent with reductions in pH measured at days 28 and 42. The amendments worked similarly in litter with or without water addition. There were no significant effects of litter amendment on bird weights or measures linked to welfare with the latter generally worsening with time and with addition of water at the beginning. Interestingly the best bird growth was observed in chickens on reused litter without amendment and the worst on new litter ( $P=0.12$ ). Cressman and Walkden-Brown (2012) also reported best growth on reused litter without amendment on each of 3 replicated experiments on commercial farms. In that study the amendment pH Natural® reduced ammonia measurements during brooding, but had an adverse effect on growth. Ranking of reused litter treatments from 1 (best) to 6 (worst) for each welfare measure, followed by the averaging of those rankings, provided mean rankings of 1.75, 2.25, 3, 3.75, 4.75 and 5.5 respectively for reused litter without amendment, bentonite, alum, sodium bisulphite, NaturClean CSM® and zeolite treatments respectively.

In conclusion, addition of either acidifying or adsorbent litter additives has potential to reduce ammonia concentrations on reused litter. Further work is required to determine optimum inclusion rates and more clearly identify effects on welfare and performance at a farm scale.

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## LITTER PASTEURIZATION: EFFECTS OF COVER AND MOISTURE LEVEL ON THE TEMPERATURE OF THE LITTER HEAP

A.F.M.F. ISLAM<sup>1</sup>, S.W. WALKDEN-BROWN<sup>1</sup> and B. WELLS<sup>2</sup>

### Summary

Two on-farm trials were conducted to examine the effects of adding extra moisture and covering on temperature profiles of broiler litter heaped in-shed for pasteurisation before reuse. Covering of litter heaps with tarpaulins increased heap temperatures by 5–10 °C particularly near the surface of the heap and also helped maintain high temperatures longer. Adding up to 10% water (w:w) also increased heap temperatures where litter moisture content was below 25%. However the two effects were not additive with no beneficial effect of covering observed at high moisture levels. This information may be useful for optimising litter pasteurization protocols to achieve early and uniform high temperatures.

### I. INTRODUCTION

Limited availability and high cost of chicken bedding materials are driving some broiler producers to reuse litter for multiple chicken batches. Partial pasteurization of litter between batches by heaping or windrowing for up to ten days can greatly assist in reducing pathogen carryover in litter to the next batch of chickens. We have previously reported litter temperatures of 50–70 °C inside broiler litter heaps between days 3 and 10 after heaping, depending on depth within the heap (Walkden-Brown *et al.*, 2010). The surface of the heaps remained relatively cool (20–40 °C) and turning at day 3 significantly increased temperatures after turning. This process was shown to eliminate or greatly reduce the infective load of viral pathogens and coccidia in the litter (Islam *et al.*, 2010). However, current industry demand is for short batch turnaround times of six days or less with a strong preference to avoid turning litter during pasteurisation if possible. Thus there is demand for a “quick uniform pasteurisation” of litter without turning. There are reports in the USA that both increased moisture level (Schmidt *et al.*, 2010) and covering (Macklin *et al.*, 2006) of litter can elevate heap temperature significantly.

The objective of the current study was to investigate the use of these methods alone and in combination to accelerate the increase in temperature following heaping, increase the maximum temperature achieved and to achieve greater uniformity of temperature in the heap, relative to untreated heaps particularly near the surface.

### II. MATERIALS AND METHODS

Experiment 1 utilized a 3 (moisture) × 2 (cover) factorial design with two replicates. Polypropylene tarpaulin covers were or were not applied to litter heaps with three levels of added water (w:w) viz. none (M0), 5% (M5) or 10% (M10). The experiment was conducted on a Sydney farm with one tunnel ventilated and two conventional open-sided sheds. The original litter was pine shavings and were preparing for the 3<sup>rd</sup> batch of chickens having an average moisture level of 21-23%. Twelve litter heaps were prepared in total, three in the smallest shed, four in the medium shed and five in the largest tunnel ventilated shed. The floor area of each shed was divided into the requisite number of sections and weight of the litter in each section was estimated. The required amount of water for each heap was added during the preparation of heaps by a hose for which the flow rate had been measured.

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Once the heaps were formed, 10 iButton temperature data loggers (Maxim Integrated, San Jose, CA, USA) were inserted in each heap at five depths (0 cm, 5 cm, 10 cm, 25 cm and 50 cm) so that each depth was replicated. Time of insertion and the location of each iButton were recorded. All iButtons were set to record temperature hourly. Six heaps, two from each moisture level, were covered with large polypropylene tarpaulin covering the entire heap to ground level. At day 7 of the experiment, all iButtons were collected and temperature data retrieved for analysis. A representative sample from each heap was also collected at the beginning (day 0) and end (day 7) of the experiment to determine moisture content.

Experiment 2 was a simpler 2×2 factorial study with two levels of moisture, no added water (M0) and 10% added water (M10) and two levels of cover (with or without). iButtons were placed inside the heaps as in experiment 1 and temperature was recorded hourly for seven days. Litter samples were collected at the beginning and end of the experiment for the determination of moisture content.

Data were analysed using analysis of variance to test the main effects (moisture, cover and depth) and their interactions. Significance of differences between levels within an effect were tested using student's T test. Least squares means and  $\pm$ standard errors of means (SEM), or simple temperature profiles without analysis are presented.

### III. RESULTS

*Experiment 1.* There were significant effects of Depth ( $P < 0.0001$ ) and Cover ( $P < 0.03$ ) but not Moisture level ( $P = 0.21$ ) on the mean temperature with a significant interaction between Moisture and Depth ( $P < 0.05$ ). As expected, temperature was significantly lower on the heap surface (0 cm) than at any other depth. The mean temperature increased with increasing depth in low moisture content litter (M0), however, with moisture level M5 and M10, temperature increased with depth up to 10 cm and 5 cm respectively and then stayed relatively constant with a tendency to reduction at 50 cm depth (Figure 1 left). Application of covers increased overall mean temperature in the M0 litter only (Figure 1 right).

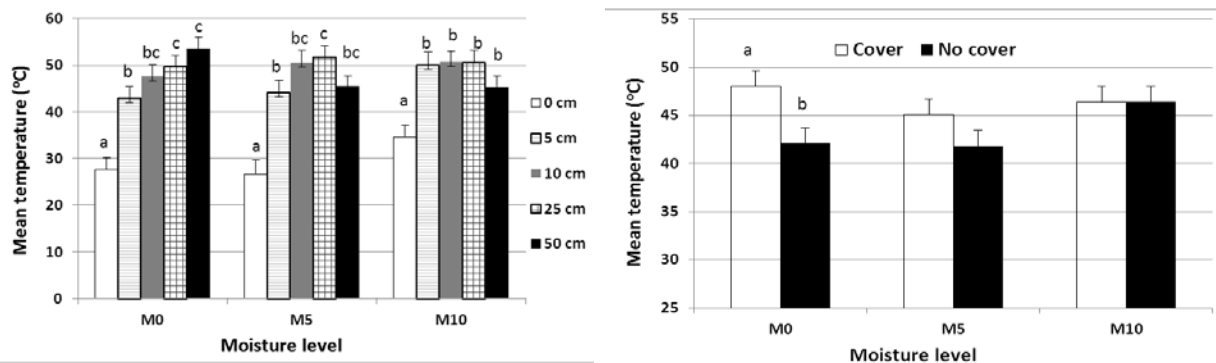


Figure 1 - Mean temperature ( $\pm$ SEM) achieved in heaps at various depths (left) and with or without cover (right) at three moisture levels. Columns within moisture treatments not sharing a common letter are significantly different ( $P < 0.05$ ).

Exploration of the temperature profiles over time revealed that, overall, covering resulted in marginally higher temperatures from day one onward (Figure 3, left). Covering increased surface temperatures by approximately 10 °C between days 2 and 7 up to the end of trial. It also increased temperatures significantly at 5 and 10 cm depths, particularly later in the experiment (Figure 2). Addition of 10% moisture to the litter achieved about an extra 5 °C mean temperature from day 4 onward (data not shown). There was no significant difference between day 0 and day 7 moisture contents ( $P = 0.07$ ) of litter, however there was a tendency towards increased moisture content following 7 days of heaping (Table 1).

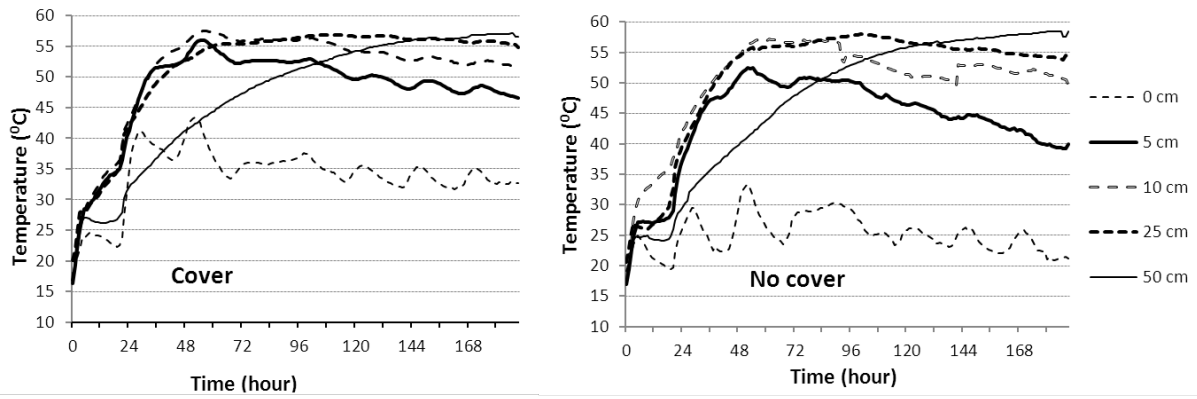


Figure 2 - Expt. 1. temperature profiles over time at various depths in heaps with (left) or without (right) covers.

Experiment 2. There was a significant effect of Depth ( $P < 0.001$ ), Moisture ( $P = 0.003$ ) and Cover ( $P = 0.003$ ) on the mean temperature of the heap with significant interaction between the effects of Depth and Cover ( $P < 0.008$ ) and Moisture and Cover ( $P < 0.0004$ ) but not Depth and Moisture ( $P = 0.61$ ). In general, mean temperature increased with the depth in the heap. The mean temperature was higher at all depths in covered heaps apart from the surface (data not shown). The interaction between the effects of moisture and cover was because the effect of covering was only evident in the M0 litter (Figure 3, right). Thus addition of extra moisture or covering the heap increased mean temperature by approximately 10°C from day three onwards, but the effects are not additive. Raw moisture content data are presented in Table 1.

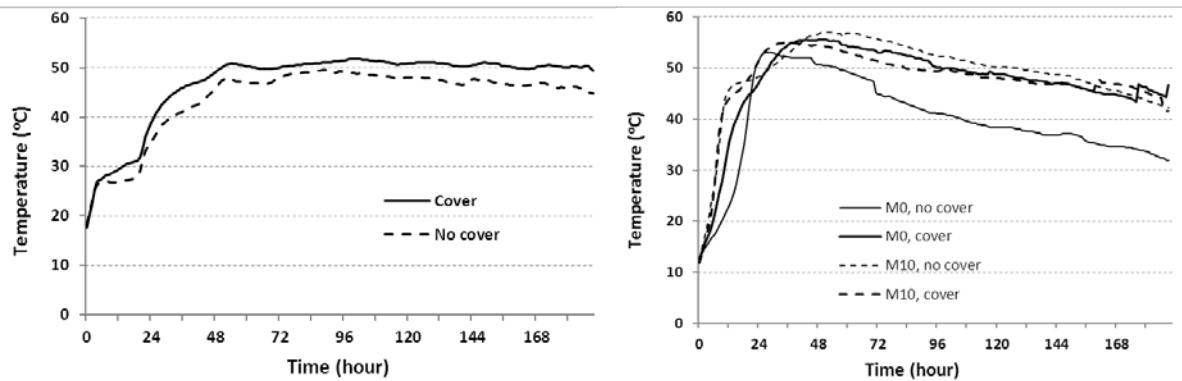


Figure 3 - Temperature profile in heaps over time showing the overall effect of covering in experiment 1 (left) and the effects of cover and moisture level in experiment 2 (right).

Table 1 - Moisture contents of litter in Experiments 1 and 2

Moisture level	Cover	Moisture level (%)					
		Experiment 1			Experiment 2		
		Day 0	Day 7	% Change	Day 0	Day 7	% Change
M0	Yes	21.61	24.37	+12.7	13.01	10.23	-13.6
M0	No	23.10	27.07	+17.0	15.36	17.90	+14.6
M5	Yes	27.68	35.30	+27.5			
M5	No	27.27	28.69	+5.2			
M10	Yes	35.46	33.39	-5.8	25.78	28.24	+9.5
M10	No	33.33	37.13	+11.4	28.17	31.90	+13.2

#### IV. DISCUSSION AND CONCLUSIONS

This study demonstrated that a higher temperature inside litter heaps can be achieved by covering the heaps due to a more rapid increase and sustained temperature, particularly at shallower depths in the heap. A mean temperature of 55 °C can be achieved within 24 hours just 10 cm beneath the surface of the heap and this can be maintained up to 7 days.

Based on a laboratory study Schmidt *et al.*, (2010) reported that moisture content between 30 and 35% provides maximum heat generation during pasteurization. Our work supports this, with good responses to additional moisture up to this range but only in uncovered heaps. A key finding in the present work is the significant interaction between the effects of additional moisture and covers. If the starting litter moisture content is below 25%, increased pasteurisation temperatures can be obtained by addition of water or covering the heaps, but the two effects are not additive. Why the beneficial effect of covering was not observed at high moisture contents is not clear, but possible reasons are condensation of moisture under the covers, and excessive ammonia trapped under the cover, inhibiting microbial action. Use of an insulating but “breathable” cover may overcome this, but at significantly higher cost.

In our earlier studies the shallowest depth below the surface at which we monitored temperature was 25 cm (Walkden-Brown *et al.*, 2010). In the present study we also included 5 and 10 cm depths and were able to show that temperatures at these depths up to day 3 were very similar to those at 25 cm, but that temperatures declined much more rapidly at the shallower depths after this, particularly in uncovered heaps.

Our observation of an increase in moisture content of litter following a 7 day heaping period is in marked contrast to a mean reduction of around 9% over the same period reported in a large farm trial (Cressman and Walkden-Brown, 2012). The reasons for the discrepancy in findings are not clear. We have previously shown that heaping of broiler litter for 9–10 days inactivates coccidial oocysts and several litter transmitted viruses (Islam *et al.*, 2010). The results from the current study suggest that more rapid inactivation of pathogens could occur by the application of additional moisture to litter if the initial moisture content is < 25% or if the litter is covered but there are no additional benefits with applying both.

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## EFFECT OF HIGH LEVEL INCLUSION OF ADSORBENT MATERIALS IN LITTER ON CHICKEN WELFARE, PERFORMANCE AND LITTER AMMONIA PRODUCTION

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M.D. CRESSMAN<sup>1,2,3</sup> and M.R. REDDING<sup>4</sup>

### Summary

Excessive nitrogen volatilization from poultry litter as ammonia (NH<sub>3</sub>) is harmful for chickens and poultry workers' health. Litter amendments such as bentonite and zeolite can reduce NH<sub>3</sub> and improve air quality in the poultry shed. Inclusion of these materials in poultry litter can reduce nitrogen volatilization and the solubility of phosphorus thus increasing the fertilizer value of the spent litter. To determine the extent to which the latter could be optimised we investigated the effect of high level inclusion of bentonite and zeolite in fresh pine shavings on broiler chicken live weight and welfare and a range of litter measurements including NH<sub>3</sub> production using the chamber acid trap method. The experiment ran for 42 days and had 5 treatments each replicated in two 2.25 m<sup>2</sup> pens with 25 chickens per pen. Treatments were nil amendment and 33% and 50% by weight addition of each of bentonite and zeolite. Inclusion of bentonite but not zeolite at these levels increased mortality of chickens during brooding, but did not adversely affect chicken live weight, footpad dermatitis, hock burn, breast blister and breast feathering of chickens up to day 42. Both amendments significantly reduced NH<sub>3</sub> in litter up to day 28. The effects of these amendments on the fertilizer value of the end of batch litter are under investigation.

### I. INTRODUCTION

The ultimate use of the large volume of spent litter produced by the meat chicken industry is as fertilizer in agriculture industry such as crop and pasture production. The estimated 1.66 million tons of spent litter produced annually in Australia (Runge *et al.*, 2007) is a mixture of poultry wastes, spilled feed and the original bedding materials, usually from plant origin (eg. wood shavings, saw dust, rice hull and chopped straw). Spent litter is a valuable but not balanced nutrient source. It releases nutrients to the surrounding plants as it decays, at a rate that may not match plant's requirements. Usually it is applied as fertilizer solely based on its nitrogen contents, which has caused phosphorus oversupply and environmental contamination.

Adsorbent litter additives such as bentonite and zeolite can retain ammonium cations in exchangeable form and increase fertilizer value of the litter (Li *et al.*, 2008). Using these litter additives as poultry bedding at a higher inclusion rate can reduce the demand for plant based litter materials for the broiler industry. These additives may thus reduce shed ammonia levels and increase the fertilizer value of spent litter. These two benefits have the potential to offset the cost of using these additives, depending on costs of the additives and their application and the additional value of the spent litter.

However, the impacts of using high inclusion rates of such additives in bedding materials on chicken health, performance and welfare are not known. Therefore, this study evaluated the effects of high inclusion rates (33 and 50% by weight) of the additives bentonite and zeolite in fresh pine shavings litter on chicken welfare such as footpad

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dermatitis (FPD), hock burn (HB), breast blister (BB) and breast feather (BF) score and live weight (LW) of chickens. Litter quality attributes such as dryness, pH and ammonia (NH<sub>3</sub>) production were also measured.

## II. MATERIALS AND METHODS

A 2 × 2 factorial experiment with 2 replicates was conducted in 1.5m × 1.5m pens with two litter amendments bentonite (Sodium bentonite, Trufeed, Unimin Australia Ltd, Miles, QLD) and zeolite (Castle Mountain Zeolites, Quirindi NSW) manually spread on the surface of fresh pine shavings at two application rates, 50% (7.1 kg per m<sup>2</sup>) and 33% (3.56 kg per m<sup>2</sup>) of litter (w:w) just before chick placement. In addition there were two control pens with fresh pine shavings without any additives. Twenty-five day-old Ross broiler chickens were placed in each pen, 10 pens in total. Due to high and uneven mortality at days 2-5, on day 7, chickens were redistributed between pens to provide 25 chickens per pen. Feed and water was provided *ad libitum*. At days 7, 14, 28, 42, 10 randomly selected chickens from each pen were individually weighed, scored for FPD. The HB, BB and BF scores were also taken at days 28 and 42. Litter samples from each pen were collected for in vitro NH<sub>3</sub> production assay (Miles *et al.*, 2008) at days 7, 14, 21, 28 and 42.

Subjective FPD scores were adapted from (Allain *et al.* 2009). Size (% area of the foot) and severity (discoloration, erosion, and necrosis) were assigned using a 10-point (0 to 9) scale. Hocks were scored using a 3-point (0 to 2) scale: 0) no discoloration, no lesion; 1) slight red discoloration and/or mild scab or lesion; and, 2) severe red discoloration and/or severe scab, lesion, or hock inflammation. Breast burn was scored using a 3-point (0 to 2) scale: 0) no discoloration, no lesion; 1) slight red discoloration, no lesion; and, 2) severe red discoloration and/or breast blistering or scab formation. Breast feathering was scored using a 4-point (1 to 4) scale: 1) <25% feather coverage; 2) 25-50% feather coverage; 3) 50-75% feather coverage; and, 4) >75% feather coverage.

Ammonia production from litter samples was measured in duplicate at 30°C using an adaptation of the chamber acid trap method of (Miles *et al.*, 2008). Briefly 660 ml subsamples of litter (44 - 326 g) were placed in a sealed 1 litre containers with air pumped through them at 215 ml/min. After equilibration at 30°C overnight, total ammonia production was determined by bubbling the output air for 6 hours through two flasks of boric acid and titrating with hydrochloric acid to a red colour endpoint using the indicators bromocresol green & methyl red. Following this, 20% additional water was added to the chamber, it was equilibrated overnight again and ammonia production measured again. Litter dry matter (oven drying for 48 hr at 105°C) and pH (pH meter, 1:20 litter solution) were assessed, the former on all collection dates, the latter on days 28 and 42.

Mortality was recorded daily from the time of placement (day 0) to the end of the trial (day 42). All animal measurements, including mortality % were averaged for each pen for each time point. Repeated animal and litter measurements for each pen were subjected to repeated measures analyses using a mixed REML model with pen fitted as a random variable. Day of measurement, litter treatment and their interaction were fitted as fixed effects. The effects of different amendment types with the treatment effect were tested by *a priori* contrasts from within the model. Ammonia production data were Log<sub>10</sub> transformed to meet the assumptions of the analysis. Data were analysed using JMP 9 (SAS Systems, Cary, NC, USA). Least squares means and SEM are presented.

## III. RESULTS

There was a significant effect of Treatment on mortality up to day 6 (P = 0.03), with higher mortality observed in bentonite-amended litter (56 %) than zeolite amended (9 %, P = 0.006)



and unamended new litter (11 %,  $P = 0.01$ ). The mortality rate was very low (0.75 %) from day 7 to 42 without any significant effect of Treatment. The early mortality was mainly on days 3 and 4 of age and mostly due to starve outs/non-starters with chicks not having fed. Among the low proportion of dead chickens that did feed, a mass of sticky grey clay material, presumably bentonite, was evident in the crop of birds from that treatment. For Live weight (LW) there was a significant effect of Day ( $P < 0.0001$ ), but not Treatment ( $P = 0.82$ ) (Figure 1, left panel) without any significant interaction between these effects ( $P = 0.93$ ). For FPD score there was a significant effect of Day ( $P < 0.0001$ ), generally worsening with the increasing age, but not Treatment ( $P=0.78$ ) with no significant interaction ( $P = 0.89$ ) between these (Figure 1, right panel). For HB, in general lower (better) scores were recorded at day 28 (0.18) than 42 (0.76) ( $P < 0.006$ ) without any effect of Treatment ( $P = 0.40$ ). Similarly, there was a lower (better) BB score at day 28 (0.56) than 42 (0.88) ( $P = 0.06$ ) without any significant effect of Treatment ( $P = 0.49$ ). There was no significant effect of either Day ( $P = 0.35$ ) or Treatment ( $P = 0.93$ ) on BF score.

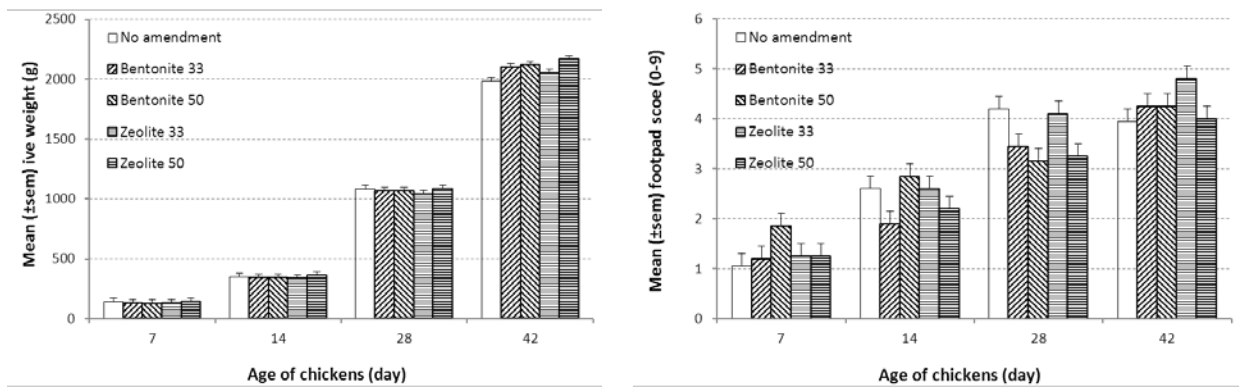


Figure 1 - Least squares means ( $\pm$  SEM) for live weight (Left panel) and footpad score (Right panel) of Ross broiler chickens reared on litters with or without additives from day 7 to 42.

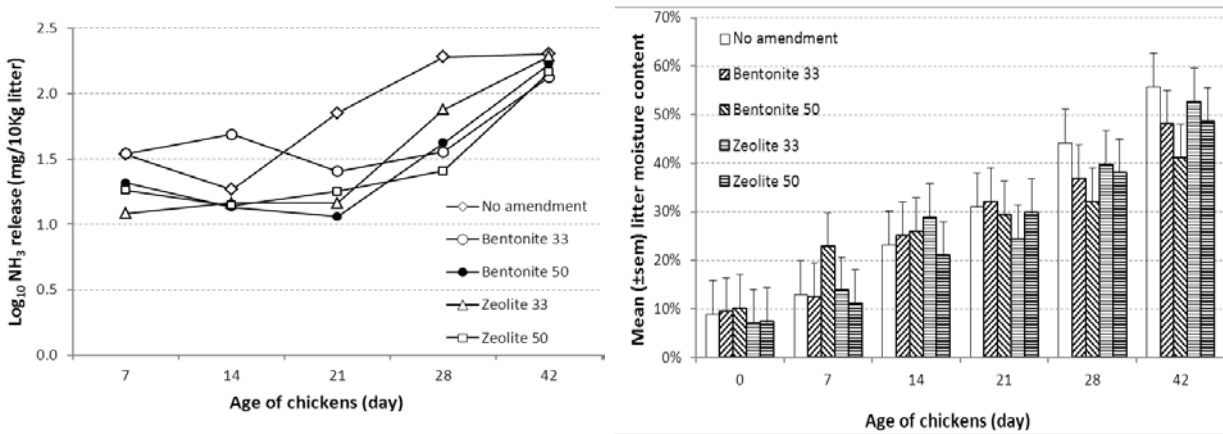


Figure 2 - Least squares means ( $\pm$  SEM) for Log<sub>10</sub> NH<sub>3</sub> content of litter of various treatments from day 7 to 42 (Left) and moisture content of litter from days 0 to 42 (Right).

For NH<sub>3</sub> production from litter there were significant effects of Day ( $P < 0.0001$ ) and Treatment ( $P < 0.0001$ ) with significant interaction between these effects ( $P < 0.0001$ ). The amended litter treatments had low NH<sub>3</sub> production up to day 21, then progressively increases at days 28 and 42. On the contrary, in unamended litter, NH<sub>3</sub> content was higher from day 21 and increased sharply by day 28 and then remained steady (Figure 2, left panel). There was a significant effect of Day ( $P < 0.0001$ ) on the litter moisture content, which increased with the progression of the experiment, but no effect of Treatment ( $P = 0.94$ ) or interaction between

these effects ( $P = 0.99$ ) (Figure 2, right panel). Litter pH at day 28 (8.00) was lower ( $P < 0.008$ ) than day 42 (8.75) without any effect of Treatment ( $P = 0.45$ ).

#### IV. DISCUSSION AND CONCLUSIONS

Inclusion of additives in new litter is not normal industry practice, this experiment was conducted to evaluate the effect of addition of a high level of additives on animal health and welfare as part of wider study looking at the effects of high levels of these amendments on the fertiliser value of spent litter. Results indicated that addition of bentonite in higher levels may cause elevated mortality in newly placed chicks due to increased non-starter levels and apparent ingestion of the amendment in a small number of birds. This is a qualified assessment because brooding conditions in the experiment were clearly suboptimal with high mortality in the control (11%) and zeolite (9%) treated groups. It may be that with optimal access to feed, water and correct brooding temperatures the excessive mortality observed in the bentonite treatment groups would not occur. Certainly there were no effects beyond the early brooding period and high level inclusion of zeolite had no adverse effects at any stage of the experiment.

High level inclusion of bentonite and zeolite reduced  $\text{NH}_3$  production from litter significantly up to day 28. In a companion study, it was shown that both bentonite and zeolite at a much lower inclusion level (13%) reduced ammonia production from reused litter but had no influence on performance or welfare of chickens (Walkden-Brown *et al.*, 2013).

Treatments in this study were applied in duplicate only. While this appears to have been adequate for the litter measurements, in future studies a higher level of replication would be desirable, particularly to detect differences in animal performance and welfare at pen level. It is important the effect of high-level inclusion of bentonite of chicks during brooding is assessed again under optimum brooding conditions and we intend to do this.

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## IMPROVED BROILER PERFORMANCE BY TRACE MINERALS

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Trace minerals are important for broiler growth and are used in various physiological, digestive and biosynthetic process of the body. Trace minerals act as cofactors in many enzymes and are associated with proteins involved in intermediary metabolism, hormone secretion path ways and the immune system (Dieck *et al.*, 2003). In broilers, organic trace minerals are better absorbed and utilized and have a greater bioavailability than inorganic trace minerals (Bao *et al.*, 2007). This experiment was conducted to examine the effect of trace mineral sources on broiler performance and carcass composition. Four hundred eight Ross 308 male d-old chicks were allocated to 24 pens and assigned to 4 dietary treatments with 6 replicate pens of 17 birds per treatment. Treatments were: inorganic trace mineral premix (IM) 750 g/t, organic yeast proteinate trace mineral premix (YP) 375 g/t, YP 500 g/t and salt encrusted trace mineral premix (SE) 1000 g/t. Between 1 and 21 d, no differences in feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR) or livability (LIV) were observed between treatments ( $P > 0.05$ ). Between 1 and 38 d, however, YP (375 g/t) and SE improved weight gain ( $P < 0.05$ ) and large improvements in FCR ( $P < 0.001$ ) were observed relative to the IM treatment. This is despite the fact that the IM was more concentrated in mineral content than other treatments. The IM diet had  $3.3 \times$ ,  $3.2 \times$ ,  $2.7 \times$  and  $4 \times$  the levels of added Zn, Cu, Fe and Mn respectively compared to YP 500. Treatment had no impact on FI or LIV ( $P > 0.05$ ). Spleen percentage of body weight at 25 d were increased in the YP (375 g/t) and SE treatments ( $P < 0.05$ ) over the IM treatment possibly reflecting improved immune status of the birds or higher iron digestion. Mineral source had no effect on thymus weight and bursa of Fabricius weight at 25 d or dressing percentage at 39 d ( $P > 0.05$ ).

**Table 1 - Growth performance and carcass attributes of broiler chickens fed various mineral sources**

	IM Min 750 g/t	YP 375 g/t	YP 500 g/t	SE 1000 g/t	P > F	Pooled CV%
FI 1-38 d	4134	4221	4147	4177	0.75	3.52
BWG 1-38 d	2824 <sup>a</sup>	3007 <sup>b</sup>	2942 <sup>ab</sup>	2985 <sup>b</sup>	0.05	3.889
FCR 1-38 d	1.466 <sup>b</sup>	1.404 <sup>a</sup>	1.409 <sup>a</sup>	1.400 <sup>a</sup>	0.01	1.838
LIV % 1-38 d	94.1	95.1	93.1	94.1	0.87	4.343
Dressing % 38 d	79.1	78.7	79.0	78.6	0.76	1.175
Spleen % 25 d	0.070 <sup>a</sup>	0.080 <sup>bc</sup>	0.073 <sup>ab</sup>	0.092 <sup>c</sup>	0.02	15.2

<sup>a, b</sup> means in rows with different superscripts are significantly different ( $P < 0.05$ ).

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## THE EFFECTS OF BEAK TRIMMING AND ENVIRONMENTAL ENRICHMENT DURING REARING ON EGG LAYING BY FREE RANGE HENS DURING EARLY LAY

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### Summary

Egg laying data for 800 ISA Brown hens (16 pens of 50 birds) housed on litter were analysed from an experiment investigating the effects of beak trimming (at day-old and repeated at 11 weeks) and environmental enrichment (deeper litter, whole oats scattered 3-5 times weekly and pecking string devices added) from 12 days to 16 weeks, on the development of feather pecking and injurious pecking. Hen-day production was higher ( $P < 0.001$ ) in enriched versus non-enriched pens in weeks 19-20. This was mainly due to an interaction ( $P = 0.05$ ) in which fewer eggs were recorded from pens of non-enriched/non-beak-trimmed birds than the other treatment combinations. In addition, during early lay there tended to be more floor eggs and fewer nest-box eggs ( $P \sim 0.1$ ) in enriched versus non-enriched pens. Opening the pop-holes to the outdoor range at 26 weeks resulted in proportionally fewer ( $P < 0.001$ ) floor eggs in the rear- and more in the front-half of the pens, away from the pop-holes. Floor eggs decreased at this time and more eggs were laid in the lower nest boxes. It is concluded that factors imposed during rearing to modify pecking may also affect egg laying characteristics.

### I. INTRODUCTION

Two issues of debate regarding the welfare of laying hens during rearing are the application of beak trimming (BT) and the provision of environmental enrichment (EE). These respective husbandry and housing practices are variously reported as insurance against, or cures for, the occurrence of feather pecking (FP) and vent pecking, which may progress to cannibalism. Opposition to BT is based on the occurrence of “short and/or long term pain and loss of function” (Gentle, 2011). The latter reviewed pain research in poultry and concluded that BT performed on “older” birds was painful, but that the literature regarding day-old chicks was inconclusive. Although Gentle and McKeegan (2007) suggested that BT performed on day-old chicks using an infra-red, laser method was not painful, the beaks of chicks had regrown to near normal length by 6 weeks, which might necessitate a second trim (with a heated blade) at around 11 weeks. Animal welfare proponents suggest that through the provision of EE, BT is not necessary for laying hens (Pickett, 2007). With the increased occurrence of free-range housing of laying hens in Australia (Cronin et al., 2012), it is important that egg producers can manage the increased risks to hen welfare from FP, injurious pecking and cannibalism that can occur under free range conditions (Rodenburg et al., 2008; Fossum et al., 2009). This paper presents preliminary egg production data from an experiment investigating the effects of rearing factors on the development of FP and injurious pecking in free-range hens. In particular, this paper investigates the occurrence of floor eggs.

