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Enquiries regarding the Proceedings should be addressed to:

The Director, Poultry Research Foundation
Faculty of Veterinary Science, University of Sydney
Camden NSW 2570

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THE SIGNIFICANT CONTRIBUTION OF JOHN L. BARNETT TO POULTRY WELFARE RESEARCH

G.M. CRONIN[1], P.C. GLATZ[2] and P.H. HEMSWORTH[3]

Summary

Associate Professor John Lawrence Barnett was an internationally-respected animal welfare scientist. This small tribute attempts to put into perspective the significance of John's contribution to the welfare of commercial poultry, and farm animals in general. John studied zoology in the United Kingdom after which he undertook post-graduate studies in Australia on the physiological and endocrine responses of birds and mammals to stressors. He was a strong advocate of a multidisciplinary approach to the scientific study of animal welfare. Throughout his career, John applied his broad scientific knowledge and skills to improve our understanding of farm animal welfare and developed new methodologies to study animal welfare. John was a keen science collaborator and communicator, with a strong commitment to building inclusive discussions between the different stakeholder groups interested in poultry welfare. His contribution to the scientific and technical literature on poultry welfare is outstanding.

I. INTRODUCTION

The tragic loss of Associate Professor John Barnett and his wife Jenny Barnett in the Victorian bush fires on February 7th 2009 deeply affected all those who knew and loved them. John was a true gentleman, a brilliant scientist, a world leader in the field of animal welfare science and, above all, a true humanitarian.

John's main area of expertise was the physiological and endocrine responses of mammals and birds to stressors, which he effectively applied to the study of domestic animal welfare. This research over 30 years provided a timely balance on discussions within science and the livestock industries on welfare methodology and interpretations. This impact will continue to improve animal welfare methodology in the future. John's research on poultry (and pigs) has also made a critical contribution to our understanding of the welfare risks associated with confinement housing, highlighting the major risks of confinement that arise from spatial and social restriction. He worked closely with the livestock industries to develop welfare components of livestock industry QA programs. This has led to improvements in awareness and practices to safeguard animal welfare standards. His outstanding scientific efforts have been highly acclaimed nationally and internationally by both science and the livestock industries.

John was a major contributor to the annual Australian Poultry Science Symposia and triennial Australian Poultry Conventions. He was also an active contributor to symposia and meetings of the WPSA European Federation Working Group 9 on *Poultry Welfare and Management.* He led the Australian Poultry Cooperative Research Centre's Welfare program from 2003 to 2009. John was also a major contributor to the Animal Welfare Science Centre's research and teaching programs at the University of Melbourne, Monash University and the Victorian Department of Primary Industries.

[1] Faculty of Veterinary Science, The University of Sydney greg.cronin@sydney.edu.au
[2] SARDI, Pig and Poultry Production Institute phil.glatz@sa.gov.au
[3] Animal Welfare Science Centre, The University of Melbourne phh@unimelb.edu.au

II. POULTRY WELFARE RESEARCH HIGHLIGHTS

A review of John Barnett's bibliographical output (see below) reveals he was a strategic and visionary thinker. John identified poultry welfare issues and applied his skills and those of collaborators to investigate the issues. He exercised a high degree of rigour in his approach to animal welfare science using well-designed, multidisciplinary experiments that provided scientists, industry and the community with evidence-based information on the extent (seriousness) of the particular issue(s). John's research combined a fundamental approach to science investigation with applied research to build the knowledge base in order to resolve practical welfare issues in the poultry industry.

John Barnett's research, however, did not remain "gathering dust on the shelf". John extended the knowledge generated through experimentation, from his own work and that of many other poultry scientists, through regular communication with industry groups. Perhaps one of John's most important achievements was the participatory approach he developed and refined for building conciliatory discussions between the different stakeholders interested in poultry welfare. Through this approach, John ensured that the range of stakeholder groups in the poultry welfare debate could voice their concerns and express other viewpoints. During this participatory process, John infused evidence-based information on poultry welfare. John was also a pioneer in the development of industry QA programmes to monitor and facilitate continuous improvement in animal welfare. A significant ancillary benefit for poultry producers was the comprehensive volume of practical information, presented in layman's terms, to support the QA documentation, thereby providing clear guidance and logic for sometimes contentious housing and husbandry procedures.

III. VALE JOHN LAWRENCE BARNETT

John Lawrence Barnett was a humble but active contributor, who provided wise counsel on matters of science as well as life. His important contributions to animal welfare science will be sadly and irreplaceably missed.

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CRUDE PROTEIN "REQUIREMENT" AND MAINTENANCE OF THE INTESTINE

E. T. MORAN, JR[1]

Summary

Reduction in feed crude protein (CP) usually follows with dietary inclusion of free essential amino acids (EAA) as a direct means to attain requirements.Although bird EAA needs are assured, reductions in CP eventually impair performance. Recovery can largely be attained by concomitantly increasing the levels of glycine-serine and proline. Not only are these conditional non-essential (NEAA) difficult to form *de novo* but prominent in mucin released during small intestinal transit. Membrance associated mucin (glycocalyx) with enterocytes and goblet cell secretory mucin co-operate to assemble the unstirred water layer (UWL) which acts as a molecular filter of pancreatic digesta accessing the microvilli surface. Large proportions of saccharides O-glycosylated to serine and threonine sterically inhibit mucin proteolysis while glycine and proline give its elastomeric character. The UWL not only protects the surface but associated sugar amination and sulfation act to stabilize the microenvironment. A pH approximating 6.5 appears to facilitate the most favorable nutrient form for membrane transfer. Given the continuous net loss of constituents associated with mucin, component sourcing for its replacement likely dominates mucosa maintenance. Vascularization of the villus is structured for immediate access of post-absorptive nutrients to cells at the top which diminishes with flow in the lamina propria to the portal system. While mucosal EAA needs are implicit in measurement of the bird's requirement, availability of NEAA in proportionate amounts for direct inclusion in mucin facilitates its resynthesis, particularly for serine, glycine and proline. Glutamine is central to N-glucosamine formation and ultimately sialic acid, thus, its presence in appropriate amounts would also be complementary to mucin replacement. Providing the array of NEAA commensurate with mucin loss when superimposed on needs for EAA is the central hypothesis for a CP "requirement". Mucin/endogenous N loss can vary with lumen conditions; in turn, CP level asssociated with the feed is expected to follow these changes in order to accommodate the variance in maintenance of the mucosal surface.

I. INTRODUCTION

The NRC (1994) indicated that the CP levels given were not to be considered as a requirement of the bird in question but to indicate an amount of N that would be sufficient to form all necessary NEAA. Inclusion of purified forms of each limiting EAA in attaining requirement levels enables a progressive reduction in the feed's CP content. A "significant" reduction in CP while maintaining EAA and all other nutrients at their minimal needs invariably leads to a loss in live performance. The only known study conducted with laying hens was a short term experiment performed by the author several years ago for another purpose and remains unpublished (Table 1). A small number of Single Comb White Leghorn hens at 28 weeks of age individually selected to be at maximal production were compared when given a 16% CP corn-soybean meal fully adequate feed with one at 12% CP that contained extensive purified EAA to assure requirements. Although both feeds were calculated to be nutritional equivalent, birds receiving the low CP for four subsequent weeks

[1] Poultry Science Department, Auburn University Al 36849 USA

decreased their egg production and lost body weight while eggs also lost weight mainly because of decreased yolk.

Considerable research has been accomplished using the growing bird on reducing CP all resulting in decreased performance and adverse effects on carcass quality. The most recent reports of Delange (2009) and Pesti (2009) indicate that there is no point at which changes become apparent but a continuum occurs without definition. Generally, attempts at fully assuring adequacy and balance of all EAA while maintaining low CP have not been fruitful. Similarly, the inclusion of easy to synthesize NEAA such as glutamic and aspartic acids to increase CP has also been of negligible value (Moran and Stilborn, 1996; Kerr and Kidd, 1999; Bregendahl et al., 2002). However, supplementation of glycine-serine where *de novo* formation is difficult has proven to be of frequent benefit (Schutte et al., 1997; Dean et al., 2006; Heger and Pack, 1996; Waguespack et al., 2009; Berres et al., 2010).

Table 1 Response of laying hens to reduced CP while maintaining EAA needs (28-32 weeks)[1]

		LIVE PERFORMANCE		
Corn-Soy CP	% Production	g Feed Cons/ Day	g BW Change	g Liver / Hen
16% w/ DLM	88.6	109	+4	38
12% w/ EAA	81.9	109	-64	38
P<	0.05	NS	0.01	NS
		EGG		
Corn-Soy CP	g Weight	g Yolk	g Albumen	g Shell
16% w/DLM	58.1	16.1	36.3	5.8
12% w/EAA	57.1	15.3	35.9	5.9
P<	0.05	0.05	NS	NS

[1] Total of 180 SCWL birds selected for equivalent high rate of lay. Feeds calculated to have 2.89 kcal/g and environmental temperature *ca*. 20 C (Moran, unpublished results).

II. COMBINING CONDITIONAL NEAA

Although the reduction in Low CP with feeds occuring with inclusion free EAA usually decreases the full array of NEAA, resulting change in feedstuffs employed is such that the conditional NEAA decrease to a greater extent than those easily formed. Allen and Baker (1974) evaluated many nonspecific N sources in their ability to improve performance of chicks fed purified EAA diets, and only glycine and proline were distinctively better than glutamic acid. Lehman et al. (2009) included gelatin into corn-soybean meal feeds with the broiler such that the levels of glycine-serine and proline increased at low CP to correspond their occurrence when CP with the control corn-soybean diets followed the NRC (1994) recommendation (Table 2). Inclusion of gelatin improved the feed conversion at low CP to agree with that obtained with the control CP having only corn and soybean meal while carcass abdominal fat decreased. The implication is that the loss in conditional NEAA occurring when CP is reduced could not meet the needs to optimize performance.

Table 2 Inclusion of gelatin in feeds to increase glycine-serine and proline, g/kg[1]

Nutrient	Corn-Soy-High CP		Corn-Soy-Low CP	
		0 – 3 Weeks		
CP	230	w/20g Gelatin	210	w/20g Gelatin
Gly + Ser	21.7	24.5	19.4	22.9
Proline	12.6	13.9	11.7	12.4
		3 – 6 Weeks		
CP	200	w/20gGelatin	180	w/20g Gelatin
Gly + Ser	19.6	21.5	17.3	19.8
Proline	11.9	12.8	10.8	12.2
		6 – 8 Weeks		
CP	180	w/20gGelatin	160	w/20g Gelatin
Gly + Ser	17.9	20.2	15.5	18.4
Proline	11.5	12.6	10.2	11.7

[1] 2% gelatin when added involved only corn-soybeam meal combination in feeds having 3.2 kcalME/g. Values were obtained from calculating contributions of analyses of individual feedstuffs. (Lehman et al., 2009)

Table 3 Broiler response to low CP feed with added gelatin to increase conditional NEAA[1]

CP	gGelatin/kg Feed	g Feed Cons	g Wt Gain	F/G	gAbdom. Fat/kg Carc
High	0	3782	6835	1.82	22.8
	20	3787	6931	1.84	25.1
Low	0	3740	7002	1.88	23.7
	20	3735	6770	1.82	21.8
SEM		19.7	77.2	0.017	0.061
CP		P < .05	NS	NS	P < .05
Gelatin		NS	NS	NS	NS
CP X Gelatin		NS	P < .05	P < .05	P < .01

[1] Ross X 708 Males, total of 64 pens of 20 birds grown 0-56 days of age at *ca*. 20 C. (Lehman et al., 2009)

III. NEAA AND MUCOSAL SURFACE

The particularly favorable response of broilers receiving low CP feed to the full array of NEAA where de novo formation is difficult but not those easily transaminated suggests a close and immediate involvement of structural glycoproteins. Birds have an endogenous ileal loss containing substantial amounts of NEAA, particularly glycine, serine and proline (Ravindran and Hendriks, 2004). Mucin loss from the small intestinal mucosa is highly representative in animal endogenous N (Montagne et al., 2000), and inclusion of gelatin in chick feed has been shown to enhance its early development (Fasina et al., 2007). Studies on absorption of all NEAA with swine have demonstrated a minimal entry into the portal system because of an extensive “consumption” by the surface (Pierzynowski and Sjodin, 1998; Lambert et al., 2006; Bertolo and Burrin, 2008) which is consistent with synthesis of mucin with its return to the lumen.

Understanding the mucosal surface and mucin's function is central to rationalizing need and function of its associated NEAA. Finalization of digestion-absorption is confined to the upper villus where a mosaic of enterocytes and goblet cells are mutually dependent. Microvilli greatly expand the enterocyte's surface while associated contractile elements facilitate convective exposure. Enzymes such as sucrase, maltase and peptidase anchored in the membrane create concentrated products capable of immediate transfer. Active transport, H^+ facilitated transport, and passive movement all exist as appropriate for the nutrient. These activities would be open to ready conflicts from the lumen except for protection by mucins. Essentially, two types of mucins provided by the surface cells co-operate to create the unstirred water layer (UWL). Microvilli have membrane associated mucins (glycocalyx) that protrude as a fibrous network from the apex. Simplistically, each glycocalyx fiber is anchored in the membrane and linearly projects multiple "bottle brush like" domains defined by considerable O-glycosylated short carbohydrate chains to threonine and serine while glycine and proline provide elastomeric character. Secretory mucin is released from goblet cells and has similar bottle brush domains, instead of being linear each is interconnected using cystine to create the equivalent of a free floating net (Bloomfield, 1983). "Entanglement" of this net with the projecting glycocalyx fibers leads to the UWL which acts like a molecular filter between the lumen and microvillus surface. The overall strategy with small intestinal digestion is for the pancreatic enzymes to reduce foodstuffs into small units capable of filtering through the UWL for finalization of digestion. Concurrently, surface enzymes are protected from proteolytic destruction while the products are inaccessible to lumen microbes.

IV. MUCOSAL MICROENVIRONMENT

The UWL also appears to provide a stable microenvironment that optimizes the terms for enzymic digestion and molecular form transversing the membrane. Sugars O-glycosylated to mucin approximate 6-8 units in length and vary with fucose, galactose, N-glucosamine, N-galactosamine, N-acetyl-glucosamine, N-acetyl-galactosamine, N-acetyl-neuraminic acid (sialic acid), and galactose-6-sulfate being among the possible participants. Not only do these chains sterically hinder proteolysis of the underlying protein chain but their side groups actively participate in stabilizing pH of the microenvironment (Shiau et al.,1985). Actual measurements of the human USWL indicated a consistent pH~6.5 at the upper villus where active digestion-absorption occurs (Daniel et al.,1985). Changes in the nature of mucin as food consumed changes (More et al.,1987; Sharma et al., 1997) and location along the tract ((Pastor et al., 1988) to suggest that goblet cells can alter the constituent sugars as mucin is produced in order to maintain consistency of pH. Of particular importance, is the glutamine:fructose-6-phosphate amidotransferase as the first and rate limiting step in formation of glucosamine-6-phosphate and all other amino sugars that follow (Li et al., 2007; Durand et al., 2008).

Prolamines in all grains are major contributors of glutamine to feed with 15-20% being provided by zein in corn (Wilson, 1987). Resulting glutamic acid may be used for transamination and formation of other NEAA (Volman-Mitchell and Parsons, 1974; Kight and Fleming, 1995) with the α-keto acid being a major source of energy for the enterocyte (Porteous, 1980; Rhoads et al., 1992; Duee et al., 1995; Wu et al., 1995; Wu, 1998). The advantage of dietary glutamine to mucosal integrity is well established among animals (Tanabe et al., 1963;Yi et al., 2005; Murakami *et al*., 2007; Zavarize *et al*., 2008) while representing a substantial amount of dietary NEAA.

Consistency of pH approximating 6.5 in the UWL would appear to enhance the overall digestive absorptive process. Products originating from pancreatic action and chaotic pH in the lumen subsequently assume a favorable "presentation" upon entering the UWL.

Events in the digestion of protein from pepsin through trypsin, chmotrypsin and the carboxypeptidases ultimately lead to EAA being in "free" form and actively transported while the NEAA end as peptides where their absorption is energized by a "proton gradient" paralleing the microenvironment (Kan, 1974; Ganapathy and Leiback 1985; Webb, 1990; Kull, 1991). Given their pK values, products of lipid digestion and organic acids have minimal charge conflict and may avail themselves of Na^+ for membrane transit (Wolffram et al., 1992). Furthermore, villi enterocytes not only have the ability to modify surface enzymes finalizing digestion according to need (Kushak et al., 1981; Ozols and Sheshukova, 1984), but these enzymes near maximize their activities corresponding to the microenvironment pH (Mizuno et al., 1982; Jamadar et al., 2003). Thus, maintaining the extent and integrity of the UWL is of primary importance to nutrient recovery from the small intestinal lumen.

V. MUCIN TURNOVER & CP

Mucin lost from the surface can vary markedly. Lehr et al. (1991) estimated the rat's mucosal mucus gel layer to have a turnover time in the range of 47 to 270 minutes. The low value is envisaged to represent the secretory mucin that is released in free form and frequently from goblet cells, whereas the high value is more likely to be membrane associated mucin of the enterocyte's glycocalyx and more refractory to loss. Overall ability of the pancreatic enzyme complement to erode mucin is expected to be far less than capabilities of microflora within the lumen. Conventional birds have greater villus area and cellular migration rate than if germ-free that demands corresponding additional mucin (Cook and Bird, 1973; Muramatsu et al., 1987). Goblet cells alter the nature of mucin in response to microbial load (Forder et al., 2007); however, rate of mucin degradation appears to be limited until entry into the large intestine and anaerobic terms necessitate access to the "bottlebrush" CHO complex (Salter and Fulford, 1974; Parsons et al., 1983). Increased microbial population, reduced partial pressure of oxygen from mucosal transfer, and facilitation of facultatives contribute to the adverse effects of feedstuffs creating viscosity of lumen contents. *Clostridia* are opportunistic in this respect while having the fucosidases, neuraminidases and proteases to collapse mucin structure (Wold et al., 1974; Chow and Lee, 2007; Olkowski et al., 2008), particularly with surface disruptions created by coccidia (Baba et al., 1997; Pederson et al., 2008). The shift from neutral to acidic type mucin by goblet cell when confronted by *Clostridium perfringens* can be interpreted as an attempt to maintain the most favorable pH of a compromised microenvironment (Golder et al., 2010). Decreasing the level of CP concurrent with mucosal assaults arising from an adverse microbial population measurably accentuates the complications (Welch *et al*., 1986; Waldenstedt et al., 2000; Drew et al., 2004; Dahiya et al., 2007).

Relative inability to recover the components comprising mucin originating from either extruded cells or eroded from the surface necessitates its complete regeneration using "new" nutrients. Villus vascularization is such that the "ease" of mucin replacement is dependent on the nutrients absorbed at that time and restricted to the immediate area. Essentially, the mesenteric arteriole terminates at the villus apex then it subdivides into many venules that decend within the lamina propria adjacent to all surfaces (Aharinejad et al., 1991). Oxygen immediately supports active absorption at the top then recovered nutrients may be employed for cell differentiation and repair with their progression to the portal system at the base. Threonine invariably represents the most limiting EAA and variation in its measurement of need follows microbial load and mucin dynamics (Kidd et al., 2003; Horn et al., 2009). Although all NEAA can be synthesized, their access in direct form and proportions releives the cells in obtaining a source of N, delayed presence and extended work to do so. Absence of glucosamine and other sugar amines also dictates a need for NEAA, specifically

glutamine. Crude protein requirement likely represents need by the mucosa for NEAA and immediate formation of mucin. Once formed, mucin enters the lumen and ultimately becomes part of the endogenous N loss. Kamisoyama et al. (2010) measured the true amino acid digestibilities (TAAD) of adult roosters given low through progressively increasing levels of CP. Reductions of TAAD for aspartic acid, threonine, glutamic acid, proline, glycine, valine, methionine, and isoleucine were observed at the mid-jejunum for the low CP feed that progressively increased with CP level. Prioritization of mucin formation and maintenance of the mucosa were likely the basis of decreases in amino acid "availability" when CP was low rather than inadequate digestion. Corrections using endogenous N loss include all amino acids associated with mucin. These amino acids have been "productively used" to maintain the mucosa; thus, their inclusion for "correction" with other N sources that are undigestible seems inappropriate to the objective.

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CORRELATIONS BETWEEN VARIABLE BROILER PERFORMANCE AND GENE EXPRESSION AND MICROFLORA IN THE GUT

R. J. MOORE[1], D. STANLEY[1], B.M. KONSAK[1], V.R. HARING[1], R.J. HUGHES[2,3], M.S. GEIER[2,3], and T.M. CROWLEY[1]

Summary

Within any flock of growing broilers there is variation in bird performance, whether measured by weight gain, feed conversion, or efficiency in the extraction of metabolisable energy from feed. From the poultry producers point of view, it is desirable to minimise such variation. Efforts to reduce variation can be purely empirically based or can be investigated by studying the underlying causes of variability, in the expectation that they can then be monitored or manipulated. Recent technological advances offer new and more detailed ways to investigate some of the possible influences on growth performance and efficiency of energy usage. We have used next generation DNA sequencing of microflora 16S rRNA genes to comprehensively characterise the structure of the bacterial populations in the gut and demonstrate that the presence or absence of certain bacteria is correlated with bird performance. Similarly, we have also applied high density microarray analysis to investigate gene expression in gut tissue and draw correlations between the expression of certain genes and bird performance.

I. INTRODUCTION

Variability in broiler bird performance can cause management issues for producers. Cost savings could be made if all birds grew at an even rate and used energy equally efficiently. Variation in performance is seen despite all birds within a flock being from the same genetic line, being in the same environmental conditions and having ready access to the same water and feed. With these external factors well controlled, we have looked to internal factors, gene expression and microflora in the gut, to find correlations with bird performance. Obviously, there are other influences that would be worth investigating, for example behavioural differences, but they were outside the scope of these studies.

The efficient operation of the chicken gut is dependent on the structural and functional properties of the gut, which in turn are determined by the metabolic activity of the tissue, which is further dependent on the expression of genes. Thus, an analysis of the gene expression patterns seen in birds of variable performance may give insights into the underlying metabolic differences in the birds. Previous work has focused mainly on the study of a few gut enzymes, either by looking at gene expression or enzymatic activity (e.g. van Hemert et al., 2004; Iji et al, 2001). Such studies gave a limited snap shot of very specific aspects of gut function. There is value in obtaining a more global view of what is happening and to this end we have used microarray analysis to interrogate the expression of all genes and investigate differences in gene expression in birds of varying performance level.

It is becoming increasingly recognised that the microflora carried by animals has a large influence on their health and normal function. It is estimated that vertebrates carry 10 times more microbial cells in and on their bodies than their own cells. A new paradigm is emerging which views the animal and its microbial population as a coevolved superorganism with a large amount of signalling and cross-talk between animal and microbial cells. Evidence is mounting that microflora is intimately involved in the development and

[1] CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong VIC 3220
[2] SARDI, Pig and Poultry Production Institute, Roseworthy SA 5371
[3] School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy SA 5271

maturation of the immune system and it has even been demonstrated that the microflora can affect behaviour. Microflora is being intensively investigated to understand its role in obesity in humans (Turnbaugh and Gordon, 2009) and so it is reasonable to assume that it may play a significant role in the growth and development of chickens. The intestinal microflora of chickens is very complex with over 600 different bacterial species identified (Apajalahti et al., 2004). The microflora can have a beneficial or detrimental effect on the host. Beneficial microbes in the gut are involved in obvious processes such as making nutrients available to the host that the host's own biochemical processes are unable to provide. They have a role in both producing and breaking down xenobiotics and can exclude pathogens. The microflora can have a negative impact in a variety of ways, for example by using excessive amounts of energy, causing diversion of too much of the host's energy towards the immune system by inducing inflammatory responses, or can be pathogens producing frank disease symptoms.

Previous work, using first generation molecular methods such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE), has suggested that certain elements of the microflora may be correlated with differential bird performance when different diets are compared (Torok et al., 2008; 2010). We have used Next Generation Sequencing (NGS) methods to investigate, in much greater detail, the microflora present in birds at the extremes of the performance continuum when a single diet is used.

II. MATERIALS AND METHODS

a) Animal experiment

Male Cobb 500 broilers (Baiada Hatchery, Willaston, SA, Australia) were raised in a rearing pen in a temperature-controlled room. All birds were given *ad libitum* access to a broiler grower diet (Table 1) which met or exceeded National Research Council guidelines for broiler chickens (NRC, 1994).

Table 1 Chicken rearing diet composition

Ingredient	g/kg
Wheat	444
Barley	150
Peas	50
Soybean meal	170
Canola meal	100
Meat meal	32
Tallow	30
Limestone	10
Salt	3.5
Lysine HCl	2.5
DL-methionine	2.3
Threonine	0.7
Vitamin and mineral premix †	5

† Included xylanase and phytase enzyme products

At 13 days post-hatch, 96 chickens were transferred in pairs to 48 metabolism cages located in a temperature-controlled room (23-25°C). Birds were placed in pairs for an initial acclimation period to minimise stress associated with isolation. Birds continued to have free

access to food and water prior to, and during, the experimental period. On day 15, birds were placed individually in 96 metabolism cages.

Apparent metabolisable energy (AME) values were determined in a classical seven-day AME study between days 15-22. The first three days enabled the chickens to adapt to solitary confinement in the metabolism cages. During the following four days, all excreta were collected daily and dried at 80°C. Feed intake was recorded during the adaptation and collection phases of the study period. Dry matter (DM) contents of feed were measured. On day 22, all birds were weighed and were retained in individual cages until day 25. Feed conversion ratio (FCR; g feed eaten/g weight gain) was calculated for each bird and the 24 birds with the highest FCR and 24 birds with the lowest FCR were identified and sampled The performance data of these birds, when grouped according to FCR, are shown in Table 2A and the performance, when grouped on the basis of AME, is shown in Table 2B. Gross energy values of feed and dried excreta were measured with a Parr isoperibol bomb calorimeter and AME values (in MJ/kg dry matter basis) were calculated as follows;

$$AME_{diet} = [(GE_{diet} \times g \text{ feed consumed}) - (GE_{excreta} \times g \text{ dry excreta})] / g \text{ feed consumed} / DM \text{ feed}$$

From all birds, a 1cm segment of tissue from the midpoint of the duodenum was collected for RNA extraction and gene expression analysis. The contents of one caecum were collected for microbial analysis by high throughput DNA sequencing (Roche/454). A 5cm length of jejunum (directly distal to the segment collected for gene expression analysis) was rinsed gently in PBS and the mucosa was collected by gentle scraping with a glass slide. The mucosal scraping was collected for microbial analysis of mucosa-associated bacteria.

Table 2A Performance data from birds grouped for feed conversion ratio study

FCR Group	BWG (g)	FI (g/bird/day)	FCR (g feed:g gain)	AME (MJ/kg)
Good	530 ± 10	102 ± 2	1.34 ± 0.01	14.57 ± 0.04
Poor	479 ± 9***	104 ± 2	1.52 ± 0.01***	14.70 ± 0.10

Table 2B Performance data from birds grouped for apparent metabolisable energy study

AME Group	BWG (g)	FI (g/bird/day)	FCR (g feed:g gain)	AME (MJ/kg)
Low	508 ± 9	103 ± 1	1.43 ± 0.02	14.38 ± 0.04
High	501 ± 13	102 ± 2	1.43 ± 0.02	14.88 ± 0.07***

Data are expressed as mean ± SEM (n = 24 birds per group). *** indicates significant difference between high and low FCR birds ($p < 0.001$). BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; AME, apparent metabolisable energy.

b) Gene expression analysis

Gene expression was measured in duodenum samples. Total RNA was isolated from the tissue using a Meridian total RNA isolation kit (Cartagen), reverse transcribed into cDNA and indirectly labelled with Cy3 dye using the Nimblegen One-Color DNA Labelling Kit. Each sample was independently hybridised to a sub array on a custom designed Nimblegen 12x135K chicken high-density microarray. The microarray contained 2 or 3 independent probes for every gene in the chicken genome and each probe was present on the array in duplicate. The gene expression profiles were compared by grouping results into high and low

AME birds or good and poor FCR birds (n = 5 for each group) in order to define genes that were differentially expressed in birds at the distribution extremes of AME and FCR values.

After hybridisation and washing all arrays were scanned and gene expression signals captured using a laser scanner. NimbleScan Software (Roche) was used to extract and pre-process the data. Pre-processing consisted of background correction, normalisation, final summarisation and quality control and was performed using the Robust Multichip Average method (Bolstad et al., 2003; Irizarry et al., 2003 and 2004; Wu et al., 2004). This method is the preferred method for high-density oligonucleotide arrays as it puts each chip's values in the context of a set of similar values (Irizarry, 2002). Statistical tests were carried out using Genowiz Version 4.0.5.3 (Ocimum Biosolutions) to determine all genes that were differentially expressed (p = 0.01) between the groups under consideration, either high and low AME groups or good and poor FCR groups. Gene ontology and pathway analysis was also performed in Genowiz.

c) Microbial profiling

Total microflora DNA from gut samples, either caecal content or jejunum scrapings, was prepared using the method of Yu and Morrison (2004). The V2-V3 region of the 16S rRNA genes was PCR amplified using primers (forward primer, 5' AGAGTTTGATCCTGG 3'; reverse primer, 5' TTACCGCGGCTGCT 3') that also included sequences to facilitate the sequencing of products in the Roche/454 DNA sequencing system and sequence barcodes so that the products from each bird could be identified,. The amplified and barcoded 16S rDNA samples from each bird were pooled and sequenced using the Roche/454 FLX Genome Sequencer and the latest Titanium chemistry. The output sequence files were quality trimmed based on sequence quality scores, homopolymer presence, ambiguous sequences, primer mismatches, and sequence length, and then analysed using a number of publically available software packages including PyroBayes (Quinlan et al., 2008), Mothur v1.12 (Schloss et al., 2009), Qiime v1.0.0 (Caporaso et al., 2010) and ARB (Ludwig et al., 2004) and databases such as the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/), the greengenes database (http://greengenes.lbl.gov/), and the SILVA database (http://www.arb-silva.de/), to phylogenetically classify the sequences, at 97% similarity, to operational taxonomic units (OTUs – similar to species level assignment but encompasses taxonomic groups that have not been named or previously characterised). The bacterial classification system has the following hierarchy; phylum, class, order, family, genus, species.

III. RESULTS

a) Differential gene expression

The comparison of RNA populations in the duodenum of high and low AME birds found that approximately one third of the 32,566 chicken UniGenes represented on the whole genome array were differentially expressed at a statistically significant level (t-test, $P < 0.01$). A similar proportion of the genes were differentially expressed when comparing birds in the good and poor FCR groups. Further filtering of the results, to find genes that were differentially expressed at a two-fold or greater level, showed 1,372 genes more highly expressed in the good FCR group than in the poor FCR group and 231 higher in the poor FCR group; there were 2,403 more highly expressed in the high AME birds than in the low AME and 1,883 higher in the low AME birds. There was a large overlap in the set of genes that were two-fold more highly expressed in the high AME group and the good FCR group with over 90% of the genes up regulated in the good FCR group represented in the high AME group (Figure 1). Only a single up regulated gene was shared between the good FCR birds and the low AME birds. This indicates that the biological processes driving the birds towards good FCR and high AME are similar. The functional significance of the differentially expressed genes was investigated by determining which biochemical pathways the encoded

proteins were part of and what biological functions they were involved in. A wide range of biological processes and molecular functions were linked to the differentially expressed genes including those involved in carbohydrate metabolism, energy metabolism, lipid metabolism, amino acid metabolism, glycan biosynthesis, immune function and insulin signalling pathways.