### II. MATERIALS AND METHODS

Hen-day egg production and the proportion of nest box (NB) eggs compared to floor eggs were measured from 17-35 weeks of age (May to September) for 16 groups of 50 ISA Brown laying hens housed in an uninsulated shed, in pens measuring 1.83 m wide x 3.25 m deep. Internal wire mesh pen divisions were covered to 0.9 m above floor height with 70% shade cloth to reduce visibility between adjacent pens. The birds were part of a 2x2 factorial

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design experiment, in which the effects of BT and EE during rearing were investigated in relation to the development of FP and injurious pecking (see paper by Hartcher et al., this proceedings). Birds in the BT treatment were trimmed as day-olds at the hatchery using a Novotech infra-red laser unit; a follow-up light-trim with a hot blade was performed at 11 weeks by a professional beak-trimmer. The non-BT treatment birds were not trimmed and were not sham-handled at that time. The provision of EE commenced at day 12 of age and consisted of providing ~5 cm deep litter of wood shavings on the concrete floor, into which ~150 g whole oats were scattered 3-5 times weekly. In addition, five pecking string devices (McAdie et al., 2005) were suspended in each EE pen, with the strings presented at head level of the birds. The non-EE treatment had ~1 cm deep wood shavings, and neither whole oats nor pecking string devices were provided. The experiment was blocked according to side of the shed (north versus south) and treatments were fully randomised to pens within blocks. Feed and water were available *ad libitum* and each pen contained a 5-rung perch unit (installed at 13 weeks) and a 10-hole roll-away nest unit with plastic mesh nest inserts (SKA). Each NB unit had 2 rows of 5 single-bird nests, above and below, which was installed and opened at 15 weeks. There were also perches at the front of each row of NB. Photoperiod was increased from 17 to 23 weeks, when 15 h light to 9 h dark was maintained. At 26 weeks each pen of birds was allowed continuous access to an outdoor run via a pop-hole measuring 0.4 m high x 0.6 m wide in the rear wall of the pen. Outdoor runs were 1.83 m wide x 10 m long and consisted of 2.1 m high wire mesh fences (without shade cloth cover). The 1.4 m closest to the shed in the outdoor runs was a metal-roofed verandah, beyond which was a 1.8 m-wide section of shade cloth overhead. The non-roofed area of the outdoor runs had wire mesh overhead. All birds were individually weighed periodically during the experiment.

The number of eggs collected from each pen was recorded on four days per week, along with egg location (upper NB, lower NB, floor and {once the pop-holes were opened} outside run). In addition, during the two weeks before and after opening the pop-holes, the number of floor eggs in the front compared to rear halves of the pen was recorded. Differences due to the BT and EE main effects on hen-day production, the proportion of eggs laid in each location to week 35 and hen live weight were determined using analysis of variance and co-variance (GenStat release 11.1, VSN International Ltd). Egg production data for successive weeks were averaged prior to analysis, and the experimental unit was the pen of birds. The number of eggs laid in different locations around the time pop-holes were opened (fixed effect) was modelled using a multinomial GLMM procedure on daily egg data.

### III. RESULTS

BT birds were heavier than non-BT birds at day 11 of age (92.7 vs 88.6 g;  $P = 0.014$ , sed 1.38) and throughout rearing (e.g. at 16 weeks: 1.478 vs 1.428 kg;  $P = 0.028$ , sed 0.0198). However, after adjusting for live weight at day 11, there were no differences ( $P > 0.05$ ) at 16 weeks due to the main effects or interactions (e.g.: non-BT/EE, non-BT/non-EE, BT/EE and BT/non-EE treatments: 1.455, 1.422, 1.471 and 1.463 kg). The pooled mean ( $\pm$ SD) weight of hens in the flock in weeks 23, 26 (day prior to opening the pop-holes) and 31 were 1.796 (0.0506), 1.919 (0.0440) and 2.017 kg (0.0584), respectively.

The first floor egg was laid at 17.6 weeks and the first NB egg at 18.6 weeks. Eggs were recorded from all 16 pens by week 20, and in weeks 21 and 23, respectively, 50% and 80% hen-day production was reached. Egg eating was regularly observed in 3 BT/EE pens, 2 non-BT/no-EE pens and 1 BT/no-EE pen, which probably reduced the overall maximum hen-day production (93% in week 35). In weeks 19-20 combined there was an effect of EE on hen-day production (30.0 and 21.7%, for EE and non-EE pens;  $P < 0.001$ , sed 1.73) and a

significant BTxEE interaction (non-BT/EE, non-BT/non-EE, BT/EE and BT/non-EE treatments: 30.4%, 18.2%, 29.6% and 25.2%;  $P = 0.05$ , *sed* 2.44). In weeks 21-22 there was no difference due to main effects, and a weak interaction (59.9%, 54.5%, 58.4% and 67.7%;  $P = 0.10$ , *sed* 5.75). Thereafter, no differences in hen-day production were found.

The change in the proportion of eggs recorded in the different laying locations per week, pooled across treatments, is shown in Figure 1. Although there were no significant differences due to the main effects on the proportion of floor (or conversely NB) eggs, in early lay there were weak tendencies for a higher proportion of floor eggs in the EE than non-EE treatment (wks 19-20: 69.1% and 54.3%, *sed* 8.41,  $P = 0.11$ ; wks 21-22: 53.0% and 41.6%, *sed* 6.35,  $P=0.10$ ).

In the 2 weeks before the pop-holes were opened, on average 41% of eggs were floor eggs, with 54% of these laid in the front half and 46% in the rear half of the pens. However, in the two weeks after the pop-holes were opened, the proportion of floor eggs decreased to 38%, of which 67% were laid in the front half and 33% in the rear half of the pens ( $P < 0.001$ ). While the proportion of eggs in the upper NB did not change between these periods (37.5% vs 38.0%, respectively), the proportion in the lower NB increased (21.1% vs 24.5%).

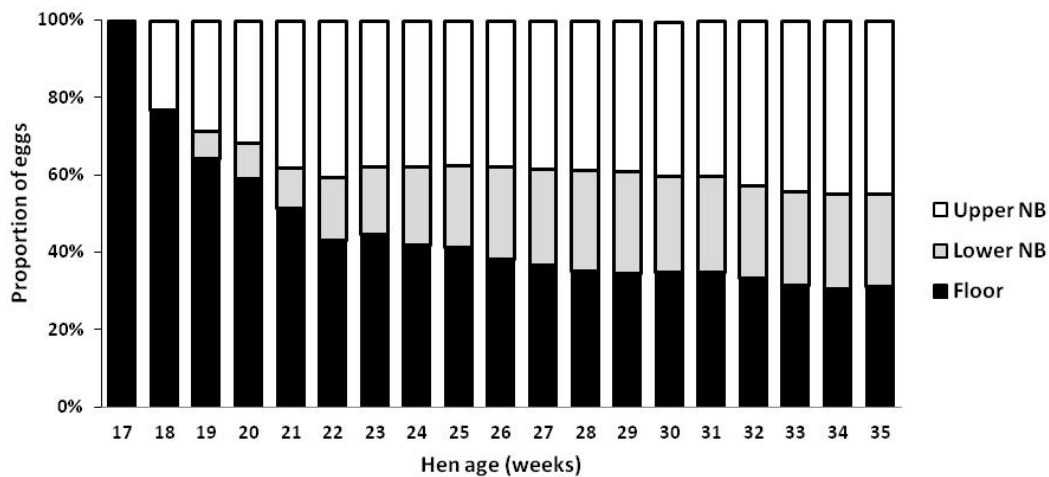


Figure 1 - The proportion of eggs laid from week 17 to 35 in different locations: upper NB, lower NB and floor. Pop-holes to the outside runs were opened in week 26 and the average proportion of eggs laid in the outdoor runs ranged from 0 to 0.3% per week.

#### IV. DISCUSSION

A practical issue for egg producers using non-cage housing systems is the proportion of floor eggs. In the present experiment, floor eggs occurred at a higher than desirable level, and in early lay there was a tendency for proportionally more floor eggs in pens with EE. Floor eggs require manual collection, are more likely to be dirty and thus down-graded and de-valued, and are possibly more prone to shell fracture and being eaten by the hens. Dirty eggs can also be a health hazard. Nicol et al. (2003) reported the findings of a survey of 100 free range egg producers in the UK, and while their results suggested that floor egg problems were more common in ISA Brown flocks, their analysis also identified that some farms restricted access to litter to prevent floor eggs. Hence the increased incidence of floor eggs in the present experiment may be due partly to the litter on the floor (rather than slatted flooring). EE in the present experiment was provided in part through increased depth of litter during rearing, with the objective to stimulate dust-bathing and ground foraging behaviours, which was relevant to the main experimental objective of investigating the development of FP behaviour. While birds in the EE treatment produced ~40% more eggs in weeks 19-20, there was an interaction

between BT and EE. Pullets in the non-trimmed/non-enriched treatment were recorded to lay fewer eggs in early lay and hence were perhaps less mature than birds in the other treatment combinations. Nevertheless, the eggs produced in weeks 19-20 were below saleable size and hence had no commercial value. It is not evident however, which component(s) of enrichment might have contributed to modify the early development of egg laying. Although there were no effects of enrichment on hen weight in the experiment, there was a strong effect of BT on weight. This effect may be due to genetic differences rather than treatment.

The high incidence of floor eggs in the present experiment is a concern as it may be difficult to induce hens to lay in nest boxes if the birds are initially attracted to lay on the floor. Cronin et al. (2007) reported that the majority young hens studied in furnished cages were consistent in their choice of egg-laying site by about the tenth egg laid, which suggests it is important to focus on managing the pullets' environment to encourage birds to lay in the nest boxes rather than on the floor. One factor that influences hens in the selection of egg-laying site is ambient light, with hens typically seeking darkened sites for egg laying. Further, Cronin et al. (2012) suggest hens may also seek egg-laying sites where they are less likely to be disturbed by other hens. An interesting finding in the present experiment was that once the pop holes were opened, the proportion of floor eggs laid in the rear half of the pens decreased, suggesting at least some hens relocated their egg laying site. While some hens continued to lay floor eggs, albeit in the front half of the pen, other hens seem to have chosen to lay in the lower level nest boxes. This shift in egg laying location towards the pen front, including the lower nest boxes, may have been influenced by increased light entering the rear of the pens via the pop holes, and/or disturbance near the pop holes at the rear of the pens.

In conclusion, the results of the experiment demonstrate that factors applied during rearing to modify FP behaviour may also affect egg laying characteristics, although the latter effects may be relatively transient.

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## EFFECT OF REARING CONDITIONS ON THE DEVELOPMENT OF FEATHER-PECKING BEHAVIOURS IN FREE-RANGE LAYING HENS

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### Summary

Despite existing as a serious welfare and economic problem in the egg industry, the cause of feather-pecking is not well-understood. This experiment investigated the effects of beak-trimming and environmental enrichment during the rearing period on the development of feather-pecking behaviours in free-range laying hens. An open-field test was also performed to investigate behavioural responses to a novel environment. A total of 800 ISA Brown laying hens (16 pens of 50 birds) were used in a 2x2 factorial experiment. Half of the birds were beak-trimmed at day-old and again at 11 weeks, and half received environmental enrichment in the form of extra litter depth, pecking strings and whole oats strewn in the litter 3-5 times weekly. Four focal birds were selected at random from each pen on day 11 and were subjected to detailed *in-situ* behavioural observations as well as tested in an open-field test during the rearing period (days 20 – 97). Birds that had been beak-trimmed performed less ground-pecking ( $P < 0.001$ ) and more gentle pecks towards other birds ( $P = 0.026$ ) than the non-trimmed birds. When tested in an open-field test, birds from enriched environments took longer to perform their initial step than those from non-enriched pens ( $P = 0.019$ ) and there was a significant interaction effect where beak-trimmed birds from enriched pens performed fewer movements ( $P = 0.024$ ). Non-trimmed birds vocalised more ( $P = 0.022$ ) and at louder volumes ( $P = 0.020$ ) than beak-trimmed birds in the open-field test. These results indicate that non-trimmed birds from non-enriched pens have more proactive coping styles in fear-eliciting situations. It has yet to be determined whether birds exhibiting proactive coping styles during rearing will be more likely to develop feather-pecking behaviours when older.

### I. INTRODUCTION

Feather-pecking (FP) is considered to be an abnormal and detrimental behavioural disorder that consists of birds pecking at and pulling out the feathers of other birds. The behaviour can lead to severe damage to the plumage as well as wounds and has been identified as a serious economic and welfare concern for the egg-production industry (Brunberg et al., 2011). Although a prevalent problem, the underlying cause of FP is not well-understood (Jensen et al., 2005; Brunberg et al., 2011) and is controlled largely by remedial measures such as reduced ambient lighting and beak-trimming (Petek and McKinstry, 2010). Previous work suggests that the rearing period is important for the development of FP behaviours later in life (Bestman et al., 2009). Feather-pecking has been linked with fearfulness and coping strategy (active or passive) which may be measured in an open-field test (OFT). Additionally, when the OFT is performed during the rearing period, the behavioural responses may be indicative of FP behaviour when older (Jones et al., 1995; Korte et al., 1997). This experiment aims to investigate the effects of beak-trimming (BT) and environmental enrichment (EE) during rearing on the development of FP in free-range laying hens.

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## II. MATERIALS AND METHODS

A total of 800 day-old ISA Brown laying hens were obtained from a commercial hatchery and randomly allocated to treatment pens in a two-by-two (BT x EE) factorial design. Each treatment was replicated four times with 50 birds per replicate pen. Beak-trimming occurred when the birds were one day-old and at 11 weeks of age. Environmental enrichment was applied in the form of additional wood shavings on the floor (i.e. litter), pecking strings and whole oats strewn in the wood shavings 3-5 times weekly. Four focal birds were selected at random from each pen at day 11 and were subjected to detailed *in-situ* behavioural observations as well as tested in an open-field test (OFT) during the rearing period (days 20 – 97). Behaviours recorded during *in-situ* observations were dustbathing, ground-scratching, ground-pecking, pecking other birds gently, pecking other birds severely, pecking at pecking strings (in enriched pens), pecking at vertical surfaces, receiving gentle pecks from another bird and receiving severe pecks from another bird. Each focal bird was observed for a 2 min period which was classified as one observation session. Thirty of these sessions were conducted opportunistically over the rearing period (days 20 – 97) for all focal birds in every pen (60 min per focal bird). In addition to *in-situ* observations, the four focal birds from each pen were subjected to an OFT in week 6 in which they were individually placed in a 1.2 x 1.2 m test arena with 0.8 m high white walls and a linoleum floor for a 5-min period. The test arena was novel to all birds. A 3 x 3 grid marked on the floor was used to quantify the activity level for each bird where each grid boundary crossed was counted as one movement. The quantity and volume of any vocalisations (mostly alarm calls) were recorded as well as the latency for the bird to perform its first movements. GenStat® was used to generate generalised linear mixed models (GLMM) in order to model the probability of behaviours being performed during the *in-situ* behavioural observations, and Linear Mixed Models were used to analyse the OFT data. A GLMM was fitted to the vocalisation data using ASReml 3.0.

## III. RESULTS

Birds that had been BT were less likely to ground-peck ( $P < 0.001$ ; 28% vs 39%; sed 7.6) and more likely to be observed performing gentle pecks towards other birds ( $P = 0.026$ ; 3% vs 2%; sed 0.12) than the non-trimmed birds. There was no significant difference between treatments for other behaviours recorded. When tested in the OFT, birds from enriched pens took longer to perform their initial step than those from non-enriched pens ( $P = 0.019$ ;  $6.17 \text{ s} \pm 1.60$  vs  $2.31 \text{ s} \pm 0.60$ ). There was a significant interaction effect of BT and EE on activity levels in the OFT where EE birds that had been BT performed significantly fewer movements in the OFT ( $P = 0.024$ ) as shown in Figure 1. Non-trimmed birds vocalised more ( $P = 0.022$ ) and at louder volumes ( $P = 0.020$ ) than beak-trimmed birds in the OFT.

## IV. DISCUSSION

### a) Ground-pecking

The most common hypothesis to explain the cause of FP is that the pecking is redirected from the environment to the feathers of conspecifics. In the present study, birds that had been beak-trimmed performed less ground-pecking, suggesting that beak-trimming reduces the motivation for birds to ground-peck. This could be due to the loss of feeling in the beak that birds experience following beak-trimming. The inverse relationship between beak-trimming and ground-pecking will be further investigated as to its relationship with FP behaviour as the experiment continues.

b) Gentle pecking

During *in-situ* observations, birds that were beak-trimmed were observed to perform more gentle pecking directed at pen mates. This could possibly be due to compensatory exploratory pecks performed as a result of reduced mechanoreception and magnetoreception in the beak following beak-trimming (Freire et al., 2010). Identified as a 'normal' behaviour, it is also acknowledged that gentle pecking at the feathers of other birds may become abnormal (McAdie and Keeling, 2002). This may occur due to an increase in frequency of pecks, hence appearing stereotypic, or for the pecks to increase in severity and develop into severe FP. Although it has been hypothesised that gentle FP may be a precursor of severe FP (Newberry et al., 2007), it has been suggested that gentle pecking at a young age may represent other motivations such as social exploration and not indicative of adult bird behaviour (Riedstra and Groothuis, 2002; Rodenburg et al., 2004). The higher incidence of gentle FP in the beak-trimmed birds in this experiment may be an expression of social exploration and investigation and not related to the incidence of severe FP, but comparisons will be conducted between the data from the rearing period and information on severe FP from the laying period.

c) Open-field Test

Freezing, number of movements and quantity and volume of vocalisations in an OFT all indicate fearfulness and the coping strategy adopted by an animal in fearful situations. The results from this experiment show that birds from enriched environments freeze for longer (i.e. took longer to perform their initial step) and beak-trimmed birds from enriched pens had lower activity levels in the OFT (indicated by fewer movements). Additionally, birds that were not beak-trimmed vocalised more and at louder volumes than beak-trimmed birds. These results could therefore indicate that beak-trimmed birds from enriched environments adopt more passive coping styles in fear-eliciting situations. It has yet to be determined whether the birds exhibiting active or passive coping styles in the OFT will be more likely to exhibit severe FP when older.

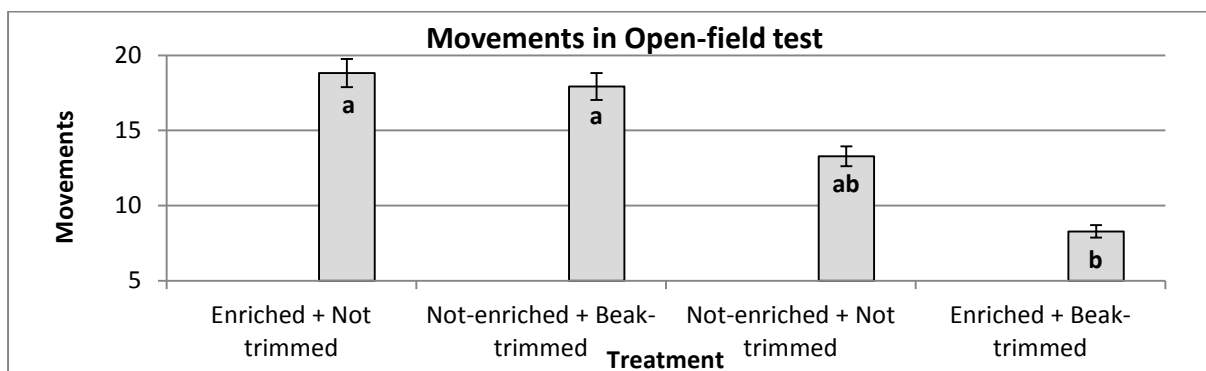


Figure 1 - Movements in open-field test were quantified by the use of a 3 x 3 grid marked on the floor. One movement consisted of crossing a grid boundary. The test was conducted for a 5 min period for each focal bird at 11 weeks of age. The BT birds from pens that received EE performed significantly fewer movements as denoted by the lower case letters generated by the least significant difference (LSD).

## V. CONCLUSIONS

As FP is a multi-factorial problem with developmental causes that are not fully understood, it is important to identify the causal factors of the behaviour when aiming to minimise its expression in commercial settings. The preliminary analysis of data from the rearing phase in the present experiment have shown some interesting effects of BT and EE on the performance of pecking behaviour and the responsiveness of birds in the open-field test. The latter is potentially indicative of how birds may react when challenged, for example when being pecked. The data from this experiment relating to gentle FP, ground-pecking and behaviour in the OFT during the rearing period, will be compared to the expression of FP in the same flock during the laying period. As the rearing period is thought to be a time of great importance for the development of behaviours later in life (Bestman et al., 2009), these findings may contribute to the current understanding of the development of FP behaviours in free-range laying hens.

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## THE EFFECT OF A BALANCED MIXTURE OF MEDIUM CHAIN FATTY ACIDS ON ZOOTECNICAL PERFORMANCES IN BROILERS

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### Summary

The objective of the current studies was to determine the effect on zootechnical performances of Aromabiotic Poultry, a carefully balanced mixture of MCFA in broilers. Trial A was designed as a complete block design with male Ross 308 broilers. The control diet was wheat/corn/soy based while the experimental diet was obtained by adding a decreasing dosage of Aromabiotic Poultry (1.7 – 1.25 – 0.8 g/kg) in the starter, grower and finishing phase. Both daily growth and feed conversion were improved (the latter not significantly). Because of a bad start of the birds in the control group, the trial was repeated. In trial B constant dosages of 0.8 and 1.2 g/kg, respectively were applied in two experimental treatments. Average daily gain and feed conversion were significantly improved with the best results using the lower dosage (ADG: 64.9 vs 62.6 g/d; WAFCR: 1.53 vs 1.58). Breast meat yield was only improved using the higher dosage (23.1 vs 22.6 %). In conclusion, Aromabiotic Poultry proved to be a valuable functional feed ingredient to sustain the rising production potential of broilers.

### I. INTRODUCTION

For years, the focus of broiler breeding companies has been the improvement of growth rate and feed conversion. To be able to reach this increased genetic potential without health problems, birds not only need a well-balanced diet but also benefit to a large extent from functional feed ingredients supporting intestinal and overall health. Poultry performance and feed efficiency are closely interrelated with the quantitative and qualitative microbial load of the host animal. Medium chain fatty acids (MCFA; C6-C12) are known to have unique nutritional, physiological and antimicrobial properties (Kabara et al. 1972; Skrivanova 2006; Batovska 2009). In pigs, a strong *in vitro* and *in vivo* regulating effect of MCFA on the proximal gut flora was obtained (Dierick et al., 2002). The use of MCFA in broilers decreases the excretion of *Salmonella* Enteritidis as measured in the cloacal swabs (Van Immerseel et al 2004). Moreover, the same research showed that MCFA are able to lower invasion of *Salmonella* Enteritidis in the intestinal organs. Finally oral supplementation of MCFA to dairy cows supports the systemic as well as the local innate immunity of dairy cows shortly after calving by improving neutrophil quality (Piepers et al., 2010). The objective of the current studies was to determine the effect on zootechnical performances of Aromabiotic Poultry, a carefully balanced mixture of MCFA in broilers.

### II. MATERIALS AND METHODS

One day old, male broiler chicks (Ross 308) were used in the described trials (Origin: Belgabroed Hatchery, Merksplas-Belgium). The birds were housed in the poultry experimental facility of ILVO/ Section Small Stock Husbandry. For trial A, 18 pens of 2.55 m<sup>2</sup> (32 birds/pen) were used whereas for trial B 21 pens of 1.90 m<sup>2</sup> were used with 30 birds/pen. The broilers were vaccinated the 1st day of age against Newcastle Disease (NDW,

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spray) and Infectious Bronchitis (Poulvac IB Primer, spray). At 16 days of age the vaccination against Newcastle Disease was repeated with La Sota (Clone 30, drinking water).

The test product in both trials was Aromabiotic Poultry, a carefully balanced mixture of 60 % MCFA (C6, C8, C10, C12) on a support of silicium dioxide. Trial A consisted of two dietary treatments with each 9 replicates of 32 birds or 576 birds in total. The control diet was a wheat/corn/soy based diet which met all dietary recommendations for chickens from 1 to 39 days of age.<sup>4</sup> To this diet, 1.7 g/kg, 1.25 g/kg and 0.8 g/kg of the test product was added to the starter, grower and finisher, respectively, to obtain the experimental diets. Trial B consisted of three dietary treatments (control and two dosages) with 7 replicates of 30 birds or 630 birds in total. The control diet was also wheat/corn/soy based.<sup>5</sup> The two dosages, 0.8 g/kg and 1.2 g/kg of Aromabiotic Poultry, respectively were added to the control diet during the whole experimental period. A randomized block design was used in both trials. A 3-phase feeding scheme was applied with periods of 13 days each. Feed was provided *ad libitum* in (finely ground) meal form by feed mangers.

Daily mortality and cullings were recorded for each pen. Corrections for mortality calculating zootechnical performances were done using the number of 'broiler days' (number of broilers x days alive). For each trial, average pen weight was recorded at day old, 13, 26 and 39 days of age. Feed intake was recorded for 1-13, 14-26, and 27-39 days. Feed conversion ratio (FCR), daily growth rate, bird-days and daily feed intake per bird were calculated. At the end of trial B from each pen 2 broilers were selected with a weight near to the average pen weight. These birds were fed their respective diet till the age of 42 days. After a 4h starvation period, they were weighed, slaughtered and cut to determine carcass and breast meat yield. The data of trial A were analysed by a linear model with the diet as fixed effect. For trial B orthogonal contrast analysis was performed (Aromabiotic Poultry diets vs. control diet) to explore the effect of the treatment (Statistica 9 program).

### III. RESULTS AND DISCUSSION

At arrival, one-day old Ross 308 birds showed a good quality with an average body weight of 41.6 g in trial A and 42.1 g in trial B). The results of trial A are summarized in Table 1. Supplementing the feed with Aromabiotic Poultry affected growth performances in a positive way. Average daily gain was significantly better in the starter and grower period. For the overall period, daily weight gain was 3.0 g higher ( $P < 0.05$ ). FCR was improved in the starter period but somewhat worse in the finishing period. However, the overall FCR was very comparable but broilers fed the Aromabiotic Poultry diet were 116 g ( $P < 0.05$ ) heavier at slaughter age. After correction for weight differences however the feed conversion ratio was two points better compared to the control group. In general, mortality rate was very low and no significant differences were observed.

In 2003, the addition of MCFA in broiler feed was evaluated by Deschepper et al. The addition of 2 kg/ton of a 50% MCFA product resulted in an improvement of body weight (not significant), average daily gain and feed conversion ratio. Feed intake was not influenced. In this 2003 study, the results were comparable with the addition of avilamycin as an antibiotic growth promoter.

<sup>4</sup> Feed analysis Trial A (starter/grower/finisher): ME (MJ/kg) = 11.5/11.9/12.3; Crude protein (%) = 21.16/20.5/20.0; Dig. Lys (%) = 1.20/1.10/1.05; Dig. Meth+Cyst (%) = 0.88/0.84/0.80; Dig Thr. (%) = 0.78/0.71/0.68

<sup>5</sup> Feed analysis Trial B (starter/grower/finisher): ME (MJ/kg) = 11.55/11.82/12.13; Crude protein (%) = 21.50/20.5/19.5; Dig. Lys (%) = 1.16/1.10/0.95; Dig. Meth+Cyst (%) = 0.85/0.84/0.72; Dig Thr. (%) = 0.75/0.72/0.62

**Table 1 - Zootechnical performances with Aromabiotic Poultry in trial A**

	<b>Control</b>	<b>Aromabiotic Poultry</b>
<b>Starter period (day 0-13)</b>		
Body weight (g/bird)	286 <sup>b</sup> ± 11.52	337 <sup>a</sup> ± 4.20
Feed intake (g/d)	32.1 ± 1.05	34.0 ± 0.75
Average daily gain (g/d/bird)	18.8 <sup>b</sup> ± 0.9	22.7 <sup>a</sup> ± 0.32
Feed conversion ratio	1.72 <sup>b</sup> ± 0.06	1.50 <sup>a</sup> ± 0.03
<b>Grower period (day 14-26)</b>		
Body weight (g/bird)	1 125 <sup>b</sup> ± 19.87	1 236 <sup>a</sup> ± 13.65
Feed intake (g/d)	111.2 <sup>b</sup> ± 1.39	120.7 <sup>a</sup> ± 0.90
Average daily gain (g/d/bird)	65.5 <sup>b</sup> ± 0.96	69.2 <sup>a</sup> ± 0.91
Feed conversion ratio	1.72 ± 0.016	1.75 ± 0.025
<b>Finisher period (day 27-39)</b>		
Body weight (g/bird)	2 562 <sup>b</sup> ± 35.06	2 678 <sup>a</sup> ± 10.79
Feed intake (g/d)	179.7 <sup>b</sup> ± 2.31	187.9 <sup>a</sup> ± 1.68
Average daily gain (g/d/bird)	110.5 ± 1.54	110.9 ± 0.69
Feed conversion ratio	1.63 <sup>a</sup> ± 0.007	1.69 <sup>b</sup> ± 0.008
<b>Overall (day 0-39)</b>		
Body weight (g/bird)	2 562 <sup>b</sup> ± 35.06	2 678 <sup>a</sup> ± 10.79
Feed intake (g/d)	107.7 <sup>b</sup> ± 1.35	114.2 <sup>a</sup> ± 0.68
Average daily gain (g/d/bird)	64.6 <sup>b</sup> ± 0.9	67.6 <sup>a</sup> ± 0.28
Feed conversion ratio	1.67 ± 0.007	1.69 ± 0.008
Feed conversion ratio (2500 g)	1.64 ± 0.018	1.62 ± 0.010
Mortality	1.04 ± 0.52	1.74 ± 0.76

<sup>a,b</sup> numbers on one row with different superscripts are significantly different (P < 0.05)

The antimicrobial activity of MCFA (Kabara et al., 1972; Skrivanova et al. 2006; Batovska et al. 2009) results in lower intestinal infection pressure, improving the intestinal morphology, resulting in better digestive and absorptive capacities (Deschepper et al., 2003). This is confirmed by the improved technical performances in the present study.

Despite the positive overall result of trial A, a comment concerning the start of the trial is in order. Average daily gain of the control group during the starter period was very low with a high feed conversion (1.72) as a consequence. With Aromabiotic Poultry, this bad start of the birds could be avoided but still was not optimal. Therefore a second trial was set-up.

Trial B, including two dietary dosages, showed a normal course of the feed conversion ratio in all the treatments. Over the whole trial, average daily gain was better using Aromabiotic Poultry and confirmed the results of trial A. However, no clear dosage effect was found for weight gain or FCR. For this reason, a contrast analysis was executed between the control and the treatment groups. Daily gain was in the 3 phases always higher (P < 0.05) in the Aromabiotic treated birds. Final weight exceeded 90 and 80 g, respectively, compared with the control birds. Because feed intake was higher in Aromabiotic treatments, FCR was not different between treatment groups. Overall weight adjusted FCR was somewhat improved (by two points) using the highest dosage and even by five points with the lower dosage. Birds receiving Aromabiotic Poultry (both treatments) showed 0.6 % higher carcass yields (69.4 vs 68.8 %; difference not significant). For the higher dosage, also the breast meat yield was improved (only numerically) by 0.5 % (23.1 vs 22.6). In this trial, the best growth performances (ADG and WAFRCR) were obtained using 0.8 g/kg. However considering the breast meat yield, the preferred dosage would be 1.2 g/kg.

**Table 2 - Zootechnical performances with Aromabiotic Poultry in trial B**

	<b>Control</b>	<b>Aromabiotic Poultry 0.8 g/kg</b>	<b>Aromabiotic Poultry 1.2 g/kg</b>	<b>P*</b>
<b>Starter period (day 0-13)</b>				
Body weight (g/bird)	347 ± 9	363 ± 8	355 ± 10	< 0.05
Feed intake (g/d)	32.5 ± 1.4	34.5 ± 1.4	33.1 ± 1.1	< 0.05
Average daily gain (g/d/bird)	23.5 ± 0.6	24.7 ± 0.6	24.1 ± 0.7	< 0.05
Feed conversion ratio	1.39 ± 0.06	1.40 ± 0.07	1.38 ± 0.05	NS
<b>Grower period (day 14-26)</b>				
Body weight (g/bird)	1 203 ± 53	1 249 ± 57	1231 ± 44	< 0.05
Feed intake (g/d)	100.0 ± 3.3	100.1 ± 3.9	99.0 ± 3.2	NS
Average daily gain (g/d/bird)	65.9 ± 4.0	68.2 ± 3.9	67.4 ± 2.7	< 0.05
Feed conversion ratio	1.52 ± 0.06	1.47 ± 0.04	1.47 ± 0.03	NS
<b>Finisher period (day 27-39)</b>				
Body weight (g/bird)	2 481 ± 31	2 571 ± 95	2561 ± 61	< 0.05
Feed intake (g/d)	164.4 ± 2.9	171.3 ± 4.6	171.0 ± 3.9	< 0.05
Average daily gain (g/d/bird)	98.3 ± 2.6	101.7 ± 3.3	102.3 ± 2.8	< 0.05
Feed conversion ratio	1.67 ± 0.04	1.68 ± 0.02	1.67 ± 0.04	NS
<b>Overall (day 0-39)</b>				
Body weight (g/bird)	2 481 ± 31	2 571 ± 95	2561 ± 61	< 0.05
Feed intake (g/d)	98.4 ± 1.0	101.0 ± 2.4	100.6 ± 2.5	< 0.05
Average daily gain (g/d/bird)	62.6 ± 0.8	64.9 ± 2.4	64.6 ± 1.6	< 0.05
Feed conversion ratio	1.57 ± 0.01	1.56 ± 0.02	1.56 ± 0.03	NS
WAFCR (2500 g)	1.58 ± 0.02	1.53 ± 0.06	1.56 ± 0.03	NS
Mortality and culling (%)	3.8	4.8	3.3	NS
Carcass yield on d 42 (%)	68.8 ± 1.5	69.4 ± 1.5	69.4 ± 1.5	NS
Breast meat yield on d 42 (%)	22.6 ± 1.3	22.5 ± 1.7	23.1 ± 1.1	NS

\* Aromabiotic treatments vs control group

#### IV. CONCLUSION

In conclusion, Aromabiotic Poultry proved to be an interesting functional feed ingredient to be used in broiler feed. The improved growth rate and FCR together with higher processing yields resulted in a lower feed cost/kg broiler meat.

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## A NEW LOW MORTALITY NECROTIC ENTERITIS CHALLENGE MODEL

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Necrotic enteritis (NE) in broilers – caused by *Clostridium perfringens* – is largely controlled by in-feed antibiotics in Australia. Alternatives to antibiotics are being sought by industry and must be tested under simulated outbreak conditions. An existing NE challenge model employed at UNE has demonstrated differences between NE intestinal lesion score between unmedicated challenged and unchallenged controls. However, up to 20% NE-related mortality was routinely observed in challenged groups without an antibiotic (Zn bacitracin). A different model was required to minimise mortality and improve the relevance of the data to industry. In the current study, a new NE challenge model was developed by removing a protein-fortified pre-challenge diet and replacing it with standard wheat-based starter and grower diets, formulated to broiler breed standard specifications.

A 2 × 2 factorial (± *C. perfringens* challenge, ± Zn bacitracin) design experiment was conducted to test the new challenge model. All birds (672 Ross 308 males divided into six replicates of each treatment) were fed the same formulations of starter, grower and finisher diets at 0-10, 11-24, and 25-35 days of age, respectively, except for Zn bacitracin which was applied according to the experimental design. The NE challenge procedure followed Wu et al. (2012). Total mortality for the 35 d study was 2.9% across all treatments, with only 1 death attributed to NE. Intestinal mucosa NE lesion scores (where; 0 = healthy, and 4 = widespread necrotic lesions) were recorded one day post challenge (table 1). Bird performance in all treatments exceeded 2012 breed performance objectives.

**Table 1 - Mean post-challenge intestinal mucosa lesion scores**

Challenge	Zn bacitracin	NE score*			
		Duodenum	Jejunum	Ileum	Average
-	+	0 <sup>b</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>
-	-	0 <sup>b</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>
+	+	0.08 <sup>b</sup>	0 <sup>b</sup>	0.25	0.11 <sup>b</sup>
+	-	0.88 <sup>a</sup>	2.17 <sup>a</sup>	0.33	1.13 <sup>a</sup>
Pooled SEM		0.10	0.07	0.06	0.05
P values					
Challenge		0.001	< 0.001	0.009	< 0.001
Zn bacitracin		0.005	< 0.001	0.682	< 0.001
Challenge×Zn bacitracin		0.005	< 0.001	0.682	< 0.001

\* Means with unlike superscripts differ significantly (P < 0.05).

Duodenum, jejunum and average intestinal NE lesion scores were higher (P < 0.001) in the challenged, unmedicated treatment than medicated or unchallenged treatments.

The new model reduced NE mortality whilst maintaining adequate lesion scores to sufficiently separate treatments (controls in challenge studies). The newly developed model will be employed in future studies to identify potential non-antibiotic candidates to control NE, improve bird welfare and increase the relevance of NE challenge study data to industry.

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## THE SUCCESSFUL USE OF PHYTOGENICS AS AGP REPLACEMENT

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Australia imports approximately 700 tonnes of antibiotics annually; of this, 550 tonnes are used either for veterinary therapy or growth promotion (El-Osta and Youil, 2012).

Consumers demand the use of antibiotic growth promoters (AGP) to be reduced/ceased and alternatives need to be found. Antimicrobial resistance is declared by the World Health Organization as one of the greatest threats to human health (Wise, 2011). Although results obtained with AGP in regards to animal performance are consistent and reproducible, the exact mechanism of how this improvement is achieved is not yet fully understood. Niewold reviewed the four major mechanisms proposed to explain AGP-mediated growth enhancement: 1) the inhibition of endemic subclinical infections thus the metabolic costs of (innate) immune system activation are reduced; 2) the reduction of growth-depressing metabolites (such as ammonia) produced by microbes; 3) the reduction of microbial use of nutrients and 4) the enhancement of uptake and use of nutrients due to the thinning of the intestinal wall of AGP-fed animals (Niewold, 2007). From all data available, the anti-inflammatory role of AGP, which results in more energy available for production, seems to be crucial. The challenge is now to find non-antibiotic compounds with similar positive properties.

Phytogenics possess flavoring, antioxidant, antifungal, antiviral, antibacterial, antidepressant, immune modulating and physiological effects which seem to work together to enhance animal performance (Windisch et al., 2009). A study in chickens was conducted at University of Agriculture and Forestry, Vietnam, to investigate the efficacy of a phytogenic mixture as an AGP replacement. One day old Cobb chicks were randomly divided into three treatment groups (nine replicates per treatment and 10 chicks per replicate) and fed one of three diets for 41 days as follows: 1) Control diet; 2) Control diet and Chlortetracycline HCl, 190 g/t as AGP; 3) Control diet supplemented with 125 g/t Digestarom<sup>®</sup> P.E.P. 125 Poultry. Results on final body weight (FW), feed intake (FI), FCR and other parameters can be observed in Table 1. Supplementation of Digestarom<sup>®</sup> P.E.P. was able to successfully replace the AGP in broiler diets.

**Table 1 – Effect of dietary supplementation of broilers with HCl and Digestarom<sup>®</sup> PEP**

	FW (g)	FI (g/day)	FCR	Livability (%)	Fecal dry matter (%)	Carcass yield <sup>1</sup> (%)	Breast meat <sup>1</sup> yield (%)	E. coli <sup>2</sup> (% fecal samples positive)	C. perfringens <sup>2</sup> (% fecal samples positive)
1	2052	102.5	2.15 <sup>a</sup>	92.2	21.3 <sup>b</sup>	70.21	25.41	67	89
2	2080	104.4	2.16 <sup>a</sup>	96.7	24.5 <sup>b</sup>	70.55	26.46	78	56
3	2151	95.2	1.90 <sup>b</sup>	95.6	26.3 <sup>a</sup>	71.82	28.05	44	33

<sup>ab</sup> Columns with different superscripts differ significantly ( $P \leq 0.05$ ) <sup>1</sup> Measured with 6 birds/treatment <sup>2</sup> Analysis carried out with 9 samples/treatment (1 sample/pen)

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## RECENT ADVANCES IN THE BIOTRANSFORMATION OF FUMONISINS

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In 1995, an unusual disease outbreak characterized by black sticky diarrhea, severe reduction in food intake, egg production and body weight followed by lameness and death was observed in two layer farms in Andhra Pradesh, India. The mortality rate was 10% and egg production was reduced by 20%. Analysis of the diets indicated contamination with fumonisin B1 (up to 8.5 ppm) in combination with aflatoxin B1 (up to 0.1 ppm) (Prathapkumar et al., 1997). Although in its recommendation (EC/576/2006) the European Commission refers to levels of 20 mg/kg of diet of fumonisins (FUM) as “safe” for poultry (EC, 2006), scientific data available acknowledge levels as low as 2 mg FUM per kg of body weight as the lowest observed adverse effects level (LOAEL). The EC recommendation, which is often confused with a regulation, may be misinterpreted in terms of what can be considered ‘safe’ for poultry. Furthermore, there is extensive literature suggesting that mycotoxins will not exist alone and that their concomitant presence will potentiate their negative effect in poultry.

So far, no single adsorbent has been tested to be effective against most types of mycotoxins (Huwig et al., 2001). An alternative way of removing non-adsorbable mycotoxins such as FUM is via enzymatic detoxification (or biotransformation). In the case of fumonisins, an FUM-degrading enzyme preparation (FDE, aminotransferase) is capable of biotransforming fumonisin B1 into the non-toxic metabolite, hydrolysed-FB1 (HFB1) (Hartinger and Moll, 2011).

In the Universidade Federal de Santa Maria, Brazil, 480 one day old Cobb male broilers were randomly divided into four treatments with 12 replicates and 10 birds in each replicate. The birds were offered one of four diets for 21 days as follows: 1) Control diet; 2) Control diet supplemented with 5 kg prototype product containing FDE (spray dried on a bentonite carrier in order to achieve 100 U/kg feed); 3) Control diet contaminated with 100 ppm FUM (68 % fumonisin B1 and 32 % fumonisins B2); 4) Combination of groups 2 and 3. Body weight (BW) and feed intake (FI) of individual birds per replicate were measured weekly. At the end of the trial, feed conversion ratio (FCR) was determined and the sphinganine:sphingosine ratio (Sa/So: a biomarker for the exposure of FUM) was measured by HPLC-MS/MS in blood collected from 12 randomly selected birds per treatment. Results of the trial are shown in Table 1.

**Table 1 - Effect of dietary supplementation of broilers with FUM and FDE**

	FI	BW	FCR	Sa/So
Control	1078 <sup>a</sup>	754 <sup>a</sup>	1.45 <sup>ab</sup>	0.2 <sup>c</sup>
5 kg FDE	1105 <sup>a</sup>	773 <sup>a</sup>	1.45 <sup>ab</sup>	0.42 <sup>c</sup>
FUM	1034 <sup>b</sup>	693 <sup>b</sup>	1.52 <sup>a</sup>	4.08 <sup>a</sup>
FUM+FDE	1043 <sup>b</sup>	739 <sup>a</sup>	1.43 <sup>b</sup>	2.57 <sup>b</sup>

Columns with different superscripts differ significantly ( $P \leq 0.05$ )

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## EFFECT OF POST-HATCH NUTRITIONAL IMPRINTING ON INTESTINAL NUTRIENT TRANSPORTER GENE EXPRESSION IN WHITE PULLETS

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### Summary

The importance of post-hatch early-life nutrition to lifelong animal health and performance is beginning to be recognized. A complete randomized design was used to study the long-term impact of a post-hatch conditioning supplement on intestinal gene expression patterns in layers after birds were switched to a common diet. White pullets (Hy-line 36) were assigned to three dietary treatments to provide eight replicate pens of 16 chicks per cage for each treatment. Real-time PCR was used to measure relative expression of select nutrient transporter genes. Results indicated that, in layers, the expression of some, but not all, nutrient carrier and transport genes that were measured can be influenced by a post-hatch preconditioning diet. Further, feeding the Alltech Alltech PN Grower Broiler premix and PN Pre-Harvest Broiler premixes, regardless of the hatch to d4 diet, resulted in the increase expression of zinc transport proteins and calbindin 1 in the intestine. Further work is needed to examine how the addition of PN Broiler premixes to the diet of layers influences the expression of these particular genes.

### I. INTRODUCTION

In recent years, it has become clear that nutrition during the neo-natal or post-hatch period has a vital impact on adult health (Wiedmeier et al., 2011). Studies in broilers indicate that diets fed early in life can have lifelong effects on birds regardless of diets fed later in life (Geyra et al., 2001; Gonzales et al., 2003; Noy and Sklan, 1999; Shira et al., 2005). Previous studies from our laboratory have indicated that a conditioning supplement fed for four days post-hatch alters gene expression patterns in the long term even after birds are returned to common diets (Brennan et al., 2012). The objective of this study was to evaluate the effects of feeding a post-hatch conditioning diet supplement on intestinal gene expression in 17-week-old pullets after birds were switched to a common grower/finisher diet. Target genes (zinc transport proteins 1, 4, 5, 6 and 7; calbindin 1; metallothionein 3 and 4; ferritin and solute carrier family 34, member 2) were chosen based on their role in mineral uptake, sensing and homeostatic maintenance.

### II. MATERIALS AND METHODS

All experimental protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee. One day old pullets were randomly assigned to three dietary treatments to provide eight replicate pens of 16 chicks per cage for each treatment. Chicks were given *ad libitum* access to feed and water. The three treatments were: 1. Corn-soy diet (Control), 2. Corn-soy diet for 4 d followed by diets formulated with Alltech PN Grower Broiler premix (d5 – wk 11) and Alltech PN Pre-Harvest Broiler premix modified to contain 2.0 g/kg available P and 7.0 g/kg Ca (wk 11 – 17, Normal-PN), and 3. Alltech PN Post Hatch Broiler chick starter for 4 d followed by diets formulated with Alltech PN Grower

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Broiler premix (d5 – wk 11) and Alltech PN Pre-Harvest Broiler premix modified to contain 2.0 g/kg available P and 7.0 g/kg Ca (wk 11 – 17, Conditioning-PN).

At 17 weeks of age, eight birds from each treatment were randomly selected and euthanized by argon asphyxiation followed by cervical dislocation. The small intestine was quickly removed, rinsed to remove contents and a sample from jejunum section 3cm proximal to Meckel's diverticulum was flash frozen in liquid nitrogen and stored at -80°C until further analysis. Total RNA was isolated from the jejunum using the RNeasy Mini Kit (Qiagen) according to the manufacturer's suggested protocol. Total RNA (0.5 µg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed in triplicate using Taqman Gene Expression Assays (Applied Biosystems) and the 7500 Real-time PCR system (Applied Biosystems). Ring finger protein 4 (RNF4) was selected as an endogenous control gene to account for any variation in the efficiency of reverse transcription and PCR. The relative quantification (RQ) was expressed as a ratio of the target gene to control gene using the delta-delta Ct ( $\Delta\Delta Ct$ ) method. Data presented are least square means (lsmeans)  $\pm$ SEM. Real-time PCR results for Normal-PN and Conditioning-PN were compared with ANOVA analysis for a completely randomized experimental design using the Proc GLM procedure of the SAS version 9.1 statistical package (SAS Inst. Inc., Cary, NC, USA). Comparisons of the treatments to the control were made using a Student's *t*-test.

### III. RESULTS AND DISCUSSION

Relative expression of zinc transport protein (ZnT) 1 and 7 did not differ between treatments (Figure 1). Expression of ZnT4 was increased in both Conditioning-PN- and Normal-PN-fed birds than control ( $P < 0.05$ ), but did not differ between birds fed the Conditioning-PN and Normal-PN treatments. Normal-PN fed birds had greater ZnT5 and ZnT6 mRNA levels compared with control ( $P < 0.05$ ), but levels did not differ from the Conditioning-PN-fed birds. Relative mRNA levels of ferritin, heavy polypeptide 1 (FTH1), metallothionein 3 (MT3) and metallothionein 4 (MT4) did not differ between treatments (Figure 2). The post-hatch conditioning supplement had no effect on calbindin 1(CALB1) mRNA levels, however, expression was greater in both Conditioning-PN- and Normal-PN-fed birds than control at 17 weeks of age ( $P < 0.05$ ). Calbindin 1 levels track directly with changes in Ca absorption with a correlation coefficient of 0.99 (Morrissey and Wasserman, 1971). Therefore birds fed either treatment in this study would likely result in an increase in Ca absorption. Somewhat surprisingly, solute carrier family 34 (sodium phosphate) member 2 (NaPi-IIb) mRNA levels were reduced 1.37-fold ( $P = 0.03$ ) in Conditioning-PN birds compared with the control. When dietary P levels are reduced, there is an adaptive response in which NaPi-IIb mRNA abundance increases (Giral et al., 2009), therefore it would be expected that NaPi-IIb mRNA abundance would be greatest in both the Conditioning-PN- and Normal-PN-fed birds where available P levels were reduced in the starter and grower phases. This may also indicate that P release by the enzyme inclusion, which among others provides 300 SPU/kg of phytase activity, in the diets containing Alltech PN premix resulted in the down regulation of NaPi-2b to regulate P uptake when more P was available.

### IV. CONCLUSION

Results from this study indicate that, in layers, the expression of some nutrient carrier and transport genes can be influenced by a post-hatch preconditioning period. Research from this lab has shown that diets fed in early life (4 d post-hatch) can have lifelong effects on expression patterns of nutrient carriers and transporters in the intestine regardless of diets fed

later in life (Brennan et al., 2012). These results indicates that diets formulated using the Alltech PN Grower (fed d5-wk 11) and Pre-Harvest (fed wk11-17) Broiler premixes but not

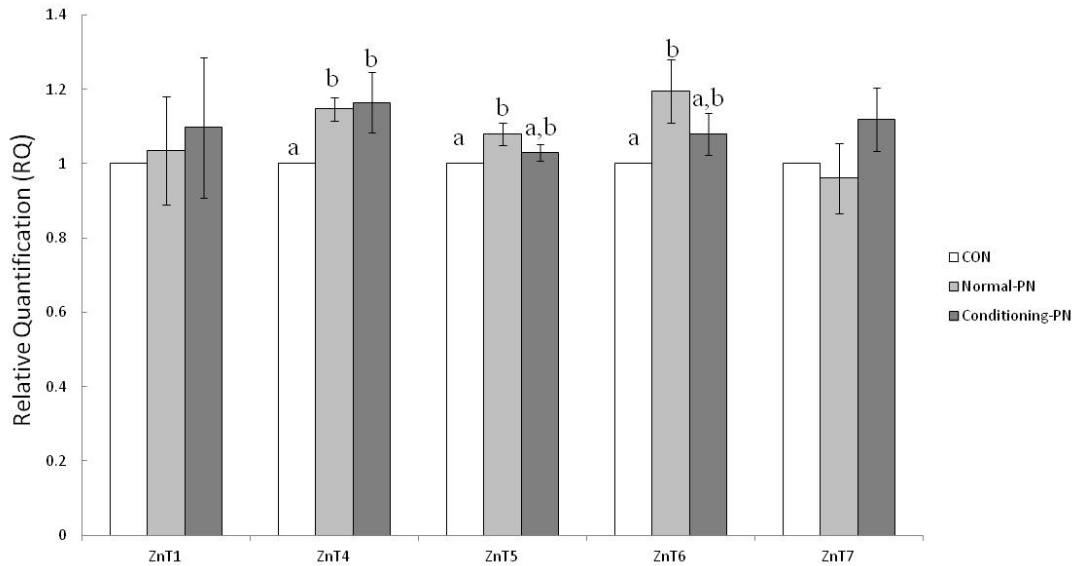


Figure 1 - Relative expression of zinc transport protein (ZnT) in the jejunum of pullets fed control (CON), Corn-soy diet for 4 d followed by diets formulated with the Alltech PN premixes (Normal-PN), and Alltech post-hatch conditioning supplement for 4 d followed by diets formulated with the Alltech PN premixes (Conditioning-PN) using real-time PCR. Values represented are least square means  $\pm$  SEM;  $n = 8$ . \*Columns without common superscripts differ at  $P \leq 0.05$

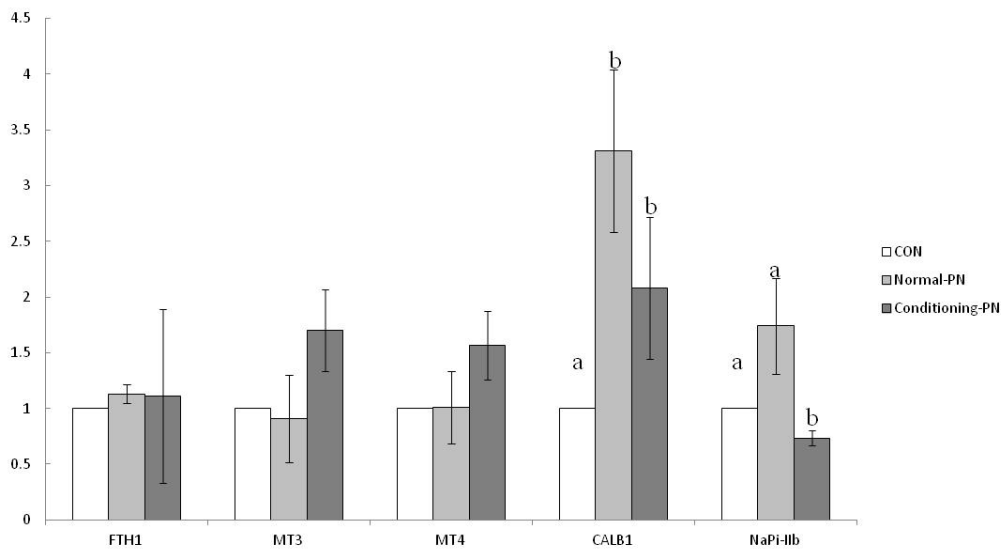


Figure 2 - Relative expression of nutrient transport genes in the jejunum of pullets fed control (CON), Corn-soy diet for 4 d followed by diets formulated with the Alltech PN premixes (Normal-PN), and Alltech post-hatch conditioning supplement for 4 d followed by diets formulated with the Alltech PN premixes (Conditioning-PN) using real-time PCR. Values represented are least square means  $\pm$  SEM;  $n = 8$ . \*Columns without common superscripts differ at  $P \leq 0.05$

the post-hatch conditioning diet, has an effect on nutrient transporter gene expression in the jejunum. This preliminary study helps us better understand nutrient-gene interactions in poultry and how different dietary strategies can influence the expression of genes involved in mineral uptake and binding.

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## IMPROVING EARLY DEVELOPMENT IMPROVES LONG-TERM PRODUCTION CHARACTERISTICS

H. KLEIN-HESSLING<sup>1</sup>

### Summary

Genetic selection in broilers has resulted, amongst other things, in substantial improvements in weight for age and there is no evidence that this phenomenon will change in the foreseeable future. Consequently, the birds have to grow faster and faster with each day. This leaves very little room for production disruptions, recovery from disease and/or mismanagement to still achieve the desired target criteria. Likewise, recent research on late-term embryonic development emphasizes the importance of the last quarter during incubation on post hatch performance. When chicks emerge from the shell, they do not have an anatomically and metabolically fully functional mature gastrointestinal system. Moreover, immediately post hatch, the chick must undergo a transition from a predominantly egg yolk- and endogenous, embryonic nutrient-based nourishment to an exogenous feed and water-based supplementary nutrition. When feed and water are provided early after hatch, the rate of gastrointestinal development and post hatch performance are improved. Seven-day body weight (BW) correlates strongly with BW at slaughter.

Several different strategies and new concepts have evolved around these scientific revelations. For example, research is ongoing on the effects of *in-ovo* injection of nutrients on post hatch performance. Secondly, new brooding concepts have begun to enter the commercial industry (Patio System, HatchBrood System, etc.) attempting to better meet the ideal, practical timing of initiation of feeding and drinking for the broiler. Finally, specific prestarter feeds for the first 4-7 days post hatch have been successfully introduced to the broiler industry that better meet the developing anatomical, metabolic and specific nutritional requirements of the newly hatched chick that otherwise regular starter diets may not fully satisfy. Specialty prestarter feeds play a progressively increasing role in successful modern-day broiler production.

### I. INTRODUCTION

Within agribusiness, the poultry industry is a very efficient and dynamic sector. Every year, advances from multiple segments (breeding companies, universities, equipment manufacturers, etc.) of the industry propel improvements down to the commercial level. Most of the progress is the result of genetic selection. However, as growth rates improve and the life cycle of broilers shorten, the more important the impact of nutrition and, especially, early nutrition. The foundation for optimal success in terms of achieving a broiler's performance potential is already established during the last days of incubation and first few days of a chick's life. Hatched chicks are considered "immature". This fact requires close attention from a nutritional point of view. Carefully designed and well-balanced prestarter diets with good physical structure, improve the performance potential of broilers and fit well with both current standard feeding and production systems, as well as the new innovative brooding systems just entering the broiler industry.

This presentation will focus on the nutritional aspects of early feeding on gastrointestinal development, immune function, thermoregulation, resistance to cold stress and how it improves the long-term production parameters of broilers. Some reference will also be made to incubation and *in-ovo* injection as it pertains to the aforementioned topics.

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## II. EFFECTS OF INCUBATION ON POST HATCH PERFORMANCE

As modern-day broilers reach the same BW earlier with every new genetic generation, the period of embryonic development during incubation becomes a more significant proportion of the bird's total lifespan (De Oliveira et al., 2008). Consequently, incubation effects and embryonic development process towards the time of hatch are of much greater importance to the efficient grow-out of meat-type poultry (Hulet, 2007). Factors that either enhance or impair growth and development during the late-stages of incubation will have a marked effect on overall production and health of meat-type poultry (Foye, *et al.*, 2007).

Numerous studies have shown the detrimental effects of high (>38.8 °C) temperatures during the last week of incubation on chick quality and performance. Elevated temperatures lead to lower chick BW (Leksrisompong et al., 2007; Laurens et al., 2007), lower heart weights (Givisiez et al., 2001; Leksrisompong et al., 2007; Molenaar et al., 2010), reduced small intestine, proventriculos and gizzard weights but higher yolk sac weights (Leksrisompong et al., 2007; Molenaar et al., 2010) when compared with chicks incubated at normal temperatures of 37.8 to 38.2 °C.

Van de Ven et al. (2011) investigated the relationship between hatch window spread (hatched early [465 h], mid-term [480 h] or late [493 h]) and hatching systems (Patio System vs. regular hatcher) on physiological parameters and post hatch performance in broilers. The results showed that chick BW at hatch was not strongly affected by the hatch window *per-se* but early growth was reduced in late hatching chicks (493 h versus 465 h), most likely influenced through lower thyroid levels which are well known regulatory metabolic hormones.

Those embryo tissues and organ systems that are the most affected by alterations to the late-stage incubation process are the highly metabolically active liver, the breast (pectoral) muscle, the hatching (pipping) muscle and the gastrointestinal tract (Moran, 2007; De Oliveira et al., 2008). In particular, the embryonic liver has a very high metabolic and enzymatic activity. Nutrients and waste products are all shunted via the vitelline circulation, the gut portal blood system and other vascular connections to the liver. Nutrients are absorbed, modified, stored or transported back out. The liver is also a major site for preparing detoxification and excretion processes of waste products.

## III. STIMULATION OF THE GASTROINTESTINAL TRACT MATURATION

When chicks emerge from the shell their digestive tract is anatomically immature and their ability to digest and absorb nutrients is not yet fully developed (Nitsan et al., 1991). Numerous morphological and physiological adaptations take place post-hatch. These can collectively be summarized as events leading to an increased gastrointestinal surface area resulting from an increase in gut length and weight. Not all segments of the gastrointestinal tract expand at the same rate. According to Maiorka et al. (2003), feed stimulates primarily the weight of the duodenum and the ileum while it increases the length of the jejunum and ileum. Consumption of feed also increases the number of crypts, villus height, villus number as well as number of goblet cells and enterocytes. The intestinal mucosa does not only respond to physical stimuli. Specific chemical characteristics of nutrients seem also to enhance its morphological development (Tarachai and Yamauchi, 2000). In chicks fed at hatch, villus height and crypt depth increase rapidly and reach a plateau after 6 days in the duodenum and about 10 days in the jejunum and ileum (Uni et al., 1999). Providing feed and water is associated with an up-regulation of pancreatic and intestinal digestive enzyme systems. When chicks were without food for 72 h after hatch, the activities of all disaccharidases (maltase, sucrase, isomaltase) in the small intestine were reduced and reductions remained evident as long as 16 days post initiation in comparison to a group



deprived of feed for only 24 h (Siddons, 1972). Taken together, these anatomical and morphological relationships suggest that feed intake, nutrient composition, intestinal growth and brush border enzyme development are intrinsically linked and dependent on each other.

#### IV. EFFECTS OF EARLY FEEDING ON IMMUNE FUNCTION, THERMOREGULATION AND RESISTANCE TO COLD STRESS

Dibner et al. (1998) studied the effect of early feeding on immune development. A hydrated nutritional supplement (HNS, Oasis hatching supplement) was provided on Days 0, 1 and 2 post-hatch while no water and feed were given to the control. Subsequently, on Day 3, all birds received a standard corn-soy based diet for *ad libitum* consumption to achieve maximum bird performance. Advancement in immune development and maturation was determined by measuring bursa weight, biliary immunoglobulin A (IgA) titers, number of germinal centers in the cecal tonsils and the ability to cope with a coccidiosis challenge. The results showed significantly greater percentage bursal weights for the HNS through 21 days ( $P < 0.003$ ; SEM=0.03), significantly greater number of germinal centers ( $P < 0.0001$ ; SEM=0.9) and a significantly better BW after coccidiosis challenge ( $P < 0.0003$ ; SEM=0.01).

The response to early feeding on thermoregulatory development and resistance to cold exposure was recently studied by Van den Brand et al. (2010). Contrary to expectation, hardly any differences occurred regarding deep body core temperature between Days 0, 1 and 2 before and following the cold exposure. Rectal temperatures for both prestarter treatments (40.7 and 40.6 °C, respectively) and the dextrose treatment (40.4 °C) were significantly higher than the rectal temperatures for albumen fed birds (39.8 °C) and the control group (39.5 °C) before cold stress ( $P < 0.05$ ). Differences in rectal temperature remained also after the cold exposure. The biggest drop in rectal temperature occurred in the albumen fed chicks (2.1 °C), followed by birds from the control and dextrose treatment (both 1.2 °C) whereas the prestarter and the prestarter plus fat lost only 0.6 and 0.7 °C, respectively ( $P < 0.001$ ). There was also a significant treatment x time interaction ( $P < 0.001$ ) suggesting that the response to the cold stress was not consistent across all treatments. It was concluded that early feeding after hatch might influence the speed of adaptation from a poikilotherm to a homeotherm physiological status and that it might improve the resistance to cold stress, likely facilitated through a higher metabolic rate when chicks are fed. This response might be further modulated through a specific diet composition. The addition of fat to the prestarter diet did not trigger any significant differences. There is strong evidence to suggest that chicks from young breeder flocks have significantly lower thermoregulatory capacity than chicks from prime and/or old breeder stock which necessitates the provision of higher house temperatures to keep these smaller animals within their thermo neutral zone and, thus, developmentally on track. Low chick rectal temperatures are positively correlated with one-week mortality, poor chick uniformity and reduced 7 day BW (Weytjens et al., 1999).

#### V. EFFECTS OF EARLY FEEDING ON BREAST MUSCLE DEVELOPMENT

Throughout the hatching process of the developing chick, skeletal muscle tissue is metabolically very active and a major contributor to the glucose homeostasis via gluconeogenic conversion of protein to glucose (Uni et al., 2005; De Oliveira et al., 2008). In this context, muscle micro satellite cell activity begins at about 18 and 25 days of incubation in broilers and turkeys, respectively. Micro satellite cell activity influences the muscle fiber numbers in the tissue. Cell activity is highest at hatch and declines subsequently significantly by 7 days post-hatch. Immediate access to feed and water post-hatch can modulate this reduction by keeping the micro satellite cell activity elevated at a high rate that supports not only the early muscle development but ensures also a high breast meat yield at market time

(Moore et al., 2005). Similar responses were observed when dietary energy was restricted as shown in studies by Mozdziak et al. (2002). A reduced cell division of micro satellite cells resulted from a lack of energy supply 2 days post hatch that was subsequently associated with a significantly lower carcass yield at slaughter time ( $P < 0.05$ ).

## VI. EARLY FEEDING STRATEGIES

### *a) In-ovo injection during incubation*

A large body of evidence exists that the developing embryo is able to utilize nutrients prior to hatching, as a complete chick during the actual hatching process but also during transport and standard placement of chicks upon arrival on the farm (Noy and Uni, 2010).

Several experiments have concluded that supplementation of 1 ml of  $\text{Na}^+$ ,  $\text{Cl}^-$ , dextrin, and HMB (2-hydroxy-4-methylthio butanoic acid) *in-ovo* led to a significant increase in liver glycogen content on day of hatch. It strongly stimulated enteric development 48 h after *in-ovo* injection (Tako et al., 2004) and was also associated with a significantly increased villi height and crypt depth, higher mRNA expression combined with a higher activity of mucosal brush-border enzymes and various nutrient transporter systems as well as a significant increase in pancreatic enzyme secretion capacity

Research on chicks originating from younger breeder flocks has shown that *in-ovo* feeding increased chick weights at hatch and breast meat yield at the end of a 25 day long test period significantly (Noy and Uni, 2010). Research and investigations on the merit of *in-ovo* injection into the amniotic fluid continue on molecules and substances like amino acids, carbohydrates, vitamins, nucleotides and hormones (McGruder et al., 2011). Provimi, for example, has its own *in-ovo* injection system and an experimental incubation facility to conduct nutritional research in this area for the development of new neonatal and/or early nutrition concepts.

### *b) New brooding concepts post hatch*

Early access to feed and water immediately upon hatch has been shown to be a key factor for improving post-hatch growth performance of broilers. The speed of development of the gastrointestinal tract strongly exceeds the rate of growth of the broiler during the early post hatch period (Potturi et al., 2005). This has significant relevance for how hatcheries are operated. In the current standard commercial hatchery system, chicks are taken out from the hatcher only when the majority of the chicks have emerged from the shell. The number of chicks this represents depends on the timespan of the “hatch window”. In either case, this leaves a significant portion of the chicks deprived of feed and water for extended periods of time with all the associated disadvantages on gastrointestinal development and growth as outlined previously. Chick services, vaccinations and transport, etc. add to the suboptimal conditions that have a negative effect on chick livability and post-hatch growth and carcass performance up to market age. Practical experience has consistently proven that controlling broiler house conditions such as room and floor temperatures, air speeds and humidity levels in narrowly defined ranges is difficult (Weytjens et al., 1999).

One new innovation in broiler production, trying to address these performance impairing factors, is the “HatchBrood” system. This equipment controls very precisely the crucial housing variables during the first few days of the chick’s life. Immediately post-hatch, chicks are placed into the HatchBrood system. Chicks have instant access to fresh water and feed. The HatchBrood system guarantees a controlled air temperature and air velocity which assures optimum chick body temperatures at all times. Importantly, the chicks will start eating and drinking almost immediately after placement. Following 4 days in the HatchBrood system, the chicks are then transported to a standard commercial farm for the remainder of their growing period.

Another innovative system is the Patio system. It combines the hatching process with the post hatch brooding- and growing phase in one and the same housing unit where feed and water are provided immediately post-hatch. The classical, physical separation between hatchery and brooding farm does not exist anymore in this system (Van de Ven et al., 2011)

c) Specific prestarter feeds for the first 4-7 days of age

To ensure maximum chick growth, the utilization of nutrients (both endogenous and exogenous) by the chick should reach optimum capacity a few days post hatch (Sulistiyanto et al., 1999). The residual yolk accounts for almost 22% of the total energy and 30% of the protein intake of a chick during the first 3 days of the chick's life (Murakami et al., 1992; Akiba and Murakami, 1995). TME, TME<sub>n</sub> and metabolisability (TME/gross energy) of raw materials were assayed in 1, 3 and 10 day old broiler chicks. The results showed that the metabolisability of dextrin and starch were low in chicks at Day 1 but increased with age up to 10 days. At all experimental ages, corn was metabolized at a significantly higher rate than wheat or sorghum ( $P < 0.05$ ). No significant differences ( $P > 0.05$ ) were found in TME, TME<sub>n</sub> and metabolisability of coconut-, safflower oil or beef tallow. Bioavailability of soybean- and fishmeal was significantly lower on Day 1 compared to Day 3 as well as Day 10. It was concluded that fat sources seem to be better utilized by the new born chick, irrespective of age, than carbohydrates and protein (Sulistiyanto et al., 1999).