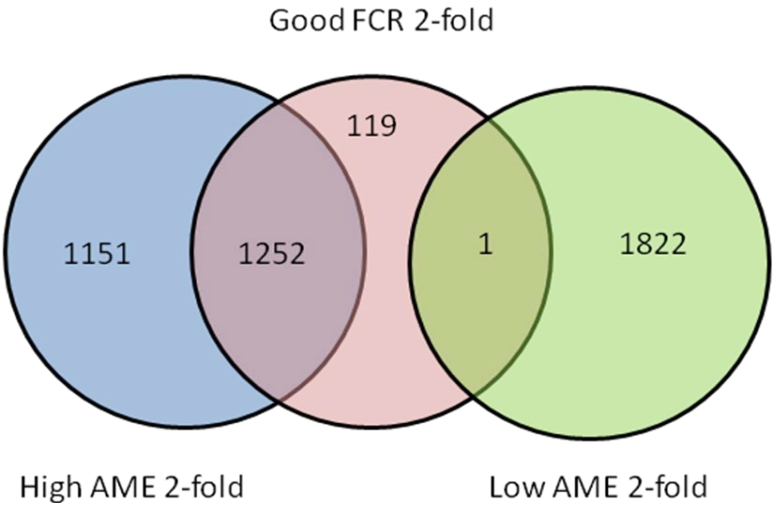

Figure 1 Venn diagram showing overlap in the gene sets up regulated in the good FCR birds compared with the high and low AME birds

b) Bacterial populations in the gut

The Roche/454 sequencing produced almost half a million sequence reads which, after quality trimming, gave approximately 10,000 reads from each bird sampled. The quality trimmed and filtered data revealed the presence of 746 OTUs at a 97% similarity level. The microflora in the jejunal mucosa was dominated by species of the genus *Lactobacillus.* In the FCR comparison, no statistically significant differences could be seen between the good and poor groups. However, the AME comparison revealed that the low AME birds carried more *Lactobacillus reuteri* than the high AME birds (p-value 0.007) and also indicated that *Lactobacillus johnsonii* is more abundant in the low AME birds (p-value 0.01).

The caecal bacterial populations were more complex with two dominant bacteria phyla; the Bacteroidetes represented by 14.3% of all sequences and the Firmicutes were present at 60.6%. 25.1% of sequences were unable to be assigned a phylum using the RDP classifier. Within the Bacteroidetes, most of the sequences could be classified down to the family level Bacteroidaceae. The Firmicutes were more diverse; approximately a third were of the class Bacilli, predominantly in the family Lactobacillus, and half were in the class Clostridia, distributed amongst the families Ruminococcaceae, Lachnospiraceae and unclassified Clostridia. A more detailed description of the Clostridia in the AME comparison is shown in Table 3. There was a statistically significant difference in the number of Clostridia sequences seen in the high and low AME birds with the high AME birds having on average well over twice as many Clostridia.

In the FCR comparison of caecal bacteria, 26 OTUs were differentially abundant between high and low FCR birds; most (22/26) were more abundant in the poor than in the good FCR birds and most (14/26) were in the class Clostridia or were unable to be classified (9/26).

Table 3 Analysis of sequences classified to the class Clostridia in six selected birds from each of the high and low AME classes.

Classification	Low AME average	Low AME SD	High AME average	High AME SD	T-test P-value
» » » » class "Clostridia"	3834.67	1831.16	9068.67	4402.66	0.03
» » » » » order Clostridiales	3399.50	555.08	8573.50	4142.16	0.05
» » » » » » family Incertae Sedis XIII	1.83	3.54	3.17	4.08	0.82
» » » » » » » genus Anaerovorax	1.67	2.83	2.67	3.30	0.91
» » » » » » family "Ruminococcaceae"	1281.50	818.69	2676.17	2196.80	0.20
» » » » » » » genus "Ruminococcaceae Incertae Sedis"	2.67	2.16	7.67	4.46	0.19
» » » » » » » genus Faecalibacterium	356.33	721.46	796.00	1683.15	0.57
» » » » » » » genus Anaerotruncus	56.33	71.20	88.17	119.21	0.76
» » » » » » » genus Subdoligranulum	79.67	144.31	132.00	137.59	0.68
» » » » » » » unclassified_"Ruminococcaceae"	784.33	494.88	1651.17	1045.33	0.11
» » » » » » family "Lachnospiraceae"	603.00	612.13	1867.00	1463.17	0.08
» » » » » » » genus Syntrophococcus	0.50	0.71	2.17	2.22	0.36
» » » » » » » genus Roseburia	10.83	6.52	81.00	84.09	0.12
» » » » » » » genus "Lachnospiraceae Incertae Sedis"	98.17	104.04	507.00	408.22	0.04
» » » » » » » genus Hespellia	2.00	2.00	3.83	2.28	0.33
» » » » » » » unclassified_"Lachnospiraceae"	485.67	556.54	1431.83	1150.29	0.11

The average numbers of sequences to a particular classification are shown as well as the standard deviation (SD) across each sample set. A Student T-test was performed to compare the high AME and low AME birds.

IV. DISCUSSION

Different cohorts of birds were ranked in the high and low groups when AME was used as the primary determinant for grouping compared with the good and poor classification when FCR was the primary determinant. When birds were ranked by FCR, the AME for the corresponding good and poor FCR birds was not significantly different. Likewise, when birds were segregated based on AME, there was no significant difference in FCR between high and low AME birds. The correlation between FCR and AME was poor ($r^2 = 0.04$, $p > 0.05$), this is likely to be due to the nature of these two parameters. AME is a measure of the energy available to the bird for metabolism, and is used for the evaluation of feedstuffs and subsequent feed formulation (Farrell, 1999). AME is the difference between the energy consumed via the feed and the energy lost via the excreta. This AME value is therefore indicative of the energy available to the bird for all necessary processes, including maintenance, energy requirements of the microbiota, and growth. AME however does not predict how effectively this energy will be utilised by the bird (Farrell, 1999), nor does it account for the quality of other essential components in the diet and their relative availability to the host. This can include amino acid profiles, vitamins and minerals. Therefore a high AME value for a bird may not necessarily indicate that the bird will perform to a high standard. Factors including intestinal villus/crypt structure, microbial composition and microbial activity can also influence AME (Hughes, 2001). Feed conversion ratio, however, is a direct indication of how efficiently a bird is converting feed consumed into body weight gained and is therefore a more accurate measure of bird performance. A bird with high AME and poor FCR indicates high energy expenditure in processes other than growth, whilst a bird with low AME and a good FCR indicates that the bird was effective at partitioning available energy towards growth and has lower energy expenditure in other areas.

An analysis of gene expression in the duodenum of birds at the extremes of both the AME and FCR distributions, showed that there are a large number of fairly subtle differences in gene expression. The differentially expressed genes encompassed a wide range of functions, mapping to many different biochemical pathways and cellular functions. Key genes involved in metabolism, nutrient and ion transport, growth, gut health and gut hormone action have been identified. No genes were highly differentially expressed (> 10-fold), suggesting that the differences seen are modulating the activity of pathways rather than turning whole pathways on or off. A large number of the genes expressed in the gut encode proteins involved in basic cellular growth and metabolism and have key roles in the ongoing rapid tissue renewal which is a prominent feature of the gut. In future analysis it may be useful to focus in more detail on those genes which encode proteins involved in direct nutrient scavenging and use. This would include sampling and analysing other organs, such as the pancreas and liver, which are producing key digestive enzymes.

Differences in gene expression may result from induction or repression, brought about by changes in the local cellular environment, or may result from intrinsic differences in expression of different alleles of the same gene. Allelic differences in expression are the result of sequence differences. The differential expression data could be used to direct the search for single nucleotide polymorphisms (SNPs) that are associated with genotypes that have favourable production characteristics.

The composition of the gut microbiota has been analysed by metagenomic analysis of 16S rRNA genes and has identified differences between the high and low AME birds and the good and poor FCR birds within the 746 OTUs (97% similarity) characterised in this study. In the caecum the class Clostridia bacteria are more abundant in the high AME birds and the poor FCR birds. These differences are due to a number of OTU groups of unknown species and also members of the genus *Lachnospiraceae*. The analysis of jejunum samples showed that *Lactobacillus* species dominate this niche. *L. reuteri* and *L. johnsonii* are more abundant in the low AME birds. These results lay a solid foundation for the ongoing analysis of the structure and function of the gut microbiota in nutritional studies. Our goal is to use such information to identify individual bacterial isolates or groups of isolates that could be targeted for use as inoculation cultures to help improve energy usage in birds.

We are currently working to determine which of the changes in gene expression and bacterial populations are reproducibly found across a series of similar trials. This will focus further studies on the most significant differences seen in differentially performing birds. Currently, the gene expression and microflora studies have been analysed as independent experiments but it will be important to perform a multivariate analysis to determine if there are correlations between the abundance of particular members of the microflora and differential expression of important genes. We anticipate that such interactions may strongly influence the function of the chicken gut.

The differences we have currently identified are interesting but it is not known whether these differences are responsible, at least in part, for driving the differences in performance or whether they are a consequence of the different levels of performance. The microflora of birds can be non-invasively characterised by analysis of the microflora present in faeces so we may be able to do prospective studies to determine if particular bacterial species that are differentially abundant (identified in the end point samples that we have analysed so far) are present before the performance differences are obvious or are only seen after the performance difference is established.

These technologies for genome wide expression analysis and deep and detailed microbial analysis offer new tools to examine aspects of bird biology. Application of these tools can help direct efforts to formulate better feeds and select birds capable of more even and perhaps higher performance. The gene expression and microbial profiling methods provide a means of determining whether feed additives are having any significant effect on the underlying biology of the bird. Further exploration of this sort of data may indicate

particular biochemical pathways and members of the microflora that can be specifically targeted in order to improve bird performance.

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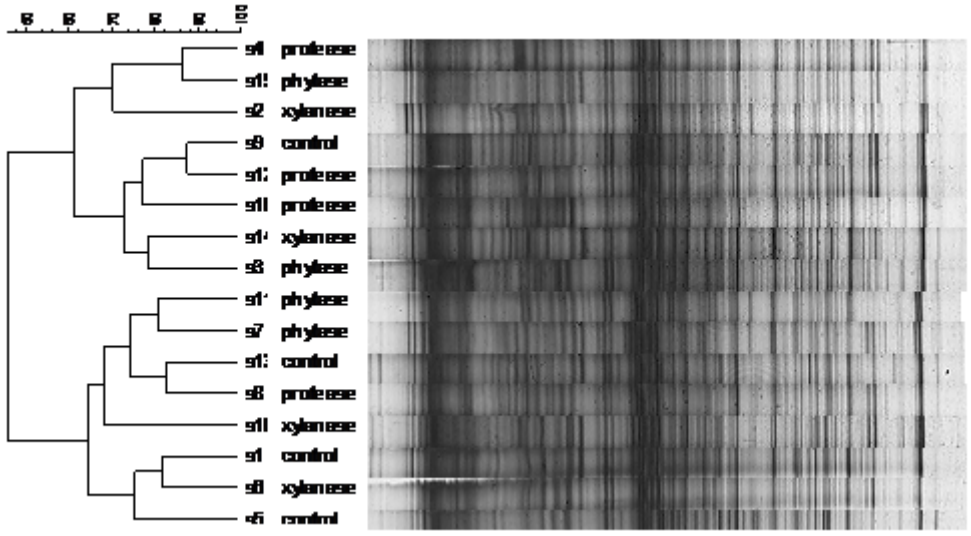
CAECAL MICROFLORA COMPOSITION OF BROILERS FED SORGHUM DIETS CONTAINING FEED ENZYMES

H.M.S. FAIZAH, A. MAGUIRE, K. HARPER, A. SULTAN, X. LI, A.V. KLIEVE and W.L. BRYDEN[1]

Sustaining a healthy gut environment is a prerequisite to efficient broiler performance. Dietary manipulation and feed enzymes have been reported to play a key role in maintaining a balanced gut microflora. Most nutrient digestion and absorption occurs in the lower ileum with some post ileal dietary fermentation. Sorghum is digested mainly in the small intestine but constituents of the grain may become available for fermentation by the caecal microflora. The addition of enzymes to sorghum-based diets could enhance nutrient utilization and limit the amount of nutrients available further down the tract for microbial metabolism. The objective of this study was to determine if dietary enzymes alter the caecal microbial profile of broilers fed sorghum-based diets.

Four sorghum-based diets (918g sorghum/kg feed) were prepared; a control diet without feed enzyme and three diets each containing a different feed enzyme (xylanase, phytase or protease). The diets were fed as mash to four replicate pens of 35-day-old broilers (8 birds/pen) in an environmentally controlled chicken house. After a week (day-42), all birds were euthanased and intestinal contents collected and pooled/pen. DNA was extracted from caecal samples using a bead-beating protocol and the V2V3 region of the bacterial 16S rRNA gene amplified by PCR. Amplicons were separated on sequence difference using Denaturing Gradient Gel Electrophoresis (DGGE) and microbial profiles generated and compared (Figure 1, below).



Pearson correlation for relative similarity of band patterns is indicated by the groupings on the dendrogram and the percentage coefficient. The DGGE profiles when analysed, indicated that there was approximately 80% similarity between gut microflora in all four dietary treatments. This indicates that there was no overall difference between any of the profiles. Therefore, the addition of different feed enzymes to a sorghum-based diet, under the conditions of this experiment, had no impact on the overall composition of the gut microflora.

[1] The University of Queensland, School of Animal Studies, Gatton QLD

QUANTITATIVE ANALYSES OF GENES ASSOCIATED WITH MUCIN PRODUCTION AND HOST INFLAMMATORY RESPONSE OF BROILER CHICKENS WITH INDUCED NECROTIC ENTERITIS

R. E. A. FORDER[1], G. S. NATTRASS[2], M. S. GEIER[1,3], R. J. HUGHES[1,3] and P. I. HYND[1]

Summary

Clostridial infection of the intestines of chickens results in necrotic enteritis (NE) and reduced production and health. Mucins play a major role in protecting the intestinal epithelium from infection. The relative roles of different mucins in gut pathology following bacterial challenge are unclear. This study was designed to establish a molecular approach to quantifying the expression of mucin and mucin-related genes, using gut samples from an NE challenge trial. A method for quantifying mucin gene expression was established using a suite of reference genes to normalise expression data. This method was then used to quantify the expression of 12 candidate genes involved in mucin, inflammatory cytokine or growth factor biosynthesis. MUC2, MUC13 and MUC5ac were the only genes that were differentially expressed in the intestine between treatment groups. Expression of MUC2 and MUC13 was depressed by challenge with *Clostridium perfringens* (*Cp*). Antibiotic treatment prevented a *Cp* induced decrease in MUC2 expression but did not affect MUC13. MUC5ac expression was elevated in birds challenged with *Cp*. This preliminary study has paved the way for more extensive studies into changes in mucin dynamics during NE challenge.

I. INTRODUCTION

Necrotic enteritis (NE), is one of the world's most prevalent poultry diseases (Van Immerseel et al., 2004). It is characterised by inflammation and necrosis of the intestine, causing losses in productivity with significant flock morbidity and mortality in acute cases (Keyburn et al., 2008). NE outbreaks and prevention cost the global poultry industry an estimated $US2 billion per year (Van Immerseel et al., 2009). Until recently, intestinal *Cp,* the causative agent of NE, has been controlled by the inclusion of in-feed antibiotics. The banning of in-feed antibiotics in the United Kingdom and European Union has increased the incidence of NE (Grave et al., 2004). As such, there is a need for the identification of effective non-antibiotic alternatives (Cooper and Songer, 2009).

The first line of defence that bacteria encounter when trying to traverse the intestinal mucosa, is the overlying mucous-gel layer. The formation of the mucous-gel is through goblet cell secretion of mucin glycoproteins (Forstner et al., 1994). Mucins possess potential binding sites for both commensal and pathogenic organisms and can be discharged in response to a wide variety of stimuli with the potential for changes in the type and quality of mucin secreted (Freitas et al., 2002). Increasing interest has been directed toward the protective properties of mucin as a barrier against epithelial attachment, and the mechanisms by which bacteria can utilise these mucin glycoproteins to facilitate adhesion and colonisation (Deplancke et al., 2001). This is important as a means to understand their involvement in the pathogenesis of intestinal diseases and to utilise and optimise their protective properties for enhanced intestinal barrier function.

[1] School of Animal and Veterinary Sciences, University of Adelaide.
[2] SARDI , Livestock and Farming Systems, Roseworthy SA
[3] SARDI, Pig and Poultry Production Institute, Roseworthy SA

In order to maximise the efficacies of potential non-antibiotic alternatives it is necessary to understand how *Cp* colonise and their consequent effects on mucosal dynamics, especially their interaction with the mucus layer. Currently, the role of mucins and the mucous layer in *Cp* infection is still elusive, with studies suggesting that specific mucin structures provide a growth advantage to the species (Collier et al., 2008). The present study aimed to develop a series of real-time PCR based assays to study mucin-related genes in the small intestine of broiler chickens and to determine whether NE challenge influenced their expression. In addition, treatments with antibiotics, organic acids and probiotics in conjunction with the NE challenge were also evaluated for their potential influence on gene expression.

II. MATERIALS AND METHODS

Twelve hundred male Cobb 500 broiler chickens were randomly allocated to 48 pens (n = 25 birds/pen) and assigned to six experimental groups (n = 8 pens/treatment); an unchallenged control (UU), an *Eimeria* spp./*Cp* challenged control (CU), and *Eimeria* spp./*Cp* challenged groups treated with; antimicrobials (45 ppm zinc bacitracin and 100 ppm monensin; CZ), organic acids (proprietary blend[4]; 2 kg/ton, CO), probiotic *L. johnsonii* (10^9 cfu/mL in PBS; CL) and probiotic sham (PBS alone; CV). *L. johnsonii* and sham-treated birds were orally administered 0.5 ml of solution using a crop needle on day 1, and 1 ml on days 3, 7 and 12. The necrotic enteritis challenge was performed as described by Geier et al. (2010). On day 18, 3 days after *Cp* challenge, a 1 cm segment from the mid-point of the jejunum was removed from 72 birds (n = 12 birds/treatment), rinsed in PBS and collected in a 5 ml tube containing 2 ml of RNAlater for gene expression analyses.

MUC13, MUC2, MUC4, MUC5ac, MUC5b, MUC16, TFF2, TNFα, IL18, KGF and TLR4 mRNA expression was quantified via real-time PCR. Total RNA was isolated with the RNeasy mini kit. Complementary DNA (cDNA) was synthesised with the High Capacity cDNA Synthesis kit using 400ng of total RNA and an oligodTV primer. Quantitative PCR was performed in triplicate on a 384 well real-time PCR machine with a modified SYTO9-based reagent. The relative quantification data were normalised against the geometric average of 2 stably expressed reference genes (TBP and GAPDH). Statistical analyses of the normalised real-time PCR data were performed with a General Linear Model in SAS (v9.1).

III. RESULTS

Prior to undertaking the qRT-PCR measurements on intestine samples, a number of the gene assays that were designed for this study were found not to be expressed in chicken intestine (MUC4, MUC5b and MUC16) or were expressed beyond the limit of detection threshold (IL18) and hence were not quantified. MUC2, MUC5ac and MUC13 were the only genes examined in this study that were differentially expressed in jejunum samples between the treatment groups ($P < 0.05$). The MUC2 mRNA levels were almost identical between the UU chickens and those treated with the antibiotic (CZ). The four other groups of challenged birds (CU, CO, CL and CV) had MUC2 mRNA levels that were 40-54% lower than the UU and CZ chickens (Figure 1A). The intestinal MUC13 mRNA level of the UU chickens was 20–35% higher than the 5 challenged groups of chickens (Figure 1B). MUC5ac expression was elevated in birds challenged with *Cp* compared to controls and antibiotic treatment (Figure 1C). The expression of other target genes analysed (KGF, TFF2, TLR4 and TNFα) did not statistically differ between treatment groups.

[4] Proprietary organic acid blend contained formic, acetic, propionic, sorbic, caprylic and capric acid.

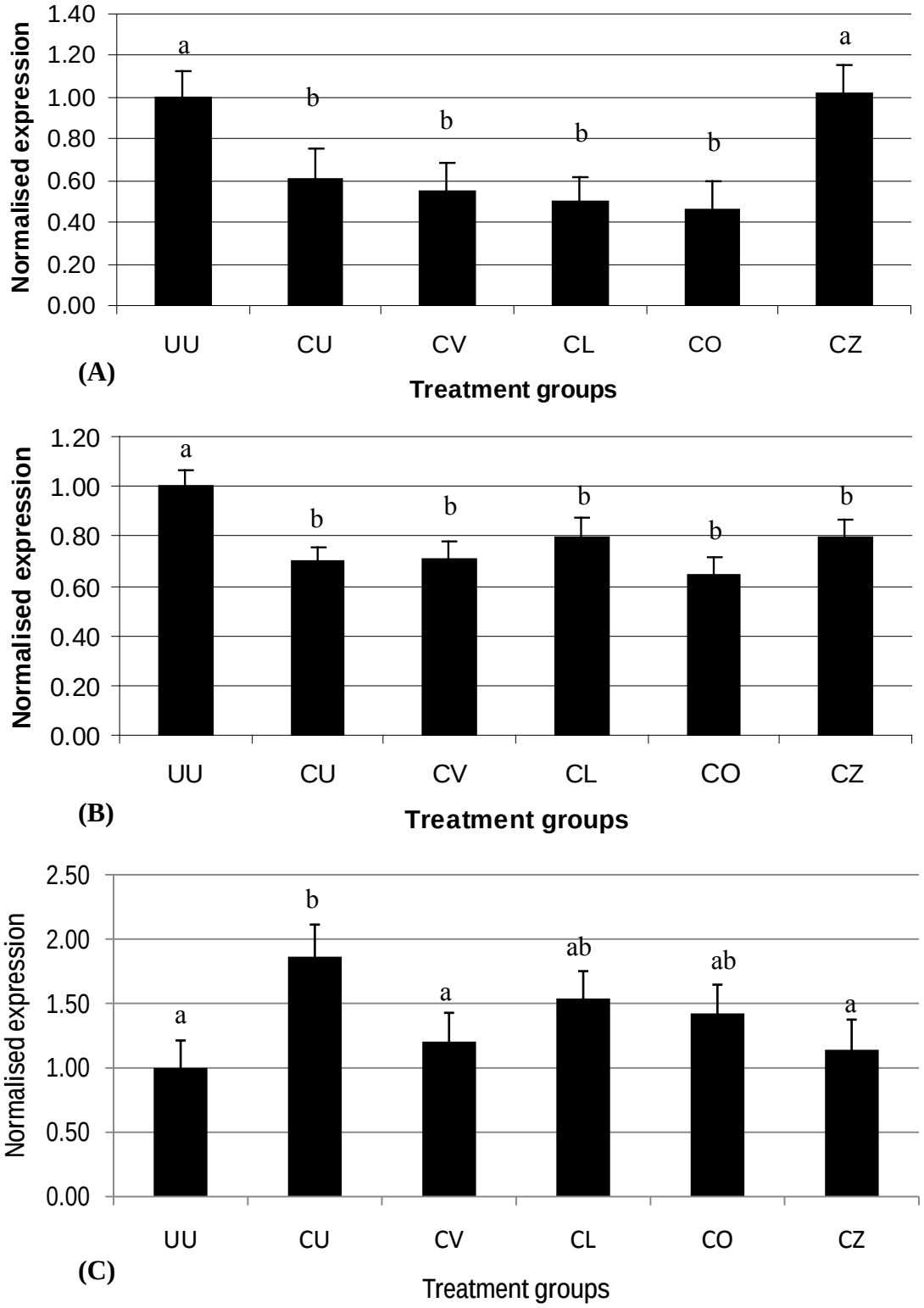


Figure 1 Relative expression of (A) MUC2; (B) MUC13 and (C) MUC5ac mRNA levels in jejunum of the unchallenged/untreated (UU) control and 5 groups of challenged birds (CU, CV, CL, CO and CZ). The UU control was assigned an arbitrary value of 1.00 and the other groups of challenged birds were expressed relative to this value. Values are least square means ± SEM.

IV. DISSCUSSION

In the present study, MUC2, MUC5ac and MUC13 were the only genes examined that were differentially expressed between treatments. Expression of MUC2 and MUC13 was significantly depressed following infection by *Cp*, whereas MUC5ac expression was elevated. *L. johnsonii* and the organic acid blend did not alter mucin production in response to a *Cp* challenge; however, the conventional antibiotic (ZnB/monensin) maintained expression levels similar to controls. Several of the mucin genes MUC1, MUC4, MUC5b and MUC16 were not detectable in jejunal tissue, regardless of challenge and treatment. Expression of these genes is regulated in a tissue and cell-specific manner. Mammalian MUC2 is observed to be widely expressed in goblet cells of the small intestine and colon, whereas MUC5ac is weakly expressed in the intestine and colon but widely expressed in the stomach (Klinken et al., 1995). Regional expression of MUC genes in human and rodent models has been well characterised (Klinken et al., 1995) however research on regional expression in chicken is limited. The differences in expression in regions of the intestinal tract suggest that each mucin has its own specific function in maintaining mucosal integrity. During infection, inflammation of the gut can cause a significant change in expression pattern having detrimental effects on the underlying epithelium. Mice deficient in MUC2 were observed to have aberrant intestinal crypt morphology with altered cell maturation and migration (Claustre et al., 2002) and that an abnormal increase in MUC5ac expression was observed in patients with colon cancer and to a lesser extent ulcerative colitis (Forgue-Lafitte et al., 2007). This study has provided informative, quantitative data on the expression pattern of mucin and other gut-related genes potentially involved in the pathogenesis of *Cp.*

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PHYTATE AND THE THERMODYNAMICS OF WATER

A.J. COWIESON[1] and N.P. COWIESON[2]

Summary

The antinutritional effect of dietary phytate has been recognised for many years but its role in contemporary poultry nutrition is poorly understood. Conventional wisdom suggests that phytate dilutes the digestible mineral content of poultry diets (notably P) as well as acting on the GI tract as an electrostatic aggressor. The mechanism for the latter effect is not well defined but may be central to the antinutritive effects of phytate in poultry nutrition and axiomatically to the benefits of microbial phytase. This paper explores the reactivity of dietary phytate with various proteins and seeks to explore how these effects may explain the extra-phosphoric effects of phytase.

I. INTRODUCTION

Phytic acid (*myo*-inositol hexakisdihydrogen phosphate; PA) was first recognised over 150 years ago as being an important phosphorus reserve in plants. PA consists of a myo-inositol ring to which six orthophosphate groups are attached by ester bonds (Costello et al., 1976). At physiological pH PA is negatively charged and so can react with positively charged nutrients such as some amino acids and minerals. Ingredients notably 'rich' in PA are fibrous by-products such as wheat and rice brans, oilseeds such as canola and soy, legumes and also some cereals. The ingestion of PA by livestock and humans is unavoidable due to its ubiquitous presence in cereals, oilseeds and legumes but the consequences of this ingestion are still obscure. The reason for this obscurity is a lack of clear understanding of the post-ingestive reactivity of phytate and its interaction with the digestive process and the immune system. However based on literature evidence, the ingestion of PA by humans and non-ruminant animals could be expected to impede mineral availability (notably the divalent cations such as iron, zinc, magnesium, manganese, copper and calcium) and also to irritate the gut via hypersecretion of digestive enzymes, bile and acids, possibly increasing susceptibility to enteric disease. These mechanisms effectively reduce the digestibility of these dietary components leading to problems associated with amino acid, energy and mineral metabolism, both in animals and humans (Harland & Morris, 1995; Cowieson et al., 2009). Recent evidence, however, suggests that PA may not interact directly with proteins but impede their solubility via alteration of the thermodynamics of the water matrix. Thus, the ingestion of PA may compromise the digestibility/availability of a range of nutrients, particularly those inherently less polar.

II. MATERIALS AND METHODS

Materials

Proteins were sourced as follows: hen egg white lysozyme, pI 9.4 (Sigma 62970), conalbumin pI 6.85, ovalbumin pI 5.19, ribonuclease A pI 8.93, carbonic anhydrase pI 6.4 (GE Healthcare 28-4038-41).

[1] Poultry Research Foundation, University of Sydney, Camden, NSW 2567, Australia
[2] Australian Synchrotron, Melbourne, Australia

Solubility assays
Proteins were used at 4 mg/ml in Tris pH 6.5 and 100 mM NaCl at room temperature. Phytate was suspended in a similarly buffered solution and varied from 100 to 0.7 mM by serial dilution. Following mixing of protein and phytate, insoluble material was removed from solution by centrifugation and final protein concentrations measured by absorbance at 280 nm.

Protein Crystallography
Lysozyme was crystallised at 12 mg/ml in 100 mM Na-acetate pH 4.8 and 1 M NaCl. Crystals of lysozyme were transferred to a similar solution containing in addition Na phytate at concentrations of 10, 25, 50 and 100 mM phytate. X-ray diffraction from the crystals was measured at the macromolecular crystallography beamline at the Australian Sychrotron. The data were reduced using XDS, pointless and scala software packages and structures were refined using Refmac5 and built using Coot.

Small-angle x-ray scattering
Small-angle x-ray scattering (SAXS) from solutions of phytate from 0 to 100 mM in deionised water were measured at the small-angle x-ray scattering beamline at the Australian Synchrotron in a Q range of 0.01 to 0.7. Degree of inter-particle correlation was calculated using the Unified fit model function of the Irena package in the Igor pro modelling software.

III. RESULTS

Increasing the concentration of phytate from 1 mM to 25 mM resulted in a decrease in the solubility of lysozyme from 100% to 50%. At higher phytate concentrations (25-100 mM), the solubility of lysozyme was restored (Fig. 1). At a fixed phytate concentration (6 mM), increasing the concentration of lysozyme protein from 1-6 mg/ml resulted in a decrease in the solubility of lysozyme (Fig. 2). The effect of phytate on the solubility of proteins was entirely uncorrelated with the isoelectric point of a series of proteins, including ovalbumin (pI 5.87), carbonic anhydrase (pI 6.4), conalbumin (pI 6.85), ribonuclease A (pI 8.93) and lysozyme (pI 9.4) (Fig. 3).

Small-angle scattering x-ray crystallography revealed no direct interaction between phytate and lysozyme but there were increasing inter-particle effects which were concentration dependent (Fig. 4). Fig. 4 shows that, in an aqueous medium, increasing the concentration of phytate from 1-15 mM resulted in an increase in molecular ‘pack’ i.e. an increase in the number of phytate molecules in close enough proximity to other phytate molecules so as to have interactive effects. This relationship appears to plateau around 15-20 mM phytate after which no further effects are noted.

IV. DISCUSSION

The interaction between phytic acid and protein is best supported by the work of Rajendran & Prakash (1993) in which they demonstrate, in an equilibrium dialysis experiment against alpha-globulin, that increasing phytate concentration results in increased loss of protein from solution. Mothes et al. (1990) observed similar effects with napin, a protein isolated from rapeseed. Putatively, phytate interacts electrostatically with the basic amino acid residues on the surface of protein, reducing solubility. However, this theory is not well supported experimentally or indeed by theoretical biochemistry. Firstly, direct binding of phytic acid to protein should increase, not decrease solubility, and secondly, if such a mechanism existed there should be a correlation between the basic amino acid concentration in, and/or the

isoelectric point of, various proteins and their propensity to interact with phytate. Finally, if direct binding between phytate and protein were the principle mechanism by which the observed effects on protein solubility are derived there would be no effect of protein concentration *per se*, another point of pivotal importance for which there is contradictory experimental evidence.