Swennen et al., (2010) studied the impact of iso-energetic substitutions between lipid, carbohydrate and protein in prestarter diets of broilers from day of hatch to 5 days of age. The three experimental diets contained the same target ME level of 13.0 MJ/kg of feed and were energetically contributed via variations in fat (4.3 vs. 10.8%), protein (12.6 vs. 24.0%) and carbohydrate levels (39.1 vs. 51.0%). Common commercial diets were fed to all groups after 5 days of age. The results showed that prestarter composition altered the BW response. The low protein diet caused statistically the lowest absolute BW from Day 3 to Day 14 ( $P < 0.05$ ) as well as percentage of BW from 0-5 days ( $P < 0.01$ ). During week two, on a percentage of BW basis, this relationship was reversed ( $P < 0.05$ ). Conversely, from 14 to 28 days, chicks fed the low protein diet were only statistically lighter than the low carbohydrate group ( $P < 0.05$ ) but not compared to the low fat group ( $P > 0.05$ ). Regardless of age, the prestarter composition had no significant effect ( $P > 0.05$ ) on percentage organ weights except for the relative intestinal weight on Days 5, 14 and 28 as well as relative liver weights on Day 5. Chicks associated with the low protein diet utilised the residual yolk sac content significantly ( $P < 0.05$ ) more rapidly only on Day 2 of the test. These chicks tried metabolically to satisfy their protein needs for growth using yolk protein but this was not even sufficient to nourish a normal small intestine- and liver development.

Based on the existing knowledge of early nutrition on growth and post hatch performance of broilers, Noy and Uni (2010) recently proposed a concept to set aside the standing traditional industry model of employing a starter, grower and finisher on-farm feeding model. Their proposal suggests providing a nutrient link approach combining the late embryonic phase with the early on-farm growth phases. Specifically, the nutrient link should include a) dietary solutions injected into the developing hatchling; b) feed and/or water provision in the classical hatcher incubation system together combined with c) some specifically designed prestarter feed followed by a standard starter, grower and finisher program.

## VII. CONCLUSIONS

Recent studies on embryonic- and post hatch development of broilers have greatly advanced our knowledge concerning the specific early brooding- and feeding requirements of the modern, high-yield genetic lines. As the lifespan of the broiler continues to shorten, more and more emphasis must be placed on early nutrition. The newly hatched chick has a physiological, morphological, and metabolically immature gastrointestinal tract which benefits from a specialized early nutrition concept. Providing feed as early as possible is associated with better development of immune function, thermoregulatory capacity, resistance to cold stress and improved breast meat yield. It is concluded that specifically designed prestarter feeds for the first 4-7 days are likely better able to accommodate the chick's nutritional requirement during this crucial stage of development rather than regular, traditional starter feeds. Current feeding practices should be adapted accordingly.

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## TURKEY FARMING – A BRIEF REVIEW OF WELFARE AND HUSBANDRY ISSUES

P.C. GLATZ<sup>1</sup>Summary

A literature review was undertaken to examine welfare issues in the turkey industry. The major issues are disease, poor locomotion due to high growth rate, behavioral problems caused by high stocking density, lack of environmental enrichment in the turkey house and in the free range and poor air quality in turkey sheds.

## I. INTRODUCTION

In Australia, vertically integrated companies Ingham's and Baiada produce more than 75% of the turkeys. Other large independent growers produce an additional 1.0 million turkeys, with the remainder (100,000) coming from small operations in each state. In 2007, turkey production comprised 22,000 tonnes of meat (\$56m) with exports valued at \$6.24m. Turkey stock of high genetic merit is imported into Australia from the USA and UK on an infrequent basis. Over the last two years, there has been an interest in developing an understanding of the major issues being faced by the Industry. A review was requested by the New Animal Products program of RIRDC in 2010 to identify the major welfare issues associated with farming of turkeys. Approximately 250 references were sourced. The key points arising from the review are summarized here.

## II. HATCHING AND HOUSING

In the hatchery, there are some negative effects of long term storage of turkey fertile eggs. Storage for more than one week increases embryonic abnormalities and chick mortality and post-hatch growth and quality of surviving birds is also affected (Fasenko, 1997).

There is a view that turkey welfare is improved if birds are housed on deep litter in naturally ventilated sheds with natural light and access to forage and shelter belts (Kijowski et al., 2005). However, problems have been observed in the outdoor system with an increase in mortality in the last few weeks of growth usually caused by very hot or cold environmental temperatures (Abdel-Rahman, 2005).

Economic losses occur every year because of mortality and decreased production due to high environmental temperatures. Stocking density is an important issue in turkey welfare with high stocking density being a major animal welfare concern. It is a very sensitive subject because the whole economic balance of turkey production is very dependent on high stocking densities. Currently, there are a wide range of recommendations for stocking densities for growing turkeys. Birds reared at a density of 8 birds/m<sup>2</sup> showed a higher incidence of hip lesions (scabs and scratches) and of foot pad dermatitis (FPD) than those reared at 6.5 or 5 birds/m<sup>2</sup>, indicating that bird welfare is compromised at the highest density (Burs and Faruga, 2006). The barren environment of turkey houses has often been identified as a major cause of poor animal welfare and predisposes cannibalism. Use of straw bales in the shed and elevated platforms gives the bird the chance to explore the environment and reduce pecking (Spindler and Hartung, 2009).

The selection of fast growing strains of turkeys has resulted in leg and locomotory problems. The weight of breast muscles have increased relative to leg muscles and some

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birds find it difficult to move. Mortality rates caused by gait problems range from 2-4%. Birds also have greater difficulty coping with heat stress which is a major welfare problem in the turkey industry. Huge economic losses occur every year because of mortality and decreased production due to high environmental temperatures (Nestor et al., 1985). However, intermittent lighting results in an increase in bird activity which results in a higher feed intake and a decrease in locomotory problems (Hester and Kohl, 1989).

### III. INJURIOUS PECKING

Under commercial conditions, domestic turkeys are often aggressive towards pen mates which leads to injuries and death. To prevent outbreaks of pecking and cannibalism in turkeys, the light intensity is usually set at 5-7 lux although 1 lux is sometimes used. At very low light intensity birds find it difficult to explore the environment and stockpersons cannot detect birds that are sick or being pecked (Barber et al., 2004).

Beak trimming is normally performed early in the life of commercial turkeys to decrease injuries caused by cannibalism, bullying, and feather and vent pecking. Beak-trimming has been used for many years as a method to prevent cannibalism but the techniques (hot blade and infrared) are coming under increasing scrutiny. A number of European countries have banned beak trimming and the development of alternatives to beak trimming and re-trimming are now being given higher priority as critics become more vocal about invasive procedures applied to domestic animals (Glatz, 2005).

### IV. HEALTH

Air quality in turkey sheds can also be a welfare issue. Poor air quality in turkey houses often occurs as farmers attempt to reduce heating costs by using high stocking densities and low ventilation rates. The key toxic gases in poultry houses are ammonia, carbon dioxide, carbon monoxide and hydrogen sulphide, together with dust. Carlile (1984) indicated that continuous exposure of poultry to ammonia increases their susceptibility to keratoconjunctivitis and respiratory infections. Wathes (1998) reported the limits are 20 ppm for NH<sub>3</sub>, 3000 ppm for CO<sub>2</sub>, 10ppm for CO, 0.5 ppm for H<sub>2</sub>S, 3.4mg/m<sup>3</sup> for total dust and 1.7mg/m<sup>3</sup> for inhalable dust (Wathes, 1998). Continuous exposure of poultry to ammonia and dust affects the respiratory system leading to health problems for turkeys (Fallschissel et al., 2009).

Foot pad dermatitis (FPD) is a common condition amongst commercially grown turkey poults and is largely caused by litter quality. The skin of the footpad becomes hard and scaly, often developing horn-like pegs of abnormal keratin. The footpad can become swollen, frequently splitting. The cause of FPD is complex, but many reasons have been suggested, such as diet, bird weight and sex, litter moisture, litter type and ventilation. Litter quality is affected by factors such as stocking density, air temperature and moisture, season, consistency and amount of faeces and drinker design. Wet litter is one of the key factors affecting FPD, followed by biotin deficiency. Turkey poults reared on wet litter have an increased incidence and severity of FPD lesions, but the problem is alleviated by replacing the wet litter with dry.

(Martrenchar et al., 2001).

Good management is essential to maintain turkey welfare including taking action to minimise contact of turkeys with wild birds and other animals. Appropriate hygiene, proper housing, and brooding and appropriate stocking density are essential when welfare of turkeys is being judged. The housing facilities and equipment used in turkey farming need to be cleaned and disinfected before restocking to prevent the carry-over of disease-causing organisms to incoming birds. Free range turkeys should not be kept on land which has

become contaminated with organisms which cause or carry disease to an extent which could seriously prejudice the health of turkeys. The potential disease/pathogen risk pathways on commercial turkey farms have shown that drinking water, movement of personnel between sites/farms and contact with wild birds were the main potential pathways for pathogen transfer to domestic turkeys (Rawdon et al., 2008).

Apart from Avian Influenza, Blackhead is one of the most serious poultry diseases in turkeys; mortality can reach 70% in some flocks. Early signs of this disease include drowsiness, drooping of the head and wings, walking with an unusual gait, soiled vent feathers due to diarrhoea and bright yellow faeces resulting from infection of the liver (Beyer and Moritz, 2000).

## V. PICKUP AND TRANSPORT

The pick-up of turkeys from sheds for transport to processing plant can result in welfare concerns. Catchers are often required to carry birds upside down through a shed to a truck outside especially when the containers cannot be taken inside the shed for biosecurity reasons. Birds are usually caught by one or both legs and then placed into the crate. During this procedure the heads or wings of the birds can be injured against the solid sides of the crates (Kannan and Mench, 1996).

Mortality has long been a concern in relation to poultry transport. When birds are being transported they are exposed to a number of stressors including temperature extremes, sudden acceleration and braking of the vehicle, vibration, abrasion on the crates, fasting, withdrawal of water, social disruption and noise. The major threat to bird welfare during transport is increased mortality due to either heat or cold stress and muscle damage (Kowalski et al., 2001).

## VI. CONCLUSION

The welfare of turkeys can be affected during most processes in the production chain. Genetic selection, housing conditions, transport and slaughter can all be causes of poor welfare. The major issues are concerns associated with disease, poor locomotion due to high growth rate, chronic pain from beak trimming, eye abnormalities from being housed under low light intensity, behavioral problems caused by high stocking density, lack of enrichment in the turkey house and in the free range and poor air quality in turkey sheds. Depopulation of sheds and transport and slaughter can also result in poor welfare for birds. Changes in the current practices may lead to higher costs which cannot be sustained by producers.

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## PRODUCTIVITY AND TONIC IMMOBILITY DURATION OF THAI CROSSBRED CHICKENS RAISED AT DIFFERENT STOCKING DENSITIES

P. NA-LAMPANG<sup>1</sup>

### Summary

The objective of this study was to investigate the effects of stocking density (8, 12 and 16 birds/m<sup>2</sup>) on productivity and tonic immobility duration (a measure of fearfulness) of Thai crossbred chickens (n=675 birds) kept at 100 birds per pen. The results showed that stocking density had no significant ( $P > 0.05$ ) effect on body weight, body weight gain, feed intake, feed conversion ratio and mortality of chickens from the wk 2 to 12. When stocking density was increased from 8 birds/m<sup>2</sup> to 16 birds/m<sup>2</sup>, tonic immobility (TI) duration of the chickens increased significantly ( $P < 0.05$ ). However, the TI duration of chickens at a density of 12 birds/m<sup>2</sup> was not significantly different from those of both the lower and the higher densities. In conclusion, Thai crossbred chickens could be stocked up to 12 birds/m<sup>2</sup> without adverse effect on productivity and welfare when compared to those kept at 8 birds/m<sup>2</sup>.

### I. INTRODUCTION

Meat of native chickens is preferred by Thai people over the same products from commercial poultry because of their taste, leanness, and suitability to Thai special dishes (Wattanachant et al., 2004). Thus, native chicken meat is more highly valued than that coming from commercial poultry. The domestic market for Thai native chickens has increased significantly and overseas markets also have strong potential. This has led to a change of practice in raising native chickens in Thailand. Cross breeding of Thai native males with egg type females, rather than pure breeding of Thai native chickens, is used to obtain higher chick production. It is recommended by the Department of Livestock Development, Thailand, that stocking density used for open houses should be 8 birds/m<sup>2</sup>. However, some producers rear their chickens at higher stocking densities in order to reduce the fixed costs of production and produce more kilograms of chickens per unit area. As it is known that a reduction in space per bird generally results in poorer productivity and welfare of the chickens (Estevez, 2007; Meluzzi and Sirri, 2009). The objective of this study was to investigate the effect of rearing at higher than recommended stocking density on production and tonic immobility duration, a measure of fearfulness (Marin et al., 2001), in Thai crossbred chickens.

### II. MATERIALS AND METHODS

A total of 675 mixed sex Thai crossbred chicks (Thai native males and ISA Brown commercial layer type females), supplied by Suranaree University of Technology poultry farm, were reared from one day old to 13 wk of age without the use of beak trimming. The experiment lasted from February to April, 2011.

The pen sizes were 12.5 m<sup>2</sup>, 8.33 m<sup>2</sup>, and 6.25 m<sup>2</sup> in area. There were 100 birds per pen. This resulted in treatment densities of 8, 12 and 16 birds/m<sup>2</sup>, respectively. The pens were bedded with approximately 5 cm of rice hulls.

Chicks were brooded for 2 wk before being randomly assigned to the treatments. At the end of the second wk, the chicks were vaccinated according to the recommendation of the Department of Livestock Development, Thailand. The chickens were fed a standard

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commercial three phase broiler diet. Feed and water were fed ad libitum throughout the rearing period. During the first 3 wk, feed was added 3 to 4 times a day. After that the feed was added twice per day (0800 h and 1630 h). The ratio of birds per feeder cup (diameter×high: 40 cm×30 cm) or water bottle (4L capacity) was 25 to one.

Natural lighting was used after the brooding period until 13 wk old. The chicken house was protected from the wind and rain with plastic sheeting, which was also used to adjust the ventilation. Before stocking the birds, the house was sprayed with disinfectant. Temperature and relative humidity in the chicken house were recorded continuously.

Data on average body weight (BW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) and mortality rate were determined at the end of the experiment when they chickens were 12 wk old.

During wk 13 (from 85 to 88 d old), 7 birds, randomly chosen from each pen, were evaluated in the tonic immobility (TI) test in a separate area of the chicken house. TI was induced as soon as the bird was caught by placing the animal on its back, with the head hanging, in a V-shaped plastic cradle (length×width×height: 30×24×20 cm). The method was similar to that described by Campo *et al.* (2008). The bird was restrained for 10 s. The observer sat in full view of the bird, about 1 m away, and fixed his eyes on the bird to cause the fear-inducing properties of eye contact. If the bird remained immobile for 10 s after the researcher removed his hands, a stopwatch was started to record latencies until the bird righted itself. If the bird righted itself in less than 10 s, and the restraint procedure was repeated (3 times maximum), then it was considered that tonic immobility had not been induced, so a 0 s score was given. If the bird did not show a righting response over the 10 min test period, a maximum score of 600 s was given for righting time.

The experimental unit considered was the pen. The experimental design used was a completely randomized design with three replicates per treatment. The data were subjected to analysis of variance with the General Linear Model procedure of SPSS 16.0. TI duration data were logarithmically transformed prior to analysis. When significance was indicated, differences among treatment means were tested by Duncan's multiple range tests.

### III. RESULTS

During the experiment, average temperature and relative humidity in the chicken house in the morning (0700 h) and the afternoon (1430 h) were (Means ± SE) 24.06 ± 0.29°C, 30.30 ± 0.46°C, 88.09 ± 0.99% and 67.05 ± 1.46%, respectively.

Different levels of stocking density did not affect BW, BWG, FI, FCR or mortality (Table 1).

**Table 1 - Effects of stocking density on body weight (BW), body weight gain (BWG), feed intake (FI) and mortality of Thai crossbred chickens (Means ± SE)**

Density (Birds/m <sup>2</sup> )	BW (g)	BWG (g)	Feed Intake (g)	FCR	Mortality (%)
8	1293.30±43.33	1187.70±43.67	3367.60±66.67	2.85±0.14	1.67±0.02
12	1242.20±70.35	1137.60±67.57	3348.20±54.54	2.96±0.14	1.00±0.01
16	1275.00±72.86	1164.50±73.28	3423.90±26.51	2.95±0.17	0.33±0.01

Stocking density affected TI duration of the chickens (Table 2). The TI duration of chickens at 16 birds/m<sup>2</sup> was higher ( $P < 0.05$ ) than that for 8 birds/m<sup>2</sup>, while that at 12 birds/m<sup>2</sup> density was not significantly different from either the higher or lower densities.

**Table 2 - Effects of stocking density on TI duration of Thai crossbred chickens**

Density (Birds/m <sup>2</sup> )	TI duration (s)
8	284±48 <sup>a</sup>
12	327±48 <sup>ab</sup>
16	432±45 <sup>b</sup>

<sup>a, b</sup> means within the same column with different superscripts were significantly different ( $P < 0.05$ )

#### IV. DISCUSSION

Temperature and relative humidity recorded during the experiment were normal for Thailand and did not cause any adverse effects on the chickens. The final BW of the chickens (at 12 wk of age) was sufficient to reach the marketable live weight of 1.2 kg which is normal for Thai chickens (Haitook et al., 2003).

The results of this experiment agreed with those of Feddes et al. (2002) and Ravindran et al. (2006) who reported similar BW and BWG for chickens reared at three levels of low, middle, and high densities. The results also agreed with those of Thomas et al. (2004) who reported that stocking density had no effect on broiler mortality. However, Hall (2001) reported a significant increase of mortality in high stocking density in commercial farms. Dawkins et al. (2004) and Jones et al. (2005) argued that stocking density itself was less important to the physical health and mortality rates of the chickens than other environmental factors. Dawkins et al. (2004) showed that the differences between producers in terms of the environment they provide to the animals had more impact on their welfare than stocking density per se.

The longer TI duration observed at the highest stocking density indicates that the chickens were more fearful. These results are similar to the findings of Andrews et al. (1997) and Onbaşlılar et al. (2008). The duration of TI response to manual restraint is widely considered to be a useful behavioral index of fear and thus welfare (Marin et al., 2001). This indicates that raising Thai cross breed chickens at 16 birds/m<sup>2</sup> can compromise one measure of chickens' welfare when compared to those raised at 8 birds/m<sup>2</sup>.

In conclusion, the results of this experiment suggest that Thai crossbred chickens could be kept at a stocking density of 12 birds/m<sup>2</sup> and maintain the same level of productivity and welfare status as those kept at the suggested 8 birds/m<sup>2</sup> by the Department of Livestock Development, Thailand.

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FIELD DATA OF THE CHANGING CLINICAL PICTURE OVER TIME OF  
INCLUSION BODY HEPATITIS IN CANADA WITH AN EMPHASIS ON  
DIAGNOSIS, PREVENTION AND TRIALS ON SUPPORTIVE TREATMENTS

D. VENNE<sup>1</sup>

Summary

Inclusion body hepatitis (IBH) has long been diagnosed in the poultry industry. It has been associated mainly with immunosuppressive conditions. It reappeared as an emerging disease in 2001 from breeder replacement stock. It was associated with the use of killed adenovirus vaccines against serotypes 8a, 8b and 1 in grand-parent flocks and an increase in cleaning and disinfection procedures of broiler replacement grow-out barns for *Salmonella* control. The development of serological monitoring tools demonstrated the susceptibility of antibody naïve flocks by the prevalence of the disease in their progeny and the importance of maternal antibodies. Measures to ensure sero-conversion before the onset of lay greatly reduced the incidence of the disease although drifts in the prevalence of different serotypes induced breaks in the control of the disease. Autogenous vaccines were developed and have helped in optimising immunity against the most prevalent serotypes in an industrial setting. In the last outbreak, clinical cases of inclusion body hepatitis were investigated during the summer of 2010 using an iSTAT-1 portable biochemical analyzer. Significant changes included metabolic acidosis, hypocalcaemia and in some cases hypoglycaemia. Glucose, sodium bicarbonate and calcium were evaluated as supportive treatments. Feed analysis indicated a low electrolyte balance (<180meq) in the feed, compatible with the metabolic acidosis. Other factors such as spiking syndrome (chick hypoglycemia) and lack of a dark period in lighting programs are compatible with the hypoglycemia. These observations may help in better understanding the pathogenicity of inclusion body hepatitis in cases where the presence of adenovirus is not sufficient *per se* to produce mortality.

I. INTRODUCTION

Inclusion body hepatitis (IBH) is caused by fowl adenoviruses belonging to group 1 avian adenoviruses in poultry, there are many serotypes and pathotypes that are well described in Diseases of Poultry (McConnell Adair et al., 2008). Poultry adenoviruses were long considered viruses without a disease, reflecting their high prevalence and low pathogenicity. The incubation period for avian adenoviruses is very short, being estimated at between 24 and 48 hours, and may play a role in the epidemiology of the IBH. The other important aspect of adenoviruses is their ability to induce a rapid immune response with detectable neutralizing antibodies within 1 week post-infection in natural and experimental conditions and may explain the association of the disease with immuno-suppressive conditions where a slow or deficient immune response may induce higher virus replication and damage to the target organs such as the liver and the pancreas. If the viral damage is greater, the capacity of the liver to regenerate itself will be more limited and mortalities will be greater. In the province of Québec, Canada, the disease has gone from not being reported in 2000, to making the number 2 most frequent diagnosis in 2010, followed by a major reduction in the number of

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cases in 2011 and 2012 following the availability of diagnostic procedures and autogenous vaccines for broiler breeders.

## II. THE FIRST CASES

The first cases I observed in 2000 and 2001 were from broiler breeder replacement stock we imported from primary breeders in the United States. Figure 1 shows the evolution of the number of cases reported at the provincial pathology laboratories since 1999. The information is compiled from the published annual reports (Revue d'épidémiologie animale du RAIZO, MAPAQ). Diagnosis was based on birds showing clinical signs of increased mortality, prostration, panting and enlarged livers and was confirmed microscopically by the presence of inclusion bodies in hepatocytes. The disease causes mortality for about 10 days following onset and is referred to as the 10 day disease by growers.

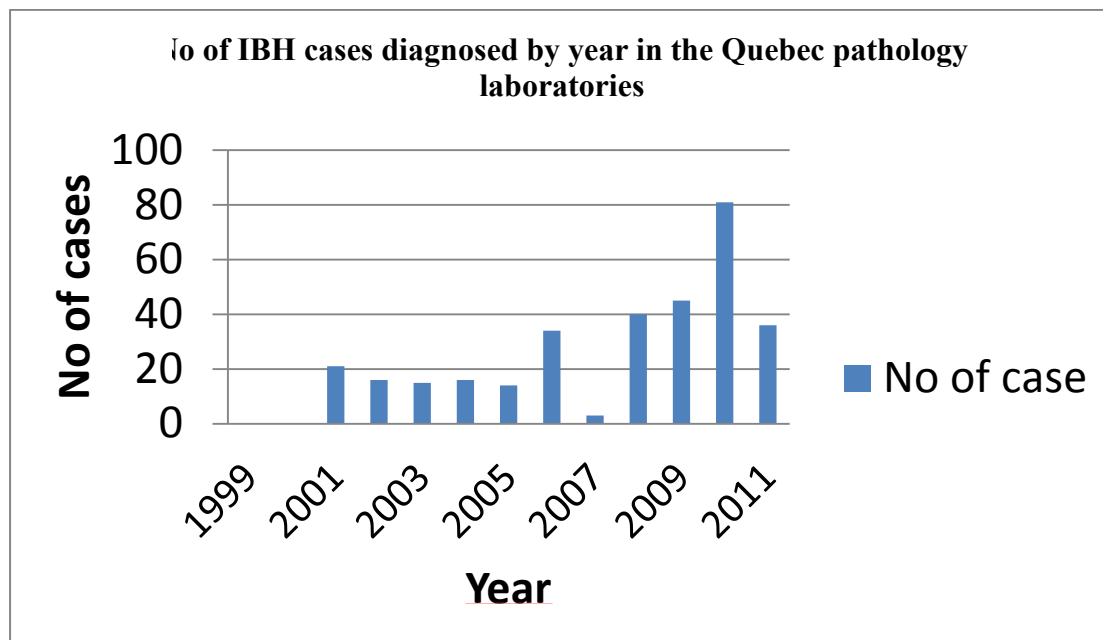


Figure 1 - No of IBH cases diagnosed by year in the Quebec pathology laboratories

Figure 1 also shows a decrease in 2007 associated with the first immunisation strategies of broiler breeder flocks, an increase in 2008 associated with shifts between the two major serotypes 8 and 11, a peak in 2010, followed by a major decrease in 2010 and 2012. This decrease resulted from the introduction of autogenous inactivated vaccination of the breeder flocks in 4 of the 5 major hatcheries with serotypes 8 and 11 and changes in feed profiles that will be discussed later in this paper.

To better understand the sudden emergence of the IBH, it is important to realise the efforts made by the Quebec egg and breeder industry to reduce the incidence of *Salmonella* Enteritidis at the end of the 1990's. The industry put in place programs to monitor and depopulate commercial egg producers, and divert the sale of

eggs not suitable for incubation from on-farm sales to further processing. At the same time, some parent broiler suppliers started vaccinating the grand-parent breeder flocks with autogenous inactivated vaccines against inclusion body hepatitis-causing adenovirus.

Our hypothesis was that there was a reduction in the vertical shedding of virus from grand-parent stock to parent stock associated with stricter biosecurity and cleaning and disinfecting procedures producing progeny without maternal antibodies as seen in Figure 2.

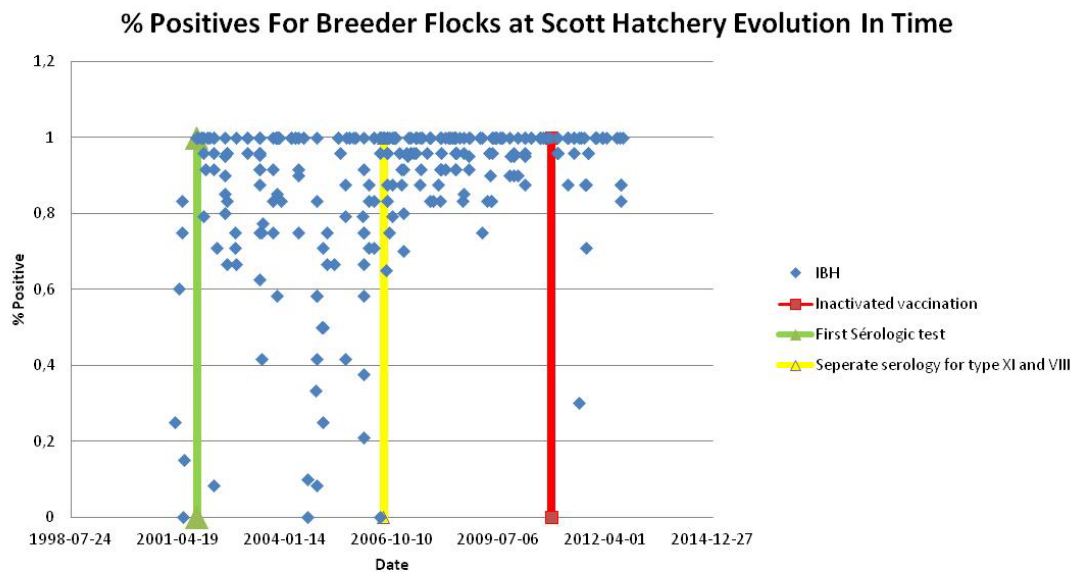


Figure 2 - Percentage positives for breed flocks at Scott Hatchery over time

Each dot on the graph indicates a flock of broiler breeders. The x axis indicates the date the flock was bled or eggs were collected and extracted for serology looking for maternal antibody. The y axis indicates the number of positive samples out of the number of sample collected (1=100% positives). The first line indicates the date at which the serological test became available. The points before this date are serologic results for the dot blot serological test that were obtained from samples from Dr. Amer Silim's sera bank to validate the test. The second line indicates when we started testing for serotype 11 and serotype 8 separately. The third line indicates the date when our hatchery started vaccinating broiler breeders with an inactivated autogenous vaccine containing serotypes 8a, 8b and 11 from local clinical cases. When the data were plotted out by age, Figure 3 was obtained. Flocks with multiple samples were investigated to see if they had generated progeny with inclusion body hepatitis for multiple hatches was used to separate them out into two groups. Breeder flocks with progeny with clinical signs of IBH are shown in red and flocks with progeny without clinical signs of IBH in green. From these data, we determined cut-off levels where we wanted 50% of the birds positive for FAV antibodies by 20 weeks of age.



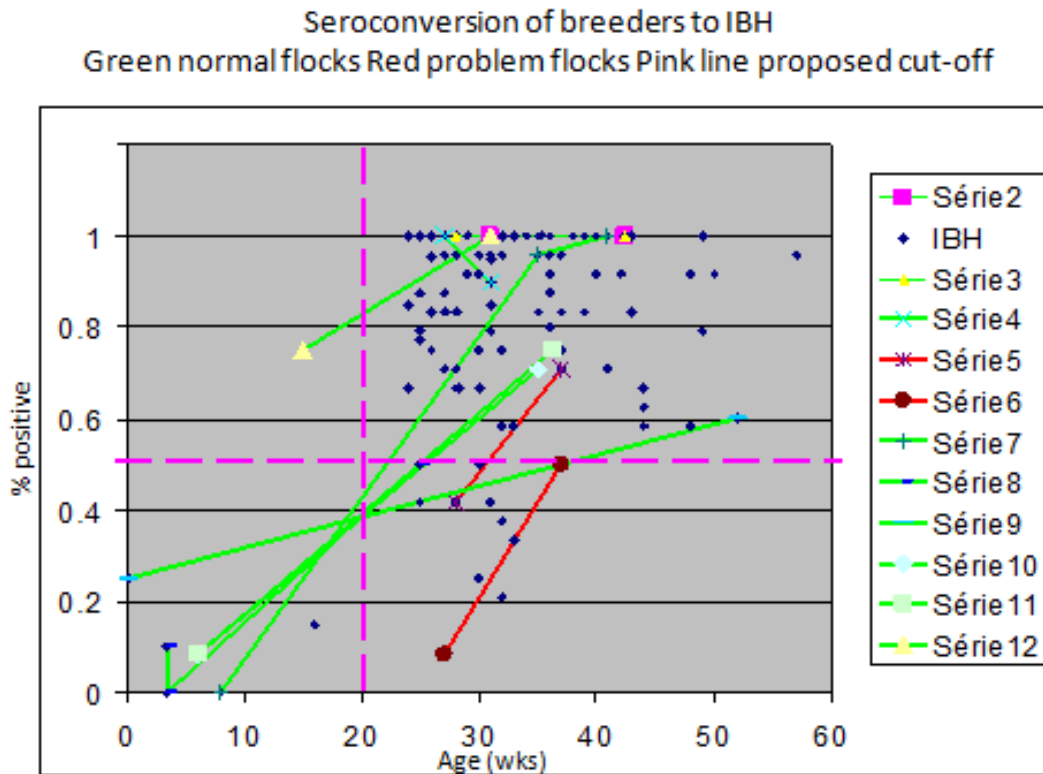


Figure 3 - Seroconversion of breeders to IBH

The serological test was also used on broilers to generate data on airsacculitis that showed sero-conversion to adenovirus of 89.6% of flocks with a low incidence of airsacculitis at slaughter (Ankouche et al. 2007), indicating a high prevalence of adenoviruses in normal broiler flocks in Quebec. We used the same test to generate broiler data according to age. The data from selected flocks showed the patterns illustrated in Figure 4. The data indicated maternal antibodies in a majority of flocks before 15 days of age, no maternal antibodies between 18 and 25 days of age and separation into two groups of flocks with and without seroconversion. Flocks bled at the beginning of clinical symptoms did not show seroconversion. Interestingly, the flocks with no seroconversion after 30 days of age were mainly from a company complex with a more thorough cleaning and disinfection program than the rest of the Quebec industry and which has never had a clinical case of IBH.

Data were also generated with the serological data for serotypes 8 and 11 showing that some serotypes are not transmitted or present if natural immunization is used (Figure 5). A review of the serotypes and genotypes isolated in Canada showed the high prevalence of serotypes 8 and 11 in these IBH outbreaks (Ojkic et al, 2008).