Based on evidence presented here, increasing phytate concentration (to a point) results in a clear reduction in the solubility of lysozyme (Fig. 1) and this could be exacerbated by increasing lysozyme concentration (Fig. 2). Further, the deleterious effect of phytate on the solubility of protein was unrelated to isoelectric point (Fig. 3). These points are indicative, not of a direct phytate:lysozyme interaction but of a generic mass effect. One possible mechanism may be that, in an aqueous medium, the negatively charged phytate molecule draws a hydration shell around itself, competing with other compounds in the water matrix. In such a context it would not be the presence of basic amino acid residues which is of importance but rather the polarity of the surface of the protein, and its ability to remain in solution as hydrating waters are drawn away by competing phytate moieties. This would explain why these effects are protein concentration dependent (Fig. 2) as higher concentrations of protein would increase the likelihood of protein:protein aggregation under conditions of low water potential. We propose that the effect of phytate on protein solubility is an indirect one, with phytate acting as a Hoffmeister anion and while it may interact directly with some proteins, this is not the main mechanism. The implications of this are considerable in that the presence of phytate in the GI tract may cause severe perturbation in the water matrix, changing thermodynamics and resulting in a change in the solubility profile of dietary and endogenous nutrients, possibly including various digestive enzymes. A greater understanding of the effect of phytate on nutrient solubility will assist in diet formulation strategy, ingredient selection (e.g. avoidance of hydrophobic proteins) and in phytase dosing.

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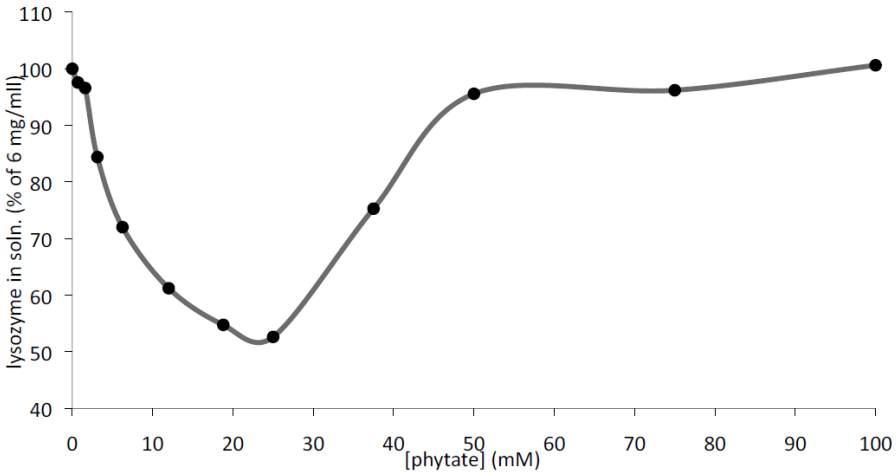

Figure 1 The effect of increasing concentration of phytic acid on the solubility of lysozyme.

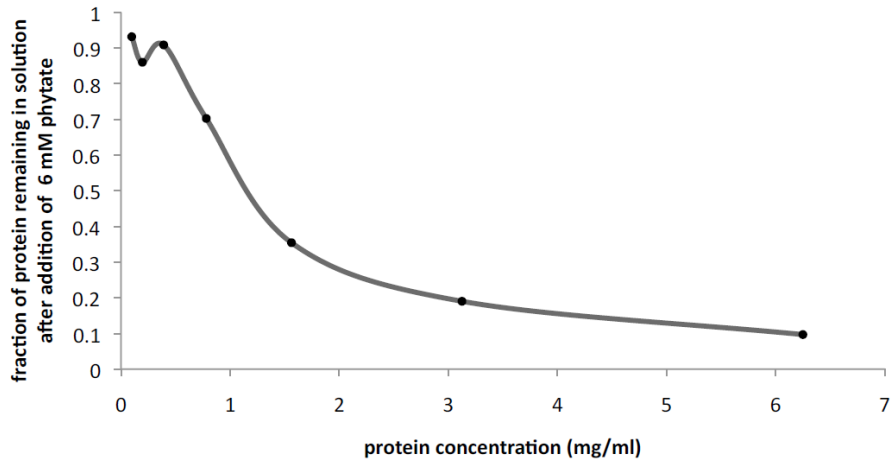

Figure 2 The effect of increasing lysozyme concentration at a constant phytate concentration on the solubility of lysozyme.

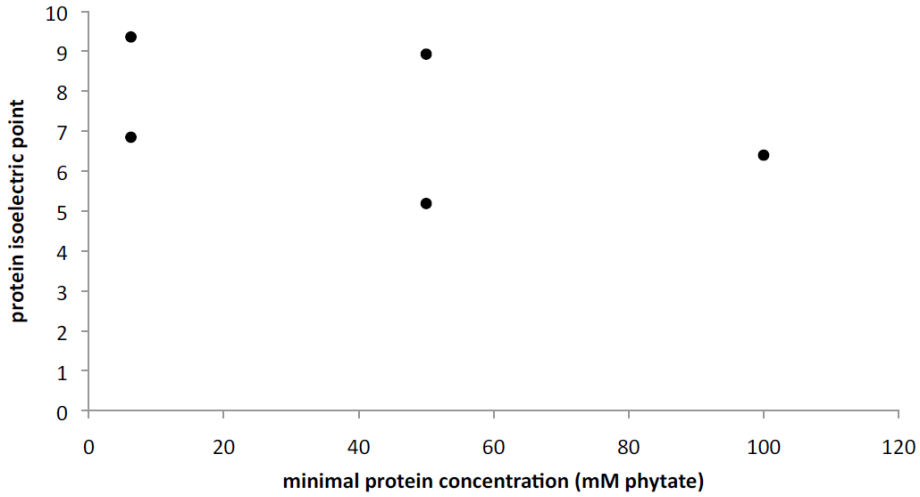

Figure 3 Correlation between the isoelectric point of protein and the concentration of phytate at which the minimum solubility was achieved.

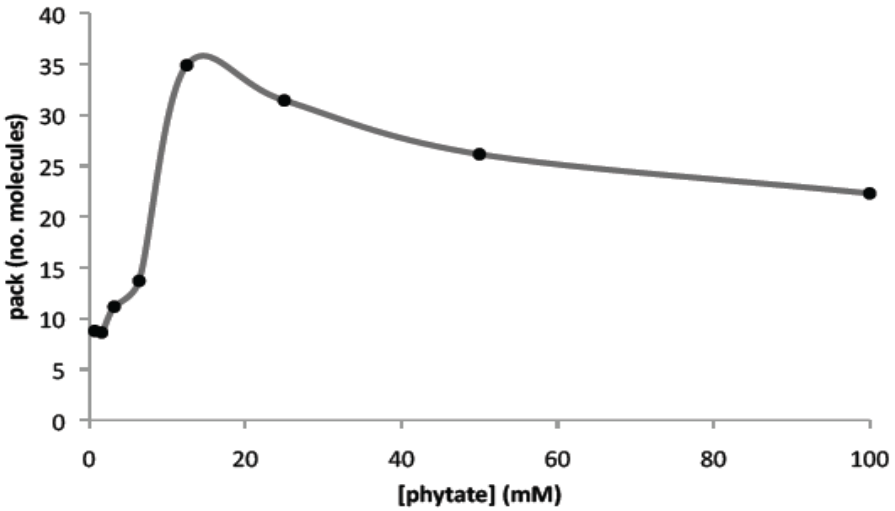

Figure 4 Relative proximity of phytate molecules in solution as measured by small-angle scattering x-ray crystallography.

EFFECTS OF NUTRITION ON WATER INTAKE AND LITTER MOISTURE ON BROILER CHICKENS

K.H. HUANG[1], C. KEMP[2] and C. FISHER[2]

Summary

A series of experiments was conducted to investigate the effects of nutrition and feed on litter moisture, litter quality and water intake of broilers. The diets were formulated with reference to Ross Broiler Nutrition Specifications (2007). Nutritional treatments involved dietary metabolisable energy, balanced protein, mineral content, feed physical quality, feed form and feed restriction. The results showed that increasing dietary energy, reducing dietary balanced protein or reducing mineral levels decreased ($P<0.05$) litter moisture and water intake and improved litter quality. Feed physical quality and feed form results showed a benefit from using reground pellets or mash feed for reducing litter moisture and improving litter quality. Feed restriction at 10% of *ad libitum* led to higher litter moisture, poor litter quality and higher water intake, compared to *ad libitum*. It was concluded that these various nutritional factors, can significantly influence litter moisture, litter quality and/or water intake of broilers.

I. INTRODUCTION

Litter conditions significantly influence broiler performance and the profitability of broiler production. Litter is defined as the combination of bedding material, excreta, feathers, spilled feed and water. Litter moisture and condition of the excreta are the most important factors determining litter conditions. Excessive litter moisture may lead to high ammonia emissions, footpad dermatitis, processing downgrades and condemnations (Martland 1985; Kenny *et al*., 2010). Both footpad lesions and litter condition are part of EU welfare legislative control. High litter moisture alone is widely considered to be the major contributor to footpad dermatitis in the industry. Amongst many factors affecting litter quality, nutrition could be one of the most important, but inconsistent reports in the literature do not facilitate clear conclusions. Recently some nutritional trials conducted by Aviagen have included the measurement of litter moisture and water intake to further understand how feed and nutrition affect these factors in modern broilers. The results reported here investigated the effect of nutrition and feed on water intake, litter moisture and litter quality of broilers. The effects on biological and economic performance were recorded in all trials but are not reported here. The best litter conditions may not be consistent with the best biological performance.

II. EFFECTS OF DIETARY ENERGY LEVEL

The results of four trials investigating the effects of dietary energy level on water intake and/or litter quality are shown in Tables 1, 2, 3 and 4. The diets were corn-soybean meal base, formulated to Ross Broiler Nutrition Specifications (2007), and pelleted in 3 mm die or fed as mash. The energy levels were 100, 95, 92.5 and 90%, where 100% was equal to Ross 2007 specification (starter (0-10d), 12.65MJ/kg; grower (11-24d), 13.20MJ/kg, finisher (25d+), 13.40MJ/kg). All other nutrients were the same as Ross 2007 specification. The decreased energy was achieved by reformulating with higher extracted rice bran and adjusted

[1] Aviagen Inc. Cumming Research Park, 5015 Bradford Drive, Huntsville AL 35805 USA
[2] Aviagen Ltd, Newbridge Midlothian, Scotland

mostly using maize, soy oil and soybean meal. All the trials were conducted in a tropical environment in Thailand in pens with rice hulls deep litter. When dietary energy decreased, litter moisture increased ($P<0.05$) in all trials (Tables 1, 2, 3, and 4) and daily water intake (0-49days or 0-42days) increased ($P<0.05$) (Tables 1 and 4). Litter quality, scored visually, improved when energy increased (Tables 2 and 3). This could be partly caused by the fibrous ingredient (extracted rice bran) used in the energy dilution. The reduced litter moisture and litter quality improvement with increased energy could reduce footpad dermatitis as seen by Kenny *et al*. (2010). However Bilgili *et al*., (2006) found that low energy diets significantly reduced the incidence of 'paw' lesions compared with high energy diets. This converse finding is probably a reflection of differences in dietary design; oil was the main ingredient changed to adjust the energy in the Bilgili *et al*., (2006) study.

III. EFFECTS OF DIETARY BALANCED PROTEIN LEVEL

The results of varying dietary balanced protein (BP) on litter moisture and water intake are shown in Table 1. The trial design was described in the previous paragraph. The BP levels were 80, 90, 100 and 120%, relative to the Ross 2007 specification (=100%), in all growing phases. All other nutrients, such as energy, minerals and vitamins, were the same as Ross 2007 specification. When BP increased in diets, the litter moisture and water intake increased ($P<0.05$). Our findings are similar to those of Ferguson et al. (1998).

IV. EFFECTS OF DIETARY MINERAL LEVELS

The effects of dietary mineral levels on litter moisture and quality, as measured by litter capping, at 26 days are shown in Table 5. The diets were wheat-soybean meal based. The control diet was formulated to Ross Broiler Nutrition Specifications (2007), and the reduced mineral diet was the same specification as the control except for Ca, avP and Na which were as shown in Table 5. All diets were pelleted in 3 mm die with phytase added. The contribution of Ca, AvP and Na from phytase was included in the calculated levels. The trial was conducted in the Aviagen trial facility in the UK in pens with wood shavings litter. The reduced mineral diet led to lower litter capping ($P=0.053$) but had no effect on litter moisture ($P=0.273$). Excessive dietary mineral intake has been shown to increase excreta moisture and water intake (Johnson and Karunajeewa, 1985). The mineral levels in current trial were considered to be balanced; however, there may be an opportunity to explore lower levels at some growing stages, if litter moisture and quality are problematic.

V. EFFECTS OF FEED PHYSICAL QUALITY AND FEED FORM

The effects of different feed physical qualities and feed form on litter moisture and litter quality are shown in Tables 2 and 3. The trial design was described above in Section II. For the fines treatment in Table 2 reground pellets were used. In Table 3, the mashes of different particle sizes were obtained by changing the screen and speed of hammer mill and they were not heat treated. The results showed that the birds fed with pellets resulted in higher ($P<0.05$) litter moisture and poorer ($P<0.05$) litter quality than the fines or mash groups. It is possible that the feed intake on fines and mash was much lower than pellets, resulting in lower water intake (data not shown).

VI. EFFECTS OF FEEDING METHODS

The effects of restricted feeding on litter moisture, litter quality and water intake are shown in Table 4. The trial design was described in section II above. The feeding methods used were *ad libitum* and a 10% feed restriction applied from day 6. The feed restriction significantly ($P<0.05$) increased litter moisture content and decreased litter quality at 35 days, although water intake was not different at week 5 (the week that litter moisture was measured). It is interesting to note that the average daily water intake for 0-42 days was significantly higher on *ad libitum* than on restricted feeding, an observation which could be related to higher feed intake. The effect of feed restriction leading to higher litter moisture and poorer litter quality has not been described previously to our knowledge. It could be that water intake was stimulated because birds felt unsatisfied during the feed restriction.

VII. CONCLUSION

Many factors will affect litter moisture in practice, including management and housing (type of litter, temperature, ventilation, stocking density, heating, drinking system), disease and health status (coccidiosis, viral and bacterial disease) and nutrition. The present results demonstrate that the nutritional factors, such as energy, balanced protein, mineral contents, feed form and feed physical quality, and feed restriction, have a significant influence on litter moisture content and litter quality and/or water intake. Litter moisture significantly contributed to poor litter conditions and footpad dermatitis, which leads to welfare issues. Controlling litter moisture to improve in-house environment is essential to improve broiler performance and nutrition has role to play in terms of improving litter conditions.

Table 1 The effects of relative energy and relative balanced protein (BP) levels on litter moisture and water intake of broilers[1]

Factors	Litter Moisture[2] 21-22 days (%)	Litter Moisture[2] 35-36 days (%)	Water Intake[2] 21-22 days (ml/bird/day)	Water Intake[2] 35-36 days (ml/bird/day)	Water Intake Average 0-49days (ml/bird/day)
Energy					
92.5	32.5[a]	44.7[a]	217.5	330.7	237.0[a]
100	30.3[b]	38.9[b]	210.1	314.7	223.5[b]
SEM	0.541	0.588	2.990	7.902	1.610
P	0.007	0.001	0.084	0.159	0.001
BP					
80	26.3[c]	34.8[d]	183.4[d]	283.9[c]	206.2[d]
90	30.4[b]	40.1[c]	206.1[c]	306.4[bc]	218.6[c]
100	32.5[b]	44.1[b]	225.9[b]	330.9[b]	240.6[b]
120	36.4[a]	48.4[a]	239.8[a]	369.6[a]	255.6[a]
SEM	0.766	0.832	4.229	11.175	2.276
P	0.001	0.001	0.001	0.001	0.001
Energy X BP	0.698	0.382	0.294	0.650	0.628

a,b,c,d values in a column with no common superscript differ significantly ($P<0.05$).
[1] The trial was in completed block randomized factorial design with 7 replicates of 16 birds each.
[2] The value was average of two days continuously measurement.

Table 2 The effects of relative energy level and feed physical quality on litter moisture and litter quality[1]

Factors	Litter Moisture 35days (%)	Litter Quality[2] 35days
Energy		
90	37.08 [a]	1.93 [a]
95	34.65 [b]	1.64 [ab]
100	31.54 [b]	1.43 [b]
SEM	1.130	0.156
P	0.005	0.090
Feed Physical Quality		
Fines[3]	31.28 [b]	1.33 [b]
Pellets	37.57 [a]	2.00 [a]
SEM	0.923	0.128
P	0.000	0.001
ME x Feed Form	0.387	0.413

a,b values in a column with no common superscript differ significantly (P<0.05).
[1] The trial was a complete block randomized factorial design with 8 replicates of 16 birds.
[2] Litter quality was scored: 1 = good (brown and dry), 2 = fair, 3 = poor (dark and wet).
[3] The fines were reground pellets*

Table 3 Effect of relative energy level and feed form on litter moisture and litter quality[1]

Factors	Litter Moisture 35-36days (%)	Litter Quality[2] 35 days
Energy		
90	40.06 [a]	1.97 [a]
95	37.09 [b]	1.53 [b]
100	34.39 [c]	1.44 [b]
SEM	0.644	0.084
P	0.000	0.000
Feed Form[3]		
Coarse Mash	34.75 [b]	1.63 [b]
Fine Mash	35.74 [b]	1.38 [c]
Medium Mash	35.84 [b]	1.33 [c]
Pellets	42.39 [a]	2.25 [a]
SEM	0.744	0.096
P	0.000	0.000
Energy X Feed Form	0.205	0.508

a,b,c values in a column with no common superscript differ significantly (P<0.05).
[1] The trial was a complete block randomized factorial design with 8 replicates of 16 birds.
[2] Litter quality was scored: 1 = good (brown and dry), 2 = fair, 3 = poor (dark and wet).
[3] The mash treatments were from day 11. All birds were fed with common crumble from 0-10 days.

Table 4 Effect of relative energy level and feeding method on water intake and litter moisture[1]

Factors	Litter Moisture 35days (%)	Litter Quality[2] 35 days	Water Intake Week 5 (ml/bird/day)	Water Intake Average 0-42days (ml/bird/day)
Energy				
95	39.71 [a]	1.89 [a]	263.89 [a]	182.99 [a]
100	35.63 [b]	1.50 [b]	254.28 [b]	176.19 [b]
SEM	1.072	0.116	2.541	1.413
P	0.010	0.020	0.010	0.001
Feeding[3]				
Ad libitum	35.24 [b]	1.46 [b]	261.24	182.68 [a]
Restriction 10%	40.09 [a]	1.93 [a]	256.93	176.50 [b]
SEM	1.072	0.116	2.541	1.413
P	0.002	0.007	0.236	0.003
Energy X Feeding	0.531	0.828	0.667	0.823

a,b,c values in a column with no common superscript differ significantly (P<0.05).
[1] The trial was a complete block randomized factorial design with 7 replicates of 16 birds.
[2] Litter quality was scored: 1 = good (brown and dry), 2 = fair, 3 = poor (dark and wet).
[3] The feeding factors were a comparison of feed intake *ad libitum* with 10% feed restriction from day 6 to 42 days. The chicks were fed *ad libitum* between day 1 to day 5.

Table 5 Effects of reduced mineral levels on litter moisture and litter quality[1]

Mineral levels[2]	Ca (g/kg)	Av.P (g/kg)	Na (g/kg)	Litter Moisture 26days (%)	Litter Capping[3] 26days
Control diet				53.21	34.29
Starter	10.5	5.0	2.0		
Grower	9.0	4.5	1.8		
Finisher	8.5	4.2	1.8		
Reduced mineral diet				48.12	42.50
Starter	9.5	4.5	1.5		
Grower	8.0	4.0	1.4		
Finisher	7.5	3.7	1.4		
SEM				3.039	2.634
P				0.273	0.053

[1] The trial was a complete block randomized factorial design with 8 replicates of 20 male birds
[2] All feeds contained phytase and Ca, avP and Na levels included the contribution from phytase.
[3] Litter capping was scored visually at 26 days.

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CALCIUM AND PHOSPHORUS REQUIREMENTS IN BROILERS AND LAYING HENS

R. ANGEL[1]

Summary

Attention to environmental stewardship and recent as well as expected new regulations have led to intense and extensive work on phosphorus (P) nutrition in broilers. Because of inconsistency in details provided in published work and differences in methodology between studies, it is difficult to arrive at P requirement concentrations for the different growth phases that would apply to different breeds and different management systems. In an effort to clarify this information, recent literature on P requirements is summarized and tabulated in this paper. Clearly, there is still not enough information on the P requirements of females or straight run birds. Even with males, the data are not conclusive. Information available on calcium (Ca) and on Ca to P ratios varies greatly between studies. In recent years, most of the published literature on P has focused on the use of phytase and rarely can the reader surmise what the actual requirements for either Ca or P are from these publications, in part because of missing information and because of the experimental design. The results of a series of studies that have been done to help clarify and start pinpointing requirements in broilers and layers will be presented. However, limitations exist in the applicability of these numbers to different strains and to different management systems.

I. DEFINITION OF TERMS

Before one can summarize Ca and P requirements, it is essential that terms be defined. Total P (P) is generally referred to as P and encompasses any and all forms of P. Available P (aP) refers to the P that is absorbed from the diet into the animal (i.e., feed P minus P in the distal ileum). From a determination standpoint, feed P minus ileal content P is often termed digestible P. Unfortunately aP has been used interchangeably with relative available P. Relative aP is determined by setting the availability of a standard P containing product as 100 and compares other P products in relation to the standard. This determination is only useful when comparing specific products but does not provide absolute values on the P that the animal can digest and absorb. Because there is so much confusion currently with the term aP, one should use digestible P, defined as feed P minus ileal P (based on a marker determination system). Retained P refers to the P that stays in the body (i.e., feed P minus excreta P). Inorganic P (iP) is any P not bound to an organic molecule. Phytic acid, *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IP6), an organic phosphate, is a phosphorylated cyclic sugar alcohol. The anion form of phytic acid, phytate, is the form present in all plants. In mature seeds, phytate is present as a complex salt of Ca, Mg and K and in some cases it is bound to proteins and starches and this complexed or chelated molecule is known as phytin (O'Dell and de Boland, 1976; Cheryan, 1980; Lott, 1984; Graf, 1986). Phytate P is organic P, specifically P that is part of a six carbon ring structure (phytic acid), found primarily in seeds in its chelated form, phytate P (PP). Non-phytate P (nPP) is total P minus PP or the P in the feed or ingredient that is not bound in the phytic acid molecule.

All terms have strengths and weaknesses. Available P has to be determined in animal availability trials that are time consuming and costly, and many factors will influence P availability. The ingredient P availability used in most formulation systems or present in tables, such as those found in the National Research Council (NRC, 1984 and 1994) Nutrient

[1] 4131 Animal Sciences Center, Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742. USA

Requirements of Poultry, are averages and are based on limited numbers of availability values. This can result in large errors associated with these values. These errors are present, in part, because biological availability is not a static number. Biological availability of P can vary depending on dietary factors such as the concentration of other nutrients (Ca, vitamin D, other macro minerals and micro minerals, vitamin K, and dietary energy and protein) in the diet as well as physiological, health and management factors (feed consumption, growth rate, sex, age, temperature, light program, etc.), choice of dietary ingredients and the availability of nutrients in these ingredients (solubility and availability of Ca and P will vary within and between different Ca and P inorganic and organic sources), the ratio between the concentration of other nutrients and P as well as the type and concentration of P in the diet. Non-PP can be chemically determined by subtracting analyzed PP from analyzed P but not all nPP is available. A key difference between aP and nPP is that the term aP includes absorbed inorganic as well as organic P (including PP), whereas the nPP excludes any potentially available PP and includes any potentially unavailable iP.

The current goal is to develop digestible Ca and P values for ingredients, an endeavor that will take time to do correctly and will involve developing the appropriate methodology that is consistently used across labs and that includes enough samples to provide the user with an average availability but also minimum and maximum values as well as a coefficient of variability or a standard deviation by ingredient. As environmental concerns have put pressure on P concentrations in animal diets, and the use of phytase has become more common in poultry diets, we have tended to formulate diets closer to requirements. Yet we still are using the concept of total Ca to either aP or nPP. As we look to the future on Ca and P requirements and formulation matrixes, we will need to look at digestible Ca and P values. We also have assumed that the matrix values we use for available P and Ca are the same for broilers, laying hens and turkeys. Comparative work between species for the same source is hard to find and this assumption is difficult to support. Thus, work will be needed to validate this assumption before digestibility matrixes can be used interchangeably.

The 1994 NRC ingredient P concentrations and P recommendations are based mostly on research done using book values of aP for different feed ingredients. The ingredient concentrations and P recommendations are very similar to those published in the 1984 NRC and the references used to derive these values overlap almost completely. Yet the 1984 NRC gives P values as aP and the 1994 NRC gives P values as nPP. Thus, it appears that NRC (1994) used aP and nPP interchangeably. Yet, available P and nPP are not equal, as specified earlier. Surprisingly, from a practical standpoint and with most common ingredients used in the US today, aP and nPP concentrations fall within a very close range of each other.

Which term should be used when determining requirements or formulating diets, aP, nPP or a different term such as that proposed by Coon and Leske (1998) of retainable P (rP) or the more recent proposed terms by the Europeans (Narcy et al., 2009), or digestible P (and Ca) is a question that remains to be answered. For the sake of clarity and to aid in comparing studies, it is essential that one system be used, but the “best” term question is still being debated.

All systems (aP, nPP, and rP) pose pitfalls when used. Requirement values for digestible Ca and P, aP and available Ca would have to be determined in trials where ingredients used should have previously had digestible Ca and P or available Ca and P determined in vivo. This makes the work almost cost prohibitive and extremely time consuming. Yet digestible or “true” available requirement values would be most useful if good values (with ranges and standard errors associated with them) for ingredients existed or could be generated accurately and quickly. On the other hand, nPP can be measured chemically indirectly through laboratory procedures. The pitfall in basing recommendations on nPP is the potential variable availability of PP between and within plant ingredients as well as variable

availability of iP from inorganic and plant sources. Pitfalls associated with rP are related to the impact of other dietary nutrients, especially Ca and vitamin D and of growth rate on the amount of P that an animal will retain. The impact of diet and growth rate when rP is being determined for an ingredient is higher than for the other two systems. Any system that is chosen should provide mean values for ingredients as well as minimum and maximum values and standard errors for these mean values.

II. PHOSPHORUS REQUIREMENT

The NRC (1994) nPP recommendations for broilers are based on peer-reviewed research published between 1952 and 1983. However, the present commercial bird is very different from commercial birds available prior to 1983, due in part to genetic selection as well as management practice changes and feed related changes (Havenstein et al., 1994; Williams et al., 2000). Work by Havenstein et al. (1994) shows that the body weight at 42 d of a 1991 commercial strain fed a 1991 diet was 2297 g as compared to the body weight of 680 g in a 1957 commercial strain fed a 1991 diet. Similarly, the body weight at 42 d of a 1991 strain fed a 1957 diet was 3.4 times the weight of 1957 strain fed a 1957 diet (1877 g vs. 538 g). Clearly, genetic changes account for the greatest proportion of the improvement in growth followed by diet changes. In 2003, similar differences were reported on 1957 vs. 2001 broiler strains (Havenstein et al., 2003) where the 1957 strain weighed 591 vs. 2903 g for the 2001 strain at 42 d of age for male broilers fed their respective year representative diet. Again, in this study the results clearly demonstrate that it is genetic change more so than diet change that accounts for the differences.

Also, the current commercial strains are more efficient in utilising nutrients, and the current commercial feeds are better formulated to meet the requirements of the rapidly growing commercial bird (Havenstein et al., 1994, 2003). At 42 d of age, body weight of a 1991 strain fed a 1991 diet was 2297 g compared to 1877 g of the 1991 strain fed a 1957 diet. The 42 d body weight of 1957 birds fed a 1957 diet was 538 g compared to the 680 g of the 1957 strain fed a 1991 diet (Havenstein et al., 1994). The greater 42 d weight of the 1957 strain fed the 1991 diet vs. the 1957 diet proves that present commercial diets are better formulated to meet the bird's requirement regardless of the genetic potential of the birds. The fact that today's broiler is different genetically and that current diets are better formulated limits the applicability of NRC (1994) nPP recommendations that were developed from work published between 1952 and 1983. The need for a new NRC as related to P requirements is clear.

A large number of the published research studies used by NRC (1994) to establish nPP recommendations are based on research where book values for aP were used. The aP values are usually determined for each feed ingredient by conducting experiments using animal models (Van der Klis and Versteegh, 1996). As mentioned previously, the availability of PP in different feed ingredients is variable and ranges from 0% (Nelson et al., 1976) to almost 50-60% (Van der Klis and Versteegh, 1996; Simons et al., 1990). Also, the concentration of PP within the same ingredient can be different as shown by Barrier-Guillot et al. (1996) in wheat. These authors found that the concentration of PP ranges from 0.092 to 0.268%, on a dry matter basis, in wheat and depended on fertilisation of the soil, time of harvesting, stage of maturity and variety. The average PP concentration in 19 samples of wheat was 2.03% on a dry matter basis. The 1994 NRC gives an average PP content for wheat grain (hard red winter wheat) of 0.13% as is or 0.15% on a dry matter basis. One single average book value that does not reference the number of values used to derive this average or gives ranges and standard errors has limited applicability in formulation systems.

Because the ingredient PP content and availability for broilers changes, aP values would also be expected to change between batches of the same feed ingredient. To base the recommendation of P for broilers on aP would mean that aP would have to be determined every time a new batch of an ingredient is used to formulate feed for a research project where aP requirements are being determined. This would be needed because the book values of aP would not be accurate and would not reflect the true availability of P of that specific batch of the ingredient to broilers. From an applicability standpoint, use of aP requirement recommendations would have to be based on an extensive database that contains ingredient averages as well as range and standard deviations. On the other hand, the nPP system has the advantage of being based on chemical analysis than can be readily done on different batches of ingredients. But the nPP system also has limitations. Chemical analysis does not provide information on P availability of the ingredient or diet and thus any changes in PP and P availability of an ingredient will not be reflected in this system.

The NRC (1994) recommends a three-age-phase feeding program whereas industry is using four-age or five-age-phase feeding programs. The nPP requirements of the rapidly growing broiler decrease as the bird ages (O'Rourke et al., 1952). Increasing the number of phases helps to more closely match dietary nutrient concentrations to the bird's requirements. Use of more feed phases during broiler production would result in a decrease in the amount of nPP and P fed to birds and a decrease in feed cost. Also, feeding birds less nPP and P translates into less P being excreted. From a feed mixing perspective, increasing the number of feeding phases leads to more feeds being mixed in a feed plant, resulting in increased cost. The reduction in the cost of feed due to less P being added to the diet, as well as the economic importance of lower litter P, would ultimately have to be balanced with the cost involved in mixing and shipping diets more often.

III. KEY INFORMATION NEEDED WHEN COMPARING PUBLISHED PHOSPHORUS REQUIREMENT

When reviewing P requirement literature, one salient fact becomes obvious. It is difficult, and at times almost impossible, to interpret some of the published results as well as compare between resulting requirement values proposed between studies. In requirement research of any type, certain information that has a large impact on the results has to be included in the published work: breed/strain; age; starting and ending body weights; replication and birds/replicate; prior nutrition (specially as it pertains to Ca, P and vitamin D); age of breeder flock where eggs came from; feed consumption (especify of corrected for mortality); formulated and analyzed Ca and P; formulated vitamin D, protein, ME, fat, and all vitamins and minerals; dietary ingredients; light and temperature schedule; where possible light intensity; pen size/animal density; actual mortality and whether data is adjusted for mortality; formulation and mixing technique (i.e., was a basal formulated, at what level was the basal included, what was substituted in, was a blending technique employed); any use of feed additives (coccidiostats, antibiotics, growth promotants, prebiotics, probiotics, and information on product name, active ingredient, inclusion concentration); vaccination program used, environmental factors (time of year; maximum and minimum temperatures if under field conditions; whether work was done in batteries or floor pens, type of litter used and whether it was previously used).

IV. OTHER CONSIDERATIONS WHEN REVIEWING CALCIUM AND PHOSPHORUS REQUIREMENTS

Most research on Ca and P requirements in growing birds is done based on the impact of the concentration of these nutrients on bone ash. This is expected given that body weight gain changes are not a very sensitive measure of Ca and P requirements (Waldroup et al., 2000; Yan et al., 2001; Dhandu and Angel, 2003). Yet, when bone ash is the requirement-defining criterion, the resulting requirement should be defined as the Ca and P requirement for maximal bone ash. From a practical stand-point, this requirement has little practical application since it is not bone ash that is the sellable product. Bone ash, as a criterion, is important because it is perhaps the most sensitive of the methods available to determine Ca and P adequacy. But its applicability is limited to where bone ash decreases start to impact performance and especially where it starts impacting processing plant downgrades that affect carcass yields and/or incidence of bone chips in deboned meat. Yet, there is no work that establishes clear correlations between lab measures of bone mineralization (bone ash, bone breaking measures, x-ray measures or bone density measures) and performance changes or processing plant losses. Without these correlations, the implications of Ca and P requirement work based on bone ash are of limited practical applications.

An area that also should be considered is that most Ca and P requirements are given, in most published literature, on the basis of percent dietary inclusion. The tendency has been to look at this percent concentration in a study or to compare percent requirement concentrations between studies without giving enough importance to other factors (feed consumption, actual intake of the nutrient and other related nutrients, dietary sources of macrominerals and the availability of the macrominerals from these sources, growth rate of the animals in the study, animal strain and its impact on requirements, Ca to P ratios and concentrations of other nutrients of key importance such as vitamin D and microminerals). Ideally, Ca and P requirements should be given as a weight unit of the mineral actually consumed within a growth range (not age) that is defined and should always be compared within the specific context of the different trials.

V. SUMMARY OF PUBLISHED CALCIUM AND PHOSPHORUS REQUIREMENTS FOR BROILERS

The following Ca and P requirement summarized in Table 1 include research done since 1992. Work performed prior to 1992 was done with broilers whose growth rate was much lower than that of current strains. Regardless of how one looks at requirements, there is poor consensus on what the needs are for Ca and P in the modern broiler.

a) Starter phase (hatch to 21 days)

Males. Requirements for Ca based on performance averaged 0.89% (SD=0.16) and 16.73 mg Ca/g gain (SD=4.50), those based on bone mineralization averaged 0.89% (SD=0.16) and 14.04 mg Ca/g gain (SD=4.73). Requirements for nPP (aP) based on performance averaged 0.35% (SD=0.07) and 6.83 (SD=2.8) mg nPP/g gain, those for nPP (aP) based on bone mineralization measures averaged 0.39% (SD=0.07) and 6.10 (SD=1.82) mg nPP/g gain. Females (no values found). Straight run. Requirements for Ca based on performance averaged 0.93% (SD=0.20) and 15.43 mg Ca/g gain (SD=3.14) those based on bone mineralization (four values) averaged 1.03% (SD=0.20) and 16.81 mg Ca/g gain (SD=3.59). Requirements for nPP (aP) based on performance averaged 0.36% (SD=0.11) and 5.84 (SD=162) mg nPP/g gain and those based on bone mineralization averaged 0.40% (SD=0.04) and 6.52 (SD=0.43) mg nPP/g gain.

b) Grower phase (21 to 42 days)

Males. Requirements for Ca based on performance averaged 0.83% (SD=0.09) and 6.73 mg Ca/g gain (SD=2.32). Requirements based on bone mineralization measures averaged 0.82% (SD=0.11) and 7.31 mg Ca/g gain (SD=2.88). Requirements for nPP (aP) based on performance averaged 0.30% (SD=0.13) and 2.23 (SD=0.78) mg nPP/g gain. Requirements based on bone mineralization measures averaged 0.31% (SD=0.10) and 2.56 (SD=1.43) mg nPP/g gain, respectively. Females or straight run (no values found).

c) Finisher phase (42 to 49 days)

Males (five values). Requirements for Ca based on performance averaged 0.60% (SD=0.17) and 10.87 mg/g gain (SD=4.04). Requirements based on bone mineralization measures averaged 0.72% Ca (SD=0.12) and 10.67 mg Ca/g gain (SD=4.82). Requirements for nPP (aP) based on performance averaged 0.11% (SD=0.01) and 1.96(SD=0.40) mg /g gain. Requirements for nPP (aP) based on bone mineralization measures averaged 0.16% (SD=0.08) and 1.96 (SD=0.40) mg/g gain. For females or in straight run trials, requirements for Ca and nPP (or aP) were the same for performance and bone mineralization and were 0.30% Ca or 7.21 mg Ca/g gain, 0.34% nPP or 2.9 mg nPP/g gain.

d) Withdrawal phase (49 to 63 days)

Males. Requirements for Ca based on performance averaged 0.77% (SD=0.040) and 13.58 mg Ca/g gain (SD=4.51). Requirements based on bone mineralization measures averaged 0.77% (SD=0.038) and 13.71 mg Ca/g gain (SD=4.37). Requirements for nPP (aP) based on performance averaged 0.14% (SD=0.075) and 6.08 (SD=0.94) mg nPP /g gain. Non PP or aP requirements for maximizing bone mineralization measures averaged 0.23% (SD=0.066) and 17.93 (SD=1.91) mg nPP (or aP) per g of gain. Females (no values found).

The summary of data has to be looked at with care. Only in the starter and possibly grower phases, for males, are there enough data to warrant an average summary. For females, the data are scarce and summaries do not reflect actual requirements. Table 2 summarizes nPP requirements for maximum bone (tibia, toe and/or femur) mineralization based on work with male broilers (Ross 308) done in battery cages and in floor pens in our lab (Angel et al., 2000a, 2000b, 2001, 2005, 2006; Angel, 2007; Dhandu and Angel 2003; Ling et al., 2000). One of the important factors to be kept in mind while determining requirements for different phases is the carry over effect of previous nutrition. When the bird is fed a sufficient amount of P and Ca during the starter, grower and finisher phases, the removal of any added nPP and most of the Ca in the withdrawal phase will not affect the performance of the bird (Skinner et al., 1992a and 1992b; Angel et al., 2000a). Bone is a dynamic tissue and is constantly being remodeled according to the needs of the body. When the bird is fed sufficient P during earlier phases and less than the P required in the later phases, then P from bone will be used to meet the other P needs of the body. This could be one of the reasons Skinner et al. (1992a and 1992b) did not find any difference in performance yet saw differences in bone breaking strength when iP was removed from the diet compared to birds fed NRC (1994) recommended nPP concentrations from 42 to 56 d. The degree and length of mineral deficiency is important as both of these determine the structural integrity of the bone.

The question remains to be answered as to what bone integrity is needed such that no changes in processing plant downgrades occur regardless of bone ash. If bone integrity is compromised by feeding low concentrations of dietary nPP, then processing losses related to breakage of femur, broken drumsticks, cartilage separation associated with the rib cage, blood splash of meats and fractures could increase (Moran and Todd, 1994; Chen and Moran,

1995). According to Moran and Todd (1994), continuous feeding of low concentrations of dietary aP will lead to processing losses. These researchers fed either a control (NRC (1984) recommendations) or a low aP (10% below NRC (1984) recommendations) diet. Three phases were studied (starter, hatch to three wk; grower, three to six wk; finisher, six to eight wk). The Ca and aP concentrations fed in these three phases were 1% Ca and 0.45% aP, 0.90% Ca and 0.40% aP, and 0.80% Ca and 0.35% aP in the control diet. The low aP diet contained 0.40% aP in the starter, 0.35% aP in the grower and 0.30% aP in the finisher phase and Ca was similar to the control diet for the respective phases.

A study was done (Angel et al., unpublished) to determine the impact of targeted Ca and nPP levels on bone mineralization and on processing losses and to determine the correlation between bone measures and processing losses. All birds (Ross 308 males) were started on the same pre-starter diet (1.2% Ca and 0.55% nPP) fed from hatch to eight days and the same starter diet (0.9% Ca and 0.45% nPP) fed from eight to 18 days. Five treatments were tested as follows: Control (containing Ca and nPP in the grower (Gr, 18 to 30 d), finisher (Fn, 30 to 36 d) and withdrawal (Wd, 36 to 44 d) 0.9, 0.9 and 0.8% Ca and 0.35, 0.35 and 0.3% nPP, respectively. The moderate high (MH) treatment contained in the Gr, Fn, Wd phases 0.8, 0.6, 0.4% Ca and 0.32, 0.25, 0.12 nPP, respectively. The moderate low (ML) treatment contained in the Gr, Fn, Wd phases 0.8, 0.5, 0.4% Ca and 0.32, 0.17, 0.12% nPP, respectively. The low moderate (LM) treatment contained in the Gr, Fn, and WD phases 0.6, 0.4, 0.4% Ca and 0.25, 0.12, 0.12% nPP, respectively. The last treatment, low low (LL) contained in the Gr, Fn, and Wd phases 0.4, 0.4, 0.4% Ca and 0.12, 0.12, 0.12% nPP, respectively. Treatments were replicated 12 times in floor pens (56 birds/pen 0.065 sq metre per bird).

The total P consumed by treatment based on analyzed diet concentrations and feed consumption was 27.8, 24.6, 24.4, 22.0, and 19.0 g/bird (0.1653 P value). There was no effect of dietary treatment on final (44 day) body weight (mean body weight was 2410 g/bird), feed consumption, feed to gain ratio (mean uncorrected feed to gain ratio from hatch to 44 days was 1.90), or mortality. Bone ash differed between treatments regardless of bone measured. Femur ash was highest for the C treatment (49.8%) similar for treatments MH, ML, and LM (48.3, 48.1, and 47.5% respectively) and lowest for the LL treatment (46.9%). Corrected (broken parts removed) carcass wt and corrected hot carcass dress percentage were lower ($P<0.001$) only in the LL treatment. Overall downgraded carcasses were highest in the LL treatment (12.5%) vs. the C treatment (3.5%) with all other treatments being intermediate but not different from either the C or the LL.

Each individual bird was followed through the processing plant and any broken parts recorded and weighed, any discarded parts weighed, and tibia and femur taken for bone mineralization work to correlate back to the individual bird yields. There was a strong correlation, even when pen was the experimental unit, between femur ash and downgraded carcasses (% downgraded carcasses = 91.53 – 1.7685 femur ash percent, ($r2 = 0.701$) and between downgrades and nPP (% downgrades = 17.2435-0.847 nPP, $r2 = 0.552$).

Work of this type that allows for prediction of losses at processing based on nPP consumption or bone mineralization measures will allow industry to better decide what P levels to feed depending on market conditions, environmental regulations and desired outcomes.

VI. LAYING HEN PHOSPHORUS AND CALCIUM REQUIREMENTS

The amount of literature focusing on Ca and P requirements for laying hens is even more limited than for broilers. Of all the poultry species, the laying hen industry feeds typically much more P relative to the requirement, largely because of concerns of inadequate

mineralization of egg shells and skeletal abnormalities resulting in poor egg production, morbidity that results in welfare as well as in productivity concerns, and mortality. Due to previous selection of certain laying hen strains for early maturation and increased egg size, hens are, therefore, typically fed 350 to 450 mg of nPP/hen/day which recent research considers to be nearly twice what is required (Table 5).

With laying hens, the length of the studies as well as the genetics of the birds, feed consumption, and rate of lay have to be considered. Ca has a very tight homeostatic control resulting in the laying hen maintaining blood ionic Ca concentrations even during deficiencies as long as these are not of extended duration. To do this, the laying hen will mobilize Ca and P from long bones (medullary bones), with P being excreted because it is not needed at the levels mobilized and Ca being used to main egg production and blood homeostatic levels until the bird simply cannot stand. This summary contains selected references that show the variability in the results published and potential reasons why.

Van der Klis et al. (1997) concluded that the requirements for White Leghorns between 18 and 50 weeks of age was 0.13 % aP or 0.14 g aP/h/d when a corn, soy bean meal, sunflower meal and corn gluten meal diet diet containing 4% Ca (4.2 g/h/d Ca consumption) was fed. Gordon and Roland (1997) reported a 0.3% nPP requirement (0.27 g/h/d nPP consumed) for Hy Line W-38 hens fed corn and soy bean meal based diets containing 2.8% Ca (2.5 g Ca/h/d consumed) for 6 weeks. Boling et al. (2000a) reported in two long term studies (20 to 60 or 20 to 70 weeks) studies a requirement of 0.15 aP (0.15 g/h/d aP consumed) in a corn soybean meal based diet containing 3.8% Ca. These authors also fed phytase and found that when phytase was added to the lowest aP diet (0.1%) at 100 U/kg, performance and bone mineralization were returned to control levels.

Recent work by Karcher et al. (2006) suggests that the requirement for aP in Hy-Line W-98 hens fed corn and soy bean meal based diets containing 3.49% Ca (4.4 g/h/d Ca consumed) was 0.16 % aP (0.2 g/h/d aP consumed) in a 120 week experiment. When the experiment was extended for 24 more weeks and the feed consumption controlled at 100 g/h/d the requirement increased to 0.24% aP (0.2 g/h/d aP consumed).

Pelicia et al. (2009) showed no differences in performance (body weight changes, feed conversion ratio/dozen or per kg of egg produced, egg quality measures) when 58 week old Hisex Brown layers were fed diets containing between 3 and 4.5% Ca and 0.34% aP for 84 days. Al-Sharafat et al. (2009) fed 23 week old Lohman-Brown Classic hens for 15 weeks a 2.6 or 3.8% Ca diet containing 0.33% total P and 0.1% nPP with or without phytase. Layers fed the 0.26% Ca diet without phytase lost weight, exhibited depressed egg production and egg mass and had an impaired feed conversion ratio and lower tibia ash as compared to those fed the 3.8% Ca. Changes in performance were not seen until 6 weeks into the experiment. Phytase improved productivity at both levels of Ca suggesting that the 0.1% nPP in the diets was deficient. Inclusion of phytase to the 2.6% Ca and 0.1% nPP diet was unable to restore performance or bone ash to control levels.

Valkonen et al. (2010) fed diets containing 4.38 or 3.73% Ca and 0.44% aP to 21 week old Lohman Selected Leghorns for 52 weeks (72 weeks of age). Laying hens fed the 4.38% Ca were lighter at 72 weeks of age but had produced more eggs with similar egg mass as those fed the 3.73% CA. Tibia ash was higher in birds fed the 3.73% Ca as compared to those fed the 4.48% Ca. The type of housing (furnished vs. conventional cages) had a greater impact on tibia ash with the hens in furnished cages having greater bone ash. This suggests that the total Ca requirement for these hens may be closer to 3.73% Ca.

As can be seen from the above short summary, requirements for laying hens are widely variable, ranging from 0.1% aP to requirement of 0.32% and consumptions ranging from 0.14 to 0.4 g/h/d As said earlier when evaluating the literature for Ca and P

requirements for laying hens, the breed, level of Ca, ingredients used in the diet, feed consumption, age of the hens and length of the study are critical.

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Table 1 Broiler calcium (Ca) and phosphorus (P), non-phytate P (nPP), and available P (aP) requirements for broilers

Reference	Criteria[1]	Breed and Sex[2]	Age, d	Gain g	Ca %[3]	P %[4]	nPP %[5]	Ca, mg /g gain	P, mg /g gain	nPP or aP, mg/g gain	P source[6]	Diet main ingredient
Starter phase (hatch to 21 days of age)												
Moran and Todd, 1994	Perf	Ross x AA, M[1]	Hatch-21	683	**1**	**0.68**	0.31	17.58	10.4	5.48	Ca_2PO_4	Corn, SBM, $CaCO_3$
Waldroup et al., 2000	BW	Cobb 500, M	Hatch-21	*628*	1	0.57	0.32					
	TbA			*678*		0.64	0.39				Ca_2PO_4	Corn, SBM, $CaCO_3$
Bar et al., 2003	Gain					**0.74**					$CaPO_4$,$KHPO_4$ $NaHPO_4$	Corn, sorghum, SBM, $CaCO_3$
	TbA	Cobb, M	8 to 21	504	**1.04**	**0.78**						
Waldroup et al, 2003	Perf	Cobb 500, M	Hatch-14	*380*	0.9	0.66	0.40				Ca_2PO_4	Corn, SBM, $CaCO_3$
Rama Rao et al., 2003	Perf	Vencob, M	1-21	*554*	0.6		0.3	9.56		4.78	Ca_2PO_4	Corn, SBM, oyster shell, Coban
	BrkF			*582*	0.7		0.4	10.57		6.04		
	TbA			*577*	0.6		0.35	9.23		5.39		
Huyghebeart, 1996	Perf	Ross, M	1-21	*613*	0.8	0.59	0.3	12.17	8.97	4.57	$CaPO_4$	Wheat, SBM
Runho et al., 2001	Perf, TbA,BrkF	Hub, M	1-21	*689*	1		0.45	16.91		7.62	Ca_2PO_4	Corn, SBM, limestone
	Perf	Hub, F[1]		*613*	1		0.45	16.7		7.52		
Hurwitz et al., 1995	Gain	Cobb, M/F	7-21	*590*	1.18	0.7					KH_2HPO_4 NaH_2HPO_4	Corn, SBM, $CaCO_3$
	TbA			*600*	1.31	0.7						
Beltran-Lopez et al., 2000	Gain, TbA	AA[3], M/F	1-21	*590*	1		0.38	14.61		5.69	Ca_2HPO_4	Corn, SBM, fish meal, $CaCO_3$
Brugalli et al., 1999	Perf, TbA, BrkF	Hub M/F	1-21	*690*	1.0	0.67	0.45	16.23	10.79	7.3	Ca_2HPO_4	Corn, SBM, oyster shell
Li et al., 2000	Perf, TbA	NS[4], M	1-21	*792*	1.0	**0.74**	0.45	13.07	9.67	5.88	Ca_2HPO_4	Corn, SBM, $CaCO_3$
	Toe ash			*574*	0.8	**0.52**	0.28	10.8	7.02	3.78		
Perney et al.,1993	Perf	Hub, M	4-14	*240*		0.54	0.32	13.08	7.07	4.17	Ca_2HPO_4	Corn, SBM, $CaCO_3$
	Tb/toe ash			*274*	1.0	0.66	0.44	12.7	8.38	5.58		
Denbow et al., 1995	Gain	Pet AA, M	1-21	*579*	1.04	0.52	0.34	14.91	7.45	4.87	DFP[5]	Corn starch, SBM, $CaCO_3$
	Perf, toe ash			*453*	0.9	0.45	0.27	12.95	6.48	3.69		
Al-Masri, 1995	Perf	Lohman, M	14-21	*231*	0.61	0.6		9.97	8.82		NS	Corn, sorghum, SBM, $CaCO_3$
Ribeiro et al., 2003	Perf, TbA	Cobb x Cobb M/F	3-15	*315*	1.00	0.66	0.36	13.81	9.11	4.96	Na_2HPO_4	Corn, SBM, Ca_2CO_3
Zyla et al., 2001	Perf	Hub, M/F	1-21	*560*	**0.66**	**0.53**	0.17	10.3	8.27	2.65 (5.17)	Ca_2PO_4	Wheat, SBM, $CaCO_3$
Al-Masri, 1995	Perf	Lohman, M	21-29	*444*	0.61	0.6		6.48	6.37			Corn, sorghum, SBM, $CaCO_3$
Brenes et al., 2003	Perf TbA	Cobb, M/F	1-21	*587*	**0.82**	**0.61**	0.35	12.88	9.58	5.49	Ca_2HPO_4	Corn, SBM,
Waldroup et al., 2003	Perf BrkF	Cobb 500, M	14-35	*1512*	0.8	0.55	0.30				Ca_2PO_4	Corn, SBM, $CaCO_3$

Kornegay et al., 1996	Gain, toe ash	Pet AA, M	1-21	*613*	1.0	0.69	0.45	15.71	10.84	7.07	DFP	Corn, SBM, $CaCO_3$
	Feed efficiency			*511*	0.88	0.44	0.20	14.38	7.19	3.27		
Reference	Criteria[1]	Breed and Sex[2]	Age, d	Gain g	Ca %[3]	P %[4]	nPP %[5]	Ca, mg /g gain	P, mg /g gain	nPP or aP, mg/g gain	P source[6]	Diet main ingredient
Grower Phase (21 to 42 days of age)												
Yan et al., 2001	Perf	Cobb 500, M	21-42	*1433*	*0.9*	0.43	0.19		7.94	3.47	Ca_2PO_4	Corn, SBM, $CaCO_3$
	TbA					0.57	0.33	16.77	10.66	6.19		
Bar et al., 2003	Gain	Cobb, (ns)	29-43	985	**1**	**0.51**		21.46	10.94		$CaPO_4$, Na and $KHPO_4$	Corn, sorghum, SBM, $CaCO_3$
	TbA					**0.64**			13.73			
Moran and Todd, 1994	Perf	Ross x AA, M	21-42	1317	**0.91**	**0.62**	0.45	13.7	9.32	5.64	Ca_2PO_4	Corn, SBM, $CaCO_3$
Chen and Moran, 1995	Perf,, TbA, FmA, BrkF	Ross, M	21-42	*1710*	**0.87**	**0.63**	0.4	16.36	11.84	7.52	Ca_2HPO_4	Corn, SBM, $CaCO_3$
Huyghebeart, 1996	Perf	Ross, M	21-42	*1676*	0.80	0.59	0.3	14.49	10.69	5.44	$CaPO_4$	Wheat, SBM
Dhandu and Angel, 2003	Perf	Ross 308, M	32-42	652	**0.69**	**0.42**	**0.15**	14.3	8.7	3.11	$CaPO_4$	Corn, SBM, $CaCO_3$
	TbA			653		**0.47**	**0.2**	13.79	9.39	4		
Finisher Phase (42 to 49 days of age)												
Yan et al., 2003	BW	Cobb 500, M	42-49	425	**0.78**	**0.30**	0.1	18.35	7.06	7.29	Ca_2PO_4	Corn, SBM, limestone
Waldroup et al., 2003	TbA	Cobb 500, M	42-56	945	0.5	0.51	0.31	5.29	12.00	2.33	Ca_2PO_4	Corn, SBM, $CaCO_3$
	BekF					0.33	0.1		3.49	1.06		
Skinner et al., 1992a	TbA	Cobb 500, M	42-49	924	0.3	0.38	0.15	5.41	4.11	1.62	Ca_2PO_4	Corn, SBM, $CaCO_3$
	Gain			478		0.34	0.12	6.28	7.11	2.51		
Skinner et al., 1992b	Gain	Cobb 500, F		416	0.3	0.33	0.12	7.21	8.17	2.88	Ca_2PO_4	Corn, SBM, $CaCO_3$
	Tb BrkF	Cobb M/F		434	0.39			8.99	7.83	2.76		
	Perf	Cobb 500, M	42-56	752	0.6			8.91	4.79	1.60		
Chen and Moran, 1995	Tb BrkF	Ross, M	42-49	737	0.6	0.45	0.24	8.11	6.11	3.26	Ca_2HPO_4	Corn, SBM, $CaCO_3$
	Performance, bone measures			541	**0.54**	**0.34**	0.1	9.98	6.28	1.85		
Dhandu and Angel, 2003	Performance	Ross 308, M	32-42	666	**0.72**	**0.36**	**0.1**	10.81	5.41	1.50	$CaPO_4$	Corn, SBM, $CaCO_3$
	Tibia ash			675		**0.42**	**0.16**	14.78	8.62	3.29		

Withdrawal Phase (49 + days of age)												
Dhandu and Angel, 2003	Performance	Ross 308, M	42-49	680	0.72	0.36	0.1	10.59	5.29	1.47	$CaPO_4$	Corn, SBM, $CaCO_3$
	Tibia ash			666		0.38	0.13	10.80	5.71	1.95		
Yan et al., 2003	Body weight	Cobb 500, M	49-56	523	**0.78**	0.30	0.1	14.91	5.73	1.91	Ca_2PO_4	Corn, SBM, $CaCO_3$
	Tibia ash					0.44	0.23		8.41	4.39		
	Body weight		56-63	403	**0.78**	0.3	0.1	19.35	7.44	2.48		
	Tibia ash					0.42	0.22		10.42	5.46		
Moran and Todd, 1994	Performance	Ross x AA M	42-56	855	**0.81**	**0.50**	0.25	9.47	5.85	2.92	Ca_2PO_4	Corn, SBM, $CaCO_3$

(Requirement values in bold are values where actual Ca and P analysis are provided.) [1] Criteria used for determining requirement; Perf (performance), BW (body weight), TbA (tibia ash), FmA (femur ash), BrkF (breaking force. [2] Sex specified as; M (males), F (females) and SR (straight run); breed Hub (Hubbard), AA (Arbor Acres), Pet (Petterson), ns (not specified). [3] Ca (calcium). [4] P (Phosphorus). [5] nPP (non-phytate P). [6] Phosphorus source; DFP (defluorinated phosphate).

Table 2 Summary of non-phytate phosphorus (nPP) requirements for male Ross 308 broilers[1,2].

Body Weight Range	Average feed consumption	Calcium			nPP		
g	g	%	g	mg/g gain	%	g	mg/g gain
40 to 170	160	1.10	1.76	13.54	0.50	0.80	6.15
171 to 700	751	0.90	6.76	12.78	0.40	3.00	5.68
701 to 1500	1300	0.75	9.75	12.20	0.32	4.16	5.21
1501 to 2100	1200	0.55	6.60	11.02	0.21	2.52	4.21
2101 to 2800	1528	0.50	7.64	10.93	0.15	2.30	3.29
2801 to 3600	1918	0.40	8.63	10.80	0.12	2.30	2.88

[1] Broilers were fed corn-soybean meal diets containing an average of 2500 ICU added D_3. Density in floor pen work ranged between 0.7 and 0.8 ft^2 per bird at placement. Requirements are based on no significant change in bone ash versus either NRC (1994) or industry average usage concentrations. Average overall feed to gain ratio 1.93 to 3600 gram body weight.
[2] From Angel et al., 2000a, 2000b, 2001, 2005, 2006; Angel, 2007; Ling et al., 2000; Dhandu and Angel, 2003.

Table 3 Laying hen calcium (Ca) and available phosphorus (aP) or non phytate phosphorus (nPP) requirements

Reference	Criteria[1]	Breed [2]	Age Start, Wk	Study lenth, Wk	Ca %[3] (Ca cons. g/h/d)	P %[4] P cons. g/h/d	nPP %[5] (P cons g/h/d)	aP, %[6] (P cons. g/h/d)	Phytase, U/kg[7]	Ca Source[8]	P source[9]	Diet main ingredient[10]
Um and Paik, 1999	Perf, EQ, SQ,	Isa Brown	21	*19*	3.7 (4.3)	0.61 (0.69)		0.37	10.69	Limestone	TCP	Corn, SCM, RSM, WB
Usayran and Balnave, 1995	Performance plasma P	New Hampshire x Leghorns	3**3**	24	3.5 (4.4)	0.57		0.32 (0.4)		Limestone	DCP	S, W, SBM
Summers, 1995	Perf.	White Leghorns	18	36	3.6 (3.6)	0.55		0.30 (.3)		Limestone	DCP	Corn, SBM,
Van der Klis et al., 1997	Perf. Tibia ash Egg quality	White Leghorn	18	50	4.0 (4.2)	0.33		0.13 (0.14)		Limestone	MCP	Corn, SBM, SFM, CGM
Gordon and Roland, 1997	Perf Egg quality Bone density	Hy-Line W-38	58	6	2.8 (2.5)		0.3 (0.27) 0.1+ (0.09)		 300	Limestone	DCP	Corn, SBM
Boling et al., 2000a	Perf Bone ash	Dekalb Delta	20	50	3.8			0.15 (0.15) 0.1+ (0.11)	 300	Limestone	?	Corn, SBM
Boling et al., 2000b	Perf Egg quality	Dekalb Delta	20	40	3.8			0.15 (0.15) 0.1+ (0.11)	 100	Limestone	?	Corn, SBM
Kershevarz, 2000	Performanc, Tibia ash P retention	Babcook B300	30-42	36	3.8	0.45+	0.2+		300	Limestone	DCP	Corn, SBM,
			42-54			0.35	0.1+		300			
			56-66			0.35	0.1+		300			
			30	20	3			0.25				
Karcher et al., 2006	Perf. Egg quality	Hy-Line W 98	23	20	3.49 (4.4)	0.38	0.16 (0.2)			Limestone	MCP	Corn, SBM
			43	24	3.49	0.46	0.24 (0.2)					

Hughes et al., 2008	Performance Tibia ash	Shaver white Bovan	21	666 675	0.72	0.36 0.42	0.1 (0.16)	10.81 14.78	5.41 8.62	$CaCO_3$	$CaPO_4$	Corn, SBM,
Pelicia et al., 2009	Perf, EQ, SQ	HS Brown	58	12	3.0			0.34		L calcitic	DCP	Corn, SBM, WB
Al-Sharafat et al., 2009	Perf, BA	L Brown Classic	23	15	3.8	0.31+	0.1+		300	CC	None +	Corn, SBM, W
Valkonen et al., 2010	Bone ash	L Selected leghorns	21	55	4.4 (5.5)	0.73 (0.86)		0		L	MCP	B, W, O, SBM

[1] Criteria used for determining requirement; Perf (performance primarily feed conversion), EQ (egg quality), SQ (shell quality), BA (bone ash). [2] Breed L (Lohman), HS (Hisex) AA (Arbor Acres), Pet (Petterson), ns (not specified). [3] Ca (calcium) value in parenthesis is analyzed. [4] P (Phosphorus) value in parenthesis is analyzed, none+ (if no inorganic source of P was added) [5] nPP (non-phytate P). [6] aP (available P). [7] Phytase use needed at the Ca and P concentrations specified and concentration of phytase in units/kg. [8] Calcium source; CC (calcium carbonate); L (limestone). [9] Phosphorus source; DFP (defluorinated phosphate), monocalcium phosphate (MCP), DCP (dicalcium phosphate), TCP (tri calcium phosphate). [10] Ingredients; SBM (soybean meal), W (wheat), B (Barley), (S) sorghum, WB (wheat bran), WM (wheat middling's), DDGS (distillers grains with soluble), O (oats), RSM (rape seed meal), corn gluten meal (CGM), sunflower meal (SFM).