% positives on IBH dot blot test for broilers

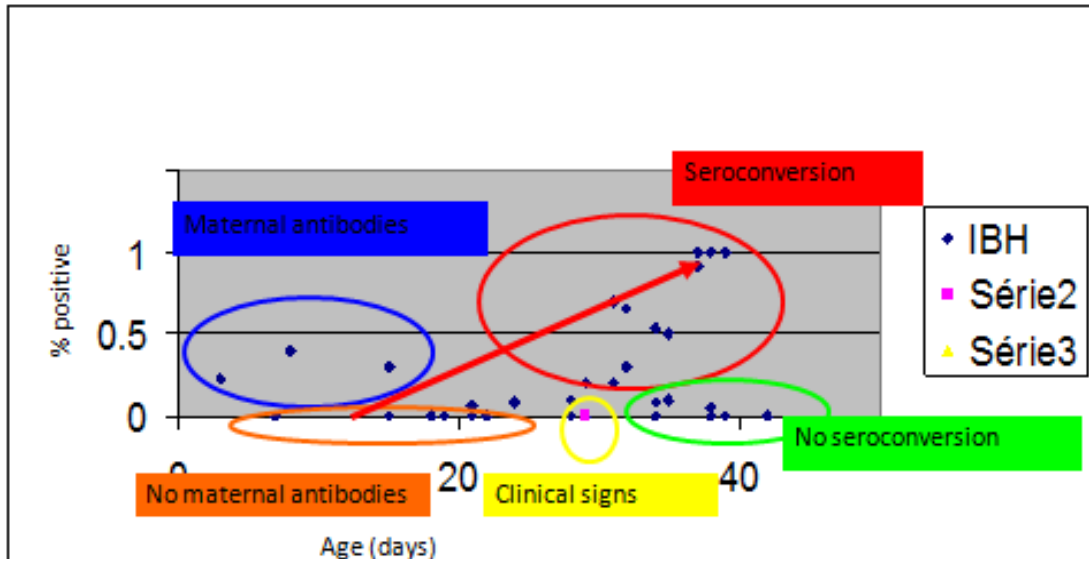


Figure 4 - Percentage positives on IBH blot test for broilers

Evolution of the intensity of the reaction against serotype VIII and XI in broiler breeders

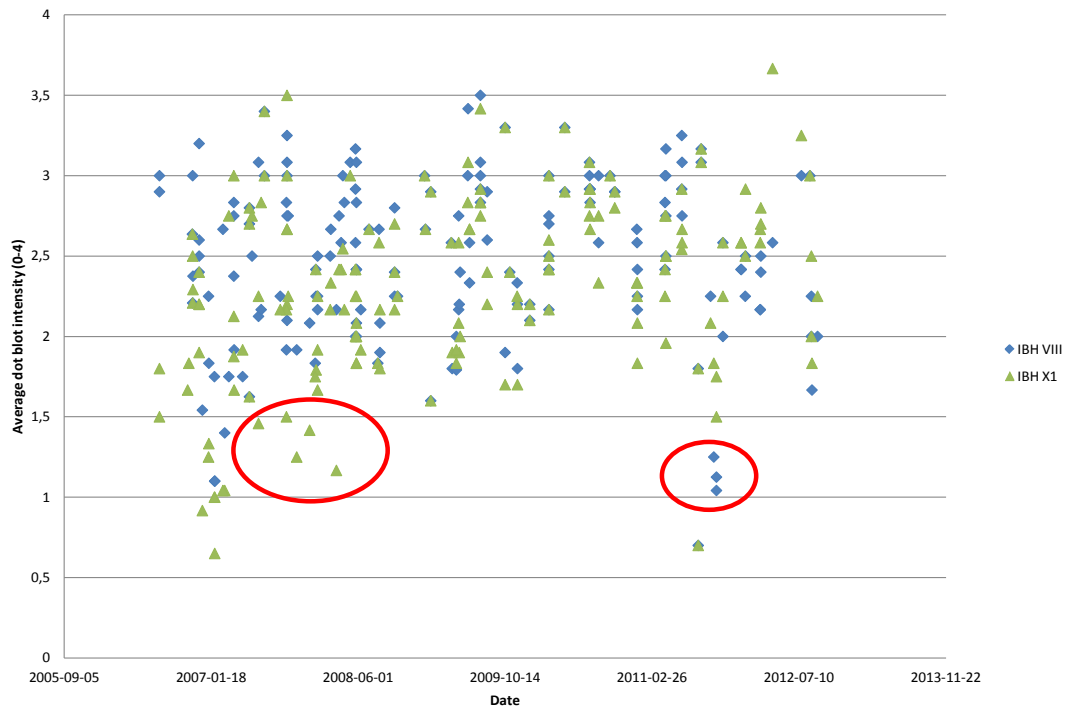


Figure 5 - Evolution of the intensity of the reaction against serotypes 8 and 11

In 2010, a new episode of IBH inclusion body hepatitis was observed in certain regions of the province of Quebec through provincial diagnostic laboratories. These cases were observed from different hatcheries and did not show the previous sequence of 5 to 6 weeks of broiler chicks with problems and were of both serotype 8 and 11. During the outbreaks, blood samples were analysed from birds showing clinical signs and birds without clinical signs. Metabolic acidosis was a consistent finding in affected birds. Hypocalcemia and hypoglycemia were also highly prevalent. The following cases were evaluated and supportive treatments were instigated for the observed biochemical changes based on variation from normal values compiled in our database. A follow-up biochemical test was evaluated and zootechnical results were recorded. Considering recent publications on the difficulty of reproducing inclusion body hepatitis with adenovirus by itself we believe that factors favoring metabolic acidosis, hypoglycemia or hypocalcemia can play a role in the severity of inclusion body hepatitis

Tables 1 and 2 show, respectively, the results of blood parameters and feed analysis.

**Table 1 - Blood parameters during 2010 IBH outbreaks**

Parameter	Database (7 to 35 days)	Birds from affected farms with clinical signs	Birds from affected farms without clinical signs	Birds from affected farms after treatment
N	166	47	29	16
Avg age (days) ±SD	19.68±8.6	20.17±5.15	19.58±6.53	21.50±7.35
Ht (%)±SD	21.43±3.45	18.93±3.90	20.48±1.97	18.63±3.42
vpH±SD	7.375±0.09	7.366±0.10	7.387±0.07	7.355±0.12
vPCO <sub>2</sub> (mmHg)±SD	46.43±9.59	34.44±14.73	43.49±8.87	40.43±11.01
vHCO <sub>3</sub> <sup>-</sup> (mmol/L)±SD	26.998±4.12	19.219±6.17	25.917±6.82	22.843±6.82
vBeecf (mmol/L)±SD	1.800±4.99	-6.135±6.48	0.931±4.78	-2.714±8.21
Na <sup>+</sup> (mmol/L) ±SD	144.29±5.36	144.17±3.93	144.90±7.16	143.38±3.81
K <sup>+</sup> (mmol/L) ±SD	5.53±0.76	5.43±0.90	5.33±0.59	5.58±0.92
Ca <sup>++</sup> (mmol/L) ±SD	1.381±0.118	1.075±0.271	1.433±0.083	1.274±0.253
Glu (mmol/L) ±SD	14.101±2.09	8.136±6.34	14.213±2.00	10.194±5.45

**Table 2 - Feed analysis during 2010 IBH outbreaks**

Parameter	Database (7 to 35 days)	Feed from affected farms with clinical signs	Feed from affected farms without clinical signs	Feed from affected farms after treatment
N	131	31	32	16
Na (%)±SD	0.24±0.02	0.16±0.02	0.14±0.02	0.12±0.03
K (%)±SD	0.73±0.08	0.71±0.08	0.70±0.09	0.68±0.24
Cl (%)±SD	0.25±0.10	0.24±0.04	0.22±0.05	0.26±0.02
Ca (%)±SD	0.94±0.14	0.88±0.08	0.87±0.12	0.96±0.43
Total P (%)±SD	0.66±0.34	0.60±0.09	0.59±0.11	0.60±0.24
Electrolyte ±SD balance (meq)	194±24	184±27	181±29	158±76

Results of birds with clinical signs from affected farms showed a metabolic acidosis with a respiratory compensation which is an attempt by the bird to regulate blood pH. The affected birds also showed a severe hypocalcaemia and hypoglycaemia. Treatment with sugar and sodium bicarbonate reduced the metabolic acidosis and increase ionized calcium in blood plasma. The treatment also reduced the previously reported 10 day spike in mortality associated with inclusion body hepatitis outbreaks.

The metabolic acidosis in this study is associated with lower sodium levels in the feed and a slightly lower electrolyte balance. Birds with hypoglycaemia in some of these inclusion body hepatitis cases were older than those reported for spiking syndrome. Inclusion bodies were also found in the pancreas of birds in some of these cases and may explain the hypoglycaemia. Birds that seroconvert to adenoviruses but do not express clinical signs or mortality associated with IBH do not show this hypoglycemia. Calcium levels in the feed do not explain the ionised calcium levels observed in the blood

The lower electrolyte balance observed in the feed of the birds after the water treatment can partially be explained by lower protein, soya and potassium as birds get older and their requirements change. The high standard deviation observed in this data set is associated with very low sodium (0.08%), potassium (0.28%) and electrolyte balance (33meq) levels in one case.

### III. DISCUSSION

Adenoviruses are highly prevalent in commercial broiler farms. The factors required for the IBH to be expressed are multiple and complex. Maternal antibody, immunosuppression, age and pressure of infection resulting from the amount of virus in the environment all contribute to the expression and severity of the clinical signs. Birds without maternal antibodies to each specific adenovirus serotype, placed in highly contaminated environments or contaminated with a rapidly-replicating strain of adenovirus, and with immunosuppressive conditions that slow the immune response, are at the greatest risk of developing the clinical signs of inclusion body hepatitis as shown in the work of other researchers (McConnell Adair et al., 2008).

Without supportive treatment, hypoglycaemia and hypocalcaemia can rapidly kill birds. Metabolic acidosis can be compensated by respiratory pCO<sub>2</sub> control that may explain the observation of panting birds as a clinical sign of inclusion body hepatitis.

Clinical expression of adenovirus-induced inclusion body hepatitis has been associated with immunosuppressive diseases or syndromes such as infectious bursal disease, chick anaemia virus or spiking. Adenoviruses are known to elicit a rapid immune response that may explain the high number of birds with antibodies and the low number of cases with high mortality. Hypoglycaemia may further increase the pathogenicity of inclusion body hepatitis by hindering the immune system and allowing more viral replication because glucose is the only energy source for cells of the immune system.

Even though the electrolyte balance was marginally lower in the feed, the very low sodium levels may have hindered compensation mechanisms to excrete excess anions. The liver plays a major role through the bile cycle in the excretion and reabsorption of bicarbonates. Low intake of sodium can become critical if acid-base compensation is needed to attain homeostasis. During this outbreak, there was a sudden change for the chickens in electrolyte balance between the starter feed and the grower feed in the region where this outbreak occurred. Changes of more than 30meq in electrolyte balance may force a sudden compensation in acid-base balance of the chickens at a critical time in the pathogenicity of this disease.

In this case, a source of calcium could have helped with the hypocalcaemia but finding sources of calcium that do not react with sodium bicarbonate in the water needs more research because most soluble calcium sources are acid-based and react with bicarbonates.

ACKNOWLEDGEMENTS: I would like to thank Dr. Amer N. Silim and Ms Diane Frenette from the Faculty of Veterinary Medicine of the University of Montreal who have recently retired and who developed the dot blot serologic test used in this presentation.

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CHARACTERISATION OF TWO AUSTRALIAN ISOLATES OF MAREK'S DISEASE  
VIRUS *IN VIVO* INCLUDING NEUROPATHOTYPING

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Summary

Virulent Marek's disease virus (MDV) induces early (1-3 weeks after challenge) neurological signs in chickens free of maternal antibody directed against MDV. A system for neuropathotyping MDV on the basis of the timing and severity of these signs has been reported in the USA and we report its application as part of the evaluation of virulence of Australian MDV isolates MPF23 and MPF57 at 3 different challenge doses in specific pathogen free (SPF) chicks over a 56-day post challenge period. MPF23 was the more pathogenic of the two viruses as assessed by a range of measures including mortality rate (81% versus 62%) and incidence of gross Marek's disease (MD) lesions (95% versus 76%). This was reflected in a clearly different pattern of clinical illness over the 56-day period with the MPF23 group showing earlier, more sustained and more severe clinical signs in the period 26-56 day post challenge (dpc). However there were few differences during the 0-23 dpc period used for the USA neuropathotyping system. The observed pattern during this period classifies both viruses as neuropathotype B consistent with a vv pathotype under the pathotyping classification of the Avian Diseases and Oncology Laboratory (ADOL) of the USDA.

I. INTRODUCTION

MDV of widely divergent virulence may be isolated from commercial flocks necessitating methods for assessing the virulence of MDV isolates. Early reports of virulence testing of Australian isolates of MDV used a variety of methods (McKimm-Breschin et al., 1990; Zerbes et al., 1994; Delaney et al., 1995) but differences in chicken genotype, maternal antibody status, challenge dose (if specified), vaccine type and dose make comparisons of isolate virulence amongst these and international reports difficult. To overcome this, more recent characterisations of MDV isolates (Walkden-Brown et al., 2007; Renz et al., 2012) have used an adaptation of a standard pathotyping protocol developed at the Avian Diseases and Oncology Laboratory (ADOL) of the USDA (Witter, 1997). This method, designed for use in chickens with maternal antibody directed against MDV, classifies MDVs into mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) pathotypes. In the Australian studies it was observed that, in SPF chickens free of maternal antibody against MDV, significant numbers of birds exhibited an acute paralytic syndrome between days 9 and 15 post challenge with MDV resulting in overall mortality rates of 2-18% (Renz et al., 2012). This syndrome was not observed in commercial layer or broiler chickens challenged with the same dose and isolates of MDV, presumably due to the protective effect of maternal antibody. Based on studies in similar maternal antibody-free SPF chickens, Gimeno et al. (2002) developed an alternative pathotyping system for MDV based on the timing and severity of neurological clinical signs following challenge with MDV.

The main purpose of this study was to evaluate the virulence of Australian MDV isolate MPF23 against the reference isolate MPF57, as determined by the incidence of MD and the neuropathotyping classification of Gimeno et al. (2002) based on neurological signs. MPF23 is a particularly pathogenic MDV that was isolated in 1985 and induced tumours in more than 80% of HVT vaccinated SPF chickens and more than 55% of chickens vaccinated

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with MDV2 or HVT/MDV2 vaccine (McKimm-Breschkin et al., 1990). MPF57, the Australian standard challenge isolate was isolated in 1994 and induced 80% mortality and gross lesions in unvaccinated SPF chickens. Both isolates were initially classified as vvMDV, but more formal pathotyping by the ADOL method has seen MPF57 re-classified as vMDV (Renz et al., 2012).

## II. MATERIALS AND METHODS

The experiment had a 2x3 factorial design with an external negative control. Experimental factors and levels were MDV challenge: MPF57 or MPF23 or unchallenged controls and Challenge dose: 500, 2000 or 8000 plaque forming units (pfu)/chick in 200 µl administered intra-abdominally on day 5 after hatch. The experimental duration was 61 days, up to 56 days post challenge (dpc). The experimental chickens comprised 51 chickens hatched at UNE from SPF eggs and transferred at hatch to positive pressure isolators. Isolator 1 held 9 unchallenged chickens, isolators 2 and 3 held 21 chickens each, challenged with MPF57 and MPF23 respectively with doses of 500, 2000 or 8000 pfu administered to 7 birds in each. Chickens were individually identified by wing tag and toe mark. The chickens were free from maternal antibody to MDV and were unvaccinated. The B-haplotype of the chicken major histocompatibility complex of these chickens is unknown, but in other experiments of ours they have exhibited some resistance to the effects of MDV challenge so likely comprise a mixture.

The challenge viruses were grown and titrated at UNE in chick embryo fibroblasts (CEF). Batch numbers were MPF23\_P4\_021209 and MPF57\_P7\_040810 respectively. CEF were inoculated with splenocytes from SPF chickens challenged with low passage virus and the isolates titrated for pfu after passage 4 and 7 respectively. Infective MPF23 material was kindly provided by Prof. Greg Tannock of RMIT University and this was the first time MP23 has been grown successfully in cell culture since the first report of McKimm-Breschkin et al. (1990).

Clinical monitoring of individual chickens to enable classification of chickens into the 5 clinical patterns described by Gimeno et al. (1999) commenced at 5 (dpc). Birds were scored 0-3 for severity of paralysis of the neck, paralysis of limbs, ataxia, torticollis and nervous tics. These were summed to provide an overall clinical score. All dead and euthanized birds were necropsied and examined for gross MD lesions including lymphomas, nerve enlargement and thymic and bursal atrophy with birds exhibiting these classified as MD positive.

Mortality patterns were investigated using survival analysis (Kaplan-Meier product-limit method) and the chi square test of independence. The latter was also used to compare the ratio of MD positive and negative chickens between groups. Treatment effects on liveweight and immune organ weights at 56 dpc were investigated by ANOVA. Analyses were performed with JMP 9 (SAS Institute, USA). A statistical significance level of  $P < 0.05$  is used throughout.

## III. RESULTS

No neurological signs were observed in the control chickens. In the challenged groups signs began at 5 dpc. These typically commenced with depression or unwillingness to move followed by leg dragging/knuckling over, drooping of wings, mild opisthotonos and then paralysis. Clinical signs then ceased at day 17, re-commencing at day 25 or 41 depending on the challenge virus (Figure 1). Paralysis during the later episodes typically involved chickens having one leg stretched forward and the other backward, together with flaccid paralysis or paresis of necks and wings. Torticollis was only induced by MPF57. There was no significant

effect of virus or dose rate on the proportion of chicks in the neurological classifications of Gimeno et al. (1999) up to 23 dpc. Overall 1 chick (2.4%) showed acute transient paralysis (ATP) leading to death, 45% showed classical transient paralysis (CTP) with signs of 4 days duration or less, 29% showed persistent neurologic disease (PND) with neurological signs over a span of more than 4 days and 23% exhibited no clinical signs (N) in this period. No chickens exhibited late paralysis (LP) in the 23-day period of Gimeno et al., (1999) but the percentage of chickens in the other categories that went on to show later neurological signs were 0, 84, 75 and 50 % respectively ( $P = 0.1$ ).

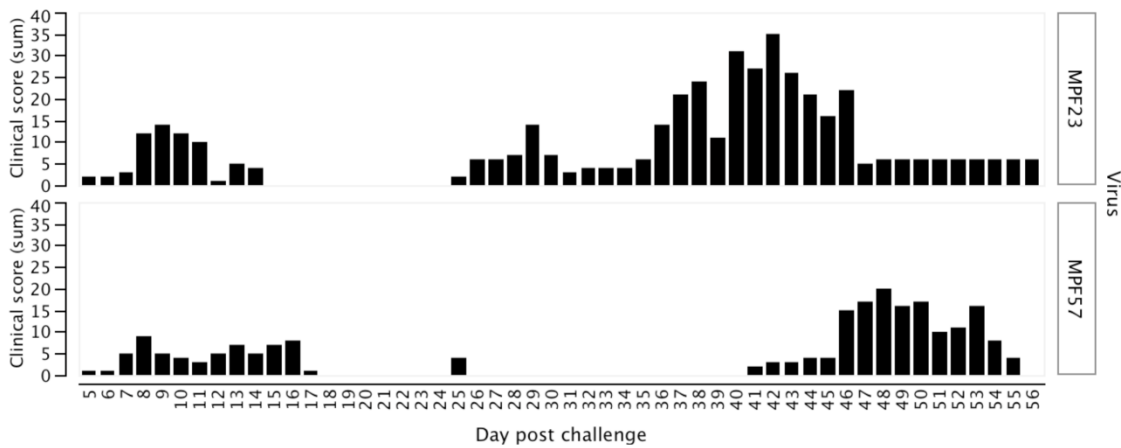


Figure 1 - Sum of daily clinical scores by challenge virus and day post challenge

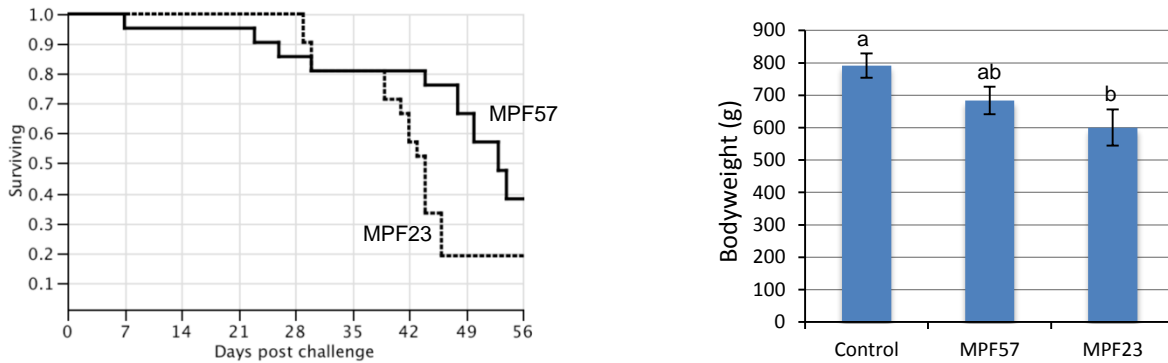


Figure 2 - Effect of challenge virus on chicken survival (Left,  $P < 0.05$ ) and on bodyweight of surviving chickens at 56 dpc (Right,  $P < 0.05$ ). No control chickens died during the experiment.

There was no mortality in the control group. The first death was at 7 dpc followed by a delay to the second death at 23 dpc and there was a significant difference in the survival curves with MPF23-challenged chickens showing a greater and more rapid late mortality rate ( $P = 0.03$ ; Figure 2). Overall mortality rate was higher in the MPF23 group (81%) than the MPF57 group (62%) but the difference was not significant ( $P = 0.17$ ; Table 1). There was a significant overall effect of challenge dose with significantly lower mortality rate in chickens challenged with 500 pfu (43%) than 2000 (86%) or 8000 pfu (86%) ( $P = 0.02$ ; Table 2). MD lesions were observed in 95% of birds in the MPF23 group compared with 76% in the MPF57 group ( $P = 0.08$ ), with no effect of dose (Table 1). At 56 dpc there were significant ( $P < 0.05$ ) effects of MDV challenge on bodyweight (Figure 2) and relative splenic but not bursal weights (data not shown).



**Table 1 - Mortality rate and incidence of MD by day 56 dpc by virus treatment and dose**

Treatment	Overall treatment effect		Challenge dose (pfu)	Effect of dose	
	Mortality	MD		Mortality	MD
MPF57	13/21 (62%)	16/21 (76%)	500	3/7 (43%)	4/7 (57%)
			2000	5/7 (71%)	7/7 (100%)
			8000	5/7 (71%)	5/7 (71%)
MPF23	17/21 (81%)	20/21 (95%)	500	3/7 (43%)	6/7 (86%)
			2000	7/7 (100%)	7/7 (100%)
			8000	7/7 (100%)	7/7 (100%)
Control	0/10 (0%)	0/10 (0%)			

#### IV. DISCUSSION

MPF23 was clearly the more pathogenic of the two viruses as assessed by mortality and induction of MD. This difference was also clearly manifested in the clinical pattern observed (Figure 1). However this difference was only evident after 25 dpc with the MPF23 group showing earlier, more sustained and more severe clinical signs in the period 26-56 dpc. This lies outside the 0-23 dpc window used by Gimeno et al. (1999; 2002) with no difference between the two groups evident during this period. The observed pattern during this period classifies both viruses in neuropathotype B consistent with a vv pathotype under the ADOL system. Renz et al. (2012) found that MPF57 was highly pathogenic in unvaccinated SPF chickens (27% mortality, 84% MD after a 500 pfu challenge) as in the present experiment, but HVT-vaccination provided 100% protection resulting in a pathotype classification of v rather than vv. The results of the present experiment indicate that if the clinical observation period was extended, a more sensitive measure of virulence may be obtained. However the practicalities of such prolonged observation and the confounding of neurological signs with the onset of MD lymphomas limit this approach.

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## THE GLOBAL AVIAN INFLUENZA SITUATION AND ASSESSMENT OF EFFECTIVE CONTROL METHODS

D.E. SWAYNE<sup>1</sup>

### Summary

The H5N1 high pathogenicity avian influenza (HPAI) virus emerged in China during 1996 and has spread to infect poultry and/or wild birds in 62 countries during the past 15 years. For 2011-2012, 19 countries reported outbreaks of H5N1 in domestic poultry, wild birds or both. The majority of the outbreaks occurred in Indonesia, Egypt, Vietnam, and Bangladesh, in decreasing order. The majority of the cases were H5N1 HPAI but outbreaks of H5N2 occurred in Chinese Taipei (chickens) and South Africa (ostriches), and an outbreak of H7N3 HPAI in Mexico (egg-type chickens). Field outbreaks of H5N1 HPAI have occurred in vaccinated flocks from both failure of the vaccines (i.e. vaccine efficacy) and failure in administration or immune response of the target species (i.e. vaccination effectiveness). Antigenic drift in field viruses has resulted in failure of protection by classic H5 vaccines strains in Mexico, China, Egypt, Indonesia, Hong Kong and Vietnam. This challenge has been met by developing new vaccine strains that provide protection against ever changing HPAI viruses.

A comprehensive review of AI control methods has been completed. From 2002-2010, >113 billion doses of AI vaccine were used in poultry in 15 countries. The majority of vaccine (>91%) was used in China while significant amounts were used in Egypt, Indonesia, and Vietnam. Implementation of vaccination in these four countries occurred after H5N1 HPAI became endemic in domestic poultry and vaccination did not result in the endemic infections. The other 11 countries used less than 1% of the vaccine. Inactivated AI vaccines accounted for 95.5% and live recombinant virus vaccines for 4.5% of vaccine used. Clinical disease and mortality were prevented in chickens, and rural livelihoods and food security were maintained by using vaccines during HPAI outbreaks. Fewer outbreaks of Low pathogenicity notifiable avian influenza (LPNAI) have been reported than HPAI and only six countries used vaccine in control programs which accounted for 8.1% of the total H5/H7 AI vaccine usage. Stamping-out without vaccination has been the preferred method for HPAI control and eradication used successfully in 27 HPAI epizootics.

### I. INTRODUCTION

OFFLU is the joint World Organization for Animal Health and Food and Agricultural Organization (OIE-FAO) global network of expertise on animal influenzas. OFFLU aims to reduce negative impacts of animal influenza viruses by promoting effective collaboration between animal health experts. OFFLU puts a strong emphasis on the importance of analyzing and sharing information, and biological material to identify and reduce health threats early. An OFFLU Global Avian Influenza Vaccine and Vaccination research project seek to define the conditions where AI vaccines can assist in control and eradication of H5N1 HPAI in Asia and Africa.

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Outbreaks of transboundary animal disease in any one country are a threat to the whole international community, as was demonstrated by the speed with which H5N1 HPAI spread across three continents affecting over sixty countries. Early detection through implementation of effective surveillance systems and immediate notification of an outbreak of HPAI to OIE is essential so that other countries can take science- based measures – laid out in the OIE Terrestrial Animal Health Code - to prevent the further spread of disease through movements of poultry and poultry products. By strengthening the capacity of National Veterinary Services to detect outbreaks early and control them quickly, benefits are provided for the whole international community now and for generations to come. In this context, the OIE promotes Veterinary Services as global public goods.

Avian influenza (AI) viruses are a diverse group divided into 144 different subtypes based on different combinations of the 16 hemagglutinin and 9 neuraminidase subtypes, and two different pathotypes (low [LP] and high pathogenicity [HP]). Twenty-nine epidemics of high pathogenicity avian influenza (HPAI) have occurred in the world since 1959. The largest of these outbreaks has been the H5N1 HPAI which has caused problems in poultry and wild birds in 63 countries in Asia, Europe and Africa since 1996. This lineage of viruses has crossed multiple species barriers to infect captive and wild birds, carnivorous mammals and humans. Human infections have been associated with direct or indirect contact with live or dead poultry while in carnivores, consumption of infected birds or their products have been associated with infections. Experimental models have suggested airborne transmission of the H5N1 HPAI virus from simulated slaughter of subclinically infected chickens or through feeding infected meat. However, the required dose to produce infection is much lower with aerosol exposure than consumption of infected meat.

## II. SCIENTIFIC ISSUES

### a) Update on High Pathogenicity Avian Influenza 2011 and 2012

Since 1959, there have been 32 HPAI epizootics in poultry (Table 1). The largest of the HPAI epizootics is the H5N1 epizootic that began in Guangdong China in 1996 and has since spread to affect poultry and wild birds in 63 countries. The period assessed is 1 January 2011 until 31 December 2012. Sources used were WAHID (OIE) for the epidemiological data and OIE Avian Influenza Reference Laboratories for sequence information and phylogenetic analysis. For this report, enzootic countries are: 1) those self-declared enzootic by the country (Egypt and Indonesia), 2) continue to report occurrences of outbreaks over multiple years (Vietnam and Bangladesh), 3) or have published data in the literature of continuous reports of infection and molecular evidence of virus persisting in country (China and eastern India).

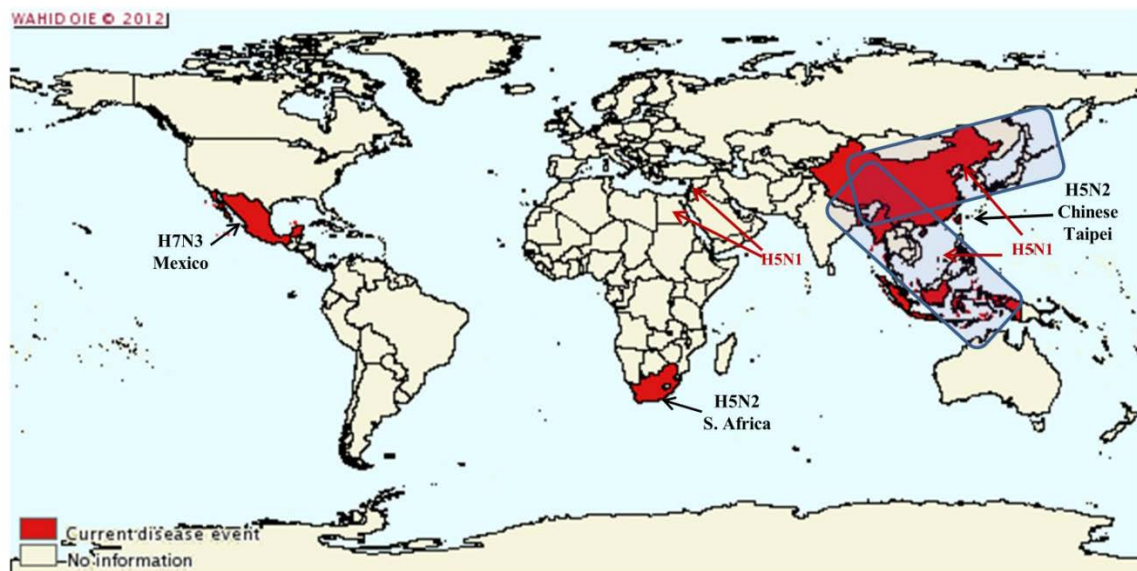
For 2011-2012 (through June 2012), 19 countries reported outbreaks of HPAI domestic poultry: 16 with H5N1 (Bangladesh, Bhutan, Cambodia, China, Egypt, India, Indonesia, Iran, Israel, Japan, South Korea, Mongolia, Myanmar, Nepal, Palestine Territories, and Vietnam); two with H5N2 (South Africa and Chinese Taipei) and one with H7N3 (Mexico) (Figure 1).

**Table 1 - 32 HPAI epizootics since 1959. All used stamping-out programs in control**

1. 1959: Scotland, H5N1	17. 1997: Italy, H5N2
2. 1961: S. Africa, H5N3	*§18. 1996-2012: Eurasia/Africa, H5N1
3. 1963: England, H7N3	19. 1999-2000: Italy, H7N1
4. 1966: Canada, H5N9	20. 2002: Chile, H7N3
5. 1975: Australia, H7N7	21. 2003: Netherlands, H7N7
6. 1979: Germany, H7N7	22. 2004: USA, H5N2
7. 1979: England, H7N7	23. 2004: Canada, H7N3
8. 1983-84: USA, H5N2	24. 2004, 2006: S. Africa, H5N2 (ostriches)
9. 1983: Ireland, H5N8	§25. 2005: N. Korea, H7N7
10. 1985: Australia, H7N7.	26. 2007: Canada, H7N3
11. 1991: England, H5N1	27. 2008: England, H7N7
12. 1992: Australia, H7N3	28. 2009: Spain, H7N7
13. 1994: Australia, H7N3	29. 2011: S. Africa, H5N2 (Ostriches)
§14. 1994-95: Mexico, H5N2	30. 2012: Chinese Taipei, H5N2
§15. 1995 & 2004: Pakistan, H7N3	§ 31. 2012: Mexico, H7N3
16. 1997: Australia, H7N4	32. 2012: Australia, H7N7

§ = vaccination also used in HPAI control, \* largest outbreak in last 50 years.

## HPAI (2011-2012): 19 countries



### H5N1 HPAI

16 countries – poultry, wild birds, humans

### H5N2 HPAI

S. Africa – ostriches  
Chinese Taipei - poultry

### H7N3 HPAI

Mexico - layers

Figure 1 - HPAI outbreaks in 2011-2012.

In 2011, there were five epicenters of H5N1 HPAI: 1) Egypt and Middle East (Israel and Palestinian Authority) with clade 2.2.1; 2) Ganges Delta (India, Bhutan, Nepal and Bangladesh) with clades 2.3.2.1 and 2.2.2; 3) Mekong Delta (south Vietnam and Cambodia) with clade 1; 4) Indonesia with clade 2.1.3; and 5) east to southeast Asia (China, northern Vietnam, Japan, Republic of Korea, Myanmar, Mongolia, and Iran) with clade 2.3.2.1.

For 2012, reports of H5N1 viruses continued in Africa, Middle East and Asia in poultry and wild birds: 1) subclade 2.3.2.1, most frequently reported with wide geographic

dispersion including northern and central Vietnam, eastern India, Bangladesh, China, Hong Kong, India, Nepal, and Bhutan); 2) subclade 2.2.1 viruses in Egypt and Israel; 3) subclade 7.2 in northern China; 4) subclade 2.1.3.2 in Indonesia; and 5) subclade 1.1 in southern Vietnam and Cambodia. Human infections were reported with clades 2.3.2.1 (Bangladesh, Hong Kong,), 2.2.1 (Egypt), 2.1.3.2 (Indonesia) and 1.1 (Vietnam and Cambodia).

Three HPAI outbreaks have involved subtypes other than H5N1. An outbreak of H5N2 HPAI began in 2011 in South Africa, affecting only ostriches. The initial cases were serologically positive but lacked clinical disease. Later, virus was identified by H5 reverse transcriptase polymerase chain reaction, and a few clinical signs appeared but without high mortality. In total, 47 outbreaks have occurred, affecting 51,518 ostriches resulting in 13,991 cases with 1178 birds being destroyed and 39,812 handled via controlled slaughter.

A second, unrelated outbreak of H5N2 HPAI occurred in Chinese Taipei with first report of mortality on 27 February 2012 on a broiler breeder farm which accumulated to 16.6% mortality rate at the time of depopulation. Additional outbreaks occurred in three chicken broiler farms and one layer farm. In total, five outbreaks occurred, affecting 46,320 chickens in 8,147 cases, resulting in 5,497 dead and 40,823 culled chickens. The H5N2 HPAI virus was closely related to H5N2 North American AIV. An outbreak of H5N2 low pathogenicity avian influenza (LPAI) virus was reported in 21 October 2008 in Hsin-Chu with the most recent case on 20 November 2011. The H5N2 HPAI virus was derived from this H5N2 LPAI progenitor lineage. The HPAI outbreak was resolved on 7 August 2012.