CHALLENGES FACING THE GLOBAL POULTRY INDUSTRY UNTIL 2020

A.M. PENZ-Jr[1] and D.G.BRUNO[1]

Summary

The increasing demand for animal products resulting from demographic factors, technical and scientific developments, diminishing resources, and increasing consumer demands for more food safety, lower environmental impact, and better animal welfare conditions will determine the development of the poultry industry during the next decade. In this scenario, the traceability of poultry products will be essential. This requires the careful selection of input suppliers, with the focus on product quality rather than on price. Monitoring flock health status will also be the key for the safe expansion of the poultry industry. As to the rearing environment, heat production by broilers should be taken into account, and its utilisation considered as an alternative energy source. In the field of nutrition and food technology, the most significant aspects will be the use of enzymes, the evaluation of non-nutritional factors, which may maximize ingredient utilization by the birds (feed processing and particle size), the utilization of new synthetic amino acids on an industrial scale, the application of new feed formulation concepts to improve dietary energy utilization, the use of nutraceuticals to modulate intestinal microbiota and the immune system as an alternative to therapeutics, and the use of special pre-starter diets. Feedstuffs should no longer be considered as commodities. Qualitative and nutritional criteria should be used for their purchase and segregation in feed mills. Technologies allowing the immediate analysis of feedstuffs, such as NIRS, will be required. Genetic engineering will become an important tool to improve feedstuff nutritional quality and, perhaps, bird performance. In this sophisticated context, growth modeling and data-analysis using computer systems will allow more robust decision-making, which will be the key for the sustainability and success of the poultry industry.

I. INTRODUCTION

In the last few years, agricultural production has experienced significant development due to an increasing demand for food by the world's population. This demand results particularly from the increase in the global population, as well as in average income and urbanization. The United Nations (UN) estimates that there will be 8 billion people on the planet by 2030, whose income will be, on average, 32% higher than in 2006. In addition, meat consumption/person per year will increase by 26% in the same period, and this increase in consumption will be chicken meat, in particular (FAO, 2010; OCDE-FAO, 2010).

However, these are not the only factors that will influence the evolution of the poultry industry in this coming decade. Technical factors and the evolution of science and technology, the availability of natural resources and water (which are becoming increasingly limited), and the maintenance of trade barriers must also be considered. The price of raw materials for feed production will also influence poultry production in the next few years. According to OCDE-FAO (2010) estimates, feedstuff prices will be higher than the historical average between 2010 and 2019, but lower than the peaks experienced in 2007 and 2008. Finally, consumer demands will have a strong influence as these demands are becoming increasingly concerned with animal welfare issues, food safety, and environmental impact relative to poultry production. New methods to assess the economic and environmental impact of poultry production have been developed. An example is the LCA (Life Cycle

[1] Provimi America Latina, Campinas, Brazil

Assessment), an ISO-standardized procedure that proposes to evaluate the impact of poultry production during the entire flock life cycle, from raw material purchase, waste production and treatment, to production surplus recycling and disposal on the environment (van der Werf and Prudêncio da Silva, 2010).

The objective of this article is to discuss some of the challenges that the poultry industry will have to face during this coming decade and the production, nutrition, and technology trends that will allow it to overcome these challenges.

II. ANIMAL WELFARE AND ITS CONSEQUENCES

The welfare of animal production can be accessed from two perspectives: through anthropomorphism, where consumers put themselves in the place of livestock and make conclusions about their welfare often based in subjective ideas, or through animal performance. Animals that are reared in poor welfare conditions are not able to express their maximum genetic potential. Consumer concerns relative to poultry welfare are becoming increasingly relevant in the meat market. There is a positive correlation between the strictness of welfare legislation and income of the citizens of a country and consequently their purchasing power (van Horne and Achterbosch,2008). These concerns are evident, particularly in the European Union, and examples are Directives 1999/74/EC and 2007/43/EG, which established the ban on conventional cages for commercial egg production after 2012, and maximum broiler density, respectively. One of the challenges for the next decade is to create standardized parameters for poultry welfare assessment and robust systems to monitor these parameters. This is the aim of the Welfare Quality project developed in the European Union, which proposes to assess animal welfare focusing on animals, and not on environmental or management factors, and using objective indicators that can be easily measured in the field, according to four principles: good feeding, good housing, good health status, and adequate behaviour (Arnould and Butterworth, 2010).

III. FOOD SAFETY

Food contamination by pathogens is the main concern of consumers (IFC, 2010). Supplying this demand for safer food requires transparency and commitment by all the parties involved in the process of food production, including governments. Each step of the food supply chain will be increasingly controlled, with emphasis on risk monitoring through preventive and corrective actions (analysis and monitoring critical control points). This requires careful selection of input suppliers, focusing on product quality and not price, which requires evaluation and maintenance plans, understanding the process and the materials used by suppliers, and technical knowledge on physical, chemical, and microbiological risks. In the feed mills during the next decades, automation will increase, with lower exposure of workers to operational risks, and more emphasis will be placed on critical control points, which will be monitored just in time, and on real-time traceability. Andree and Schwaegele (2010), who participated in the development of a project to analyze existing or potential vulnerable points in food production chains, said the loss of information or of traceability is the main risk factor for the entrance of contaminants into the process of animal feed production. Giving proper attention to these new requirements is of great importance for the poultry industry, particularly considering the exporting countries, which must comply with the increasing demands of the importers.

Health monitoring of the flocks is and will become increasingly important, not only to prevent foodborne disease, but also to avoid performance losses and to ensure bird welfare. Compliance with health programs (cleaning and disinfection, vaccination, pest control,

disease monitoring), immediate notification and record of abnormal situations, health monitoring programs, and measures for infection control and eradication must be put in place, particularly in a scenario where the use of antimicrobial compounds is increasingly restricted.

IV. ENVIRONMENT

Thermal comfort inside poultry facilities is essential, as unfavourable environmental conditions significantly affect production. Both excessive cold and heat may cause production losses and impair bird health and welfare and, in extreme situations, increase bird morbidity and/or mortality. The evolution of technology and of the knowledge on thermoregulation physiology and behaviour will reduce mistakes in poultry house design and in bird management that can cause thermal discomfort. The development of information technology allows new techniques in the study of broiler thermal comfort, such as the use of real-time image analysis using video cameras, image-acquisition hardware, and image-analyzing software programs to acquire, process, and evaluate information (Moura et al., 2010). Interestingly, inside broiler houses, 80% of the heat is not produced by lamps or brooding systems, but by the birds themselves. Proper evaluation of this heat production may allow creating mechanisms for the utilization of this energy, which could be translated in significant cost savings.

V. NUTRIENT UTILIZATION AND FEED FORMULATION

Out of the trends currently observed and that will define how nutritionists are going to formulate diets in the next ten years, is the increasing cost of raw materials and the pressures to reduce feed costs and nutrient environmental excretion will be emphasised. These factors will cause diets to be formulated more accurately, avoiding large safety margins. The biofuel industry will compete for raw materials used for animal feeding, and will require the utilisation of its byproducts. In this case, knowledge of the analysis of the nutritional content and digestibility of these materials, which are not yet standardized, as in the case of distillers dried grain solubles (DDGS**)** for example in the US, should be developed.

In this context, enzymes will be increasingly used, as they improve ingredient digestibility and nutrient absorption (Cowan et al., 1996), as well as reduce the detrimental effects of anti-nutritional factors, thereby allowing higher flexibility in the use of feedstuffs as well as reducing feed costs (Ferket, 2009) and pollutant excretion in animal waste (Penz-Jr and Bruno, 2010).

Higher emphasis will also be placed on anti-nutritional factors that change energy and nutrient availability for broilers, using particle size and diet processing to maximize nutrient supply. Better pelleting, expansion, and extrusion processes, among others, will be developed, in terms of physical aspects (temperature, moisture, pressure, time) and their effects on nutrient utilization (Ferket, 2009).

Skinner-Noblet et al. (2005) observed that pelleting improves effective dietary energy value by changing the behaviour of broilers, which includes higher feed intake of birds fed pelleted feed. Methodologies to evaluate the impact of heat stress during corn (Métayer et al., 2009) and soybean meal drying (Helmbrecht et al., 2010) on their nutritional quality are currently available. Corn particle size and density may also result in different nutrient digestibility, and should be better evaluated. Hetland et al. (2002) observed higher starch digestibility in broilers fed whole wheat grain as compared to those fed ground wheat. Parsons et al (2006) concluded that higher particle sizes promote a linear increase in the feed

efficiency of broilers. Figueiredo et al. (2009) observed that corn density is directly related to its metabolizable energy content.

As to protein nutrition, new synthetic amino acids, produced at competitive prices, will become commercially available. In addition to lowering feed costs, this will also reduce nitrogen excretion in the environment (Nahm, 2002). Research on the next limiting amino acids after threonine will be extremely important, and their requirements will have to be evaluated not only relative to lysine, but also as to minimum intake and impact of their use under practical broiler production conditions (Kidd, 2009). For instance, the use of valine for broilers, whose beneficial effects were demonstrated by Corzo et al. (2009), is becoming a reality.

Energy is usually the most expensive nutritional component of poultry diets. Therefore, a higher efficiency in its utilization will result in lower feed cost. One of the strategies to be considered is formulating diets not only takes into account a feedstuff's metabolizable energy, but also its net energy defined as metabolizable energy minus energy loss due to heat increment, that is, the energy that is effectively used for production. This strategy may allow reducing feed cost and nutrient excretion (Mohen et al., 2005) and it is currently being discussed in Australia by the Poultry Cooperative Research Centre (Clements, 2010).

The utilization of trace minerals will be determined by a better understanding of their interaction with the immune system, as well as on the quality of their sources, preventing final product contamination with residues. In addition, further research on the differences between organic and inorganic sources is also needed.

VI. INTERACTION BETWEEN NUTRITION AND INTESTINAL HEALTH

a) Use of additives: an economic, political or technical issue?

The restrictions on the use of antimicrobials as growth promoters, due to consumer demands and to the recent understanding of the interaction between nutrients and intestinal health, intestinal microbiota, and the immune system, will require nutritionists to change their paradigms. It seems that there will be an increasing need to concentrate efforts in the modulation of the intestinal microbiota and immune system through the use of nutraceutics, instead of controlling enteric diseases with therapeutic compounds (Ferket, 2009). Moreover, complying with the recommendations related to flock health management and farm biosecurity will become increasing critical. Today, there is a wide range of nutraceuticals available in the market, including acidifiers, prebiotics, probiotics, essential oils, enzymes, osmoregulators, nucleotides, zinc oxide, etc.

b) Perinatal nutrition

Due to genetic improvement and a reduction in market age, the perinatal period of broilers corresponds to 50% of their life cycle. Therefore, nutritional management during this stage, aimied at ensuring the proper supply of water and feed to the birds, will become increasing critical. Studies have shown the consequences of feed and/or water restriction during the first hours of the chick's life, resulting in intestinal villi damage (Geyra et al., 2001; Viola et al., 2009). It was demonstrated that access to energy and nutrients immediately after hatching accelerates intestinal development, and consequently, broiler growth (Uni et al 1998). In this context, the supply of specific nutrients and the establishment of specific management practices dedicated to this phase will become increasingly important, and pre-starter diets will be extensively supplied. Another technology that is becoming more popular is *in-ovo* nutrition, when nutrients are injected into the amniotic fluid of embryos during the last stage of incubation, stimulating the development and maturation of the intestinal villi before hatch.

Foye et al (2007) observed that chicks submitted to *in-ovo* feeding had higher concentrations of digestive enzymes. Kornasio et al (2010) also found higher breast muscle yield in broilers fed *in ovo* caused by the influence of nutrients on muscle satellite cells.

VII. FEED MILL

Regarding feed mill structure, in the near future there are at least two challenges for the animal protein industry.

The first one is related to the regulatory issues implemented by different countries, looking forward to product traceability and production sustainability. In 2005, the EU nations established the regulatory 183/2005/CE that was implemented at beginning of 2006. The main objective was related to animal feed hygiene, to guaranty animal and human feed and food safety. Regulations like these stimulated different countries, especially the meat exporter ones, to start implementing good manufacturing practices (GMP) locally in their feed mills. All these new regulations require investments in feed mill structures and good database information to preserve traceability of the final product that the consumers would have available.

The second one is the understanding of the segregation of ingredients concept. So far, corn and soybean are mainly considered by their traders as commodities. The final nutrient composition does not always make an important difference in negotiations. In the future, this oversimplification will not be acceptable, once feedstuffs will continue representing at least 70% of the final cost of a business that more and more will be tied by cost efficiency. So, ingredient nutrient variations caused by plant cultivar, processing, harvest year, nutritional density, presence of mycotoxins, etc., will need to be more seriously considered if the main purpose of the business will be reaching the precision nutrition concept. As an example, Zhou et al. (2010) observed that amylose to amylopectin ratio is one of the main factors that determines true metabolisable energy of corn, and can be used to predict available energy for poultry. Li et al. (2000), through genetic engineering, were able to improve the nutritional quality of corn, developing low-phytate varieties. Neoh and Ng (2006), studying soybean meal coming from Malaysia, USA and Argentina, were able to identify differences in apparent metabolisable energy of the samples which influenced the performance of the broilers. Therefore, it is clear that at least these ingredients can no longer be considered as commodities, and their qualitative and quantitative aspects must be taken into account when someone is making purchasing decisions. To accommodate the ingredient differences, the segregation concept must be implemented in feed mills. This will demand investments in silos to store different batches, according to nutritional characteristics of the ingredients. For corn and other cereals, besides investment in silos, feed mills will need to implement cleaning structures and gravity separators should become a common practice to separate them based on their densities. However, the implementation of ingredient segregation is limited to wet chemistry techniques, which are usually expensive and time-consuming. This limitation may be overcome by the use of NIRS (Near Infrared Reflectance Spectroscopy), that allows immediate analysis of energy as well as amino acid composition and digestibility of each feedstuff batch (Penz-Jr et al., 2009). So, the design of new feed mills will have to consider the use of NIRS, providing more storage, dosing, and milling flexibility, which will allow savings that are not feasible today due to the lack of this physical infrastructure.

VIII. UTILIZATION OF THE AVAILABLE KNOWLEDGE AND TECHNOLOGICAL INNOVATION

The progress in information technology will allow the application of growth models and several related mathematical equations, which will estimate animal growth according to rearing conditions. The ultimate objective will be optimizing the rearing process as a function of the company's or farmer's needs. Feed intake and broiler growth prediction models under different scenarios, such as those developed by Emmans, Fisher and Gous, will allow better definition of strategies that will favor production efficiency. Gous (2005) also mentioned that the idea of abandoning the conventional formulation proposal and adopting a dynamic proposal, based on several factors in addition to those considered in least-cost formulations, is not new.

In addition to growth models, simulation models could also be used to evaluate risks and to optimize financial return using data mining (correlations, classifications, associations, neural networks, and clustering) and data analysis by bioinformatics, meta-analysis, and holo-analysis techniques (Ferket, 2009). Geographic information systems (GIS) are already used for production zoning and viewing, allowing correlation of performance parameters with the geographical location of poultry houses in terms of altitude, latitude, and longitude. These tools are becoming increasingly important to make decisions as to which product should be used to maximize the economic performance of birds under different rearing conditions. However, the efficient use of these tools depends on the availability of detailed and accurate data, with a complete house inventory.

There are even more "futuristic" tools that control animal performance in real time. The IMS technique (Integrated Management Systems) aims at providing a completely on-line and real-time system, with no human interference, except when a problem is detected. This technique is operated by a "visual image analysis (VIA)" system that, using video cameras placed inside the poultry house, allows the continuous collection of images. By measuring bird area and length, bird body weight and carcass yield may be determined with an accuracy similar to that of conventional tables (Penz-Jr et al 2009). This technique is already used for pigs in Europe, and prediction measures for broilers are still under study because the feathers make the true measure of meat surface area difficult (Green and Parsons 2006).

In scientific innovation, a new field of knowledge nutrigenomics must be considered. Nutrigenomics studies the molecular relationships between nutrition and gene response, and aims at understanding how gene expression is induced by nutrients or feeding regimes, with consequent influence on performance parameters.

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UTILIZING THE LATEST DEVELOPMENTS IN ANIMAL NUTRITION, NUTRIGENOMICS, TO OPTIMIZE THE ANTIOXIDANT STATUS OF BROILERS

A. LEARY[1] and A. KOCHER[2]

Summary

Traditionally, the animal feed industry has relied on growth or performance studies in animals as a means of investigating the effect of a dietary change. Unfortunately, these trials generally take an extended period of time and provide information on only a few parameters. A new technology, nutrigenomics, has provided a means of developing a more detailed understanding of changes caused by a specific nutrient because it considers changes in expression of thousands of different genes. Practical application of nutrigenomics technology within the animal feed industry has resulted in development and evaluation of novel feed additives. The following paper will discuss this new technology and how it is currently being applied in the animal feed industry.

I. CLASSICAL NUTRITION

In recent years, the focus of most nutritional research has been on parameters related to animal growth performance, reproduction, intestinal health or the utilisation of specific nutrients as a mean to reduce excess waste. Classical nutritional studies, which are commonly used, involve a considerable number of individual animals and replication of treatments in order to establish statistical differences between individual treatments. Depending on the actual hypothesis, nutritional studies usually take a considerable time to deliver a final conclusion. The measured parameters are often closely interrelated, hence it is difficult to demonstrate the effect of a specific dietary ingredient. For example, damage in the intestine as a result of bacterial overgrowth will result in reduced nutrient absorption and, subsequently, reduced animal performance. The question remains if the excess availability of nutrients leads to more bacterial growth or if bacterial growth leads to a lower availability of nutrients.

The key to understanding the relationship between specific nutritional intervention and the impact on health and performance lies in a deeper understanding of the impact of these nutrients on the expression of specific genes or specific metabolic pathways. The development of molecular tools which enable researchers to study the effects of specific nutrients on the whole genome or, in other words, the effect of thousands of genes simultaneously, has opened a completely different avenue for nutritional research (Muller and Kersten, 2003).

II. WHAT IS NUTRIGENOMICS

Nutrigenomics is the science of the relationship between nutrition and the specific effects of nutritional additives on the response of genes. In other words, the science of nutrigenomics investigates the activity of thousands of genes and what happens if they are switched on, switched off or remain unchanged in response to a specific nutrient or to a diet (Kaput, 2004). The application of this science provides researchers with a much better understanding of the

[1] Alltech Biotechnology, 209/1 Sukhumvit 21 Road, 17th Floor K Tower B, Wattana, Bangkok 10110, Thailand
[2] Alltech Biotechnology, 64-70 Nissan Drive, Dandenong South Vic 3175, Australia

effects of specific feed additives and their actual value for commercial livestock and poultry production (Dawson, 2006; Murphy, 2007).

The numerous gene expression changes observed within a specific organ or tissue are much more subtle than can be measured in animal growth and physiology. It is therefore not surprising that, when applying these techniques, the numbers of individual animals used in an experiment is much lower, the actual duration of the animal experiment is much shorter and the data output from a single experiment can be enormous.

a) Nutrigenomics: how does it work?

Nutrigenomics combines a number of different sciences in order to understand the effects of nutrients on physiological responses. In nutrigenomics, nutrients are signals to specific cells in the body, when interpreting the information of such a nutrient, the cell reacts by sending biochemical information from DNA or genes (the science of genomics) to RNA (the science of transcriptomics) which is in turn translated into specific proteins (science of proteomics) and ultimately determines the metabolic pathways (science of metabolomics) (Moody, 2001). All biological processes depend on the flow of information in this sequence. Although these processes are controlled by the basic genetic make-up, external factors such as disease challenge, environmental toxins or specific nutrients can have an influence on these pathways.

The greater understanding of the genome of humans and animals alike is the foundation of nutrigenomics. Although techniques to measure the expression of individual genes have been available for some years, only the development of gene chips (DNA microarrays) have allowed researchers to measure the expression of thousands of genes simultaneously. Gene chips are simply a collection of thousands of microscopic spots of specific DNA sequence.

Rather than measuring the effect of a specific nutrient on animal performance or the physiological response at the end of the experiment, researchers can extract specific messenger RNA (mRNA) from tissue at any stage. The amount of mRNA present relates to the relative amount of copies of known genes, measured by using gene chips. Using contrasting colour labels, it can be determined if a gene is up-regulated, down-regulated or unaffected as a result of a specific dietary manipulation.

III. NUTRIGENOMICS AS A TOOL TO EVALUATE FEED ADDITIVES

Nutritional studies have demonstrated that the use of specific nutrients or feed additives can significantly influence animal health and performance. It is generally not possible to demonstrate significant differences in feed additives of a similar nature without the use of extensive, large scale and very costly experiments. As a result, producers rely too often on small scale trials conducted by individual manufacturers or suppliers of these supplements when evaluating the benefit of products. The understanding of the effects of specific feed additives at gene level is a new and very exciting tool for evaluating nutritional strategies for livestock and poultry. This information can be used to compare different forms of a particular nutrient and evaluate the benefits to animal performance (Burton, 2002).

a) Comparison of different sources of selenium on gene expressions

Classical nutrition has established that selenium supplementation can significantly improve many production and reproduction parameters. More recently, it has been demonstrated that the form in which the selenium is presented to the animal can significantly affect

performance. However, until now there has been little information available on the effect of selenium source on gene expression.

Recent work compared the effects of three types of selenium in mice: sodium selenite, selenomethionine and Sel-Plex® (Alltech Inc.). This study clearly showed that the addition of Sel-Plex altered the expression of over 1,100 genes compared with only 900 genes when selenomethionine was added. The use of gene chip technology also identified that more beneficial genes were up-regulated while the expression of stress associated genes was suppressed. This expression pattern is consistent with previous studies showing lower levels of oxidative stress in tissue when Sel-Plex was fed. (Murphy, 2007; Murphy, 2010). In poultry, at mucosal surfaces, secretory IgA is the primary defense against invasion of bacteria and viruses (Snyder, Edens, Ashwell, Cantor and Pescatore, 2010). This first line of defense is through interaction between the mucosal B cell system and the polymeric immunoglobulin receptor (pIgR) with its secretory component. pIgR gene expression was investigated in tissues from enteric avian reovirus-challenged broiler chickens fed diets with no supplemental selenium, selenium yeast (Sel-Plex®), or sodium selenite. Selenium yeast induced pIgR mRNA in avian reovirus-challenged chickens on a transitory basis along with increased pIgR mRNA to a level significantly greater than that found with selenite or no supplemental selenium (Snyder et al., 2010).

b) Developing and evaluating a new ingredient

The development of new functional nutrients is generally a very lengthy and costly affair. Although the adoption of nutrigenomics cannot completely replace the necessity of conducting animal trials, it is a valuable tool to screen nutrients, determining their effect on gene regulation in target tissue, thus producing potential performance benefits (Dunshea, 2006).

It is well documented that the use of a specific source of organic selenium (Sel-Plex®, Alltech Inc.) lowers the antioxidative stress in tissue. Increased antioxidative stress has a detrimental effect on optimal growth, immunity and reproduction. In addition to the use of selenium, traditionally nutritionists have added vitamin E in excess of up to 10 times the actual animal requirement to lower antioxidative stress. With the opening of the Alltech Nutrigenomics Centre in Lexington (Kentucky, USA), Alltech Biotechnology has now developed a novel product (EconomasE™) that influences the antioxidant status of the animal. Gene expression models have shown that EconomasE helps to maximise synthesis, recycling and response of the animal's antioxidant system. Studies in poultry confirmed that EconomasE can be added to poultry diets in place of supplemental Vitamin E and selenium.

IV. CONCLUSIONS

Clearly nutrients included in the normal diet or specifically added to a diet influence multiple known, as well as unknown, physiological actions. Until now, the identification of these processes with traditional animal experiments was lengthy and often inconclusive. The science of nutrigenomics is a young and emerging tool for rapid and effective evaluation of nutritional strategies.

It provides a valuable tool for producers to differentiate and identify nutritional strategies to maximize not only animal performance but ultimately profitability.

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GROWTH PERFORMANCE AND ENERGY UTILIZATION OF BROILER CHICKENS ON TRITICALE-BASED DIETS

A.P. WIDODO[1], J. V. NOLAN[1] and P. A. IJI[1]

Triticale will become an increasingly important cereal grain in some areas because of its high yield potential, drought stress tolerance and disease resistance (Todorov, 1988). However, it is important to assess the nutritive value of the grain in order to establish its potential as an energy source. The concentrations of nutrients and antinutritive factors in some of the new high-yielding cultivars are also variable and need to be documented. The next level of research will be to determine the nutritive value of these cultivars including the availability of energy in these grains. The present study was conducted to investigate the effect of high-yielding cultivars of triticale in diets on production performance and utilization of dietary energy by broiler chickens.

Semi-purified diets were formulated to contain 70-75 % of five cultivars of triticale (Bogong, Canobolas, Jackie, Tobruk and Endeavour) and two control diets based on maize and wheat. All diets were iso-caloric and iso-nitrogenous and were offered without any microbial enzyme supplements to 336 day-old chicks (initial weight, 37.2±0.90 g) until 22 days of age. Each diet was replicated 6 times, with 8 chicks per replicate. The chicks were reared in cages in an environmentally controlled house. The AME of the diets was measured through total faecal collection between 18 and 22 days of age. Feed intake, body weight and FCR were measured weekly. At the end of the trial, 2 chicks per replicate were randomly selected and slaughtered. The carcasses were minced, freeze-dried and sub-sampled for protein, fat, and energy analyses. The data were used to derive values for nutrient retention, net energy of production (NEp), heat production and efficiencies of utilization of protein, fat and metabolizable energy based on the methods of Olukosi *et al* (2008).

From 1 to 7 days of age feed intake, weight gain and FCR were poorer ($P < 0.05$) on the wheat-based diet than on the triticale- and maize-based diets. When assessed to 21 days of age, feed intake and weight gain of birds fed on the diets based on wheat and Canobolas were also lower ($P < 0.05$) than those of chicks on the other diets but FCR was similar for all diets.

The ME intake and NEp from 1 to 22 days was lower ($P < 0.05$) on the wheat-based diet than on the triticale- and maize-based-diets. Chickens on all five triticale-based diets retained more ($P < 0.05$) energy in form of protein and fat than those on the wheat based-diet. The efficiencies of ME use for energy and protein retention were relatively similar among the diets, however, the efficiency of ME use for lipid retention on the wheat- and maize-based diets was lower ($P < 0.05$) than on diets containing Bogong, Canobolas, Jackie and Tobruk but relatively similar to Endeavour.

It can be concluded that all but one of the tested triticale cultivars was similar to maize and superior to wheat, without microbial enzyme supplementation. Further studies are underway to measure the response of the cultivars to supplementation with microbial enzymes and physical processing.

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[1] School of Environmental and Rural Sciences, University of New England, Armidale, NSW, 2351, Australia.

EFFECTS OF A COMMERCIAL PELLET BINDER AND MOISTURE ADDITION ON PELLET QUALITY AND, THE PERFORMANCE AND NUTRIENT UTILISATION OF BROILERS

M.R. ABDOLLAHI[1], D.V. THOMAS[1], T.J. WESTER[1], G. RAVINDRAN[1] and V. RAVINDRAN[1]

Summary

A trial was conducted to examine the effect of a commercial pellet binder and moisture addition on the quality of pellets and performance and nutrient utilisation of broilers. The experimental treatments were as follows: Treatment 1, basal diet steam-conditioned at 60 ºC; Treatment 2, basal diet with added 0.3% commercial pellet binder steam-conditioned at 60 ºC; Treatment 3, basal diet with added 2.42% moisture steam-conditioned at 60 ºC; Treatment 4, basal diet with added 0.3% commercial pellet binder plus 2.42% moisture steam-conditioned at 60 ºC; Treatment 5, basal diet steam-conditioned at 90 ºC; Treatment 6, basal diet with 0.3% commercial pellet binder steam-conditioned at 90 ºC. The results demonstrated that it is possible to manufacture high quality pellets through the addition of pellet binder and/or moisture to mash diets without applying high conditioning temperatures.

I. INTRODUCTION

Feeding pelleted diets *per se* is not enough to ensure optimum performance of broilers. The quality of pellets must also be taken into account (Briggs et al., 1999). While assessing the cost-effectiveness of investment in high pellet quality, consideration should also be given to whether or not the strategies employed to improve pellet quality may have a negative effect on nutrient availability (Moritz and Lilly, 2010). More specifically, conditions of high heat and moisture may induce non-favourable reactions that lead to reduced nutrient availability (Moritz and Lilly, 2010). A previous study (Abdollahi et al., 2011) in our laboratory has shown that, while steam-conditioning of mash diets at 60 ºC improved growth response, increasing conditioning temperatures above 60 ºC had negative effects on nutrient utilisation and performance of broiler starters. On the other hand, in pelleted diets, conditioning at 90 ºC improved pellet quality and the better pellet quality resulted in the restoration of deteriorated performance. Based on these results, the present study evaluated possible ways of simultaneously maintaining high nutrient utilisation as well as achieving a reasonable pellet quality without using high conditioning temperatures.

II. MATERIALS AND METHODS

Wheat-soy based broiler starter and finisher diets were formulated to meet the Ross 308 strain recommendations for major nutrients. The experimental treatments were as follows: Treatment 1, basal diet steam-conditioned at 60 ºC; Treatment 2, basal diet with added 0.3% commercial pellet binder steam-conditioned at 60 ºC; Treatment 3, basal diet with added 2.42% moisture steam-conditioned at 60 ºC; Treatment 4, basal diet with added 0.3% commercial pellet binder plus 2.42% moisture steam-conditioned at 60 ºC; Treatment 5, basal diet steam-conditioned at 90 ºC; Treatment 6, basal diet with 0.3% commercial pellet binder steam-conditioned at 90 ºC. Conditioning temperature was altered by adjusting the steam flow rate and measured at the outlet of the conditioner. Conditioning time of the mash was 30

[1] Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand

seconds. Pellet binder and moisture were added as a top-dressing to the basal mash diet in a single-screw paddle mixer. All diets were pelleted using a pellet mill (Richard Size Limited Engineers, Orbit 15, Kingston-upon-Hull, UK) capable of manufacturing 180 kg of feed/h and equipped with a die ring (3-mm hole and 35-mm thickness). Each of the six dietary treatments was offered ad libitum to six replicate cages (eight birds per cage). Body weight and feed intake were recorded weekly. From d 17 to 20, feed intake and excreta output were measured quantitatively per cage for the determination of apparent metabolisable energy (AME). On d 35, ileal digesta were collected for determination of apparent ileal digestibility of nitrogen (N) and starch. Pellet durability index (PDI) and pellet hardness were determined using a Holmen Pellet Tester (New Holmen NHP100 Portable Pellet Durability Tester, TekPro Limited, Norfolk, UK) and a Stable Micro Systems Texture Analyser (TA-XT Plus, Godalming, Surrey, UK), respectively. The cage means were used to derive performance data. The data were analysed by a one-way ANOVA using the General Linear Models procedure of SAS (2004) in a completely randomised design.

III. RESULTS AND DISCUSSION

During the starter period (d 1 to 21), birds fed diets conditioned at 60 ºC, regardless of pellet binder or/and moisture addition, gained more ($P < 0.05$) weight than those fed diets conditioned at 90 ºC with or without pellet binder (Table 1). Addition of pellet binder or/and moisture to the diets conditioned at 60 ºC had no effect ($P > 0.05$) on weight gain, feed intake and feed per gain. Pellet binder addition to the diet conditioned at 90 ºC increased ($P < 0.05$) weight gain and feed intake and improved ($P < 0.05$) feed per gain compared to the diet conditioned at 90 ºC with no pellet binder. Birds fed the diet conditioned at 90 ºC without pellet binder had similar ($P > 0.05$) feed per gain to those fed the diet conditioned at 60 ºC without pellet binder or moisture addition, but higher ($P < 0.05$) than those fed other dietary treatments.

Over the whole trial period (d 1 to 35, Table 1), birds fed the diet conditioned at 90 ºC with no pellet binder addition had lower ($P < 0.05$) weight gains than those fed other dietary treatments. Birds fed the diet conditioned at 90 ºC with no pellet binder addition tended ($P = 0.06$) to have a similar feed intake to those fed the diets conditioned at 60 ºC without or with pellet binder, but lower than those fed other diets. The dietary treatments had no effect ($P > 0.05$) on feed per gain.

In starter diets, pellet durability of the diet conditioned at 60 ºC with pellet binder and moisture addition was similar ($P > 0.05$) to the diet conditioned at 90 ºC with pellet binder, but higher ($P < 0.05$) than other dietary treatments (Table 2). Among starter diets conditioned at 60 ºC, moisture addition, individually or in combination with pellet binder, had higher ($P < 0.05$) PDI and pellet hardness compared to diets with and without pellet binder addition. Starter diets conditioned at 90 ºC with pellet binder had the highest pellet hardness followed by the diet conditioned at 90 ºC without pellet binder.

Among finisher diets, the diets conditioned at 90 ºC, regardless of pellet binder addition, had higher ($P < 0.05$) PDI and pellet hardness than all the diets conditioned at 60 ºC (Table 2). In finisher diets conditioned at 60 ºC, while the diet with combination of pellet binder and moisture had the highest PDI, moisture addition increased ($P < 0.05$) the PDI compared to the diets without or with pellet binder. Among finisher diets conditioned at 60 ºC, moisture addition, individually or in combination with pellet binder, increased ($P < 0.05$) the pellet hardness compared to the diet without pellet binder and moisture.

All starter diets conditioned at 60 ºC were determined with similar ($P > 0.05$) AME values. However, conditioning at 90 ºC, regardless of pellet binder addition, resulted in lower

($P < 0.05$) AME compared to diets conditioned at 60 ºC (Table 2). Apparent ileal digestibility of N and starch in finisher diets was unaffected ($P > 0.05$) by dietary treatments (Table 2).

Based on these observations, it may be concluded that the effectiveness of high pellet quality in improving broiler growth responses depends on the effects of conditioning temperature on nutrient utilisation of the diets. It seems that similar AME values of the diets conditioned at 60 ºC did not leave any room for the higher pellet quality achieved by addition of pellet binder and moisture, individually or in combination, to improve the performance further. It is plausible that pellet binder addition to the diet conditioned at 90 ºC may have created pellets of high quality to offset lower AME values of diets conditioned at 90 ºC and resulted in higher weight gain and feed intake and an improved feed per gain compared to the diet without pellet binder. Moritz *et al*. (2003) showed that feeding broilers on low energy (5% less than National Research council [NRC, 1994] recommended levels) maize-soy diets with moisture addition produced feed efficiency values equivalent to those fed NRC-recommended energy levels. It was speculated that higher pellet durability achieved through moisture addition to low energy diets, compared to adequate energy diets, may have reduced bird activity relating to feed prehension and resulted in expending substantially less energy in ingestion of pellets.

In conclusion, the negative effects of higher conditioning temperature on weight gain, and to some extent feed intake, of broilers were not only limited to the starter period, but also can be seen over the whole trial period unless pellet quality improves. The current study also illustrates possibilities for high quality pellets to be manufactured by the addition of pellet binder or/and moisture to a mash diet without applying high conditioning temperatures.

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Table 1 Influence of the dietary treatments on weight gain (g/bird), feed intake (g/bird) and feed per gain (g feed/g gain) of broilers during starter (d 1 to 21) and overall (d 1 to 35) periods[1]

	Starter period			Overall period		
	Weight gain	Feed intake	Feed per gain	Weight gain	Feed intake	Feed per gain
60 ºC	1090[a]	1442[ab]	1.353[ab]	2697[a]	4097	1.557
60 ºC + Pellet binder	1090[a]	1455[a]	1.340[b]	2674[a]	4091	1.543
60 ºC + Moisture	1103[a]	1459[a]	1.331[b]	2745[a]	4196	1.547
60 ºC + Pellet binder + Moisture	1095[a]	1461[a]	1.342[b]	2735[a]	4207	1.559
90 ºC	975[c]	1343[c]	1.376[a]	2553[b]	3942	1.554
90 ºC + Pellet binder	1047[b]	1398[b]	1.335[b]	2684[a]	4148	1.545
Probabilities, P ≤	***	***	*	***	0.065	NS
SEM[2]	8.4	17.6	0.0103	26.3	63.2	0.0119

[a,b,c] Means in a column not sharing a common superscript are significantly different (P < 0.05).
NS, not significant; * P < 0.05; *** P < 0.001.
[1] Each value represents the mean of six replicates (eight birds per replicate).
[2] Pooled standard error of mean.

Table 2 Influence of the dietary treatments on PDI[1] (%), pellet hardness[2] (Newton), AME (MJ/kg DM), and apparent ileal N and starch digestibility in broilers[3]

	Starter diet			Finisher diet			
	PDI	Pellet hardness	AME	PDI	Pellet hardness	Ileal N digestibility	Ileal starch digestibility
60 ºC	56.5[d]	14.9[d]	13.50[a]	74.1[d]	24.3[c]	0.774	0.850
60 ºC + Pellet binder	63.1[c]	18.0[d]	13.49[a]	73.9[d]	27.3[bc]	0.794	0.850
60 ºC + Moisture	67.2[b]	23.9[c]	13.54[a]	84.7[c]	30.8[b]	0.775	0.858
60 ºC + Pellet binder + Moisture	70.2[a]	23.4[c]	13.48[a]	89.7[b]	29.4[b]	0.774	0.841
90 ºC	63.2[c]	28.4[b]	13.26[b]	92.8[a]	41.7[a]	0.774	0.869
90 ºC + Pellet binder	69.6[ab]	37.8[a]	13.19[b]	93.1[a]	45.7[a]	0.760	0.861
Probabilities, P ≤	***	***	*	***	***	NS	NS
SEM[4]	0.86	1.27	0.0759	0.51	1.63	0.0132	0.0226

[a,b,c,d] Means in a column not sharing a common superscript are significantly different (P < 0.05).
NS, not significant; * P < 0.05; *** P < 0.001.
[1] Each value represents the mean of six replicate samples.
[2] Each value represents the mean of 15 replicate samples.
[3] Each value represents the mean of six replicates.
[4] Pooled standard error of mean.

PREDICTING VARIATIONS IN TOTAL AND PHYTATE PHOSPHORUS IN RAW MATERIALS OF PLANT ORIGIN

C. G. OLNOOD[1], Y. G. LIU[1] and C. GADY[2]

Summary

A total of 227 samples (mainly cereals, cereal by-products, oilseeds and oilseed meals) was collected worldwide, and analysed for concentrations of total phosphorus (P) and phytate P using mineralization and enzymatic methods, respectively. Using Near Infrared Reflectance Spectroscopy (NIRS), prediction models were then established to determine correlation between absorbances to both total P and phytate P. Results showed that phytate P content represents from 18% in cassava to 83% in rice bran of the total P content. There were large variations within crop species in terms of phytate P/total P ratio which ranged from 64 to 87% in maize (corn), 64 to 80% in wheat, 66 to 95% in rapeseed meal and 59 to 69% in soybean meal. Preliminary results showed that NIRS prediction models are able to capture 93 and 92% of the variations measured in total P and phytate P with standard error 0.05 and 0.04%, respectively, suggesting the technique may contribute to better master P variations in major feed ingredients.

I. VARIATION OF PHOSPHORUS IN FEED INGREDIENTS

It is known that the contents of both total phosphorus (P) and phytic P possess substantial degrees of variation within the same ingredients as well as among different feed ingredients (Tran and Skiba, 2005). The lack of knowledge in P contents often leads to over-formulation of inorganic sources of P in animal diets (Maguire et al, 2005). For instance, for laying hens, research published during the last decade estimated that the true requirements of available P (av.P) or non-phytic P (NPP) fall between 150 and 200 mg/hen/day, which is well below the actual dietary supplies in the industry of 350–500 mg/hen/day (Keshavarz, 2000) based on average feed intake (110 g/hen/day). It is clear that there is an over-use of NPP in layer diets by some 40-50%. The main reason for the large safety margin is probably due to inability to determine actual NPP contents in the feed ingredients at the time of formulation. Needless to say, over-formulating inorganic P is neither economical nor environmentally correct. Hence, there has been considerable interest in obtaining accurate data of phytic and non-phytic P in major feedstuffs.

In this study, a total of 227 samples was collected worldwide, mainly cereals, cereals by-products, oilseeds and oilseed meals, and analysed for concentrations of total phosphorus (P) and phytate P using mineralization and enzymatic methods, respectively. The results (Table 1) indicate that phytate P content represents from 18% (cassava) to 83% (rice bran) of the total P content. There were large variations within crop species in terms of phytate P/total P ratio which ranged from 64 to 87% in maize (corn), 64 to 80% in wheat, 66 to 95% in rapeseed meal and 59 to 69% in soybean meal. These results are well in line with data published by Tran and Skiba (2005).

[1] Adisseo Asia Pacific Pte Ltd, Singapore 179803
[2] Adisseo France SAS, Antony France 92160

Table 1 Variability of total P and phytate P in raw materials (CERN) compared with INRA data

	Number of Samples	Total P (% as fed)				Phytate P/Total P (%)			
		INRA*		CERN **		INRA*		CERN**	
		Mean	SD	Mean	SD	Mean	Range	Mean	Range
Cassava	3	0.09	0.03	0.07	0.02	25		18	10-31
Barley	13	0.34	0.03	0.35	0.03	55	40-79	65	58-69
Maize	23	0.26	0.03	0.26	0.04	75		77	64-87
Oat	7	0.32	0.04	0.33	0.05	55		69	61-74
Triticale	5	0.35	0.04	0.35	0.01	65		68	64-75
Wheat	24	0.32	0.03	0.31	0.03	65	30-95	72	64-80
Wheat bran	9	0.99	0.11	1.00	0.18	80		79	67-95
Rice bran	4	1.61	0.21	1.57	0.08	85		83	78-91
Full-fat soybean	5	0.55	0.05	0.52	0.02	60		68	65-73
Rapeseed	7	0.66	0.09	0.64	0.07	70		78	69-85
Rapeseed meal	21	1.14	0.09	1.03	0.13	60		80	66-95
Soyabean meal	38	0.62	0.05	0.67	0.06	60		64	59-69
Sunflower meal	16	1.01	0.14	0.99	0.15	85		81	73-89

* INRA 2005 (Institute of National Research Agriculture, France), with raw materials harvested in 2004; **: CERN (Centre of Expertise and Research in Nutrition, Adisseo France), with raw materials harvested in 2009.

II. NIRS PREDICTION FOR TOTAL AND NPP IN RAW MATERIALS

The prevalence of wide variations of P content of feed ingredients creates a need for a rapid prediction or screening tool to predict both phytic and NPP in major feedstuffs. Our study investigated the feasibility of using NIRS to predict P content, by measuring the absorbance of the 227 samples in NIRS from 1100 to 2500 nm wavelength. NIRS prediction models were then calculated to determine correlation between absorbance to both total P and phytate P, using mPLS regression.

For total P contents, Figure 1 shows correlation between NIRS prediction and laboratory analytical values, Figure 2 displays correlation of phytic P values. These preliminary results suggest that NIRS prediction models are able to explain 93 and 92% of the variations measured in total P (Fig. 1) and phytate P (Fig. 2) with standard errors of 0.05% and 0.04%, respectively.

For independent validation, we applied the above NIRS prediction models to 8 independent samples of soybean meal (SBM) and results are illustrated as Figure 3. Laboratory analyses showed the average content of total P as 6.2 g/kg and phytic P as 3.7 g/kt, with a ratio of approximately 60% of total P. Moreover, these results clearly suggest variations of both phytic P and NPP among different SBM samples, and NIRS prediction results are closer (SEP 0.04) to lab analytical results than the book values (SEP 0.07).

Figure 1 NIRS calibration of total P (%, as fed) in a range of raw materials.

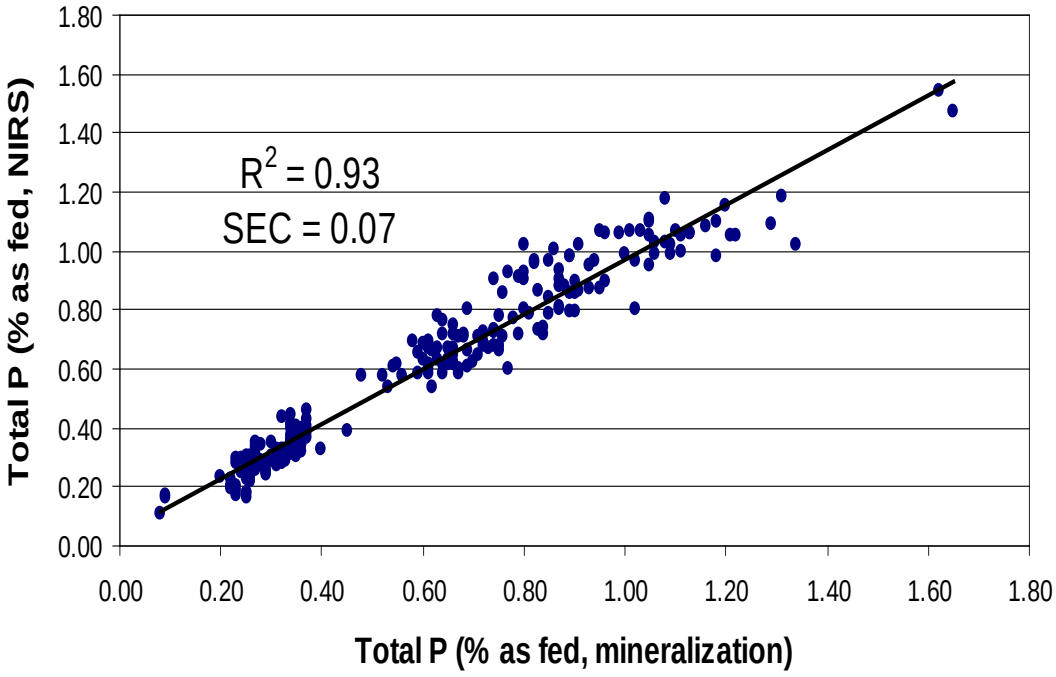


Figure 2 NIRS calibration of phytate P (%, as fed) in a range of raw materials.

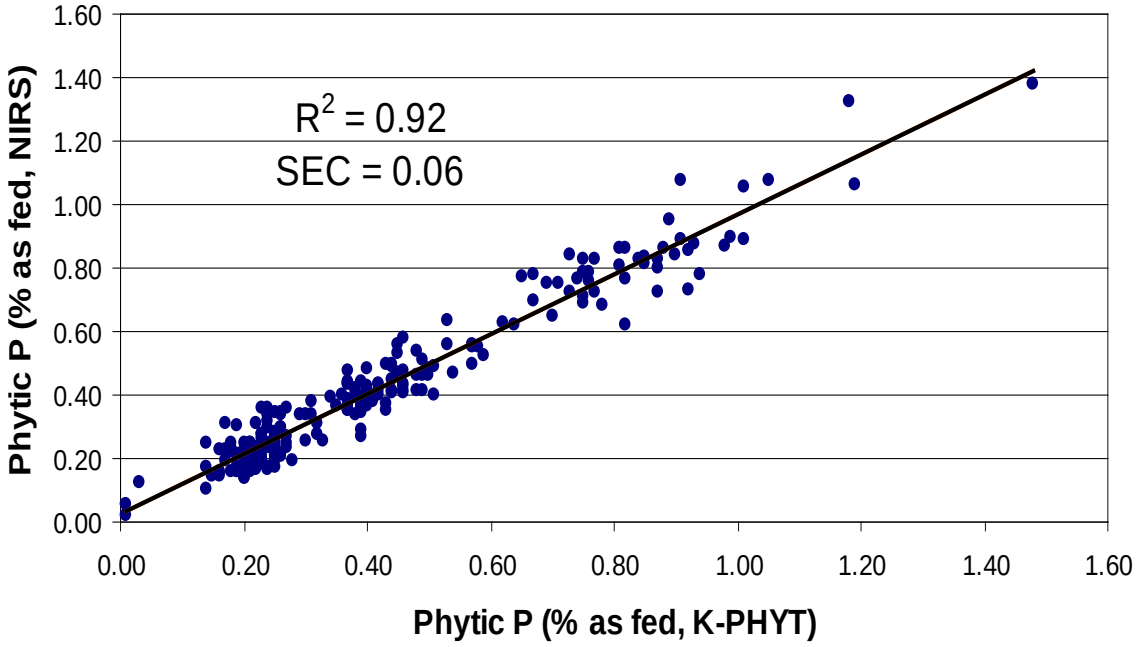
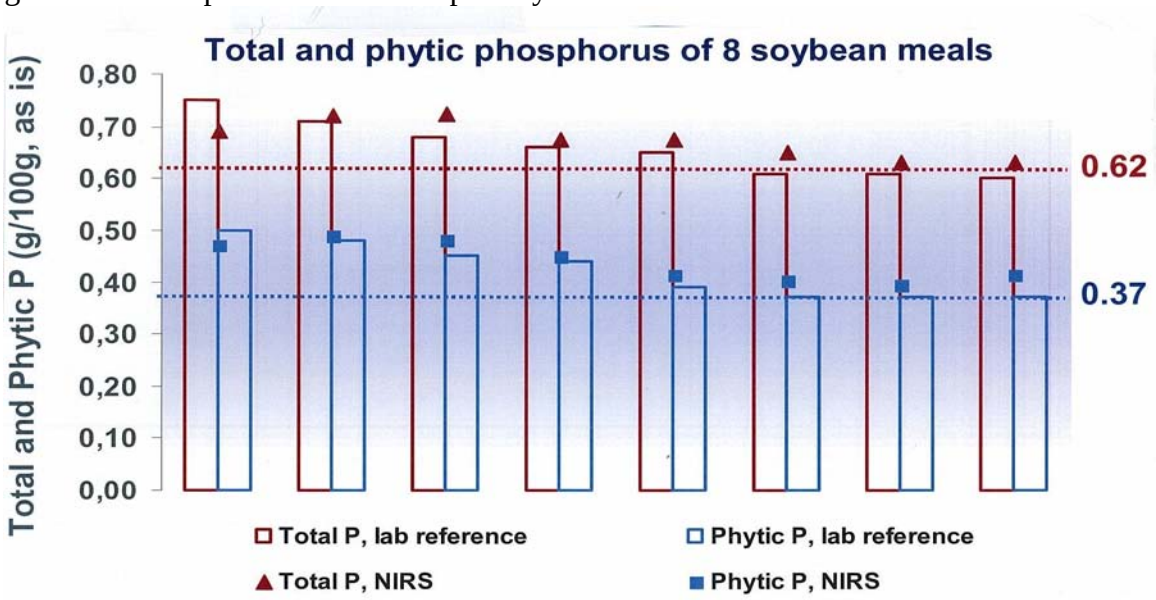


Figure 3 Comparison of NIRS capability to screen P value versus lab test and book value



	NIRS vs. lab ref	Book vs. lab ref.
Total P (g/100g)	0.04	0.07
Phytic P (g/100g)	0.03	0.07

III. CONCLUSIONS

The NIRS technique has unique advantages of being non-destructive and rapid in screening the quality of any given feed ingredient. Upon establishing reliable calibrations, not only can it predict chemical composition but also biological values of any materials after various processing, such as heat treatments that alter the organic structure of materials. Successful examples of NIRS applications are digestible amino acids and metabolisable energy.

The results of this study demonstrated that there are considerable variations of both phytic and non-phytic P contents in the same ingredients, and NIRS technique is able to provide accurate data of the true contents of phytic P and NPP in major feed ingredients.

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MATURATIONOF FOWL SPERM BINDING CAPACITY TO THE EPITHELIUM OF THE SPERM STORAGE TUBULES AND LONGEVITY IN THE MALE REPRODUCTIVE TRACT

M.U. AHAMMAD[1], C. NISHINO, H. TATEMOTO, S. OKAMOTO[2], Y. KAWAMOTO and T. NAKADA

Summary

In this study we examined sperm binding potentiality to the epithelium of the utero-vaginal (UV) and infundibular sperm storage tubules (SSTs) and survivability in vitro. Spermatozoa from the testis, epididymis and vas deferens were incubated with either the UV-junction or distal infundibulum at 39^{o}C for 30 min. After incubation and washing, bound spermatozoa were counted using fluorescent stain DAPI. Aliquots of the spermatozoa were stored at 4^{o}C for 24 and 48 h in Lake's solution. Sperm viability was measured using fluorescent stain CFDA and PI. A significantly greater number of epididymal spermatozoa bound to the epithelium than did the testicular spermatozoa. A gradually increased number of bound spermatozoa per 0.25mm^{2} was observed from the epithelium of the infundibular SSTs than to the UV-SSTs. The percentage of viable sperm from the testis to distal vas deferens stored for 48 h increased progressively. This study under *in vitro* conditions suggests that fowl spermatozoa acquire the ability to bind to the SSTs epithelium and survive while migrating through the male reproductive tract.

I. INTRODUCTION

Fowl spermatozoa, after natural mating or artificial insemination, are stored in the SSTs, located in the UV junction and in the infundibulum of the oviduct, with a fertile period of about 21 days in chicken (Brillard, 1993). Many studies using in vitro models have shown that sperm survive longer when in direct contact with the epithelium of SSTs (Van Krey et al., 1967; Ashizawa and Nishiyama, 1983). Binding to the epithelium stabilizes the spermatozoa and greatly enhances their survival in a relatively hostile environment. Therefore, ability to bind to the oviduct is a very important sperm function with respect to prolonged survival.

Testing of sperm ability to bind to the oviducal epithelium, therefore, measures sperm functional ability to survive. On the other hand, it is likely that the prolonged survival of fowl spermatozoa in the SSTs is not due entirely to the function of the hen oviduct. Rather, the spermatozoa might acquire the potential to survive in the oviduct for a longer period of time. There is also much early literature describing the prolonged survival of spermatozoa in the oviduct but few studies concentrated on the acquisition of potential longevity of spermatozoa in the male reproductive system. The present study was, therefore, aimed at examining the binding capacity of spermatozoa to the epithelium of SSTs and survivability of the same sperm samples under *in vitro* condition.

[1] Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan
[2] Faculty of Agriculture, Kagoshima University, Kagoshima, Japan

II. MATERIALS AND METHODS

a) Sperm-epithelium (sperm storage tubules) binding assay

A small piece of epitheilium (1.0 cm x 1.0 cm), from either the UV or infundibular-SSTs, was incubated in 1ml MEM containing spermatozoa, at 1 x 10^7 cells/ml, taken from the testis, epididymis, proximal, middle and distal vas deferens at 39^{o}C for 30 min. in 5% CO_2 humidified atmosphere. After incubation, epithelium samples were washed vigorously in PBS to remove the loosely attached spermatozoa. The washed epithelium was then incubated in 500µl PBS containing fluorescent stain DAPI for 15 min at 39^{o}C. Sperm binding to the epithelium was observed under the fluorescence microscope. The average number of sperm bound to 0.25mm^2 of surface area was counted.

b) Detection of live and dead spermatozoa using dual fluorescent staining

Spermatozoa obtained from the testis, epididymis and vas deferens were diluted with either MEM or Lake's solution, pH 8.0 (Lake, 1960), and concentration of spermatozoa was adjusted to 1x10^8 cells/ml. Aliquots of the respective diluted spermatozoa from the testis, epididymis and proximal, middle and distal vas deferens were subjected to storage at 4^{o}C for 0, 24, and 48 h in Lake's solution. The protocol used for assessing sperm viability using dual fluorescent staining with 6-carboxy-fluorescein diacetate (CFDA, 10µg) and propidium iodide (PI, 10µg) was adopted from the method described by Bayyari et al. (1990) with modification.

c) Statistical Analysis

Values are presented as the mean of three independent experimental replicates. All statistical analyses were subjected to the Statistical Analysis System "R" software package (http://www.r-project.org). For evaluation of different groups, the percentage data on sperm viability were carried out by analysis of variance (ANOVA) followed by the Tukey-Kramer test for non-parametric multiple comparisons using generalized linear model (GLM, binomial error distribution).

III. RESULTS

Table 1 Number of spermatozoa (mean ± SEM)/0.25 mm^2 bound to the epithelium of utero-vaginal (UV) and infundibular sperm storage tubules (SSTs) after incubation at 39^{o}C for 30 min with 1x10^7 sperm/ml

Binding site (epithelium)	Source (s) of spermatozoa				
	Testis	Epididymis	Vas deferens		
			Proximal	Middle	Distal
UV-SSTs	23.4 ± 1.5^a	39.6 ± 2.1^b	48.3 ± 2.4^c	55.8 ± 1.9^d	67.4 ± 1.6^e
Infundibular SSTs	9.7 ± 2.2^a	21.8 ± 1.9^{b*}	33.6 ± 2.3^c	41.5 ± 2.3^d	51.2 ± 1.5^e

Values are expressed as mean ± SEM (n=5). $^{a-e}$Values are significantly different among spermatozoa from different sources by one way ANOVA ($P<0.05$).

Spermatozoa from the testis to the distal vas deferens exhibited a gradually increased capacity for binding to the epithelium of either the UV or indundibular SSTs. Spermatozoa irrespective of their sources in the male reproductive organ, in general, had higher binding affinity to bind to the epithelium of UV SSTs than to the infundibular SSTs (Table 1).

Viability of spermatozoa during storage in Lake's solution at 4°C

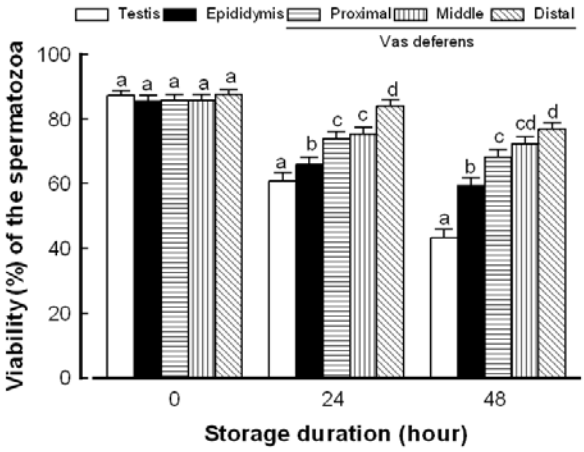

Figure 1 Mean percentage of viable spermatozoa in the testis, epididymis, and proximal, middle and distal vas deferens after storage for 0, 24, and 48 h at 4℃ in Lake's solution. Bars indicate mean value ± SEM (n=5). Letters above bars indicate significant differences by one-way ANOVA ($P < 0.05$).

Figure 1 shows that spermatozoa, irrespective of source lose survivability with increased storage period, but the rate of decline in viability decreased from the testicular to distal vas deferens samples. Spermatozoa from the testis, epididymis and vas deferens exhibited minimal, medium and maximal viability ($P<0.05$), respectively, when stored for 24 h. The mean viability (%) of the spermatozoa obtained from the testis, epididymis and proximal, middle, and distal vas deferens stored for 48 h were 45.7 ± 2.5, 59.8 ± 2.5 and 67.0 ± 2.4, 71.4 ± 2.3, and 75.3 ±2.2, respectively.

IV. DISCUSSION

The results of the current study demonstrate that testicular spermatozoa had the lowest capacity to bind to the epithelium of SSTs and the lowest survivability. Spermatozoa from the epididymis to the distal vas deferens exhibited gradually increased binding ability and vitality. It has been shown by Ashizawa and Nishiyama (1983) that longevity of spermatozoa depends, physiologically, on the degree of close association between spermatozoa and oviducal cells. Using spermatozoa from the testis, epididymis and vas deferens, we were also able to demonstrate an existence of parallel development between the development of binding capacity and survivability: cells that have better binding ability can survive. The development of spermatozoal binding ability to SSTs epithelium and vitality occurs during transportation through the descending tract of the male reproductive system. Bedford (1983) demonstrated that spermatozoa can recognize the binding sites and remain bound to the zona pellucida by the function of protein on its surface. This has another function in protecting against the attack by the female's immune system. Viability is directly related to the integrity of the plasma membrane, and the changes in viability may imply surface changes of the fowl spermatozoa that take place during their passage through the male reproductive tract. Moreover, Esponda and Bedford (1985) also demonstrated plasma membrane associated maturational changes in the chicken. It is concluded from the present study that fowl spermatozoa acquire the ability to bind to the SSTs epithelium and survivability while migrating through the male reproductive tract.

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SCOPE FOR THE HIGH INCLUSION OF SORGHUM DISTILLERS' DRIED GRAINS WITH SOLUBLES IN BROILER CHICKEN DIETS

M. R. BAREKATAIN[1], M. CHOCT[2] and P. A. IJI[1]

Distillers' dried grains with solubles (DDGS) are increasingly becoming important in poultry feeding. Research into the use of this material has focused largely on maize DDGS from North America and less work has been done on the predominantly sorghum DDGS that are produced in Australia. In preliminary tests, we investigated the variation in nutrient composition of sorghum DDGS and then conducted a feeding trial on broiler chickens. The trial involved 432 day-old male broiler chicks in a 4 x 2 factorial layout (4 levels of DDGS inclusion - 0, 100, 200 or 300 g/kg with or without a xylanase enzyme, Ronozyme WX), over 21 days on starter diets and subsequently on finisher diets to 35d of age. Compared to the control diet, feed intake was significantly increased ($P<0.001$) with the inclusion of dietary DDGS in the diet during the first three weeks and the entire period of this study. There was no effect on body weight gain (BWG) from the addition of dietary DDGS or xylanase. Feed conversion ratio (FCR) deteriorated significantly ($P<0.05$) with the rising level of DDGS in diets during the first 3 weeks of feeding. Over that period, the effect of xylanase supplementation was not significant at up to 200 g/kg DDGS inclusion. However, in birds fed 300 g/kg DDGS, the FCR was significantly improved ($P<0.05$) by the addition of xylanase, over the starter and the entire feeding period of the study, with birds ending up with similar body weight but tending to consume less feed as a result of xylanase addition.