An H7N3 HPAI outbreak occurred in central Mexico in the state of Jalisco. The outbreak was diagnosed on 21 June 2012; in total 44 farms were affected with 1,016,844 chickens dead and 10,251,595 poultry were culled. The outbreak involved only layers and layer breeders in the commercial sector. The incidence rate was 25%, mortality rate 9.6% and fatality rate was 39.2%. An emergency vaccination program was initiated with 128m doses used by mid-October. Surveillance in the region has involved 64,498 samples from 537 premises with 44 farms having H7N3 isolations. There were no H7N3 HPAI viruses identified in commercial broilers or village poultry within the control and surveillance zones. Initially, farmers thought the high mortality was a return of H5N2 LPAIV or Fowl Cholera.

#### b) H5N1 Field Viruses Resistant to Classical H5 Vaccine Seed Strains

Vaccines and vaccination have emerged during the past two decades as essential tools for controlling AI in poultry because they increase resistance to infection, prevent illness and death, reduce virus replication and shedding from respiratory and alimentary tracts, and reduce virus transmission to birds and mammals, including humans (Swayne & Kapczynski, 2008b). However, as H5N1 viruses have become entrenched and outbreaks prolonged, field outbreaks have been reported in flocks that are well vaccinated with early classical H5 AI vaccines in Central America, China, Egypt, Hong Kong, Indonesia and Vietnam (Abdelwhab *et al.*, 2011; Grund *et al.*, 2011; Swayne & Kapczynski, 2008a). These failures can be the result of failure of the vaccines (i.e. vaccine efficacy) or failure in administration or immune response of the target species (i.e. vaccination effectiveness).

Vaccination Effectiveness. Lack of adequate protection in the poultry population in the field has been associated with a variety of application and related issues including: attempting to vaccinate all poultry in the national herd, improper vaccination technique; trying to get field protection from a single vaccination; maternal and active immunity interference; immunosuppressive population; improper storage & handling of vaccines; administration of reduced vaccine dose; high environmental exposure to virus; farmer resistance to vaccination of domestic ducks; high population turnover rate in poultry; logistics problems with administration; and vaccination “burn-out.” For example, attempts to

vaccinate village poultry in Indonesia and Egypt have only resulted in 20-40% and 20% vaccine coverage rate, respectively (Government of Egypt, 2010; Mariner, 2011).

Vaccine Efficacy. Low antigenic mass in H5 AI vaccines is a less common problem today than it was 10 years ago. The market place has demanded high potency vaccines and most manufacturers have provided such to stay in business. However, in some regions, antigenic drift of the field viruses has occurred such that older classic H5 vaccine seed strains have lost efficacy and continual evaluation of vaccine seed strains against field viruses is needed to maintain relevant protective vaccine seed strains.

As early as 1998, H5N2 LPAI field viruses were identified in Mexico and Guatemala that had escaped immunity induced by the 1994 Mexican H5N2 vaccine seed strain (Lee *et al.*, 2004). Subsequently, study of inactivated vaccines obtained from the field identified six which used the 1995 original Mexican seed strain and three that utilized genetically recent H5N2 seed strains (Eggert *et al.*, 2010). The latter three vaccine strains, along with the recombinant fowl poxvirus vaccine with A/turkey/Ireland/83 hemagglutinin gene insert, provided protection while the 1994 Mexican vaccine strain did not prevent replication and shedding of the field viruses in the infected vaccinated poultry.

In research on H5 AI vaccines used against H5N1 HPAI outbreaks in Asia and Africa, emerged H5N1 field viruses were examined by genetic sequencing and analysis, and antigenic cartography. Three H5N1 HPAI field viruses from Indonesia (A/chicken/West Java/PWT-WIJ/2006 [PWT/06], A/chicken/West Java/SMIHAMD/2006 [HAMD/06] and A/chicken/Papua/TAS/2006 [Papua/06]) were selected, because of genetic diversity, for intranasal challenge of chickens vaccinated with different classic H5 inactivated vaccine seed strains including Mexico/94, England/73, Legok/03. The results are as follows:

- All vaccines resulted in antibody production but the titers varied with the individual vaccines and seed strains. The lowest average hemagglutination inhibition (HI) titers per group (24-64 geometric mean titer [GMT]), when using homologous vaccine strain as HI antigen, were from the local Legok/03 vaccine strain and the highest average titers per group (294-955 GMT) were produced using the Mexican H5N2 vaccine. When using the challenge virus as HI antigen, only the Legok/03 vaccine groups had consistent average HI titers (5 and 8, PWT/06; 16, Papua/06; 26, HAMD/06 GMT) while other H5 vaccines lacked titers to PWT/06 and had lower titers to HAMD/06 and Papua/06 than respective vaccine seed strains.
- Most vaccines protected chickens against HAMD/06, some protected against Papua/06 but many vaccines did not protect against PWT/06 challenge
- Experimental vaccine made from inactivated PWT/06 HPAI virus ( $\beta$ -Propiolactone inactivated water-in-oil emulsified vaccines [generic emulsion]) produced HI antibody titers of GMT >100 in SPF chickens and 80-100% of birds were protected following challenge with PWT/06, Papua/06 and HAMD/06
- Inactivated PWT experimental vaccine strain performed best against PWT challenge along with the recombinant fowl poxvirus with AI virus H5 insert vaccine (rFPV) + inactivated Legok/03 prime-boost regime
- A reverse genetic generated virus was produced using the HA of PWT/06 and the remaining 7 gene segments from PR8 vaccine strain. When used in an inactivated vaccine, 90% of vaccinated chickens survived lethal challenge, and when oropharyngeal swabs were examined at 2 days post-challenge, significantly few vaccinated chickens shed virus and the titers were significantly lower when compared to sham vaccinated chickens that were challenged.

An antigenic drift variant H5N1 HPAI virus (PWT/06) has been identified in Indonesia that was not protected against by classic H5 AI vaccines. Usage of vaccines constructed from

the parent HPAI virus or reverse genetic strain using hemagglutinin of PWT/06 protected chickens from lethal challenge.

In December 2008, Hong Kong had an outbreak of severe clinical disease in a flock of broilers that had been vaccinated with 1995 Mexican H5N2 vaccine. In experimental studies, the 1994 Mexican vaccine strain did not protect against this 2.3.4 lineage of H5N1 HPAI field virus. China has successively changed its H5 vaccine seed strains as genetic and antigenic variant field viruses appeared, first using the A/turkey/England/73 H5N2 seed strains, then to a reverse genetic (rg) strains of A/goose/Guangdong/1996 virus (RE-1) and then to an rg strain A/chicken/Shanxi/2/2006 and most recently to rg strain A/duck/Anhui/1/2006 (Chen, 2009). Egypt has identified a few field viruses from chickens on well-vaccinated commercial farms that are resistant to 1994 Mexican and 1973 England vaccine seed strains.

#### c) OFFLU Vaccine and Vaccination Project

Since 2003, the H5N1 HPAI virus has spread from China to affect poultry and/or wild birds in 62 additional countries. Most countries have focused control strategies in poultry on stamping-out programs which have been successful in many countries. In addition, vaccination was added to the control programs in some countries when stamping-out alone was not effective in achieving eradication or to prevent infections in domestic poultry. Based on detail to World Organization for Animal Health (OIE), Dr Swayne has conducted a comprehensive review of control methods used in HPAI outbreaks, focusing especially on vaccines and vaccination (Pavade *et al.*, 2011; Swayne *et al.*, 2011).

There are variations between countries' responses to AI outbreak situations based on their economic status, diagnostic capacity and other factors (Pavade *et al.*, 2011). The objective of this study was to ascertain the significant association between HPAI control data and a country's poultry density, the performance of its Veterinary Services, and its economic indicators (gross domestic product, agriculture gross domestic product, gross national income, human development index and Organisation for Economic Co-operation and Development [OECD] status). Results indicate that, as poultry density increases for least developed countries, there is an increase in the number and duration of HPAI outbreaks and in the time it takes to eradicate the disease. There was no significant correlation between HPAI control and any of the economic indicators except membership of the OECD. Member Countries, i.e. those with high-income economies, transparency and good governance, had shorter and significantly fewer HPAI outbreaks, quicker eradication times, lower mortality rates and higher culling rates than non-OECD countries. Furthermore, countries that had effective and efficient Veterinary Services (as measured by the ratings they achieved when they were assessed using the OIE Tool for the Evaluation of Performance of Veterinary Services) had better HPAI control measures.

During the 2002 to 2010 period, more than 113 billion doses of AI vaccine were used in at-risk national poultry populations of over 131 billion birds (Swayne *et al.*, 2011). At two to three doses per bird for the 15 vaccinating countries, the average national vaccination coverage rate was 41.9% and the global AI vaccine coverage rate was 10.9% for all poultry. The highest national coverage rate was nearly 100% for poultry in Hong Kong and the lowest national coverage was less than 0.01% for poultry in Israel and the Netherlands. Inactivated AI vaccines accounted for 95.5% and live recombinant virus vaccines for 4.5% of the vaccines used. Most of these vaccines were used in the H5N1 HPAI panzootic, with more than 99% employed in the People's Republic of China, Egypt, Indonesia and Vietnam. Implementation of vaccination in these four countries occurred after H5N1 HPAI became enzootic in domestic poultry and vaccination did not result in the enzootic infections. Vaccine usage prevented clinical disease and mortality in chickens, and maintained rural livelihoods

and food security during HPAI outbreaks. Low-pathogenicity notifiable avian influenza (LPNAI) became reportable to the World Organisation for Animal Health (OIE) in 2006 because some H5 and H7 low-pathogenicity avian influenza (LPAI) viruses have the potential to mutate to HPAI viruses. Fewer outbreaks of LPNAI have been reported than of HPAI and only six countries used vaccine in control programmes, accounting for 8.1% of the total H5/H7 AI vaccine usage, as compared to 91.9% of the vaccine used against HPAI. Six countries have used vaccine to control LPNAI, with the majority being used in Mexico, Guatemala, El Salvador and Italy. In countries with enzootic HPAI and LPNAI, development and implementation of exit strategies has been difficult.

#### d) Future

To improve the success of HPAI and LPNAI control and eradication, several aspects have been identified as having highest impact:

1. Move from mass vaccination campaigns to targeted vaccination of the reservoir species or species that have mostly asymptomatic infection. This concentrates resources to the critical control points;
2. Improve vaccines so that they can be administered easily to the poultry population such as by aerosols or water vaccination systems;
3. Develop recombinant vaccines for ducks and geese that will protect against significant disease such as duck viral enteritis, and have an H5 gene insert that will protect from H5 HPAI;
4. Market and production systems need to be upgraded to improve biosecurity, encourage one-way movement of live birds to markets, and reward production and distribution systems that are AI-free;
5. Develop realistic exit strategies for vaccination that are based on risk models and current production systems in country.

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RAPID *IN VITRO* ANTIMICROBIAL SCREENING ASSAY AGAINST  
*CAMPYLOBACTER*.

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The antimicrobial activity of some plant compounds and extracts could be used to combat *Campylobacter* colonisation of poultry. Research was undertaken to adapt a multi-well antimicrobial assay (Sultanbawa et al., 2009) as a fast and reliable method to screen large numbers of plant extract treatments against multiple *Campylobacter* strains.

*Campylobacter* cultures isolated from chicken faecal droppings were used to trial and validate the assay. The strains were recovered by growing in 7% sheep blood agar at 42°C for 24 hours in 5% CO<sub>2</sub>. Cultures were then placed in nutrient broth N° 2 (Oxoid CM0067) enriched with *Campylobacter* growth supplement (Oxoid SR0232E). An inoculum of 10<sup>4</sup> cfu/ml was obtained by dilution in the broth and measured by Optical Density (OD). Samples of 10 essential oils, 6 phytochemicals, 2 plant by-product extracts and 5 organic acids were evaluated for activity against all the *Campylobacter* strains. Essential oils were diluted with agar and the rest of compounds in sterile water.

The screening assay was carried out using flat bottom 96-well sterile microtiter plates. Each plate had rows of wells containing the treatments and their replicates with the first and last row containing sterile culture media and bacteria culture tested as negative and positive controls respectively. OD of the wells was determined at 595 nm prior to incubation for 48 hours. The growth/inhibition value was calculated by the following formula: % Inhibition =  $(1 - (OD_{T_{48}} - OD_{T_0}) / (OD_{C_{48}} - OD_{C_0})) \times 100$ , where T is the treatment and C the positive control. The minimum inhibitory concentration (MIC) was considered the concentration of the compound tested that first gave 100% inhibition of the growth of the bacteria.

The MIC of *Campylobacter* by treatments was relatively independent of the strain. Oregano, thyme and their purified constituents carvacrol and thymol showed the strongest inhibition with the MIC > 0.0004% w/w. Cinnamon, cinnamaldehyde and anise myrtle had MIC values between 0.001% and 0.004%. The rest of the essential oils and phytochemicals tested (lime, sage, lemon, garlic, tea tree oil, eugenol, citral, and vanillin) all had MIC > 0.01% w/w. The MIC of the sorghum extract was > 5% while olive leaf extract showed no antimicrobial activity. Of the organic acids citric, lactic and benzoic gave MIC values between 0.03% and 0.06%, while ascorbic and caprylic acids were higher than 0.25%. The results obtained with this new screening method were in agreement with existing literature (Kollanoor et al., 2010) validating the assay. Future research will survey more strains with combinations of extracts and acids to find MIC compositions that can be achieved *in vivo* in the chicken intestinal tract and caecum.

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## EGG QUALITY AND FOOD SAFETY OF TABLE EGGS

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This study is an update of data presented previously (Roberts & Chousalkar, 2012) from a study which investigated egg quality at different stages of lay. Although eggs produced in Australia are considered medium to low risk for food borne illness, the egg industry in Australia is periodically implicated in cases of food poisoning. Egg shell defects may potentiate the movement of bacteria into the egg. Two egg shell characteristics were targeted: the extent of cuticle cover and the incidence of translucency. Eggs were collected from commercial caged layer flocks at different stages of lay: early (<25-40 wks), mid (40-55 wks), late (55-65 wks) and very late (>65 wks). Eggs were candled and scored for translucency. Cuticle cover was estimated using MST cuticle stain and a Konica Minolta hand-held spectrophotometer (L\*a\*b\* colour scale). Traditional measures of egg quality were determined using specialised equipment (TSS, U.K.) Shell ultrastructural features were scored following plasma ashing of shell samples and viewing under a benchtop scanning electron microscope. Translucency score increased in late lay and then decreased in the very late flocks, which had been moulted (Table 1). As flock age increased, egg shell quality and egg internal quality decreased although quality was improved to some extent in late lay flocks following an induced moult. Cuticle cover, as measured by a\* or the single score (Leleu et al., 2011) was not significantly affected by flock age although the difference between before and after staining,  $\Delta a^*$ , was highest for the mid and late lay flocks indicating a trend to better cuticle cover at these ages. The incidence of unfavourable shell ultrastructural features was higher for older flocks and the incidence of favourable ultrastructural features was lower. The differences in mammillary layer characteristics among flock ages is consistent with the findings of previous researchers (Brackpool, 1995; Solomon, 1991) and with the traditional measures of shell quality. The limited number of correlations between egg shell translucency scores and the scores obtained from examination of the mammillary layer of the egg shells suggest that the ultrastructure scoring system only partly accounts for the phenomenon of shell translucency. Cuticle blue dye was a reliable indicator of the presence of cuticle.

Table 1 - Egg Quality Measurements

Measurement	Early Lay	Mid Lay	Late Lay	V Late Lay	P Value
Translucency score	<sup>b</sup> 2.52	<sup>b</sup> 2.59	<sup>a</sup> 3.07	<sup>b</sup> 2.53	<0.0001
Shell reflectivity (%)	<sup>c</sup> 28.0	<sup>b</sup> 30.5	<sup>a</sup> 31.6	<sup>a</sup> 32.1	<0.0001
a*	1.861	0.876	1.007	1.400	NS
$\Delta a^*$	<sup>ab</sup> 14.02	<sup>a</sup> 15.04	<sup>a</sup> 14.94	<sup>b</sup> 13.31	0.0060
Single Colour Score	16.10	16.53	16.55	15.73	NS

Values are Mean  $\pm$  SEM. Values across a row with different superscripts are significantly different. NS is not statistically significant.

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EFFECT OF WIDELY DIVERGENT VACCINATION-CHALLENGE INTERVALS ON PROTECTION PROVIDED BY RISPENS CVI988 VACCINE AGAINST VERY VIRULENT MAREK'S DISEASE VIRUS CHALLENGE IN ISA BROWN CHICKENS

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Summary

Rispens CVI988 is a highly effective serotype 1 Marek's disease virus vaccine currently used to vaccinate layer and breeder chickens in Australia and worldwide. We tested the effects of Rispens CVI988 on the level of protection provided to commercial ISA Brown layers following challenge with very virulent Marek's disease virus (vvMDV) isolate 02LAR at various intervals before and after vaccination. Protective index was measured for vaccination challenge intervals of 0, 5, 10, -5 and -10 days with the negative values indicating challenge prior to vaccination. All treatments were tested in duplicate with 25 ISA Brown chickens per isolator and appropriate controls. Chickens were challenged by injection with 400 pfu of MDV and/or vaccinated with 3200 pfu of the Rispens vaccine virus subcutaneously, and the presence of gross MD tumours was assessed up to 56 days post challenge (dpc). MDV challenge in unvaccinated chickens resulted in tumours in 52% of chickens. Rispens vaccine provided no significant protection when the birds were challenged prior to vaccination, i.e. at VCI -5 (-4%) and -10 (21%). Rispens CVI988 provided 85% and 100% protection at VCI 5 and 10 respectively, Interestingly it provided significant protection (60%) when challenge and vaccination occurred simultaneously.

I. INTRODUCTION

Rispens CVI988 is a live attenuated serotype 1 Marek's disease virus (MDV-1) which is widely considered to be the most efficacious vaccine used worldwide at present against Marek's disease (MD) (Baigent et al. 2006; Witter et al. 1995). For this it is used routinely to vaccinate layer and breeder chickens against Marek's disease in Australia. Although Rispens vaccine is known to provide excellent protection against MD, it is unknown to what extent this protection is influenced by the vaccination to challenge interval (VCI). As part of a wider investigation into interaction between vaccinal and pathogenic MDV-1 viruses administered at different times relative to each other, we investigated the effect of VCI on protection provided by Rispens vaccine against MD. The pathogenic MDV-1 challenge isolate used (02LAR) has been previously classified as very virulent MDV-1 (vvMDV, Walkden-Brown et al. 2007) The experiment was designed to test the following hypotheses:

*1. Vaccination after MDV challenge will not provide significant protection; 2. Rispens vaccination at or after challenge will provide significant protection and the protection will be positively correlated with VCI.*

II. MATERIALS AND METHODS

The complete experiment utilised 600 commercial female ISA Brown (year of hatch-2010) from parents vaccinated with Rispens, and thus positive for maternal antibody directed against MDV (mab+). They were placed in 24 positive pressure isolators with treatments as shown in Table 1. There were 25 chickens/isolator placed initially in each isolator, with 2 isolators for each treatment. The experiment commenced on the day of hatch (day 0) and was

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terminated at 56 days post-challenge, giving different termination dates for the different treatments. Chickens were vaccinated or challenged by sc injection in 200 µl diluent on days 0, 5 or 10. Unvaccinated or unchallenged chickens received diluent only.

The factors in the design were: MDV challenge 02LAR @ 400pfu/bird; vaccination Rispens CVI988 @ 3200pfu/bird (Vaxsafe® RIS Vaccine, Bioproperties, Ringwood, Vic); and Vaccine-challenge interval (VCI) of -10, -5, 0, 5 or 10 days (Table 1).

**Table 1 - Treatment groups, vaccination and challenge details. Each treatment used two isolators containing 25 chickens initially.**

No	Treatment	Vaccination day	MDV challenge day	VCI (days)
1	MDV d0	-	0	-
2	MDV d5	-	5	-
3	MDV d10	-	10	-
4	RIS d0	0	-	-
5	RIS d5	5	-	-
6	RIS d10	10	-	-
7	VCI -10	10	0	-10
8	VCI -5	5	0	-5
9	VCI 0	0	0	0
10	VCI 5	0	5	5
11	VCI 10	0	10	10
12	Negative control	-	-	-

Ten chickens were removed from most treatments at day 14 (data not reported) providing a maximum of 40 chickens per treatment for the protection study. Diagnosis of MD was based on detection of gross tumours morphologically resembling lymphomas observed on the skin, eye muscles and internal organs on post-mortem examination of all dead and euthanised chickens. All MD lesions were scored 1-3 subjectively for severity based on the size and extent of the lesion. The protective index (PI) provided by vaccination at various VCI was calculated as:  $(\%MD \text{ in unvaccinated chickens} - \%MD \text{ in Rispens-vaccinated chickens}) \div (\%MD \text{ in unvaccinated chickens}) \times 100$  where %MD is the percentage of birds “at risk” of exhibiting MD lesions, in which lesions are present. The latter was taken as the population of chickens alive at the time the first gross MD lesion is detected (31 dpc).

Statistical analyses were performed with JMP10 (SAS Institute Inc. NC, USA). Analysis 1 tested the effect of VCI in those treatments with a VCI, while analysis 2 tested the effect of all 12 treatments. Discrete data were analysed using a generalized linear model with a binomial link function (logistic) fitting the effect VCI or treatment, and differences between different levels within main effects were tested by specific contrasts within the model. For MD incidence (% tumour positive) and PI data, which could only be measured on a whole isolator, data were analysed using a Standard Least Squares model fitting the effect of VCI for analysis 1. Significance of difference between levels within the main effects of both analyses were determined using Student’s t test protected by overall significance of the effect.

### III. RESULTS

The first MD tumours were detected at 31 dpc. Analysis 1 revealed significant effects of VCI ( $p < 0.0001$ ) for both mortality and mortality with MD lesions. Significantly more birds died in the VCI -5 treatment (55%) than in all other treatments. This was also the case for mortality with MD lesions (52.5%) (Table 2). Analysis 2 showed that overall treatment also

had a significant effect ( $p < 0.0001$ ) on both mortality and mortality with MD lesions. Unvaccinated birds challenged at different days showed higher mortality (27.5%, 22.5% and 37.5% after challenge at days 0, 5 and 10 respectively) than unchallenged vaccinated birds at different days (7.7%, 0% and 0% at day 0, 5 and 10 respectively) (Table 2). Mortality with MD lesions showed similar trends (Table 2). Of the 18 chickens that died or were euthanased without gross MD tumours, nine were non-starters/dehydrated, five had yolk sac infection and four were accidentally injured or killed (crushed behind feeder).

**Table 2 - Mortality, mortality with MD, incidence of MD to 56 dpc and protection index by VCI and treatment. Mortality is for eligible chickens from 2 dpc; MD incidence from 31 dpc.**

VCI	Treatment	Vacc	Chall	Total mortality (%)	Mortality with MD lesions (%)	MD incidence (%)	Protection Index (%)
	MDV d0	Unvacc	02LAR	11/40 (27.5)	8/40 (20)	21/37 (56.8) <sup>a</sup>	
	MDV d5	Unvacc	02LAR	9/40 (22.5)	8/40 (20)	17/39 (43.6) <sup>a</sup>	
	MDV d10	Unvacc	02LAR	15/40 (37.5)	11/40 (27.5)	22/38 (57.9) <sup>a</sup>	
	RIS d0	Risp	Unchall	3/39 (7.7)	0/39 (0)	0/39 (0) <sup>c</sup>	
	RIS d5	Risp	Unchall	0/50 (0)	0/50 (0)	0/50 (0) <sup>c</sup>	
	RIS d10	Risp	Unchall	0/48 (0)	0/48 (0)	0/49 (0) <sup>c</sup>	
	Neg Cont	Unvacc	Unchall	2/20 (10)	0/20 (0)	0/18 (0) <sup>c</sup>	
-10	VCI -10	Risp	02LAR	6/40 (15)	6/40 (15)	18/40 (45) <sup>a</sup>	20.9 <sup>c</sup>
-5	VCI -5	Risp	02LAR	22/40 (55)	21/40 (52.5)	23/39 (59) <sup>a</sup>	-3.6 <sup>c</sup>
0	VCI 0	Risp	02LAR	10/40 (25)	8/40 (20)	9/40 (23.1) <sup>b</sup>	60.4 <sup>b</sup>
5	VCI 5	Risp	02LAR	1/30 (3.3)	1/30 (3.3)	2/30 (6.7) <sup>bc</sup>	84.8 <sup>ab</sup>
10	VCI 10	Risp	02LAR	2/30 (6.7)	0/30 (0)	0/28 (0) <sup>c</sup>	100 <sup>a</sup>
	Total			81/457 (17.7)	63/457 (13.8)		

<sup>abc</sup>Means within columns not sharing a common letter in the superscript differ significantly.

The combined incidence of gross MD lesions in chickens that died with MD or had MD lesions on post mortem after euthanized at 56 dpc is summarized by treatment and VCI in Table 2. Overall, there was a significant effect of VCI on MD incidence ( $P = 0.001$ ) with a higher incidence in treatments VCI -10 (59%) and -5 (45%) than VCI 0 (23.1%), VCI 5 (6.7%) and VCI 10 (0%). VCI had a significant effect on PI ( $P = 0.002$ ) with VCI 0, 5 and 10 showed significantly higher protective indices (60.4%, 84.8% and 100% respectively) than VCI -5 (-3.6%) and -10 (20.9%) (Table 2). On an individual isolator basis there was a significant ( $p = 0.0008$ ) linear association between VCI and PI ( $PI = 52.27VCI + 4.93$ ;  $R^2 = 0.77$ ). Treatment also had an overall significant effect on MD incidence ( $p < 0.0001$ ). Unchallenged birds showed no MD while 52.6% challenged unvaccinated birds showed MD (Table 2).

#### IV. DISCUSSION

This study has demonstrated that VCI has a significant effect on the protective efficacy of the Rispens CVI988 vaccine with full protection observed for a VCI of 10 days. With regards to the first hypothesis this study demonstrated that vaccination with Rispens, 5 or 10 days after challenge with vvMDV provided no significant protection.

Our second hypothesis was that vaccination together with, or after, challenge with MDV would enhance the protection rate and that VCI would be positively associated with PI. This was clearly supported with PI of 60.4%, 84.8% and 100% at VCI of 0, 5 and 10 days respectively and a highly significant positive linear association between PI and VCI on an individual isolator basis. These data suggest that maximal protection is obtained by day 10 following vaccination. This is supported by the findings of Baigent et al., (2007) of uniformly

high PI (>90%) in mab- chickens vaccinated with Rispens CVI988 at 1 day of age and challenged with MDV at 14, 21 and 28 days post vaccination. The positive association between VCI and PI has also been reported in other studies. Islam et al., (2007) reported that vaccination of mab+ broilers with a range of HVT doses induced higher protection against MDV challenge at a VCI of 5 (mean PI of 79%) than a VCI of 2 (mean PI of 15%). Similarly Islam et al., (2008) reported PI of HVT vaccination against MDV challenge in mab+ broilers of 48, 69 and 77 % for VCI of 2, 4 and 7 days respectively. In a second experiment they reported PI of 66, 33, 53, 76 and 76 % for VCI of 0, 2, 4, 7 and 10 days respectively, concluding that for HVT, no improvement in protection is obtained beyond a VCI of 7 days. Interestingly the latter experiment also showed significant protection when the vaccine and challenge virus were co-administered (VCI = 0) an observation also seen in the present experiment. In all studies using mab+ chickens those with longer VCI are challenged later, and thus have lower levels of mab at challenge time than those with shorter or negative VCI. It is well known that mab slows the pathogenesis of MD (Chubb and Churchill 1969), presumably by slowing the MDV replication rate. It is therefore possible that part of the observed effect of VCI is mediated by a reduced mab inhibition of challenge virus.

The protection provided by the Rispens vaccine in the experiment is numerically greater than provided by HVT or HVT/MDV2 against the same challenge virus in the same chicken strain some years ago. Renz (2008) reported PI of 27.2 and 63.1 % for HVT and HVT/SB1 vaccines administered to ISA brown chicks at hatch, followed by challenge with 500 pfu of 02LAR at day 5 (VCI=5). This compares with a value of 85% for VCI 5.

In summary, we can conclude that a) Rispens provides increasing levels of protection with increasing VCI up to 10 days at which 100% protection was observed, b) Rispens provides no significant protection if chickens are challenged 5 or 10 days prior to vaccination c) Rispens appears to provide greater protection against vvMDV than that provided by HVT or HVT/MDV2.

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## FLY THE COOP! VERTICAL STRUCTURES INFLUENCE THE DISTRIBUTION AND BEHAVIOUR OF LAYING HENS IN AN OUTDOOR RANGE

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Free-range farms have increased greatly in the Australian egg industry, up by 64% over the last 5 years and representing 34% of the egg retail sales in 2011. Nonetheless, free-range systems offer particular challenges. The use of the outdoor range is variable among hens, with some hens never going outside in some systems. The hens' distribution is also usually not uniform across the range. Hens tend to stay close to the indoor shed or to features such as walls or fences. This causes issues in terms of loss of grass cover and increased stocking density in particular areas, which may contribute to feather pecking, land overstocking and parasite contamination. Several causes have been suggested for laying hens not utilising the outdoor range, such as genetics, experience, fear, or lack of cover. Yet, this practical issue remains unsolved.

We investigated the effect of implementing vertical structures in the range on the hens' number, distribution and behaviour. We hypothesized that providing a gradual reduction in the visual continuity of the vertical structures rather than an abrupt change from the shed to the open outdoor area could attract hens onto the range. This study was performed on one flock of 17000, 67 weeks-old, Hy-Line brown hens during summer. The hens had access to a 2.5 m wide winter garden and a 130 m × 60 m outdoor range as of 28 weeks of age from 1200 to 2200 h daily. The two treatments were presence or absence (control) of vertical structures using two matched-pairs design. The vertical structures were erected as two parallel, 1 m apart, fences that extended 17.4 m into the range. The first 5 m was made of heavy shade cloth ('Zone 1'), the next 5 m was moderate shade cloth ('Zone 2'), and the last 5 m was conventional chicken wire ('Zone 3'). Behavioural observations were carried out from day 11 to day 15 using video recordings. The number and distribution of hens was collected using instantaneous scan sampling at 30 min intervals from 1200 to 2200 h. The hen behaviour was collected at 5 min intervals in the periods 1200-1300 h, 1530-1630 h, and 1900-2000 h. Data were analysed using Proc MIXED in SAS with a model that included treatment, time of day, their interaction, and accounted as repeated measures over days.

The vertical structures in Zone 1 attracted hens, but this effect varied with time of day (treatment\*time:  $P < 0.001$ ) with the vertical structures attracting more hens from 1530 to 2000 h ( $P < 0.05$ ). For Zone 2, the vertical structures attracted more hens (treatment\*time:  $P < 0.001$ ) at 1230 h and from 1630 to 2030 h ( $P < 0.05$ ). For Zone 3, the vertical structures attracted more hens (treatment\*time:  $P = 0.02$ ) but only from 1800 to 1830 h ( $P < 0.05$ ). Hence, vertical structures could attract hens up to 18 m from the shed. The potential for longer or different types of structures to enhance the distribution of the hens further away and over greater areas requires additional research.

The hens were observed pecking at the structures for 40% of the time, suggesting that it stimulated the hens' interest. The shorter time spent in locomotion, lying and pecking at the ground (all  $P < 0.01$ ) around the vertical structures likely reflected this interest in pecking at the structure. Hens spent more time preening in the control treatment ( $P < 0.01$ ). Further research is needed to elucidate the reasons behind these behavioural changes.

The vertical structures proved successful at attracting the hens in a commercial setting, although replication on more than 1 flock is warranted. Elucidating which physical features fulfil the hens' biological needs could improve outdoor range use in free-range production systems.

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## COMPARATIVE BIOEFFICACY OF QUANTUM GLO™ IN EGG YOLK PIGMENTATION

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### Summary

Encapsulation of active ingredients using microemulsion offers exciting possibilities for enhancing the solubilization, stability and bioavailability of feed additives. We have formulated microemulsified Yellow or Red carotenoid products (Quantum GLO™ Y or R, respectively) using emulsifying systems which are comprised of homogenous liquid mixtures of oil, surfactant and co-emulsifier, to solubilize pigment molecules. These components spontaneously form microemulsion of below 1 µm in size upon interaction and dilution with an aqueous phase.

In the dose-dependent trials conducted, microemulsified products were found to offer enhanced bioavailability and absorption. The first trial was conducted using a corn-soybean basal diet supplemented either with 0.25, 0.5, 0.75, 1.0 and 1.25 kg/T of microemulsified Yellow or non-microemulsified preparation. The second trial involved feeding microemulsified Red or non-microemulsified preparation using a similar dosage range. Results showed that the color and carotenoid content of egg yolk increased ( $P < 0.05$ ) with increasing amount of carotenoids in the diet. The colors of egg yolks from layers fed similar concentrations of microemulsified or non-microemulsified preparation were significantly different ( $P < 0.05$ ). At the dose of 1.0 kg/T non-microemulsified yellow or red product, the microemulsified product is able to provide the equivalent yolk color at a 20-30% lower dose. The trial results supported our hypothesis that a desired yolk color score is achievable at a significantly lower inclusion rate, when carotenoid molecules are emulsified using the microemulsion nanotechnology.

### I. INTRODUCTION

Yolk pigmentation is one of the important factors in the evaluation of egg quality. The extent of the pigmentation required depends on consumers' expectations and can vary from market to market, from farm to farm. Yolk pigmentation can be achieved via synthetic ingredients, where they serve purely an aesthetic purpose. However, yolk pigmentation using natural pigments, such as those derived from marigold oleoresin and paprika oleoresin, enhances the egg as a functional food and may improve its taste. These natural pigments contain carotenoids which can be converted physiologically to vitamins or act as antioxidants *per se* (Dutta *et al.*, 2005). Unfortunately, carotenoids cannot be synthesized *de novo*. Birds need to obtain an exogenous source of carotenoids, through feed ingredients such as corn, corn gluten meal, etc. (Irwandi *et al.*, 2011).