Table 1 Growth performance of broiler chickens

Treatments	Xylanase	BWG(g/bird)		Feed intake (g/bird)		FCR	
DDGs (g/kg)		1-21	1-35	1-21	1-35	1-21	1-35
0	-	679.5	1843.3	884.8^{b}	2574.6^{c}	1.31^{cd}	1.40^{c}
	+	698.5	1850.8	894.8^{b}	2605.3^{c}	1.30^{d}	1.41^{c}
100	-	696.8	1853.9	1020.5^{a}	2884.7^{ab}	1.48^{ab}	1.56^{ab}
	+	698.2	1858.0	1013.9^{a}	2836.3^{b}	1.45^{abc}	1.53^{ab}
200	-	682.8	2004.1	1016.7^{a}	3022.5^{ab}	1.50^{ab}	1.51^{ab}
	+	699.3	1984.2	992.7^{a}	2947.4^{ab}	1.42^{abcd}	1.49^{bc}
300	-	660.0	1926.0	1017.2^{a}	3064.9^{a}	1.54^{a}	1.59^{a}
	+	704.3	1925.0	971.6^{a}	2864.5^{ab}	1.38^{bcd}	1.49^{bc}
	SEM	8.64	16.86	9.27***	25.05***	0.018*	0.01**

Means in a column sharing different superscript differ significantly. (*P<0.05, **P<0.01, ***P<0.001)

While dietary DDGS or enzyme supplementation did not alter digestibility of starch, protein digestibility was adversely influenced ($P<0.001$) by increasing level of DDGS, which was in line with the results shown by Youssef et al. (2008). Increasing DDGS to 300 g/kg in the diet tended to increase ileal digesta viscosity, which was reduced as a result of enzyme addition to the diet. While there was no effect on pancreatic enzyme activities, sucrase and maltase activities were reduced ($P<0.001$) when birds received 200 and 300 g/kg DDGs. Xylanase may help to limit the detrimental effect of high DDGS inclusion especially in the starter phase of feeding.

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[1] School of Environmental and Rural Sciences, University of New England, Armidale, NSW, 2351
[2] Australian Poultry CRC, Armidale, NSW, Australia

RESPONSIVENESS OF HIGH SCREENINGS AND COMMERCIAL CEREAL GRAINS TO A BLEND OF XYLANASE AND PHYTASE ENZYME PRODUCTS

R.J. HUGHES[1,2], M.S. GEIER[1,2], J.L. BLACK[3], A.M. TREDREA[4], S. DIFFEY[5] and S.G. NIELSEN[6]

Summary

Two experiments evaluated the apparent metabolisable energy (AME) values for cereal grains fed with and without a blend of xylanase and phytase enzyme products to broiler chickens. The first experiment involved 30 high screenings grains (10 wheats, 11 barleys, 3 triticales, and 6 sorghums). The second experiment involved 50 grains (20 wheats, 13 barleys, 3 triticales and 14 sorghums) donated by commercial companies throughout Australia. Each experiment included "connectivity" grains used in previous experiments that contributed to the AusScan near infrared reflectance (NIR) calibration database. The inclusion of "connectivity" grains (15 in Experiment 1 and 17 in Experiment 2) enabled data to be analysed statistically for valid comparison across many experiments conducted in the period 1998 to 2010. These grains were also fed with and without the blend of enzymes. In general, the results show that the enzyme blend improved AME values for some wheats and only one barley, and had little or no effect on other types of grain.

I. INTRODUCTION

Black et al. (2009) described calibrations based on NIR spectroscopy for estimating the AME content and AME Intake Index of cereal grains for broiler chickens. The calibrations were developed from results obtained in the Premium Grains for Livestock Program. Updated NIR calibrations were reported by Black et al. (2010). These new calibrations included results from an extra 55 grains, comprising 30 high screenings grains and 25 grains donated by industry. This greatly improved the ability to predict values for unknown grains and the accuracy of prediction. Further improvements are anticipated when results from an additional 25 industry grains are included in the database.

Two large AME experiments funded by RIRDC Chicken Meat were required to evaluate a total of 80 grains, which included 30 high screenings grains (Experiment 1) and 50 industry grains (Experiment 2). All grains were fed with and without a blend of xylanase and phytase enzyme products. This paper describes the responses in grain AME to enzyme products.

II. MATERIALS AND METHODS

The AME values of grains were determined across a series of conventional energy balance studies involving measurements of feed intake and excreta output as described by Mollah et al. (1983) with minor modifications, and subsequent measurement of gross energy values of feed and excreta by bomb calorimetry. High screenings and other weather damaged samples (total 30 comprising 10 wheats, 11 barleys, 3 triticales, and 6 sorghums) collected as part of a Pork CRC project were examined in Experiment 1. The second experiment involved 50 grains (20 wheats, 13 barleys, 3 triticales and 14 sorghums) donated by commercial companies

[1] SARDI, Pig and Poultry Production Institute (PPPI), Roseworthy, SA 5371
[2] School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, SA 5371
[3] John L Black Consulting, Warrimoo, NSW 2774
[4] University of Sydney, Plant Breeding Institute, Narrabri, NSW 2390
[5] NSW Industry & Investment, Orange, NSW 2800
[6] Charles Sturt University, Wagga Wagga, NSW 2678

throughout Australia. Experiment 1 required four batches of day-old feather-sexed broiler chickens, while Experiment 2 used eight batches. Each batch was raised in floor pens on a commercial broiler diet to 22 days of age and then transferred in single-sex groups of five to metabolism cages in controlled temperature rooms. Air temperature was maintained at 26^{o}C at the start of the 7-day experiment and lowered daily until it was 23^{o}C at the end. Experimental diets contained grain, casein, dicalcium phosphate, limestone, DL-methionine, mineral and vitamin premix, salt, and choline chloride. All grains were fed with and without a blend of xylanase (Porzyme 93010 at 50 g/tonne for wheat, barley and triticale, or Rovabio Excel at 200 g/tonne for sorghum) and phytase (Phyzyme TPT at 50 g/tonne) enzyme products. Dietary treatments were replicated four times (two cages of males and two cages of females). Cold-pressed diets were fed for seven days. The first three days enabled the chickens to adapt to the feeds. During the following four days, all excreta were collected and dried at 85^{o}C. Feed intake was measured during the adaptation and collection phases of the study. Birds were weighed at the start and end of the 7-day period. Dry matter contents of samples of pelleted and milled feeds were measured. Gross energy values of dried excreta and milled feeds were measured with a Parr isoperibol bomb calorimeter. AME of the grain was calculated by subtracting from the total energy intake the energy contribution of casein, which was assumed to be 20.1 MJ/kg dry matter (Annison et al., 1994).

III. RESULTS

In Experiment 1, there were significant 2-way interactions between grain type and enzyme ($P < 0.001$) and grain type and sex ($P < 0.05$) on AME values for high screenings grains (Table 1). The effects of enzyme and sex on individual wheats are summarised in Figure 1. The proportion of grains passing through a 2 mm sieve averaged 17% and ranged from 1.4% for wheat 1753 and 53.1% for wheat 1762 in Figure 1. Regression analysis indicated AME values for grain declined by about 0.2 MJ/kg for each 10% rise in screenings, in both male and female chickens, when given enzymes. In the absence of enzyme, the responses to screenings were highly variable, particularly for male chickens.

Table 1 Effects of interactions between grain type and enzyme, and grain type and sex on AME (MJ/kg dry matter) values for high screenings grains (Experiment 1). Effects of enzyme or sex within a grain type are not significantly different ($P > 0.05$) when followed by the same letter

Enzyme	Barley	Sorghum	Triticale	Wheat
-	12.72 a	16.09 a	14.69 a	14.00 b
+	12.84 a	16.24 a	14.76 a	14.88 a
Sex	**Barley**	**Sorghum**	**Triticale**	**Wheat**
Female	12.98 a	16.23 a	14.74 a	14.71 a
Male	12.57 b	16.10 a	14.70 a	14.15 b

In Experiment 2, there was a significant 3-way interaction between individual grains, enzyme and sex. Enzymes improved AME for only one sample of barley fed to male chickens. The effects of enzyme and sex on individual wheats are summarised in Figure 2

The soluble non-starch polysaccharide (NSP) values (in g/kg dry matter) for wheat ranged from 9.8 for wheat 1876 to 15.2 for wheat 1860 in Figure 2, and averaged 12.1. These soluble NSP values are considerably lower than 20 g/kg dry matter normally found in "low AME" wheats (Choct et al., 1996). The insoluble non-starch polysaccharide values (in g/kg dry matter) for wheat ranged from 70 for wheat 1861 to 115 for wheat 1878 in Figure 2, and

averaged 83. Regression analysis indicated negligible change in AME values for grains due to soluble NSP level, however, for each 10g/kg increase in insoluble NSP, AME declined by about 0.3 and 0.4 MJ/kg dry matter, respectively, for males and females given enzymes.

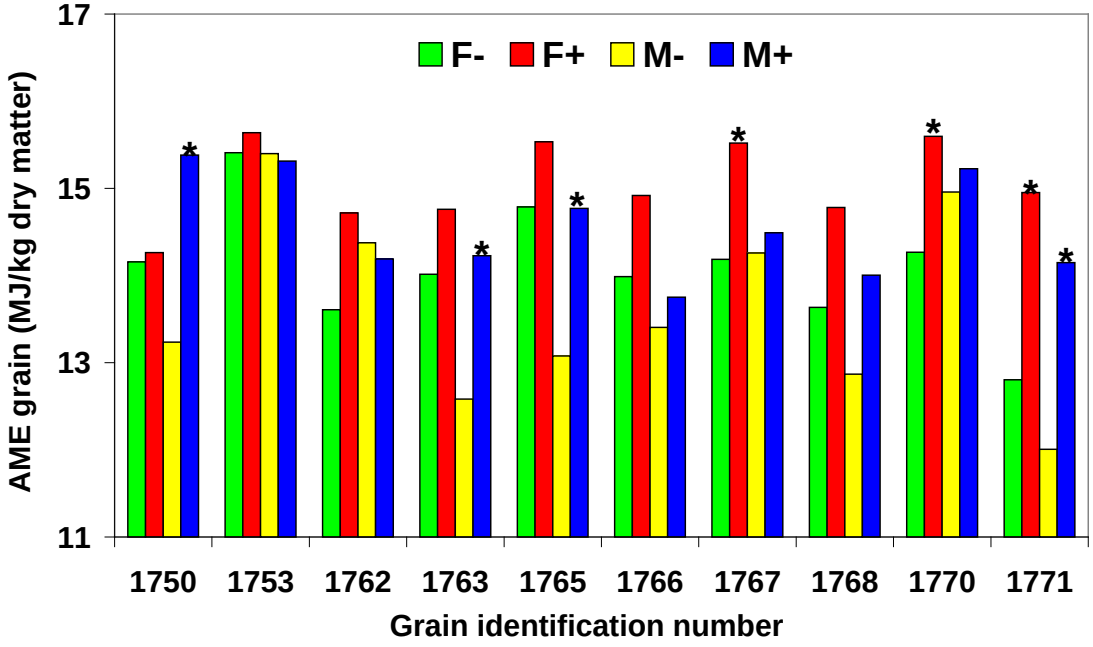

Figure 1 AME (MJ/kg dry matter) values for individual high screenings wheats fed with and without the xylanase and phytase enzyme blend to male and female broiler chickens (22-29 days of age) in Experiment 1. An asterisk represents a significant effect ($P < 0.05$) of enzyme within sex.

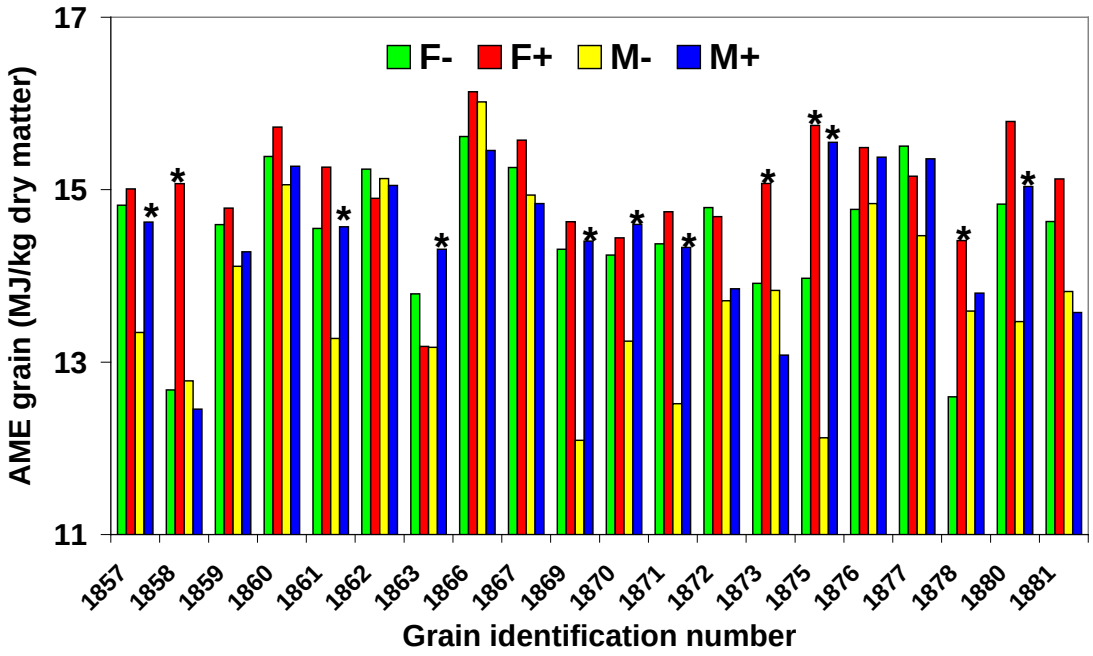

Figure 2 AME (MJ/kg dry matter) values for individual wheats donated by commercial companies and fed with and without the xylanase and phytase enzyme blend to male and female broiler chickens (22-29 days of age) in Experiment 2. An asterisk represents a significant effect ($P < 0.05$) of enzyme within sex.

IV. DISCUSSION

The results of both experiments showed that the blend of xylanase and phytase enzymes improved AME values for some, but not all wheats, and only one sample of barley. Other types of grain were unaffected. Variable responses of different wheats to enzymes were observed in high screenings grains with presumably higher than usual concentrations of soluble and insoluble NSP. Increasing concentrations of insoluble NSP, but not soluble NSP, depressed AME values for wheat sourced from commercial feed mills. The lack of response to this enzyme blend in some wheats (high screenings and industry-sourced) probably reflects differences in cell wall matrices in grains and the way NSP are bound to other cell wall components (Choct et al., 1996), with net effects of xylanase and phytase in the enzyme blend differing between grains. These experiments were not designed to distinguish between the effects of xylanase and phytase components of the blend, but the respective effects are likely to be additive in wheat diets (Cowieson and Bedford, 2009).

The results of both experiments show that males fed barley and wheat can have lower AME values than females, whereas there were no differences between males and females given triticale and sorghum. These results are consistent with earlier work by Hughes et al. (2001) who found similar reductions in AME of wheat and barley fed to male chickens, but no differences when fed triticale or sorghum. This phenomenon may indicate that the b-glucan and arabinoxylan NSP affect males more so than females. Hughes (2003) conjectured that sex differences may have arisen through establishment of different gut microflora profiles as a result of differential flow of undigested nutrients into the hindgut acting as differential growth media for bacteria, or by exchange of chemical messages between host tissue and gut microflora. These possibilities warrant further investigation.

In conclusion, the results show that the particular blend of xylanase and phytase enzymes improved AME values for some, but not all wheats, and only one sample of barley. Other types of grain were unaffected by enzymes. Significant responses to enzymes in wheat diets were not always associated with high NSP levels.

ACKNOWLEDGMENTS

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THE RESPONSES OF A COMBINED NSP-DEGRADING ENZYME AND PHYTASE IN LAYING HENS FED ON CORN BASED DIETS

Y. G. LIU[1], C.G. OLNOOD[1], P. DALIBARD[2], and P. GERAERT[2]

Summary

Two field studies were conducted to investigate the responses of a combined NSP-degrading enzyme and phytase (Rovabio® Max) in laying hens fed on corn-soybean meal based diets (Positive Control, PC, available phosphorus or AP 2.6 and 2.3 g/kg) with reformulated diets (Negative Control, NC, with reduced ME 0.18 and 0.21 MJ/kg, AP 1.2 and 1.5 g/kg, Ca 1.0 and 1.5 g/kg, respectively) in which dicalcium phosphate (7.5 and 8.9 kg/mt) was entirely replaced. The results showed that hens fed on the NC diets performed significantly worse, with lower feed intake and laying rate and increased mortality, whereas the enzyme treatment fully restored these parameters to the levels of the PC diets. The results suggest an intake of AP at 160 mg/hen/day is insufficient and the combined enzymes contribute ME 0.18-0.21 MJ/kg and AP 1.2-1.5 g/kg.

I. INTRODUCTION

Non-starch polysaccharides (NSP) are found in almost all ingredients of vegetable origin. These NSP are polymeric carbohydrates with different composition and structure from starch and considered anti-nutritional factors with poor digestibility in poultry (Bedford, 1993). Soybean meal contains a significant quantity of NSP (210-220 g/kg dry matter), including 55% of pectins mainly in the form of rhamnogalacturonans, as well as oligosaccharides of α-galactoside series (raffinose, stachyose and verbascose), for which poultry do not secrete relevant enzymes for hydrolysis. The NSP-degrading enzymes target the cell walls of vegetable ingredients, not only for cereals such as corn, wheat or barley, but also for soybean meal and other vegetable protein meals, especially the insoluble components, to facilitate the release of nutrients encapsulated by the cell walls or incorporated into the cell wall itself, resulting in an easier access of digestive enzymes (Choct, 2006). Numerous studies have demonstrated that the negative effects of NSP can be overcome by supplementation with suitable exogenous enzyme preparations (Gracia *et al*., 2003).

Despite the use of feed ingredients containing substantial amounts of phosphorus (P), an inorganic source of P is routinely added to layer feeds to ensure sufficient P supply because phytate P is largely unavailable. A number of studies have demonstrated that supplementing layer diets with microbial phytase results in improved performance (Gordon and Roland, 1997). Furthermore, the use of phytase can reduce the P and nitrogen excretion into manure thus benefitting the environment.

Conversely to energy deficiency, which prompts increased feed intake, P deficiency strongly reduces feed intake, which can be restored by phytase supplementation. Most of the phytase effects result in restoring production performance without having much affect on feed efficiency. Thus it was of interest to investigate the combination of carbohydrates and phytase on feed intake, egg production rate, and bone mineralization in chickens (Musapuor et al., 2005; Francesch and Geraert, 2009).

[1] Adisseo Asia Pacific Pte Ltd, Singapore 179803
[2] Adisseo France S.A.S., Antony France 92160

Rovabio® Max AP is a stabilized multi-enzyme combination of 19 NSP-degrading enzymes naturally occurring in a non-genetically modified fungus *Penicillium funiculosum*, further fortified with phytase of *E coli* origin. The objective of these studies was to determine the efficacy of the enzyme preparation on performance and egg quality in laying hens fed on corn soybean meal diets. The enzyme combination (xylanase 22,000 visco unit/kg, β-glucanase 2,000 AGL unit/kg and phytase 10,000 FTU/kg) was tested in the negative control diets at levels of 50 g/mt feed.

II. MATERIALS AND METHODS

Two experiments were conducted with brown egg layers, Experiment 1 from 57-77 weeks of age and Experiment 2 from 20-44 weeks of age, respectively. Diets were in mash form and the enzyme was added through the premix. The negative control (NC) diet was derived from the positive control (PC) diet, by reducing ME, digestible amino acids and total and available P. These reductions in nutrient content were achieved by removing or lowering vegetable oil, removing dicalcium phosphate completely and slight reductions in soybean meal. At the end of the two experiments, one bird per replicate was sacrificed to test bone mineralization (tibia ash, Ca and P contents).

Exp. 1. A balanced reference corn-soybean meal diet (Positive Control, PC-1) was compared with two reformulated diets (Negative Control 1, NC1) with total removal of dicalcium phosphate (7.5kg/mt) plus reduction of ME 0.21 MJ (44 Kcal)/kg, available P 1.2 g/kg and Ca 1.0 g/kg, without and with addition of combined NSP-degrading enzymes and phytase (Rovabio® Max) at 50g/mt). The NC-l diet had complete removal of vegetable oil. The three dietary treatments used six replications of 18 Lohman Brown hens per cage from 57 to 77 weeks of age, with starting egg production around 85%. Ingredients and composition of the experimental diets are shown in Table 1. The experiment was performed in the Institute of Animal Nutrition, Sichuan Agriculture University, China.

Table 1 Main composition and characteristics of the control diets (Exp. 1)

Major Ingredients (g/kg)	PC-1	NC-1	Calculated nutrients	PC-1	NC-1
Corn (maize)	644.0	646.0	Crude protein (g/kg)	155.0	152.7
Soybean meal (48%)	154.8	151.0	ME (kcal/kg)	2,650	2,606
Rapeseed meal	99.0	96.6	ME (MJ/kg)	11.09	10.90
Vegetable oil	4.0	-	Lysine (g/kg)	7.0	6.9
Limestone (fine)	39.0	40.0	Methionine (g/kg)	3.2	3.2
Limestone (coarse)	39.0	39.5	Calcium (g/kg)	34.0	33.0
Dicalcium phosphate	7.5	-	Total P (g/kg)	4.90	3.70
DL-methionine	0.6	0.6	Available P (g/kg)	2.60	1.40

Exp. 2. This experiment was conducted to compare standard corn-soybean meal based diet (Positive Control 2, PC-2) with two reformulated diets (Negative Control 2, NC-2) with total removal of dicalcium phosphate plus reduction of amino acids by 1.5-3.0%, without and with addition of Rovabio® Max AP (50 g/mt). The experimental diets are shown in Table 2. The three dietary treatments used nine replicates of 15 HyLine Brown hens per pen from 20 to 44 weeks of

age. Performance and egg quality parameters were recorded during six consecutive four-week periods. This experiment was conducted at IRTA, Department of Animal Nutrition (Reus, Spain).

The performance data include means of six replicates of 18 laying hens in Exp. 1 and nine replicates of 15 laying hens in Exp. 2. In the bone mineralization analysis, data are means of six birds per treatment in Exp. 1 and nine birds per treatment in Exp. 2.

Table 2 Main composition and characteristics of the control diets (Exp. 2)

Major Ingredients (g/kg)	PC-2	NC-2	Calculated nutrients	PC-2	NC-2
Corn (maize)	600.6	624.0	Crude protein (g/kg)	160.0	156.0
Soybean meal (48%)	188.0	179.0	ME (kcal/kg)	2,750	2,700
Sunflower meal (34%CP)	80.0	80.0	ME (MJ/kg)	11.51	11.30
Soybean oil	27.5	16.2	Dig. lysine (g/kg)	7.4	7.3
Calcium carbonate	86.0	91.8	Dig methionine (g/kg)	3.7	3.5
HCI-lysine 98	1.0	1.1	Dig met + cystine (g/kg)	5.9	5.8
DL-methionine 99	1.0	0.9	Dig threonine (g/kg)	5.3	5.2
Dicalcium phosphate	8.9	-	Calcium (g/kg)	36.0	36.0
Sodium chloride	3.0	3.0	Total P (g/kg)	5.20	3.60
Vit/mineral premix	4.0	4.0	Available P (g/kg)	2.30	0.10

III. RESULTS AND DISCUSSION

Feeding layers with reduced nutrients depressed feed intake, rate of lay and egg weight, impaired feed conversion ratio and increased mortality in comparison with the PC diets in both experiments. Significant differences were observed between the NC and PC groups in terms of feed intake (113.8 *vs* 117.8g/hen/day in Exp. 1 and 100.9 *vs* 109.6 g/hen/day in Exp. 2), percentage production (77.59 *vs* 84.38% in Exp. 1 and 77.20 *vs* 88.70% in Exp. 2) and mortality (13.89 *vs* 2.78% in Exp. 1 and 8.88 *vs* 3.70% in Exp. 2) (Table 3). Differences between the two groups became significant after eight weeks for production and after four weeks for feed intake, with the effect lasting until the end of the experiments.

The very high mortality of NC-1 indicates a more severe effect of P deficiency for the older hens, compared with that of the NC-2, even though the calculated available P was higher for the older hens. The addition of the enzyme fully restored the mortality in both experiments. The live weight of the birds was clearly affected by the dietary treatments. The hens on NC-1 and NC-2 lost weight whilst the enzyme addition improved the utilisation of nutrients. The influence of dietary treatments on bone ash, Ca and P contents appears to be inconsistent due partially to different test and expression methods used in these two experiments. More studies are required to further illustrate the effect on bone mineralization in laying hens.

In conclusion, for laying hens fed on corn soybean meal diets, Rovabio® Max is efficient in compensating the reduction of available P (1.5 g/kg), AME (up to 0.21 MJ or 50 kcal/kg) and CP-dAA (up to 1.5%). It restored the productivity to the level of a nutritionally adequate diet. The inclusion of Rovabio® Max AP supported laying performance, egg production and live weight of the birds fed a diet without inorganic phosphorus.

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Table 3 Laying performance in response to the enzyme application

Treatments	PC	NC	NC+ Max 50g/t
Exp. 1 – Lohman (57-77 weeks)			
Laying rate (%)	84.38[a]	77.59[b]	84.80[a]
Feed intake (g/hen/day)	117.8[a]	113.8[b]	116.5[ab]
Egg weight (g)	63.10	62.50	62.30
Egg Mass (g)	53.0[a]	48.5[b]	52.8[a]
Feed conversion	2.18	2.28	2.18
Mortality (%)	2.78[b]	13.89[a]	2.78[b]
Body weight (kg/bird)	1.87[a]	1.68[b]	1.93[a]
Ash content (% Fat free)*	52.44	50.42	50.03
Calcium (% ash)**	35.51	34.23	36.02
Phosphorus (% ash)**	16.59	16.81	16.60
Exp. 2 – Hy-Line Brown (20-44 weeks)			
Laying rate (%)	88.70[a]	77.20[b]	88.60[a]
Feed intake (g/hen/day)	109.6[a]	100.9[b]	110.0[a]
Egg weight (g)	59.70	58.80	59.60
Egg mass (g)	52.9[a]	45.4[b]	52.8[a]
Feed conversion	2.07[c]	2.22[b]	2.09[c]
Mortality (%)	3.70[b]	8.88[a]	2.22[b]
Body weight (kg/bird)	1.83[a]	1.67[b]	1.78[a]
Ash content (% DM)	49.3[a]	46.6[b]	46.4[b]
Calcium (% DM)	17.20[a]	16.20[b]	16.30[b]
Phosphorus (% DM)	7.70[a]	7.04[b]	7.28[b]

[ab] Means within a row not bearing a common superscript differ significantly ($P < 0.05$)
* Expressed as % of fat-free, moisture-free tibia; **: Expressed as % of tibia ash.

DIETARY ENZYME COMBINATIONS IMPROVE SORGHUM ILEAL PROTEIN AND STARCH DIGESTIBILITY DURING THE BROILER STARTER PHASE

A SULTAN[1], CY GAN, X LI, D ZHANG and WL BRYDEN

Sorghum is an important grain crop for poultry but it has been shown that broilers offered sorghum-based diets often have sub-optimal performance when compared to birds fed wheat-based diets. The cause of poor performance appears to relate to reduced energy availability (Bryden, et al 2009). The aim of this study was to determine if dietary enzymes improve protein and starch utilization of sorghum by 21-day-old broilers.

The basal experimental diet contained a commercial red sorghum (918 g/kg) as the only source of starch and protein with celite (20 g/kg), a source of acid insoluble ash (AIA), added as an indigestible marker. To the basal diet, various enzymes were added to produce 8 experimental diets (Table 1) that were fed as mash to four replicate pens of 14day-old broilers (12 birds/pen) in an environmentally controlled house. After a week (day-21), all birds were euthanased and intestinal contents collected. Protein and starch ileal digestibility coefficients were calculated and are shown in Table 1.

Table 1 Dietary enzymes and ileal protein and starch digestibility of sorghum in 21day-old broilers.

Treatment	Treatment Number	Ileal digestibility coefficients	
		Protein	Starch
Control	1	0.72^{c}	0.75^{c}
Xylanase	2	0.75^{bc}	0.83^{b}
Phytase	3	0.78^{ab}	0.86^{ab}
Protease	4	0.75^{bc}	0.83^{b}
Xyl + Phytase	5	0.81^{a}	0.89^{a}
Xyl + Protease	6	0.79^{ab}	0.86^{ab}
Phyt + Protease	7	0.78^{ab}	0.88^{a}
Xyl +Phyt +Prot	8	0.78^{ab}	0.87^{a}
Pool SEM	-	0.01	0.01

Means with different superscripts are significantly different ($p<0.05$).

The ileal protein and starch digestibility coefficients were improved with the addition of dietary enzymes, especially phytase and protease combinations. Interestingly, xylanase combined with phytase resulted in the greatest numerical improvement in both starch and protein digestion. The combination of all three enzymes improved the protein (8.3%) and starch (16.0%) coefficients, substantially, when compared with the control diet. A strong positive correlation ($r=0.61$, $p<0.01$) between protein and starch digestibility coefficients was observed.

From these results, it can be concluded that application of dietary enzymes to sorghum-based broiler starter diets has the potential to increase protein and starch digestion and hence energy availability. Further studies are required to delineate the mechanisms by which sorghum anti-nutritive factors impede energy utilisation.

Bryden, WL, Selle, PH, Cadogan, DJ, Li, X, Muller, ND, Jordan, DR, Gidley, MJ and Hamilton, WD (2009) Publication 09/077. RIRDC Barton, ACT.

[1] The University of Queensland, School of Animal Studies, GATTON QLD

DIETARY ENZYMES MODULATE SORGHUM STARCH DIGESTION KINETICS IN BROILERS

A. SULTAN[1], C.Y. GAN, X. LI, D. ZHANG and W.L. BRYDEN

Energy in poultry diets is mainly provided by starch; a major component of cereal grains. Sorghum starch is present in the endosperm of grain in the form of tightly packed, polygonal granules, shielded by spherical protein bodies and matrix that decrease its digestibility. Hydrolysis of protein in sorghum grains exposes starch granules to both host and dietary enzymes thus increasing its utilization. The present study was undertaken to examine the effect of dietary enzymes on the pattern and kinetics of sorghum starch digestion in different sections of the intestinal tract.

Four mash diets, containing a commercial red sorghum (918g sorghum/kg diet), were prepared as a control diet and three containing a different commercial feed enzyme (xylanase, phytase or protease) included at the rate recommended by the manufacturer. Each dietary treatment was offered to four replicate pens of 35-day-old broilers (8 birds/pen) in an environmentally controlled house. After a week (day-42), all birds were euthanased and intestinal section contents collected and pooled/pen Starch and acid-insoluable ash in feed, intestinal and faecal samples were analysed and starch digestibility coefficients and fractional rate of disappearance were calculated (Table 1).

Table 1 Influence of dietary enzymes on intestinal sorghum starch digestion kinetics in 42-day-old broilers

Diet	Starch digestibility coefficient				Potential starch digestibility coefficient (DST)*	Fractional starch digestion rate (h^{-1})*
	Jejunum	Upper ileum	Lower ileum	Faeces		
Control	0.557^{4}	$0.840^{b,3}$	$0.905^{d,2}$	$0.942^{d,1}$	0.947^{b}	2.85^{c}
Xylanase	0.621^{4}	$0.857^{ab,3}$	$0.917^{c,2}$	$0.948^{c,1}$	0.954^{ab}	3.08^{bc}
Phytase	0.607^{4}	$0.878^{a,3}$	$0.927^{b,2}$	$0.956^{a,1}$	0.958^{a}	3.63^{ab}
Protease	0.620^{4}	$0.880^{a,3}$	$0.929^{a,2}$	$0.950^{b,1}$	0.954^{ab}	3.66^{a}
Pooled SEM	0.02	0.008	0.004	0.002	0.002	0.183

[abcd] Means in column followed by different superscripts are significantly different.
[1234] Means in rows followed by different superscripts are significantly different.
* DS = DST x $(1 - e^{-kds \times t})$ (Ørskov & McDonald 1979)

Starch digestibility coefficients increased down the tract. All three enzymes increased the ileal starch digestibility coefficient and this correlates with fractional starch digestion rates, determined by analysis of residual starch contents in different sections of the digestive tract. These effects were most apparent ($p<0.05$) with phytase and protease. Faecal starch digestibility coefficients for all the treatments were significantly ($p<0.05$) greater than ileal values; possibly due to hind gut fermentation. These results show that the application of dietary enzymes can improve sorghum starch digestion and suggest that this reflects hydrolysis of both protein and phytate. These components of the sorghum caryopsis appear to impede amylase access to the starch granules and/or reduce activity of the enzyme.