Encapsulation of active ingredients using microemulsion (Spernath *et al.*, 2002) offers exciting possibilities for enhancing the solubilization, stability and bioavailability of feed additives in general. We have formulated microemulsified Yellow and Red using emulsifying systems which are comprised of homogenous liquid mixtures of oil, surfactant and co-emulsifier to solubilize pigment molecules. In this paper, we report the outcome of two dose-dependent trials designed to test the hypothesis that the microemulsified products show improved bioavailability over the corresponding non-microemulsified preparation, leading to greater yolk pigmentation at lower dosages.

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## II. MATERIALS AND METHODS

The microemulsified Yellow or Red carotenoid products (Quantum GLO™ Y or R, respectively) and non-microemulsified Yellow and Red products were prepared using Kemin Industries (Asia)'s pilot production facility. Dosages of each preparation were expressed in kg/T of feed and a typical corn-soybean basal diet was utilized for the ration.

A research farm in Malaysia was utilized for the dose-dependent carotenoid studies. Layers used in the experiment were Lohmann Brown (Age: 54 weeks and 60 weeks respectively) obtained from a local hatchery. Layers were placed in individual wire-floored cage under 14 L; 10 D lighting regime, and fed a typical corn soy-based diet for 2 weeks before the initiation of each trial. The first trial was conducted using corn-soybean basal diet supplemented either with 0.25, 0.5, 0.75, 1.0 and 1.25 kg/T of microemulsified Red or non-microemulsified Red. The second trial involved feeding microemulsified Yellow or non-microemulsified Yellow using a similar dosage range. The layers were divided into four replicates of 8 layers each (32 layers per treatment). The eight cages of layers were fed from a single feed trough. Feed and water were provided *ad libitum* throughout the trial. Each week, eggs were collected. The whole liquid egg color was determined by means of a commercially available yolk color fan. Where required, HPLC-based analysis of trans-capsanthin or trans-lutein equivalents using the AOAC method were carried out. Data were statistically analyzed by one-way ANOVA method using Statgraphics.

Eggs were cracked without whites onto plastic petri dishes of 6 cm in diameter and placed on a flat white background under total spectrum fluorescent light. A team of eight trained observers was asked to evaluate the eggs utilizing a commercial yolk color fan. The trans-capsanthin and trans-lutein concentration for each treatment was determined by extracting two yolks per treatment using a modified AOAC method.

## III. RESULTS AND DISCUSSIONS

The microemulsified Yed and Red carotenoids were found to be  $\sim 0.5\mu\text{m}$  in size, as analyzed by electron microscopy and light-scattering diffraction study (Figure 1). This was a dramatic reduction from the  $\sim 20\mu\text{m}$  measured for the non-microemulsified crude carotenoids.

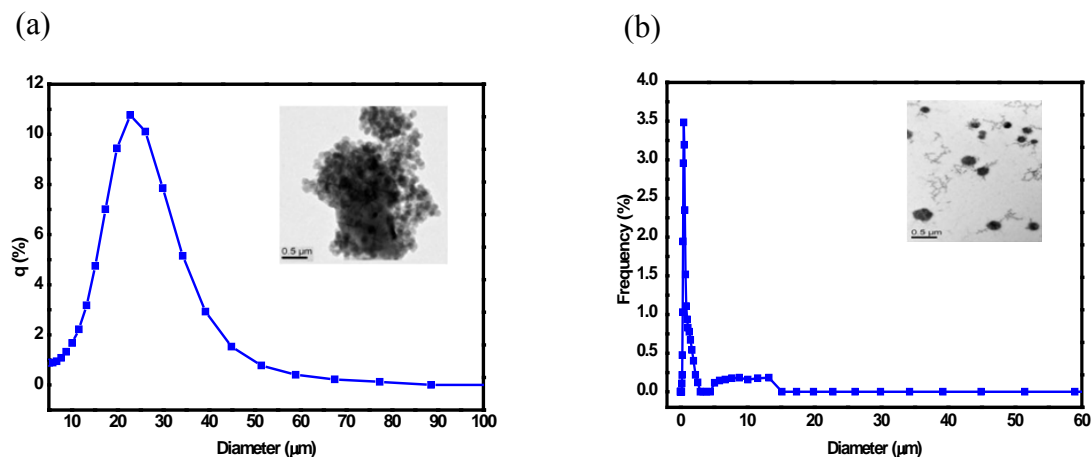


Figure 1 - Particle size analysis by transmission electron microscopy and light scattering diffraction study for (a) non-microemulsified carotenoids and (b) microemulsified carotenoids.

The yolk color fan score results for the microemulsified Red and Yellow, shown in Figure 2, demonstrated a significant improvement in the color score with increasing pigment supplemented in the diet, as compared to the non-microemulsified preparation. The data

points were fitted to a polynomial curve, and the curve fit equations were used to determine the least amount of microemulsified Yellow and Red carotenoid products needed to achieve a similar yolk score by the non-microemulsified versions. From Table, based on the current recommended dosage of 1 kg/T, the results showed that a similar yolk color score is now achievable with ~0.7-0.8 kg/T of microemulsified Yellow and Red carotenoid products, respectively. This reflects a ~20-30% reduction in the amount needed.

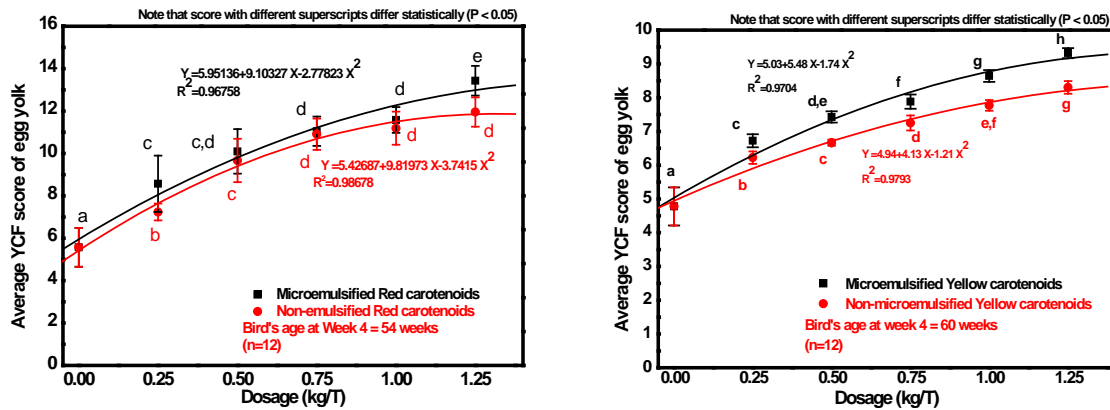


Figure 2 - Yolk color score at different inclusion rates of microemulsified Red and Yellow and non-microemulsified Red and Yellow carotenoids preparation (in comparison to the control). Note that scores with different alphabet superscripts differ statistically (P < 0.05)

Table 1 - The amount of microemulsified Red and Yellow carotenoids required to achieve a similar yolk color score compared to non-microemulsified preparation.

Microemulsified Red (kg/T)	YCF score of egg yolk	% <u>Decrease</u> in amount of Microemulsified Red needed to achieve the same YCF score (kg/T)
0.25	7.64	21.02
0.5	9.39	12.83
0.75	10.67	13.84
1	11.49	19.29
1.25	11.84	29.10

Microemulsified Yellow (kg/T)	YCF score of egg yolk	% <u>Decrease</u> in amount of Microemulsified Yellow needed to achieve the same YCF score (kg/T)
0.25	5.89	33.18
0.5	6.70	31.51
0.75	7.35	32.55
1	7.86	34.90
1.25	8.21	38.57

Microemulsified Red and Yellow carotenoid products were deposited more efficiently than the non-microemulsified pigments. There was a dose-related increase in trans-capsanthin and trans-lutein content of the egg yolk from layers fed with either the microemulsified or the non-microemulsified pigments. However, the trans-capsanthin and trans-lutein equivalents in egg yolk from the non-microemulsified pigments treatment group were significantly lower (P

< 0.05), and the non-microemulsified Red seemed to show a yolk color saturation at higher concentration (1 kg/T).

From the current study, bioavailability of carotenoids prepared using microemulsion systems was increased. There are a number of possible reasons for this increase. Firstly, microemulsified pigment molecules present a larger surface area, and may be acted on more quickly, physiologically. Secondly, reducing the size may enable carotenoid molecules to better penetrate the intestinal epithelium, increasing their residence time and enhancing absorption. Thirdly, smaller pigment particles may be more efficiently transported across the cellular epithelium by either paracellular or transcellular mechanisms. One thing is for sure, the ratio of surface area to volume of the smaller carotenoid structures was found to have increased dramatically after the emulsification, by 40 orders of magnitude. As such, improved efficiency in carotenoid delivery, uptake and utilization was expected to be significant.

Apart from an increase in pigment deposition, color intensity of the egg yolk is possibly dependent also on the pigment particle size. Perhaps at the nanometer range, the number of carotenoid molecules that can be packed at the surface has increased, leading to an increase in light absorption and scattering coefficient. A similar observation was reported (Fu *et al.*, 1998). This could possibly explain why a richer redness or yellowness was observed for the egg yolks obtained from layers treated with the microemulsified pigments.

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## EGG PENETRATION BY *SALMONELLA* TYPHIMURIUM IN WASHED AND UNWASHED EGGS

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### Summary

Egg or egg product related *Salmonella* poisoning is a major concern for the Australian egg industry. Salmonellosis can be acquired by the ingestion of raw or undercooked eggs. *Salmonella* Typhimurium (ST) is the most common serovar notified in *Salmonella* food poisoning cases in Australia. The objectives of the current study were to examine the effect of egg washing on the survival of *Salmonella* on the eggshell surface, to investigate the penetration ability of four different *Salmonella* Typhimurium phage types (ST PT), previously isolated from Australian layer farms, and to study the effect of egg washing on bacterial eggshell penetration. Our results indicated that there was no significant difference in survival of ST PTs on the egg shell surface of washed and unwashed eggs. Survival rate on inoculated eggshell surface was highest for ST PT 9 (83.33%) followed by ST PT 44 (53.33%), ST PT 193 (43.33%) and ST PT 170 (43.33%). All these phage types are able to penetrate the eggshell and they can survive in the egg internal contents at 20°C and 37°C in both washed and unwashed eggs. ST PT 44 penetration was significantly higher in washed eggs as compared to unwashed eggs. However, for other ST PT (PT 9, 170 and 193), we did not find any significant difference in the penetration of washed and unwashed eggs. The internal contents of whole eggs were most frequently contaminated by ST PT 44 (23.33%) followed by PT 170 (20%), PT 9 (10 %) and PT 193 (10%). It was also found that there was no significant effect of incubation temperature (20°C and 37°C) on *Salmonella* penetration.

### I. INTRODUCTION

Food borne illness costs Australia an estimated \$1.2 billion per year (Hall et al., 2005). The annual report of the OzFoodnet network (2009) reported 9,533 cases of *Salmonella* infection. Although the eggs produced in Australia are of good quality, the egg industry is often blamed for cases of food poisoning due to Salmonellosis. Salmonellosis can be acquired by the ingestion of raw or undercooked eggs. As well, cross contamination of ready to eat meals with *Salmonella* also plays major role in food poisoning cases. Intact eggs can be contaminated by *Salmonella* by either vertical (which is common in case of *Salmonella* Enteritidis) or horizontal transmission. *Salmonella* Enteritidis, which is of major concern for the food industry worldwide, is not prevalent in Australian layer flocks. According to Humphrey (1994), horizontal transmission is the most common route for Salmonellae other than *Salmonella* Enteritidis. The eggshell can be contaminated by any surface with which the egg comes in contact such as faeces, water, caging material, nesting material, insects, hands, broken eggs, dust on egg belt, blood and soil (Board and Tranter, 1995; Davies and Breslin, 2003). Egg washing is used to reduce eggshell contamination in many countries such as the United States, Australia and Japan (Hutchison et al., 2004). Egg washing can reduce the microbial load on the eggshell surface (Messens et al., 2011) and thus may lower the rate of horizontal transmission of *Salmonella* across the eggshell. However, egg washing chemicals

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may affect the cuticle of the eggshell (Wang and Slavik, 1998). Hence, benefits and losses due to egg washing are under debate. As ST is the most common serovar notified in Salmonellosis food poisoning cases in Australia (Oz Foodnet, 2009), the objectives of current study were to examine the effect of egg washing on the survival of *Salmonella* on the eggshell surface, to investigate the penetration ability of different ST PTs and to study the effect of egg washing on bacterial eggshell penetration. All PT used in this study have been reported in egg product related *Salmonella* poisoning cases in Australia.

## II. MATERIALS AND METHODS

In the present study, the eggshell penetration ability of four isolates ST, each of a different PT (PT 9, 44, 170 and 193) was investigated. All of the isolates were from Australian layer farms and were obtained from the Australian Salmonella Reference Centre, Adelaide. For each PT, 90 eggs were collected from hens in early lay. These eggs were divided into two groups: washed (30 eggs) and unwashed (60 eggs). For egg washing, a commercial detergent (a hydroxide and hypochlorite based washer used at concentration of 0.45% (v/v) which equates to a pH of ~12 and ~200 ppm hypochlorite in the working solution) and the compatible sanitiser (at concentration of 0.16% (v/v) which equates to ~200 ppm hypochlorite in the working solution) were used. The pressure of the sprays was 3 pounds per square inch (psi) without brushes. Eggs (90) were divided into 9 different treatment groups based on washing, dose of infection and incubation temperature. All eggs were dipped in 70% ethanol for 30 sec in order to kill bacteria present on the eggshell surface. After drying, each egg was dipped in a solution containing PBS (control),  $10^3$  Colony forming units (CFU)/ml or  $10^5$  CFU/ml dose of ST for 90 sec followed by incubation at either 20°C or 37°C for 21 days. After incubation, the survivals of *Salmonellae* on the eggshells were studied; egg was put in a whirl-pak bag containing 10 ml of BPW (Oxoid, Australia) and was massaged for 1 min. A 10 ul aliquot of the BPW was plated onto XLD plates and the plates were incubated at 37°C overnight. Next day, the colonies on the plate were observed for *Salmonella*. Similarly, to investigate the penetration and survival of *Salmonella* in internal contents, a 2 ml aliquot of the internal contents was transferred into 8 ml of BPW and 10 ul of this BPW was plated on XLD plates and the plates were incubated at 37°C overnight. After incubation, the colonies on the plate were observed for red colonies with a black centre of *Salmonella*. Finally, statistical analysis was conducted using Fisher exact test using Graph pad software.

## III. RESULTS

In the first experiment, all the controls worked well as all the eggshell surfaces and internal contents of these control group eggs were found negative for *Salmonella*. Results indicated that ST PT 44 (at  $10^5$  CFU) penetration in washed eggs was significantly higher compared to unwashed eggs (Table 1). However, in the cases of other ST PTs (PT 9, 170 and 193), there were no significant difference in the *Salmonella* penetration of washed and unwashed eggs. At 20°C, in total, 20% (16/80) of the washed eggs and 10% (8/80) of the unwashed eggs were penetrated. Our results also indicated that the internal contents of whole eggs were contaminated by ST PT 44 (23.33%) followed by PT 170 (20%), PT 9 (10 %) and PT 193 (10 %). As the penetration of bacteria across the eggshell is dependent on the survival of bacteria on the eggshell, we compared the survival of ST PT on the eggshell surface of washed and unwashed eggs; however, there was no significant difference in *Salmonella* survival on the egg shell surface of washed and unwashed eggs (data not shown). Survival rate on eggshell surface was highest for ST PT 9 (83.33%) followed by ST PT 44 (53.33%), ST PT 193 (43.33%) and ST PT 170 (43.33%). It was also found that there was no significant effect of

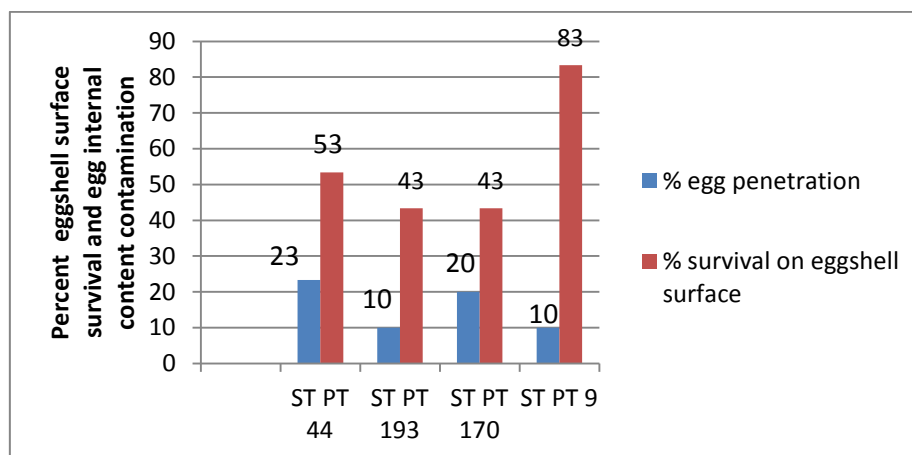
incubation temperature (20°C and 37°C) on the penetration ability of any of the ST PT used in this study (data not shown).

#### IV. DISCUSSION

In Australia, there is limited information available regarding the survival ability of different ST PTs on the eggshell surfaces and egg penetration ability of these phage types and also the effect of egg washing on *Salmonella* penetration. The present study showed that; if eggs are stored at 20°C or 37°C, ST can survive on the eggshells for three weeks following

**Table 1 - Whole egg penetration by different *Salmonella* Typhimurium phage types: comparison between washed and unwashed egg at 20°C**

Phage type	CFU	Group	Number of penetrated eggs	Number of non-penetrated	P-Value
ST PT 44	10 <sup>3</sup>	Washed	4	6	0.6285
		Unwashed	2	8	
	10 <sup>5</sup>	Washed	8	2	0.0007
		Unwashed	0	10	
ST PT 193	10 <sup>3</sup>	Washed	2	8	0.4737
		Unwashed	0	10	
	10 <sup>5</sup>	Washed	0	10	0.4737
		Unwashed	2	8	
ST PT 9	10 <sup>3</sup>	Washed	2	8	0.4737
		Unwashed	0	10	
	10 <sup>5</sup>	Washed	0	10	0.4737
		Unwashed	2	8	
ST PT 170	10 <sup>3</sup>	Washed	0	10	1.000
		Unwashed	0	10	
	10 <sup>5</sup>	Washed	0	10	0.4737
		Unwashed	2	8	



*Figure 1 - Percent eggshell surface survival and egg internal content contamination by various *Salmonella* Typhimurium phage types*

contamination. This finding underlines the importance of proper storage, careful handling of eggs in the food industry and the domestic environment. These findings are in agreement

with De Reu et al. (2006) who found a high survival rate of *Salmonella* Enteritidis on the eggshell surfaces 21 days after infection. They also reported that cuticle deposition and specific gravity may have an impact on the survival of bacteria on the eggshell surfaces. In order to study the egg penetration ability of ST PT, a more holistic approach was used in the current study as compared to some other studies where *Salmonella* culture was artificially inoculated into the albumen using a syringe. Results indicated that all phage types used in the present study are capable of penetrating the eggshells and are also able to survive in the egg albumen which is considered to be a hostile environment for the survival of bacteria. In the current study, it was found that ST PT 44 penetration was significantly higher in washed eggs than unwashed eggs, which could be due to damage to the cuticle by egg washing chemicals. However, to draw concrete conclusions, it is essential to conduct more experiments to investigate the effect of egg washing on the ultrastructure of the cuticle. Findings in the present study are in agreement with earlier findings by Wang and Slavik (1998) who reported that bacterial invasion was increased as a result of alteration in the eggshell surface due to the washing chemical alkaline sodium carbonate. Although Leleu et al. (2011) reported that egg washing had no effect on cuticle quality, eggs used in their study were from old hens and cuticle thickness decreases with increasing hen age (EFSA 2005). Other ST PTs (PT 9, 170 and 193) penetrated washed and unwashed eggs equally. There was no significant effect of storage temperature (20°C and 37°C) on penetration of ST across the eggshell. A good quality eggshell protects the internal contents from bacterial penetration. A cracked or damaged egg encourages bacteria to move across the eggshell and into the contents which is likely to increase the risk of food poisoning. Hence, in order to study the effect of eggshell quality on bacterial penetration, an agar penetration approach will be used for all of these ST PT in further studies. In the present study, only one isolate per PT was used, hence further investigation using multiple isolates of same PT is essential in order to confirm the variation in penetration ability of different phage types.

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## EGG WASHING – TRIALS AND TRIBULATIONS

D. MAY<sup>1</sup>, M. SEXTON<sup>2</sup>, G. HOLDS<sup>1</sup> and A. KIERMEIER<sup>1</sup>Summary

Up to 5% of the 333.9 million dozen eggs produced in Australia annually are discarded because they are classified as ‘black’ eggs, deemed so visually contaminated that they cannot be recovered using current washing methods. Therefore, improved washing methods may result in substantial cost recovery. This study investigated ways to increase recovery of black eggs through improved washing conditions, resulting in recovery of more than 80% of black eggs when washed multiple times. It was found that the biggest limitations on recovery were: egg washer design; being able to make appropriate adjustments; inadequate spray temperature at egg surfaces; inappropriate chemical application resulting in incorrect levels of chemical being used at incorrect temperatures; operation issues including blocked spray nozzles; brushes interfering with sprays and/or not touching the eggs; and inadequate cleaning of washing and grading machinery. Improvements in these areas along with better wash chemicals will lead to greater egg recovery and reduction in potential for foodborne illness outbreaks related to eggs.

## I. INTRODUCTION

The annual production of eggs in Australia totalled 333.9 million dozen in 2008/09, of which 67.8% were cage eggs, 5.5% were barn laid and 26.8% were free range (Australian Egg Corporation Limited, 2009). The vast majority of eggs are washed in Australia prior to packing to remove dirt and faecal material and to reduce the microbial contamination of the egg shell (Anonymous, 2009). Visually dirty eggs after washing are diverted for pasteurisation or discarded, along with cracked eggs, resulting in lower returns to processors.

The washing process consists of several stages: pre-washing, washing with the aid of a surfactant/cleaner, sanitising and blow-drying, and can take less than 30 seconds. Hence, the effectiveness of the surfactant’s ability to penetrate and effectively remove dirt and faecal matter is critical for the recovery of table eggs. An effective cleaner can also assist in the removal of bacteria while a suitable sanitiser, together with a clean post-wash processing environment, will assist in maintaining the hygienic status of the eggs. It is estimated by the authors that dirty eggs constitute between 5 and 20% of total production, depending on the production system and management practices and up to 50% of these may not be recovered. In addition, up to 5% of all eggs are ‘black’ eggs. These are deemed so visually contaminated that they cannot be recovered using current washing protocols and hence are discarded.

The primary objective of this project was to improve the effectiveness of current washing practices to reduce the proportion of eggs downgraded or disposed. The secondary objective was to reduce potential food safety risks through the reduction of enteric microorganisms on the egg shell surface.

## II. METHODS

Three egg processing plants were visited to assess the recovery of black eggs using two chemical/temperature combinations. These combinations had previously been assessed in laboratory-based trials and were determined to yield the best black egg recovery. At each

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processing plant, black eggs (60 eggs per chemical/temperature combination) were washed, repeatedly if needed, until they were visually clean to a maximum of four washes (30 sec. per wash cycle). Fifteen black eggs were left unwashed as a control.

a) Cleaner/sanitiser combinations

Two cleaner/sanitiser combinations were used for in-plant trials, one on each of the two days on which trials were conducted after normal production finished: *Chemical 1*: A liquid alkaline (pH 12) chlorine based product to be used at 1% solution (v/v) and 40°C (or as near as practical). This was used with a quaternary ammonium (QAC) based sanitiser (0.25% (v/v), final QAC concentration 400 ppm) at 42°C (or as near as practical); and *Chemical 2*: A liquid sodium hydroxide based product to be used at 0.45% solution (v/v) at 40°C (or as near as practical) with a sodium hypochlorite based sanitiser (0.16% (v/v), final hypochlorite concentration of 200 ppm) at 32°C (or as near as practical).

Final pH was determined using pH Test Strips (Sigma). Final QAC concentration was determined using Hydrion Papers QT-40 (Microessential Laboratories Inc). Final hypochlorite concentration was determined either using Precision Chlorine Test Paper (Precision Laboratories) or by titration.

b) Microbiological assessment of washed and unwashed eggs

Total Viable Counts (TVC, hygiene indicator) and Enterobacteriaceae counts (these are used as a faecal indicator and include *Escherichia coli* and *Salmonella*) were determined for 15 unwashed (control) and 15 washed eggs by placing eggs aseptically into individual sterile stomacher bags. Sterile peptone saline solution (PSS) (10 mL) was added and the bag gently shaken by hand for two minutes. Serial decimal dilutions (1 mL) were plated onto 3M<sup>TM</sup> Petrifilm<sup>TM</sup> Aerobic Plate Count Petrifilm and incubated at 35°C for 48 hours to determine the TVC per mL of rinse. To determine the total Enterobacteriaceae count per mL of rinse, serial decimal dilutions of the rinse were plated onto 3M<sup>TM</sup> Petrifilm<sup>TM</sup> Enterobacteriaceae Count Plates and incubated at 35°C for 24 hours.

c) Statistical Analysis

TVC and Enterobacteriaceae counts were log<sub>10</sub> transformed for analysis and results below/above the lower/upper limit of detection (LoD) were set equal to the respective LoD. Results were analysed separately for each plant using a two-way analysis of variance.

### III. RESULTS

a) Recovery of black eggs

Under the conditions used in this trial and irrespective of the chemical used, an average of 29, 57 and 85% of black eggs were visually clean after two, three and four washes, respectively. There was no clear relationship between plants or chemicals, although variability was large. This indicates that in-plant optimisation and validation of the wash system and chemicals is required to achieve best results.

b) Microbiological analysis of eggs

Box plots of the  $\log_{10}$  TVC (cfu/egg) are shown in Figure 1. For Plant 1, there was a significant interaction between chemical/day and process step ( $P < 0.001$  – excluding dirty eggs from the analysis). This interaction was due to graded eggs being higher on Day 1 than on Day 2, which coincided with the plant being cleaned overnight. For Plants 2 and 3, excluding dirty eggs prior to washing, there were no significant differences between process steps ( $P = 0.09$  and  $0.15$ ), chemical/day ( $P = 0.75$  and  $0.85$ ), nor their interaction ( $P = 0.43$  and  $0.35$ ). The average TVC was 4.18 and 4.11  $\log_{10}$  cfu/egg, respectively.

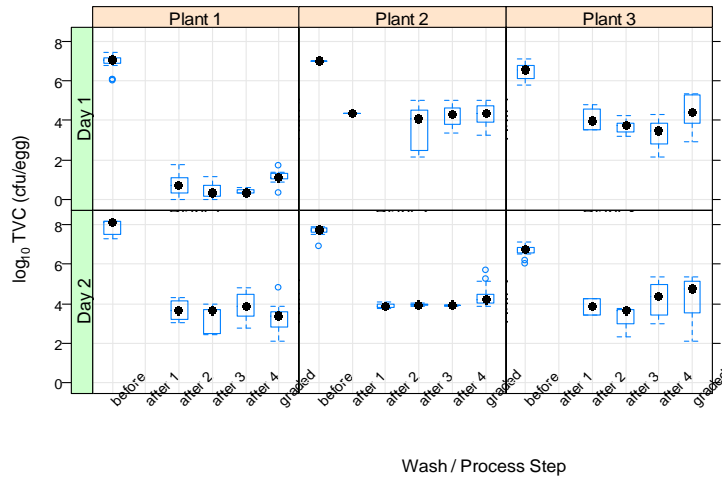


Figure 1 - Box plots of the  $\log_{10}$  TVC (cfu/egg) on eggs before washing, after washing one to four times, and after grading. Eggs were collected over two days from three plants.

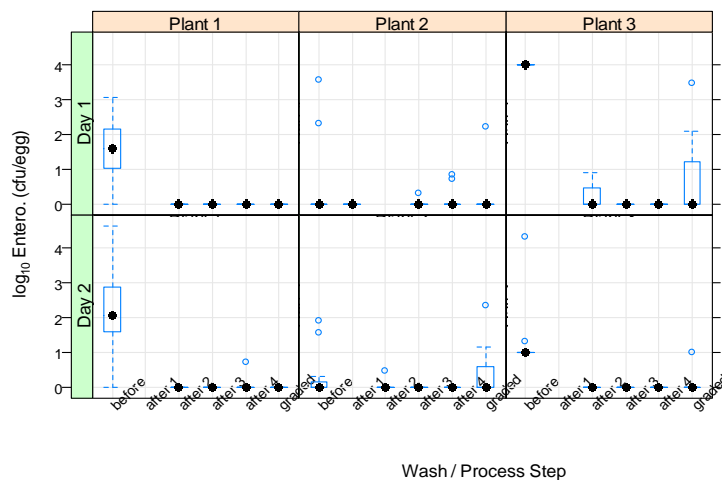


Figure 2 - Box plots of the  $\log_{10}$  Enterobacteriaceae (cfu/egg) on eggs before washing, after washing one to four times, and after grading. Eggs were collected over two days from three plants.

Box plots for Enterobacteriaceae counts per egg are shown in Figure 2. Because of the limited post-washing Enterobacteriaceae detections, no formal statistical analysis was undertaken, but the following key observations can be made:

- Unwashed eggs can be substantially contaminated with Enterobacteriaceae.
- Washing removes most Enterobacteriaceae to below detectable levels ( $< 1$  cfu/egg).
- At Plant 1, only one graded egg had detectable levels of Enterobacteriaceae (too numerous to count). The other two plants resulted in multiple detections of Enterobacteriaceae, which may be related to the hygiene of the post-wash equipment.

#### IV. DISCUSSION

Washing black eggs up to four times resulted in an 85% recovery, which equates to a potential recovery of 14.2 million dozen eggs per annum. With the increases in free range production and production figures increasing annually, this figure can be expected to rise. However, whether washing eggs for up to two minutes is economically beneficial will depend on individual processors and their ability to slow down the process or return dirty eggs for rewashing. Therefore, it is difficult to accurately measure the overall potential cost saving to industry, although improved washing systems and chemicals will make this process easier.

The reduction in TVCs (up to 6 log<sub>10</sub>) and Enterobacteriaceae (up to 4 log<sub>10</sub>) achieved are similar to those of Musgrove *et al.* (2005) who achieved a 4 log<sub>10</sub> reduction in TVC and 1 log<sub>10</sub> reduction in Enterobacteriaceae post washing. These results highlight how washing can reduce potentially harmful bacteria on the egg surface.

Correct maintenance of egg washing machinery, application of detergents/sanitiser during washing, and cleaning and sanitation of grading equipment is essential for maintaining egg quality and safety. However, the results from the in-plant trials suggest that these may be an issue for many egg washing and grading facilities. Shortcomings result in poor quality washing of eggs and wastage of chemicals and water. They are the result of a large variation between (measured) input water temperatures and actual temperature at the egg surface; inability to accurately measure and adjust active chemical concentrations; and inadequate agitation of chemical solution on the egg due to blocked spray nozzles and brushes that are ineffective or interfere with sprays.

It was clear from these site visits that cleaning of washing and grading equipment is often difficult and hence is not as thorough as it could be. This could potentially result in microbial cross-contamination of eggs following washing. Furthermore, Enterobacteriaceae were found in swabs taken from grading machinery from multiple premises. However, following instruction on how to better manage the cleaning regime, subsequent swabbing of Plant 3 did not recover any Enterobacteriaceae (data not shown). This indicates that, with appropriate guidance surrounding plant hygiene, the risks associated with faecal contamination of eggs can be greatly reduced.

A better understanding by plant managers on correct chemical use, machine setup, temperature control and plant hygiene is likely to lead to improved egg recovery and safety. Importantly, after discussions with the project team, one plant was able to reduce the presence of Enterobacteriaceae on post-wash eggs and equipment on Day 2 after thorough cleaning of all equipment. Such improvements industry wide can lead to better egg recovery and improved safety of eggs and egg products. This, in turn, will help make eggs safer for the consumer and reduce the potential for foodborne outbreaks.

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## PROTOPORPHYRIN IX IN SHELL AND CUTICLE OF BROWN SHELLED EGGS

S. SAMIULLAH<sup>1</sup> and J.R. ROBERTS<sup>1</sup>Summary

Eggs were collected from Hy-Line brown flocks aged 33, 50 and 67 wk. Thirty eggs from each flock were analyzed to determine the reliability of MST (MS Technologies, U.K.) cuticle blue stain as an indicator of the presence of cuticle and the effective removal of cuticle by use of an EDTA solution. Another 30 eggs, collected at the same time from each flock, were processed for the quantification of protoporphyrin IX (PP IX) from the eggshell with and without the presence of cuticle. The L\*a\* components of the colour space system were significantly different among the age groups. There was a high degree of correlation between the extent of MST cuticle blue staining and the amount of cuticle on the eggshell as recorded by scanning electron microscopy. PP IX pigment was quantified by spectrophotometric analysis of digested eggshell solutions. The average amount of PP IX in the shell without cuticle ( $8.046 \times 10^{-8}$  g/g of shell) was higher than the amount present in cuticle ( $1.669 \times 10^{-8}$  g/g of shell). The amount of PP IX in the cuticle of 1g of shell in the 33 wk eggs was significantly lower than for eggs from the 50 wk and 67 wk flocks whereas, in the shell without cuticle, it was not significantly different among flock ages. PP IX in cuticle, as a percentage of total PP IX in shell was 13% in 33 wk, 20 % in 50 wk and 18 % in 67 wk eggs.

## I. INTRODUCTION

Brown, white or tinted eggs are linked to the genotype of the hen. The eggshell colour of brown eggs is a quality aspect for consumers (Curtis *et al.*, 1985; Jones *et al.*, 2010) and may be related to shell ultrastructure (Richards and Deeming, 2001). Eggshell pigment has been shown to have some antimicrobial properties (Ishikawa *et al.*, 2010). Protoporphyrin IX (PP IX) is believed to be the main eggshell pigment, but other pigments such as zinc porphyrin, biliverdin and zinc biliverdin (Kennedy and Vevers, 1976) may contribute to shell colour. Previous research has suggested that most of the PP IX is located in the cuticle (Miksik *et al.*, 2007). In contrast, however, the study of Nys *et al.* (1991), which investigated the kinetics of PP IX deposition, found that approx. 75% of the PP IX was laid down in association with the calcareous part of the shell. Therefore, the purpose of the present study was to quantify the amount of PP IX from the cuticle and true shell layer in brown shelled eggs.

## II. MATERIALS AND METHODS

Eggs were collected from conventional cage flocks (Hy-Line brown) which were 33, 50 and 67 wk of age. Eggs were divided into two groups and used for the following two investigations.

The first experiment was conducted to verify the reliability of MST cuticle blue dye as an indicator of the presence of cuticle. It also investigated the effectiveness of the use of EDTA to remove the cuticle without eroding into the true shell. Thirty eggs from each age group (33, 50 and 67 week) were included in this study. Shell colour (L\*a\*) was measured before staining using a Konica Minolta spectrophotometer (CM-2600d). "L\*" has a maximum of 100 (white) and a minimum of 0 (black). For "a\*", green is towards the negative end of the scale and red towards the positive end. Eggs were soaked in MST Cuticle blue dye for 1 minute and rinsed in distilled water to remove excess stain. After drying, shell colour

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(L\*a\*) was measured on the stained eggs. For the thorough removal of the cuticle, the method described by Leleu *et al.* (2011) was followed with slight modification. Each egg, individually, was soaked in an EDTA solution (0.34 M, pH 7.5) for 5 minutes and the cuticle was carefully scrubbed off in running tap water using a small soft brush. Shell colour (L\*a\*) was measured on the eggs without cuticle. Eggs were restained as described earlier and shell colour (L\*a\*) was again measured. A small piece from around the equator of the shell was cut out, mounted on an aluminium stub using silver paint, sputter coated with gold for 5 min in a Neocoater (Nikon, Japan) and viewed under a Scanning Electron microscope (SEM) at various magnifications.

The second experiment measured the amount of protoporphyrin IX (PP IX) in the eggshell. Eggs from the 33, 50 and 67 week flocks were analyzed for PP IX in whole eggshell including the cuticle as well as in eggshell from which the cuticle had been removed. A method described by Poole (1965) was used with some modification. Following removal of egg contents via a hole at the blunt end of the eggshell, a clean thin stick was passed across the long axis of the eggshell in such a way as to immerse one longitudinal half of the shell in a glass container of 0.34 M EDTA (pH 7.5) for 5 minutes at the same time as maintaining the other half outside the solution. The cuticle of the soaked longitudinal half side of the eggshell was washed away in running tap water. The eggshell was cut off longitudinally into two equal halves; one having cuticle and one without cuticle. A 0.250 g sample from the equator of each dried shell (without shell membrane) was weighed into a clean 10 mL plastic centrifuge tube into which 4 mL of methanol- concentrated HCl (2:1) solvent was added. All the tubes were wrapped in aluminium foil and placed in a refrigerator for 12 hours. The samples were centrifuged at 3000 rpm for 1 hr. After centrifugation, the supernatant solution was decanted into spectrophotometer cuvettes (4mL) and the absorbance of the supernatant read at 412 nm (Shimadzu, UV-1201). In order to confirm that the shell had dissolved completely, the sediment remaining in the bottom of the centrifuge tubes was viewed under a light microscope at various magnifications.

A standard stock solution was prepared by dissolving 0.0018 g of powder PP IX disodium salt (Sigma Aldrich Australia) in 30 mL methanol-concentrated HCl (2:1) solvent. Serial dilutions were prepared until a 1:128 dilution was reached and their absorbance was read in a spectrophotometer. A standard curve was constructed by plotting the concentrations of protoporphyrin in the standards diluted 1:16 ( $6.87 \times 10^{-6}$  mM), 1:32 ( $3.43 \times 10^{-6}$  mM), 1:64 ( $1.72 \times 10^{-6}$  mM), and 1:128 ( $8.59 \times 10^{-7}$  mM) from the stock solution against the absorbance reading for each standard. The absorbance values were converted into concentration of PP IX in the sample solvent in mmol/L and the amount of protoporphyrin in 1 g of eggshell (with and without cuticle present) was calculated. For determination of the amount of PP IX in the cuticle, the values of the eggshell samples without cuticle were subtracted from the values of the eggshell samples with the cuticle still present. Data were analyzed using Statview Software (SAS Institute Inc., Version 5.0.1.0).

### III. RESULTS

In Experiment 1, the Specular Component Included (SCI) L\* component of the L\*a\* space system was not significantly ( $P=0.1860$ ) affected by flock age when the cuticle was intact. However, following the removal of the cuticle, there were differences among flocks with values being highest for the 50 wk flock. There was a significant effect of cuticle treatment on mean values of L\* among each age group. Staining of eggs with intact cuticle resulted in lower L\* values whereas cuticle removal increased these values. There was a significant effect of flock age on the SCI a\* reading for all cuticle treatments with the 67 wk flock having the highest values. There was also a significant effect of cuticle treatment on values

for SCI a\* for all flocks. The a\* reading decreased following staining in eggs with intact cuticle. Removal of the cuticle with EDTA resulted in lower readings than for shells with intact cuticle. Staining with cuticle blue dye of eggs with cuticle removed resulted in reduction in the a\* value for all flocks. A high correlation between the presence of cuticle blue stain and the amount of cuticle as viewed under the scanning electron microscope was recorded. Eggs with good quality intact cuticle stained well; eggs with patchy cuticle acquired patchy stain whereas, in the absence of the cuticle, the eggs did not stain at all. SEM observations of the restained eggshell after cuticle was removed by EDTA confirmed that, in the absence of cuticle, eggshells did not stain with cuticle blue dye.

In Experiment 2, for a 1 g piece of eggshell, there was more PP IX in the shell with cuticle intact, as compared with a piece of shell from the same egg with cuticle removed. When the difference between the two was calculated, there was more protoporphyrin present in the true (calcareous) shell than in the cuticle from the same amount of shell, as shown in Table 1. The total amount of PP IX in 1 g of shell with cuticle intact was not significantly different among the flocks. The total amount of PP IX in 1 g of shell without cuticle was not significantly different among the three flocks, although it tended to be lowest in the 55 week and highest in 33 week flock with the 67 week flock intermediate. However, when the amount of PP IX in the cuticle alone of 1 g of total eggshell was calculated, it was significantly higher ( $P < 0.02$ ) in the 50 and 67 week flocks as compared with the 33 week flock. For a given weight of whole eggshell, the percentage of total PP IX found in the cuticle was 13, 20 and 18% in 33, 50 and 67 wk flock eggs, respectively. Microscopic observations of the digested shell precipitates showed only shell membranes which confirmed that all shells had been dissolved in the solvent.

**Table 1 - Protoporphyrin IX in cuticle and true eggshell of Hy Line brown eggs**

Variable	Flocks age (weeks)			P Value
	33	50	67	
PP IX in cuticle of 1 g of eggshell mM	<sup>b</sup> 1.304 x 10 <sup>-8</sup>	<sup>a</sup> 1.898 x 10 <sup>-8</sup>	<sup>a</sup> 1.806 x 10 <sup>-8</sup>	0.0195
PP IX in 1 g of shell without cuticle mM	8.406 x 10 <sup>-8</sup>	7.569 x 10 <sup>-8</sup>	8.164 x 10 <sup>-8</sup>	0.0581

<sup>a, b</sup> Across a row, values with different superscripts are significantly different from each other  
Values are Mean

#### IV. DISCUSSION

In Experiment 1, as expected, a significant change in L\*a\* components of the colour space system with the various cuticle treatments confirmed the effectiveness of the procedure used for cuticle removal. For unstained eggs with cuticle intact, there was no difference among the flocks for L\* values. Following cuticle removal (but prior to staining), L\* values were highest for the 50 wk flock. The SCI a\* value of eggshells stained with MST cuticle blue dye is the most important indicator of the amount of cuticle present on an eggshell. The higher SCI a\* values for +cuticle, +stain eggs in the 67 wk flock indicated the presence of less cuticle. When cuticle blue stain was applied to shells with cuticle removed, all flocks showed slight reductions in a\* values, indicating a small amount of staining which was occurring where cuticle was present in crevices and pores. Experiment 1 also verified that the MST cuticle blue dye is a reliable indicator of the presence of cuticle on eggshells. SEM of shells with cuticle removed confirmed that the EDTA treatment reliably removed the cuticle without eroding the calcium carbonate of the eggshell.

The results of Experiment 2 showed that there is more pigment (protoporphyrin IX) in the calcareous components of the eggshell than in the cuticle of commercial brown eggs. These results are in contrast to previous research that reports more pigment in the cuticle than in the calcareous layers of the eggshell (Miksik *et al.*, 2007; Wang *et al.*, 2009). However, the results of the present study are consistent with the findings of Nys *et al.* (1991) who reported that 75% of the protoporphyrin was found in the calcareous layers of the shell of brown eggs. In the present study, the amount of protoporphyrin IX in the eggshell with the cuticle removed ( $P=0.0581$ ) and shell with the cuticle ( $P=0.4363$ ) was not significantly different among the three age group eggs which supports the suggestion of Odabasi *et al.* (2007) of a constant rate of secretion of PP IX in the shell gland throughout the production cycle of laying hen. However, there was a statistically significant ( $P=0.0195$ ) difference among the three flocks in the amount of PP IX in the cuticle alone. A reduced amount of PP IX in the cuticle of the shell does not necessarily indicate reduced cuticle cover as, in the current study, the 33 week flock eggs, which had the lower amount of PP IX in per gram of shell compared to 67 wk, showed more cuticle than 67 week eggs, measured by spectrophotometer ( $L^*a^*b^*$  space system). Our finding that there is a greater percentage of total pigment in the calcareous part of the shell than in the cuticle raises questions about the stages of eggshell formation and the times at which protoporphyrin deposition is maximal. Most authors have suggested that pigment is secreted in the last hour of oviposition and deposited in the cuticle (Kennedy and Vevers, 1976; Poole, 1965).

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## THE GROWTH AND SEXUAL MATURITY OF THE AUSTRALIAN MEAT-TYPE JAPANESE QUAIL

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### Summary

This study was conducted to describe the growth pattern of 5 meat-type Japanese quail strains of primary breeder flocks. We hypothesised that the growth rate will vary between strains and that growth of males will be different to females. The pattern of growth was consistent with reports in the literature for meat-type quail; highest growth rate occurred between week 2 and 4. The 5 strains displayed similar growth patterns and varied little in live weight. After 4 weeks of age, the difference in growth of strains was mainly due to females having a 20% higher live weight as compared to males. Analysis of gonad size suggests males were becoming sexually mature earlier than females.

### I. INTRODUCTION

Japanese quail are farmed for egg and meat production. They are attractive for farming because of their small body size, fast growth, early sexual maturity and short generation interval. The quail industry has been flourishing steadily over the last 40 years with over a billion quail being produced around the world. Australia alone produces about 6.5 million quails per year which constitutes 47% of the total game birds (other than turkey and ducks) slaughtered in the country (Foster, 2009). The Rural Industries Research and Development Corporation (RIRDC) in Australia predicts a favourable future for Australian quail meat production to supply Asian and Middle East countries; France and China are likely to be the major competitors. This scenario highlights the need for efficient quail production, which can only be achieved with development of high yielding breeds/strains, while maintaining low costs of production to compete in national and international markets.

Japanese quail are known to exhibit sexual dimorphism; males are smaller than females. This difference in growth pattern may impact on genetic gains, nutrient requirements, feed conversion ratio, socio-sexual interactions, and fertility of the breeding flocks. By controlling sexual dimorphism in growth and synchronizing the age of sexual maturity of the breeder flocks, production performance may be improved. Unfortunately no particular studies are available to describe the reproductive and productive potential of the Australian meat type quails. To fill this gap the present study investigated the growth pattern, sexual dimorphism in growth, and sexual maturity in Australian meat type quails. This report is characteristic of the major proportion of Australian meat type quails because the studied strains represent approximately 75% of Australian meat type quail population.

### II. MATERIAL and METHODS

The birds representing the primary breeding lines were provided by the Game Farm Pty Ltd. (Galston NSW, Australia) and were housed in special quail breeder cages (335 cm x 85 cm x 25 LWH, Venturi Valter 8-47016 Predappio, Italy) at the Native Animal Facility of the University of Western Australia. The housing environment of the breeder quail room was

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maintained at 22-26°C (using negative air pressure with automatic temperature control system), 14/10-h light/dark cycle (continuous fluorescent white light, 90-110 lux at the cage level) with adequate ventilation, 45-55% humidity and *ad-libitum* feed and water. The quail were fed a quail breeder diet containing 20.0% CP (crude protein) and 11.5 MJ/kg ME (metabolisable energy) (Inghams Enterprises Pty. Ltd., Liverpool, NSW, Australia).

The parent birds representing 5 strains produced 1482 chicks in eight subsequent hatches (April, 2011- December, 2011) from eggs laid by pair-mated quails that were 8, 16, 26 & 36 weeks of age (n = 225 parent birds). Each male was mated with 4 females one by one (8 hours/female) for two weeks at each age. The eggs were stored in a cool room at 15 °C for 3-6 days and then incubated (GQF 1202 sportsman incubator) at 37.5°C and 55% relative humidity with automatic turning every 2 hours. At day 15, eggs were transferred to a hatcher which was maintained at 37°C and 75% relative humidity. Chicks were hatched using special hatcher boxes partitioned to hatch chicks separately.

Immediately after hatching, chicks were weighed, tagged, and then transferred to the brooding cage (240 cm x 106cm x 30cm, Venturi Valter 8-47016 Predappio, Italy). The brooding cage provided 50cm<sup>2</sup> floor space/bird during the first two weeks and 120 cm<sup>2</sup>/bird thereafter. The cages were equipped with light (incandescent 25 watt light source) and an automatic heating source (separate heating unit installed at the roof of each cage). The temperature on the first day after hatching was 33-35°C which was decreased by 0.5°C per day during the first week and then at a rate of 1°C per day during the 2<sup>nd</sup> week. After the 2<sup>nd</sup> week, a room temperature of 22-25°C was maintained. Fresh air was provided at 0.5 m<sup>3</sup>/kg per hour (maintained by negative air pressure) and 45-55% humidity was maintained inside the room. During rearing and brooding a continual 24-hour photoperiod was provided with a 100-120 lux light intensity at the cage level. From 0 to 1 week of age, chicks were maintained on a quail starter diet (26% CP and 12 MJ/kg) and from 2-6 weeks of age on a finisher diet (22% CP and 12 MJ/kg). All diets were provided in a pelleted form. Live weight was measured individually at 0, 2, 4, 5 and 6 weeks of age. The birds (696 males and 786 females) were sacrificed at 6 weeks of age and the paired testes and ovaries were collected and weighed immediately after death was confirmed. All procedures were approved by the Animal Ethic Committee of the University of Western Australia.

The data were analysed with PASW Statistics 18, (SPSS©, Inc., 2009, Chicago, IL, USA). Analysis of variance procedures were applied to test the effect of strain, gender, and week of age. Means were compared by LSD (Least Significant Difference test) and statistical significance was assessed at  $P < 0.05$ .

### III. RESULTS

Table 1 shows the increase in body weight up to 6 weeks of age. Both gender and strain had a significant effect ( $P < 0.01$ ) on live weight at all ages (except gender body weight at 0 weeks of age). Between-strain differences ( $P < 0.01$ ) in live weight were first observed at hatching (Week 0) and persisted throughout the 6-week growth period. However, there was no consistency in strain performance in the first 4 weeks of age, as ranking of strains was changing. In the final weeks, one strain lagged behind four other strains ( $P < 0.05$ ). A significant ( $P < 0.05$ ) strain by gender interaction was found for body weight in Week 5 and 6. The maximum difference in live weight between strains changed from 8 and 10 grams for males and females in Week 4, to 10 and 15 grams at Week 5, and 8 and 12 grams at Week 6 respectively.

**Table 1 - The mean ( $\pm$ SE) live weight at 0, 2, 4, 5 and 6 weeks of age and the gonads weight of 6-week-old Japanese quails.**

Strain	Chick Gender	Live weight (g)					Ovaries/ Testes weight
		0 weeks	2 weeks	4 weeks	5 weeks	6 weeks	
1	Female	8.9 $\pm$ 0.07 <sup>c</sup>	72 $\pm$ 1.0 <sup>a</sup>	201 $\pm$ 1.9 <sup>bcA</sup>	243 $\pm$ 1.9 <sup>bA</sup>	280 $\pm$ 2.0 <sup>bA</sup>	8.6 $\pm$ 0.3 <sup>bc</sup>
2		9.0 $\pm$ 0.07 <sup>bc</sup>	76 $\pm$ 1.0 <sup>bA</sup>	197 $\pm$ 1.9 <sup>cA</sup>	235 $\pm$ 1.9 <sup>cA</sup>	274 $\pm$ 2.0 <sup>cA</sup>	9.3 $\pm$ 0.3 <sup>b</sup>
3		9.1 $\pm$ 0.07 <sup>b</sup>	74 $\pm$ 0.9 <sup>b<sup>a</sup></sup>	205 $\pm$ 1.8 <sup>abA</sup>	244 $\pm$ 1.8 <sup>bA</sup>	280 $\pm$ 1.9 <sup>bA</sup>	10.2 $\pm$ 0.3 <sup>a</sup>
4		9.0 $\pm$ 0.07 <sup>bc</sup>	76 $\pm$ 1.0 <sup>b<sup>A</sup></sup>	207 $\pm$ 1.9 <sup>aA</sup>	250 $\pm$ 1.9 <sup>aA</sup>	286 $\pm$ 2.0 <sup>aA</sup>	10.2 $\pm$ 0.3 <sup>a</sup>
5		9.4 $\pm$ 0.07 <sup>a</sup>	75 $\pm$ 1.0 <sup>b<sup>a</sup></sup>	202 $\pm$ 1.9 <sup>abcA</sup>	241 $\pm$ 1.9 <sup>bA</sup>	275 $\pm$ 2.0 <sup>cdA</sup>	8.0 $\pm$ 0.3 <sup>c</sup>
1	Male	8.7 $\pm$ 0.08 <sup>c</sup>	70 $\pm$ 1.0 <sup>b</sup>	189 $\pm$ 2.0 <sup>bB</sup>	221 $\pm$ 2.0 <sup>cB</sup>	241 $\pm$ 2.1 <sup>bB</sup>	5.3 $\pm$ 0.34
2		8.8 $\pm$ 0.08 <sup>b<sup>c</sup></sup>	71 $\pm$ 1.0 <sup>bcB</sup>	188 $\pm$ 2.0 <sup>bB</sup>	217 $\pm$ 2.0 <sup>cB</sup>	241 $\pm$ 2.1 <sup>bB</sup>	5.5 $\pm$ 0.34
3		9.3 $\pm$ 0.08 <sup>a</sup>	74 $\pm$ 1.0 <sup>a</sup>	196 $\pm$ 2.0 <sup>aB</sup>	225 $\pm$ 2.0 <sup>bB</sup>	249 $\pm$ 2.1 <sup>aB</sup>	5.2 $\pm$ 0.33
4		9.0 $\pm$ 0.08 <sup>b</sup>	71 $\pm$ 1.0 <sup>bcB</sup>	193 $\pm$ 2.0 <sup>aB</sup>	223 $\pm$ 2.0 <sup>bcB</sup>	247 $\pm$ 2.1 <sup>aB</sup>	5.6 $\pm$ 0.34
5		9.3 $\pm$ 0.08 <sup>a</sup>	73 $\pm$ 1.0 <sup>ac</sup>	195 $\pm$ 2.0 <sup>aB</sup>	227 $\pm$ 2.0 <sup>aB</sup>	247 $\pm$ 2.1 <sup>aB</sup>	5.5 $\pm$ 0.34

<sup>a-d</sup> Means without a common superscript within a gender specified column (strain body weight) differ ( $P < 0.05$ )

<sup>A-B</sup> Means without a common superscript in columns (gender body weight with in strain) differ ( $P < 0.05$ )

Between-gender difference was not significant at hatch ( $P > 0.05$ ). From Week 2, the females were significantly heavier than males ( $P < 0.001$ ). The difference increased from 6 gram at Week 2 to 45 grams at Week 6. From Week 4, differences in growth were mainly due to higher female growth.

The mean testes weight between strains ranged from 5.2 to 5.6g but no significant differences were found between strains ( $P > 0.05$ ). However, ovary weight differed significantly ( $P < 0.05$ ) between strains, which ranged from 8.0 to 10.2 grams .

#### IV. DISCUSSION

The pattern of growth observed in this study is consistent with that reported for meat-type quails selected for high body weight (Aggrey et al., 2003; Reddish, 2003; Hyankova and Novotna 2007). In this study, significant differences in live weight (between strains) were first observed at hatch and persisted throughout the 6-week growth period. The live weight changes show the highest growth rate in the first 4 weeks of age, especially between 2 and 4 weeks and a reduced rate of growth thereafter although chicks kept gaining weight up to 6 weeks of age. This increase in live weight occurred mainly in females, which resulted in females having a 20% higher live weight than males at 6 weeks of age.

The greater variability in ovary weight (CV = 58%) compared to testes weight (CV = 25%) supports the view that a considerable proportion of females would not have reached maturity at 6 weeks of age. This corroborates other reports (Reddish, 2003; Hyankova and Novotna 2007) that have shown that females tend to reach sexual maturity at about 7 weeks of age.

The between gender differences in the attainment of sexual maturity and reaching final body weight may have two major implications for the breeding flocks. i) Males attaining sexual maturity earlier (5 weeks of age) will start mating; consequently more energy is expended in activities associated with reproduction rather than increasing body reserves which may be utilized in the later stages. Additionally, early attempts at mating with females before they reach sexual maturity may mean sexual harassment and increased stress levels, resulting in poor growth and reproductive output. Sexual harassment by males during the mating has already been reported to lower the probability of laying fertile eggs by the females (Persaud and Galef 2005). Moreover, where females are heavier, their dominance over males may cause injuries to males. ii) Higher body weight differences between male and female at

sexual maturity may reduce the ability of the male to copulate and fertilize eggs successfully (which stress the optimization of body weight difference between the genders to attain mating success in the first step before any change in the growth pattern of males or females is introduced to the selection programs).

This study concluded that i) the pattern of sexual dimorphism for growth is the same as described for Japanese quail present elsewhere. ii) The between strain variations for 6 week body weight, and testes and ovaries weight, might be useful for breeding purposes to enhance the productivity of the breeding flocks.

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PROTECTION PROVIDED BY RISPENS CVI988 VACCINE AGAINST VERY VIRULENT MAREK'S DISEASE VIRUS CHALLENGE IN ISABROWN CHICKENS

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Summary

As part of a wider study into kinetics of vaccinal and wild type serotype Marek's disease virus (MDV) serotype 1 viruses in the same host, we tested the protection offered by the Rispens CVI vaccine to MDV challenge in Isa Brown chickens. Apart from the vaccine used, the protocol largely followed that of the Avian Diseases and Oncology Laboratory (ADOL) of the USDA. In a 2x3 factorial isolator experiment with two replicates we tested the effects of Rispens vaccination (vaccinated, unvaccinated) and MDV challenge (MPF57, FT158 at 500pfu/chicken, unchallenged) in 236 female Isa Brown chicks. Vaccination was at hatch, challenge at day 5 and the experiment duration was until 56 days post-challenge. The very virulent isolate FT158 induced a significantly higher level of mortality (33%) and incidence of MD (65%) than the virulent isolate MPF57 (10% and 39%). Rispens vaccinated challenged chickens had lower mortality (13%) and MD incidence (28%) than unvaccinated chickens (30% and 76%). The protective index provided by Rispens against FT158 (61%) did not differ significantly from that provided against MPF57 (66%). These results demonstrate that current Australian isolates of MDV can induce significant MD and mortality in Rispens vaccinated chickens at a vaccination challenge interval of 5 days. However the protective index is higher than reported for HVT and bivalent vaccine in previous experiments using the same virus (MPF57) and protocol in Isa Brown chickens.

I. INTRODUCTION

Marek's disease (MD) has been controlled since 1970 mainly by vaccines which consist either of attenuated oncogenic serotype 1 MDV (MDV) or the apathogenic HVT or serotype 2 MDV (MDV-2). The virulence of MDV has increased markedly since the 1950s (Witter, 1998) probably due to the effects of reduced chicken lifespan and vaccination with imperfect vaccines that do not prevent co-infection of the host with pathogenic MDV (Atkins et al, 2013). The increase in virulence over time has led to sequential failure of vaccinal control in the USA so it is important to monitor the vaccinal protection provided by current vaccines against the most virulent recent isolates of MDV. The most recent Australian isolates of MDV have been pathotyped using an adaptation of the pathotyping protocol of the USDA Avian Diseases and Oncology Laboratory (Walkden-Brown et al., 2007; Renz et al., 2012). This protocol, designed to be used in chickens containing maternal antibody directed against MDV, measures protection provided by HVT and bivalent HVT/MDV-2 vaccines and classifies MDVs into mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) pathotypes (Witter, 1997). However, Rispens CVI988 vaccine, the most effective current MDV vaccine that is used routinely to vaccinate all long-lived commercial chickens, has not been included in such experiments.

As part of a wider study into kinetics of vaccinal and wild type serotype Marek's disease virus (MDV) serotype 1 viruses in the same host, we therefore tested the protection offered by the Rispens CVI988 vaccine to MDV challenge in Isa Brown chickens using the adapted ADOL method in previous reports from our group (Walkden-Brown et al., 2007; Renz et al., 2012). The main hypothesis under test was that Rispens will protect against MD

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and that protection will be greater against MPF57 (vMDV) than FT158 (vvMDV) isolates of MDV.

## II. MATERIALS AND METHODS

The experiment used a 3x2 factorial design with two replicates. The experimental factors and levels of each factor were:

- 1) Challenge virus (3 levels): MDV isolates MPF57 and FT158, 500pfu per bird in 0.2 ml diluent injected subcutaneously. The unchallenged treatment groups received 0.2 ml diluent per bird only, subcutaneously at the day of hatch (day 0).
- 2) Vaccine (2 levels): Rispens vaccine (Vaxsafe® RIS Vaccine, Bioproperties, Ringwood, Vic), 4000 pfu per bird in 0.2 ml diluent injected subcutaneously. Unvaccinated treatment groups received 0.2 ml diluent per bird subcutaneously. Chickens were challenged at 5 days of age.

The experimental chickens comprised 236 female Isa Brown chicks purchased from a commercial hatchery at hatch. Chicks were not vaccinated at the hatchery, but came from parents vaccinated against MD with the Rispens CVI988 vaccine so contained heterologous maternal antibody for MDV. They were placed in 12 positive pressure isolators with approx. 20 birds per treatment combination. The experiment started on the day of hatch (day 0) and was terminated at 61 days of age (56 days post challenge, dpc). Chickens were maintained at the isolator facility at the University of New England (UNE), Armidale and the experiment was approved by the UNE Animal Ethics Committee (AEC No. UNE 12-020). The challenge viruses were grown and titrated at UNE in chick embryo fibroblasts (CEF). Batch numbers were FT158\_P4021209 and MPF57\_P4181109 respectively. CEF were inoculated with splenocytes from SPF chickens challenged with low passage virus and the isolates titrated for pfu after passage 4. Virus origins are reported by Renz et al. (2012).

On 14 day post challenge (dpc), 5 birds from each isolator (10/treatment) were sacrificed and body weight and the weights of the bursa of Fabricius and spleen were recorded. The thymus and bursa were also scored subjectively for atrophy. All chickens that died or were euthanised during the experiment were examined post mortem for gross MD lesions and thymic and bursal atrophy. Chickens were classified as MD positive when a visible MD lymphoma was present. All remaining chickens were sacrificed at 56 dpc and similarly examined.

The protective index (PI) provided by vaccination was calculated as:

$$= \frac{\%MD \text{ in unvaccinated chickens} - \%MD \text{ in Rispens vaccinated chickens}}{\%MD \text{ in unvaccinated chickens}} \times 100$$

Mortality patterns were investigated using survival analysis (Kaplan-Meier product-limit method) and the Chi square test of independence. The latter was also used to compare the ratio of MD positive and negative chickens between groups. Treatment effects on liveweight, immune organ weights and protective index were investigated by ANOVA. A statistical significance level of  $P < 0.05$  is used throughout. Statistical analyses were performed using JMP 10 (SAS Institute Inc., Cary NC, USA).

## III. RESULTS

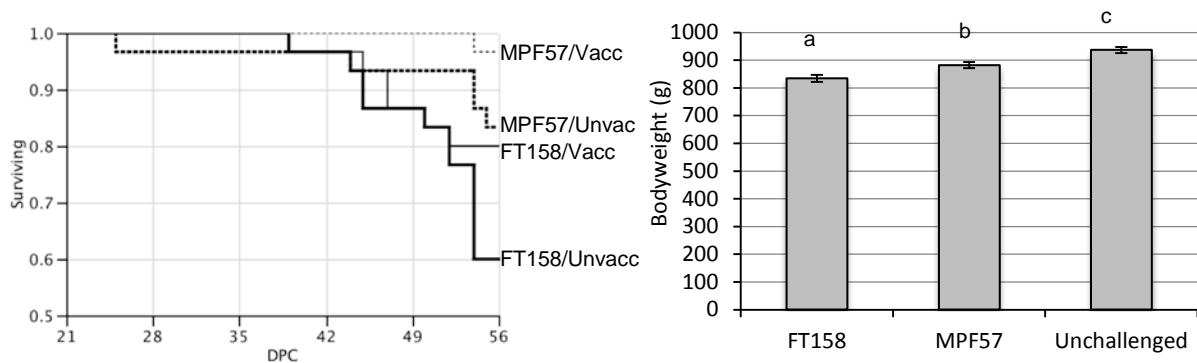
Mortality by treatment and overall incidence of MD are summarized in Table 1 and Figure 1 shows the survival curve of chickens in the challenged treatments. There was no mortality or MD lesion in the unchallenged treatments. The first mortality was at 25 dpc. All subsequent mortality was associated with the presence of MD lesions and commenced at 39 dpc. Overall

mortality was higher in chickens challenged with FT158 (33%) than MPF57 (10%) ( $P = 0.002$ ). In challenged chickens it was also higher in unvaccinated (30%) than vaccinated (13%) ( $P = 0.002$ ). Similarly the incidence of MD lesions was higher in chickens challenged with FT158 (65%) than MPF57 (39%) ( $P = 0.005$ ) and higher in unvaccinated (76%) than vaccinated (28%) ( $P < 0.0001$ )

**Table 1 - Mortality and incidence of MD in the experiment**

Treatment	Numbers and percentages of affected chickens		
	Mortality	Mortality with MD	MD lesions
FT158/Unvacc	13/30 (43%)	13/30 (43%)	28/30 (93%)
FT158/Vacc	7/30 (23%)	7/30 (23%)	11/30 (37%)
MPF57/Unvacc	5/30 (17%)	4/30 (13%)	17/29 (59%)*
MPF57/Vacc	1/30 (3%)	1/30 (3%)	6/30 (20%)
Vacc/Unchall	0/26 (0%)	0/26 (0%)	0/26 (0%)
Unvac/Unchall	0/30 (0%)	0/30 (0%)	0/30 (0%)
Overall	26/176 (15%)	25/176 (14%)	62/145 (43%)

\*One chicken excluded as it died early due to a clearly non-MD related cause.



**Figure 1** - Effect of challenge virus and vaccination on chicken survival (Left,  $P = 0.005$ ) and on bodyweight of surviving chickens at 56 dpc (Right,  $P < 0.0001$ ). No unvaccinated or Rispens vaccinated control chickens died during the experiment.

The mean protective index provided by Rispens vaccine against FT158 (61%) did not differ significantly from that provided by MPF57 (66%).

At 14 dpc, the mean body weight was  $189.3 \pm 15.3$  g with no significant effect of vaccination ( $P=0.45$ ) or challenge ( $P=0.17$ ). At 56 dpc body weight was significantly influenced by vaccination ( $P < 0.0001$ ) and challenge ( $P < 0.0001$ ; Figure 1). Relative bursal weight (% of bodyweight) at 14dpc was higher in vaccinated ( $0.45 \pm 0.01$ ) than unvaccinated ( $0.37 \pm 0.01$ ) but there was no effect of challenge ( $P=0.48$ ). There was no significant association between mean relative bursal weights at 14 dpc and MD incidence at 56 dpc ( $R^2 = 0.14$ ,  $p=0.21$ ). At 14 dpc relative spleen weight was higher in unvaccinated ( $0.20 \pm 0.006\%$ ) than vaccinated ( $0.17 \pm 0.006$ ) and higher in challenged ( $0.21 \pm 0.008\%$ ) than unchallenged ( $0.15 \pm 0.008\%$ ) birds. There was a significant positive association between mean relative spleen weights at 14 dpc and MD incidence at 56 dpc ( $R^2 = 0.76$ ,  $p=0.0002$ ).

## IV. DISCUSSION

Australian layers and breeders have been vaccinated with Rispens CVI988 since 1997 to provide long-term protection against MD. Despite Rispens CVI988 being the superior vaccine used worldwide against MD, this study has shown that Australian pathogenic MDV1 isolates have the capability to overcome the protective effects of Rispens vaccination when challenge is 5 days after vaccination. This was particularly evident with the vvMDV isolate FT158 which induced 23.3% MD mortality in the Rispens vaccinated groups. Nevertheless the protective index provided by Rispens did not differ between the two MDV isolates because of the lower level of MD induced in unvaccinated chickens by the vMDV MPF57. Thus our hypothesis of better protection against vMDV than vvMDV is not supported. This finding lends further support to our previous observations that virulence and vaccine resistance are different traits, with the ADOL method of pathotyping being more a measure of the latter than the former (Walkden-Brown et al., 2007; Renz et al., 2012).

In a similar pathotyping experiment in Isa Brown chickens MPF57 induced a similar level of MD in unvaccinated chickens (69% cf. 59% in the present experiment) but the protective indices provided by HVT vaccine (35%) and bivalent HVT/MDV2 vaccine (59%) were numerically although not statistically lower than the PI of 66% observed in the present experiment (Walkden-Brown et al., 2007). This suggests superior but not absolute protection provided by Rispens at a vaccination challenge interval (VCI) of 5 days. In a more recent experiment in Isa Brown chickens challenged with another vvMDV isolate (02LAR), Islam et al. (2013) reported a protection index provided by Rispens of 85% at a VCI of 5 days, rising to 100% at a VCI of 10 days. This suggests that the standard VCI of 5 days used in the ADOL pathotyping model may not allow full expression of protection provided by the Rispens vaccine.

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