Orskov ER, and McDonald I (1979) *J. Agric. Sc.*, **92**: 499-503.

[1] The University of Queensland, School of Animal Studies, Gatton QLD

EFFECT OF PHYTIC ACID AND PH VALUE ON THE ACTIVATION OF CHICKEN PEPSINOGEN *IN VITRO*

N. LIU[1] and A.J. COWIESON[2]

Summary

The objective of this study was to investigate the activation of chicken pepsinogen by HCl solutions *in vitro,* and the possible interference of phytic acid. Five concentration gradients of phytic acid (0, 1.0, 2.0, 3.0 and 4.0%) and different pH values (0.8, 1.8 or 2.8) of hydrochloric acid solutions were used in experiments I, II and III. Pepsinogen was prepared from the proventriculus of broiler chickens and activated to pepsin by hydrochloric acid, and the activity of pepsin was determined. Each treatment was replicated 5 times. The results showed that the activation of pepsinogen was depressed by phytic acid at 2.0, 3.0 and 4.0% under pH values of 0.8, 1.8 or 2.8, compared with the treatment without phytic acid. The results suggest that phytic acid negatively influences the activation of pepsinogen and the activity of pepsin *in vitro*.

I. INTRODUCTION

Phytate and protein may form binary complexes through electrostatic links between its charged phosphate groups and basic amino acid residues or with the terminal amino group of proteins (Mothes et al., 1990). At pH below the isoelectric point, proteins possess a net positive charge and as phytate is negatively charged there is potential for a strong electrostatic phytate-protein interaction. The pH range in the gizzard-proventriculus of the chicken would facilitate the formation of phytate-protein complexes. The interaction between proteins and phytate may influence the enzymatic digestion of proteins in the gizzard-proventriculus of poultry. It is also noteworthy that the gizzard-proventriculus in chickens has been identified as the main sites of phytate hydrolysis (Liebert et al., 1993).

Chicken pepsin contains 39 free carboxyl groups and 16 basic groups, and its optimal activity is at pH 1.8 (Bohak, 1969). Further, the isoelectric point of chicken pepsinogen is 3.7 (Krebs et al., 1971). The range of pH in chicken proventriculus is about 0.5 to 2.5 (Dingle, 1990), which is lower than the isoelectric point of pepsinogen and may cause the pepsinogen to possess a net positive charge. The acidic conditions of the proventriculus hypothetically contribute to the formation of a phytate-pepsinogen complex. In this context, it is noteworthy that the peptide which activates pepsinogen contains 13 basic residues out of a total of 44 amino acids (Dunn et al., 1978), thus phytate may readily bind this activation peptide and impede the conversion of the zymogen to pepsin. Indeed, Dykes & Kay (1977) have shown that modifying lysine and histidine residues of this peptide significantly depressed activation of pepsinogen. Thus the possibility of phytate interacting with this activation peptide may be another avenue whereby phytate influences pepsin activity.

Thus, as there are few reports on the effect of phytic acid on the activation of chicken pepsinogen, the present study is designed to investigate the factors of pH value and phytic acid on the *in vitro* activation of chicken pepsinogen.

[1]School of Animal Science, Henan University of Science and Technology, Luoyang 471000, China
[2]Poultry Research Foundation, University of Sydney, Camden, NSW 2567, Australia

II. MATERIALS AND METHODS

a) Experimental design

Three experiments were conducted, each using five concentrations of phytic acid (0, 1.0, 2.0, 3.0 and 4.0%) and three pH values, 0.8, 1.8 and 2.8 for experiments I, II and III respectively. Solutions of phytic acid was purchased from Sigma and prepared with deionized water and pH was adjusted with HCl. Each treatment included 5 replicates and each replicate was measured in duplicate.

b) Pepsinogen preparations

Before sampling, broilers (Arbor Acres, male) at 21 days of age were offered diets (ME 13.14MJ/kg, CP 20%, Ca 0.90%, Available P 0.45%, Phytate P 0.20%, Na 0.21%, Cl 0.17%, K 0.40%) and then killed by electrocution. The proventriculus was excised and washed with cold deionized water to remove traces of feed. The lumen of the proventriculus was cut longitudinally to expose brush border cells. Mucosa was gently scraped off with a glass microscope slide and pooled. Each mucosal sample was homogenised (1:4, wt/vol) with cold deionized water and then centrifuged at 8000 g for 3 min at 4°C. The supernatant was immediately used to prepare the zymogen. Chicken pepsinogen was extracted from the forestomach of chicken and purified by the consecutive application of acetone precipitation, chromatography on diethylaminoethyl cellulose, and gel filtration on Sephadex G-100 (Bohak, 1969).

c) Pepsin activity assay

Pepsin activity in the proventricular mucosa was determined by the method of Anson (1938). Bovine haemoglobin (2%, 100 ml; Sigma-Aldrich) was used as a substrate in 60 mM HCl (pH 2.0) and was incubated with 25 ml of mucosal homogenate in a microcentrifuge tube for 30 min. The reaction was stopped by adding 200 ml of 5% trichloroacetic acid, and the reaction mixture was centrifuged at 4200 g at 4°C for 6 min. The absorbance of L-tyrosine released was read at 280 nm and pepsin activity was determined using an L-tyrosine (Sigma-Aldrich) standard curve. Pepsin activity was expressed as nanomoles of L-tyrosine liberated per min per milliliter supernatant.

d) Statistical analysis

Data from experiments I, II and III were analysed using the one-way ANOVA of SPSS (version 11.5). Differences of variables were separated using LSD test at $P<0.05$ level of significance. Values in the figures are means and standard deviation (SD).

III. RESULTS

The activity of pepsin was decreased ($P<0.05$) by the inclusion of phytic acid at 2.0, 3.0 and 4.0% by 27.6 to 55.5% at pH 0.8 (Fig. 1), compared with the treatment without phytic acid. Similarly, phytic acid depressed ($P<0.05$) the activity of pepsin by 6.9 to 21.8% at pH 1.8 (Fig. 2) and by 18.8 to 33.5% at pH 2.8 (Fig. 3). There were no differences in the activity of pepsin between the treatments without and with phytic acid at 1.0% when the pH values were at 0.8, 1.8 and 2.8 ($P\geq0.05$).

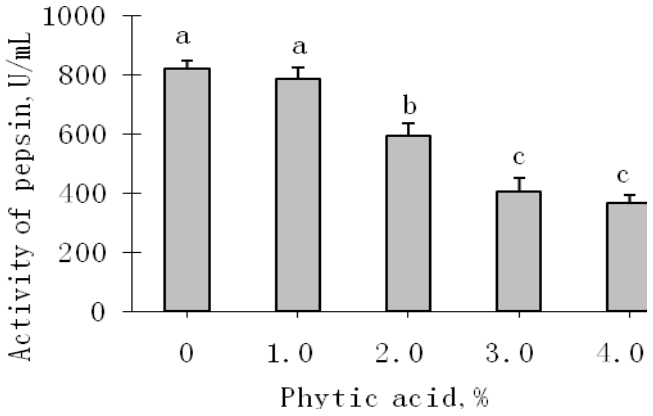

Figure 1 Effect of phytic acid on in vitro activation of pepsinogen at pH 0.8.

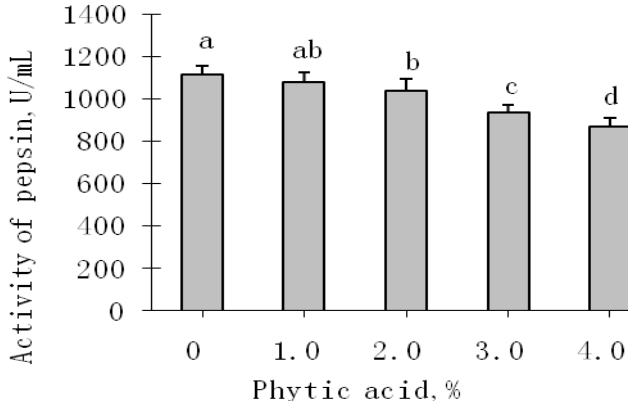

Figure 2 Effect of phytic acid on in vitro activation of pepsinogen at pH 1.8.

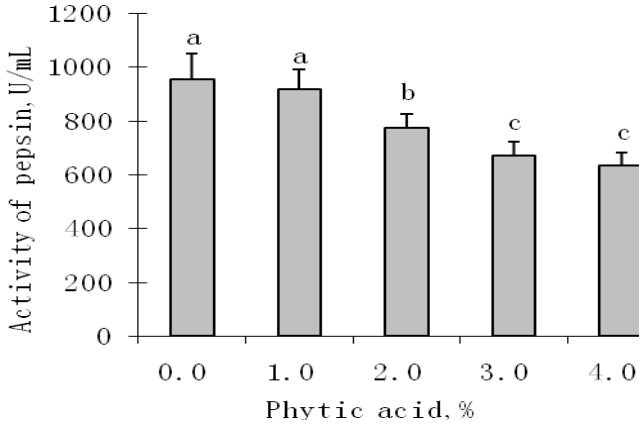

Figure 3 Effect of phytic acid on in vitro activation of pepsinogen at pH 2.8.

IV. DISCUSSION

Phytate and protein may form binary complexes through electrostatic links between charged phosphate groups and either the free amino acids arginine or lysine, basic amino acid residues present within protein or with the terminal amino group on proteins at a pH lower the isoelectric point of protein (Mothes et al., 1990). The pH range in the gizzard-proventriculus of the chicken is about pH 0.5 to 2.5 (Dingle, 1990), which is lower than the isoelectric point of pepsinogen, and may facilitate the formation of phytate-protein complexes. Indeed, the extent of activation of pepsinogen at pH 0.8 was lower than that at other pH values, possibly

indicating greater interaction between phytic acid and zymogens at lower pH. Additionally, the negative effect of phytic acid on the activation of pepsinogen at pH 1.8 was least among the observed pH values in the present study, most likely because the optimal activity of pepsin is at this point. Vaintraub and Bulmaga (1991) reported that phytate inhibits the action of pepsin on protein by binding with the substrate and this inhibition is maximal at pH 2-3 and drops to zero when the pH increased to 4.0-4.5. In the present study the effect of phytic acid on the activity of pepsin at higher pH was not tested and this warrants further study.

Of importance here is that it has recently been shown (Cowieson and Cowieson, 2011) that phytic acid may not bind directly to protein, regardless of its isoelectric point, basic amino acid residue composition or ambient pH. This recent work suggests instead that phytic acid reduces protein solubility by competing with nutrients in an aqueous medium for water. Putatively, phytate draws a hydration shell around itself by nature of its negative charge and this reduces water potential, causing less polar proteins to precipitate and self aggregate. It is beyond the scope of this article to expand on these mechanisms in detail but it is possible that the effects of phytic acid on the activation of pepsinogen in the current study may be associated not with direct phytic acid binding to the zymogen (or activation fragment) but with competition as a Hoffmeister anion for water.

It can be concluded that phytic acid has the capacity to interfere with the activation of pepsinogen to pepsin *in vitro*. If these effects are representative of *in vivo* conditions then one possible explanation for the so-called 'extra-phosphoric' effects of phytase may be restoration of optimal digestive enzyme kinetics.

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EFFECTS OF MICROBIAL PHYTASE ON NUTRIENT DIGESTIBILITY AND ENERGY UTILISATION IN YOUNG BROILERS FED PHOSPHORUS-ADEQUATE DIETS

F. ZAEFARIAN[1], L.F. ROMERO[2] and V. RAVINDRAN[1]

Summary

The influence of microbial phytase on the performance and digestibility of lipids and other nutrients of young broilers fed phosphorus-adequate diets based on maize or wheat was evaluated. In both diet types, supplemental phytase improved the weight gain and feed conversion ratio, and increased the apparent ileal digestibility of nitrogen, lipids and phosphorus. Improvements in the apparent ileal digestibility of palmitic, stearic, oleic and linoleic acids were also observed. Phytase supplementation had no effect on the nitrogen-corrected apparent metabolisable energy in both diet types, but tended to improve the ileal digestible energy in wheat-based diets.

I. INTRODUCTION

The effectiveness of microbial phytase in increasing the availability of phytate-bound P, and several other important minerals, protein, and amino acids in poultry diets based on a range of plant derived ingredients, is becoming increasingly accepted (Selle and Ravindran, 2007). However, literature on the influence of phytase on the digestibility of lipids and fatty acids is limited. Therefore the study reported herein was carried out to determine the effects of phytase supplementation in phosphorus-adequate maize- and wheat-based diets on the performance and nutrient digestibility of young broilers.

II. MATERIALS AND METHODS

The experimental design was a 2 × 2 factorial arrangement of treatments using two diet types of grain (maize- or wheat-based) and two inclusion levels of microbial phytase (0 or 500 FTU/kg diet; Phyzyme XP, Danisco Animal Nutrition, Wiltshire, UK). Both basal diets were formulated to meet the Ross 308 recommendations for major nutrients, including calcium (10 g/kg) and available phosphorus (4.5 g/kg), for young broilers. The diets contained 29-38 g/kg animal fat:soybean oil (50:50) blend; and titanium dioxide (3 g/kg) was included as an inert marker for the estimation of nutrient digestibility. Each of the four dietary treatments was randomly assigned to six cages, each housing eight birds (Ross, 308; n = 192). The experimental diets were offered *ad libitum* from day 1, and water was freely available. Body weights and feed intake were recorded on a cage basis at weekly intervals throughout the 21-day trial. Birds received 20 h of fluorescent illumination per day. The temperature was maintained at 31 ºC during the first week and then gradually reduced to 24 ºC by the third week.

From day 17 to 20 post-hatching, feed intake and total excreta output were measured quantitatively per cage for the determination of nitrogen-corrected apparent metabolisable energy (AME_n). On day 21, the birds were euthanised and digesta from the lower half of the ileum was collected for analysis. Digestibility coefficients of nutrients (protein, calcium, phosphorus, fat and fatty acids) and energy utilisation were calculated using marker ratios in

[1] Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand
[2] Danisco Animal Nutrition, Marlborough, Wiltshire, SN8 1XN, UK

the diet and digesta. The procedure reported by Sukhija and Palmquist (1988) was used to measure fatty acid composition in diet and digesta samples. The samples were subjected to solvent extraction, purification and esterification followed by gas chromatography analysis. Two-way analysis of variance was employed to test the main effects (diet type and phytase) and their interaction by using the General Linear Model procedure of SAS (2004).

III. RESULTS AND DISCUSSION

Addition of microbial phytase to both maize- and wheat-based diets improved weight gain ($P < 0.01$; 810 g vs. 858 g in diets without and with phytase, respectively) and feed conversion ratio ($P < 0.001$; 1.382 g/g vs. 1.345 g/g in diets without and with phytase, respectively) of birds, compared to those diets without phytase during the experimental period. Growth responses to phytase supplementation to phosphorus-inadequate diets are mediated mainly through increased feed intake (Selle and Ravindran, 2007). In this study, however, neither the main effects of grain type and phytase nor the interaction between grain type and phytase was significant ($P > 0.05$) for feed intake (data not shown). These data suggest that the effects of phytase on weight gain and feed conversion ratio were independent of its effect on phosphorus availability.

Birds fed maize-based diets had higher ileal digestibility coefficients of nitrogen ($P < 0.001$), calcium ($P < 0.05$), phosphorus ($P < 0.01$), and gross energy ($P < 0.001$), apparent ileal digestible energy (AIDE; $P < 0.001$), and AME_n ($P < 0.001$) than those fed wheat-based diets (Table 1). Addition of microbial phytase increased ($P < 0.05$) the ileal digestibility coefficient of nitrogen and phosphorus. No grain type × phytase interaction ($P > 0.05$) was observed for these parameters. However, for ileal energy utilisation and AIDE, the grain type × phytase interaction tended ($P = 0.09$) to be significant. Ileal energy utilisation and AIDE were unaffected by phytase supplementation in maize-based diets, but tended to increase in wheat-based diets.

Ileal digestibility coefficients of all fatty acids and total lipid were higher in birds fed maize-based diets compared to those fed wheat-based diets (Table 2). Phytase supplementation increased ($P < 0.05$) the ileal digestibility of lipid ($P < 0.001$), and palmitic ($P = 0.06$), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids. No grain type × phytase interaction ($P > 0.05$) was observed for the ileal digestibility coefficient of fatty acids and total lipid.

In the present study, addition of microbial phytase in phosphorus-adequate diets caused significant improvements in ileal digestibility coefficients of nitrogen and phosphorous in both maize- and wheat-based diets. These responses may be attributed, in part, to the capacity of phytase to release these bound nutrients by hydrolysing phytic acid. Recent studies suggest that phytate increases endogenous protein losses (Cowieson and Ravindran, 2007), and can cause hypersecretion of mucin and depression of enzyme activities in the digestive tract of birds (Cowieson et al., 2004; Liu et al., 2008). Amelioration of these negative effects may also have contributed to the observed improvements in nutrient digestion.

Inclusion of microbial phytase had no effect on the AME_n, but tended to increase the AIDE of wheat-based diets. The lack of effect of phytase on AME_n may be related to the fact that the classical excreta-based AME measurements do not reflect the actual responses because of the modifying effects of caecal microorganisms on energy utilisation and the contribution of microbial mass to energy output in the excreta. The variable and modifying effects of hindgut microflora on protein digestion have been previously demonstrated (Ravindran et al., 1999).

Table 1 Influence of grain type and phytase on ileal digestibility coefficients of nitrogen (N), calcium (Ca), phosphorus (P), gross energy (GE), apparent ileal digestible energy (AIDE, MJ/kg DM) and AME_n (MJ/kg DM) of broiler chicks to 21 days of age.

		Ileal digestibility coefficient					
	Phytase	N	Ca	P	GE	AIDE	AME_n
Maize	-	0.832	0.449	0.522	0.758	14.74	13.48
	+	0.841	0.466	0.611	0.759	14.75	13.48
Wheat	-	0.771	0.360	0.453	0.665	12.91	12.76
	+	0.805	0.366	0.497	0.696	13.53	12.79
SEM		0.0079	0.0378	0.0242	0.0087	0.1691	0.073
Main effects							
Grain type							
Maize		0.836[a]	0.458[a]	0.566[a]	0.758[a]	14.74[a]	13.48[a]
Wheat		0.788[b]	0.363[b]	0.475[b]	0.680[b]	13.22[b]	12.78[b]
Phytase							
-		0.801[b]	0.405	0.487[b]	0.711	13.83	13.12
+		0.823[a]	0.416	0.554[a]	0.727	14.14	13.14
Probabilities, P ≤							
Grain type		***	*	**	***	***	***
Phytase		*	NS	*	0.08	0.08	NS
Grain type × Phytase		NS	NS	NS	0.09	0.09	NS

[a,b]Means within a column not sharing a common superscript are significantly different (NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

The significant improvement observed in ileal digestibility coefficient of lipid with phytase is consistent with the findings of Camden et al. (2001). However, this is the first report demonstrating the effects of phytase on the digestibility of fatty acids in maize- and wheat-based diets. The findings from previous work, listed here, may explain improvement in lipid and fatty acid digestibility observed in current study. Hydrolysis of phytate by phytase may have (i) reduced the formation of metallic (calcium-phytate-fat) soaps in the gut lumen (Ravindran et al., 2000), and (ii) overcome the adverse effect of phytate on lipase activity (Knuckles, 1988) and hypersecretion of bile. Bile is a rich source of phospholipids and there is suggestive evidence that bile output is increased in the presence of phytate (Cowieson et al., 2009).

Table 2 Influence of grain type and phytase on the ileal digestibility coefficients of fatty acids and total lipid of broiler chicks to 21 days of age.

		Ileal digestibility coefficient				
	Phytase	C16:0	C18:0	C18:1	C18:2	Total lipid
Maize	-	0.822	0.779	0.845	0.843	0.831
	+	0.832	0.805	0.851	0.861	0.852
Wheat	-	0.734	0.721	0.775	0.762	0.756
	+	0.772	0.781	0.816	0.801	0.801
SEM		0.0120	0.0162	0.0091	0.0104	0.0073
Main effects						
Grain type						
Maize		0.827[a]	0.792[a]	0.848[a]	0.852[a]	0.842[a]
Wheat		0.753[b]	0.751[b]	0.795[b]	0.782[b]	0.778[b]
Phytase						
-		0.778	0.750[b]	0.810[b]	0.803[b]	0.794[b]
+		0.802	0.793[a]	0.833[a]	0.831[a]	0.827[a]
Probabilities, P ≤						
Grain type		***	*	***	***	***
Phytase		0.06	*	*	*	***
Grain type × Phytase		NS	NS	0.06	NS	NS

[a,b]Means within a column not sharing a common superscript are significantly different (NS, not significant; * P < 0.05; *** P < 0.001)

Overall, the present findings demonstrated that phytate is an anti-nutritive factor that can impair the availability not only of phosphorus and protein but also of lipid and fatty acids, resulting in the depression of growth and feed conversion efficiency of young broilers fed phosphorus-adequate maize- and wheat-based diets. These adverse effects can be overcome by supplemental phytase.

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EFFECT OF A NEW XYLANASE/BETA-GLUCANASE ENZYME COMBINATION ON THE PERFORMANCE OF BROILER CHICKENS FED WHEAT/BARLEY-BASED DIETS

A. PÉRON[1], A. OWUSU-ASIEDU[2] and A. KUMAR[3]

Summary

This study investigated the efficacy of a new product containing both xylanase (xyl) and beta-glucanase (bgl) activities in broilers fed wheat/barley-based diets. 2000 day-old Ross male broilers were allocated to 4 dietary treatments, with 20 replicate pens per treatment (25 birds/pen). The treatments (T) comprised a control diet either unsupplemented (T1) or supplemented with 3 levels of the xylanase and beta-glucanase product to supply guaranteed minima of 610 U xyl and 76 U bgl /kg feed (T2); 1220 U xyl and 152 U bgl /kg feed (T3) or 2440 U xyl and 304 U bgl /kg feed (T4). Diets were offered *ad libitum* in two phases either as crumbles (starter, 0-21 days) or pellets (grower-finisher, 22-42 days). Feed intake and bird weights were measured on days 21 and 42, and feed conversion ratio (FCR) calculated. Data were analyzed using the SAS programme (SAS Inc., 2000) to determine statistical differences between treatments for the measured parameters. Results showed that xylanase and beta-glucanase supplementation improved ($P < 0.05$) bird performance during both starter and grower-finisher periods. From 0 to 42 days, the addition of the enzymes (T2-T4) increased ($P < 0.05$) bodyweight gain by 4.5, 2.9 and 3.8%, respectively and the corresponding FCR values were improved ($P < 0.05$) by 2.9, 3.5 and 4.2%, respectively. There was no difference ($P > 0.05$) in mortality between treatments. The study demonstrated the efficacy of a new xylanase and beta-glucanase product in broilers fed viscous cereal-based diets. These benefits will be related to the breakdown of both soluble and insoluble fibre fractions from wheat and barley grains.

I. INTRODUCTION

Feed enzymes have been used commercially in poultry diets for over 20 years. The first generation of products were developed in Europe in order to address issues related to the presence of non-starch polysaccharides (NSPs) in cereal-based diets. NSPs from viscous grains such as wheat and barley are mainly composed of arabinoxylans and beta-glucans, polymers which are known for their anti-nutritive effects in poultry. High levels of soluble NSPs result in increased gut viscosity. High levels of insoluble NSPs result in increased water-holding capacity, reduced access for digestive enzymes (nutrient packaging) and increased endogenous secretions. Ultimately, these effects lead to reduced performance and nutrient utilisation. They can also result in increased microbial proliferation in the gut and poor litter quality (Annison 1991; Choct and Annison, 1992; Guenter, 1993; Maisonnier et al., 2001; Steenfeldt, 2001; Carré et al., 2002). The use of xylanase and beta-glucanase activities to break down the NSP polymers and improve the nutritional value of poultry feeds has been extensively demonstrated (Bedford and Schulze, 1998; Dänicke et al., 1999a,b; Partridge, 2001; Péron and Partridge, 2009). However, due to characteristics such as affinity for substrate, pH range of activity or susceptibility to cereal endogenous inhibitors, the bio-efficacy of glycanases (carbohydrate degrading enzymes) can vary widely (Choct et al., 2004; Murphy et al., 2009; Péron and Kumar, 2010). The aim of the present study was to

[1] Danisco Animal Nutrition, 61 Science Park Road, Singapore
[2] Danisco Animal Nutrition, Westmound Drive, Waukesha USA
[3] The University of Queensland, Gatton QLD 4343 Australia

investigate the effect of a new xylanase/beta-glucanase product on the performance of broilers fed a wheat/barley-based diet.

II. MATERIALS AND METHODS

A total of 2000 Ross male day-old chickens were purchased from a commercial hatchery, selected according to average weight and allocated to 80 deep-litter floor pens (25 birds/pen) arranged in a completely randomised block design in a standard broiler rearing house. The broilers were housed at a density of 7 birds per square meter. Standard husbandry practices were followed during the brooding and growing stages of the experiment. The study comprised four experimental treatments (T1 to T4) with 20 replicate pens per treatment. The control treatment (T1) was based on wheat, barley, rye and soybean meal, and did not include any products of animal origin or antibiotics (Table 1). The other three treatments were supplemented with graded levels of a new enzyme product (Danisco Animal Nutrition, UK) containing xylanase (xyl) and beta-glucanase (bgl) activities that supplied a minimum guaranteed units (U) of 610 xyl and 76 bgl U/kg feed (T2); 1220 xyl and 152 bgl U/kg feed (T3) or 2440 xyl and 304 bgl U/kg feed (T4).

Table 1 Experimental diets for the control treatment (T1)

Ingredients (g/kg, as fed)	Starter phase (0 to 21 days)	Grower-finisher phase (22 to 42 days)
Wheat	351.7	372.6
Barley	200.0	200.0
Rye	60.0	60.0
Soybean meal (48% CP)	298.2	263.4
Soybean oil	47.6	63.9
L-Lysine	3.0	2.0
DL-Methionine	3.1	2.4
L-Threonine	0.6	0.6
Salt	3.6	3.4
Limestone	8.1	5.6
Dicalcium phosphate	19.1	21.2
Vitamins and minerals	5.0	5.0
Nutrient provision		
ME, MJ/kg	12.34	12.87
CP, g/kg	210.0	195.0
Lysine, g/kg	13.1	11.4
Methionine, g/kg	6.0	5.2
Methionine+Cysteine, g/kg	9.5	8.5

Diets were offered as crumble during the starter (1-3 weeks) phase, and pellets during the grower-finisher (4-6 weeks) phase. Feed and water were offered *ad libitum* during the entire trial period. Extract viscosity of the control feed was measured using the Danisco Avicheck™ method. All chickens received 23 hours light with a minimum light intensity of 100 lux and 1 hour dark period as recommended by the breeder. The lighting programme and the environment in the house were controlled by timers and temperature sensors respectively. This study was conducted at the Poultry Teaching and Research Unit, University of Queensland, Australia, to the standards set by the Australian Model Code of Practice for the Welfare of Animals – Domestic Poultry 4th Edition, 2002.

III. RESULTS AND DISCUSSION

Proximal analysis of the diets did not reveal any major difference with the nutrient provision calculated during the feed formulation. Extract viscosity of the control diet was relatively high, with a value of 13.7 cPs. Performance data for the starter (0-21 days), grower-finisher (22-42 days) and overall (0-42 days) periods are presented in Table 2. Performance data for the control treatment were in agreement with the breed targets at this age (2.85 kg bodyweight and 1.70 FCR). During each production period, addition of the enzyme complex to the basal diet significantly improved ($P < 0.05$) bird performance. Supplementation of feed with xyl/bgl increased bodyweight gain by an average of 7.2, 2.5 and 3.7% during the starter, grower-finisher and overall periods, respectively. The corresponding FCR values were improved ($P < 0.05$) by an average of 5.7, 2.7 and 3.6%, respectively. Bodyweight gain and FCR improvements were more pronounced during the starter period. This is probably due to the greater sensitivity of young birds to high dietary NSP levels. During the starter phase, the best feed conversion response was observed for the lowest inclusion rate. However, during the grower-finisher phase, the best response was generated with higher inclusion rates. This result suggests that older/heavier birds could benefit from higher inclusion rates of NSP-degrading enzymes. This may be related to greater feed consumption, leading to increased amounts of substrate (NSPs) in the gut. There were no significant differences in mortality between treatments. Finally, it can be noted that enzyme supplementation improved the homogeneity of the flock at 42 days: the coefficient of variation for broiler final bodyweight was 3.3% for the control treatment T1, versus 1.4, 2.0 and 2.3% for the xyl/bgl treatments T2, T3 and T4 respectively.

Table 2 Bird performance from 0-21, 22-42 and 0-42 days of age.

Treatment	T1	T2	T3	T4
From 0 to 21 days				
Bodyweight gain, g	775^{c}	864^{a}	819^{b}	810^{b}
Feed intake, g	1147	1159	1158	1154
FCR*	1.48^{a}	1.35^{d}	1.41^{c}	1.43^{b}
From 22 to 42 days				
Bodyweight gain, g	2298^{b}	2348^{a}	2343^{a}	2379^{a}
Feed intake, g	4061^{ab}	4128^{a}	4013^{b}	4026^{b}
FCR*	1.79^{a}	1.78^{a}	1.74^{b}	1.71^{b}
From 0 to 42 days				
Bodyweight gain, g	3073^{c}	3211^{a}	3161^{b}	3189^{ab}
Feed intake, g	5208^{b}	5287^{a}	5170^{b}	5180^{b}
FCR*	1.71^{a}	1.66^{b}	1.65^{b}	1.64^{b}

^{a,b,c,d} Means with different superscripts are significantly different ($P<0.05$)

* Corrected for mortality

IV. CONCLUSION

Results of the present study confirmed the efficacy of the new xyl/bgl enzyme product in broiler chickens fed wheat/barley-based diets. In the conditions of this trial, the lowest dose (0.05 kg/tonne) of the new xyl/bgl enzyme gave the highest biological response in the bird. This positive effect is related to the breakdown of both soluble and insoluble fibre fractions from viscous grains.

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SELECTING FOR SUSTAINABILITY

D. ELFICK[1]

Summary

Sustainability is built on the pillars of economic, environmental and social issues. Aviagen, as a global leader in poultry genetics, understands that these issues cannot be viewed as separate but rather as inter-related and interlocking parts of a whole. The broiler breeding industry is striving to produce a low cost high quality product available to all and that meets the ethical demands of governments and consumers, and reduces the impact of the industry on the environment. The use of advanced statistical techniques, medical technologies and direct testing of birds has been championed by Aviagen, amongst others. This has resulted in birds that have low levels of physiological issues, when properly fed and managed, with the lowest output of Green House Gas per unit of meat and the best feed efficiency of any farmed land animals. Selection today goes far beyond issues such as growth rate, into areas of behaviour, welfare, resource utilization and the requirement for long term viability in breeding populations. It should be noted that factors such as management, nutrition and biosecurity can have much more dramatic impacts on individual flocks that genetics can.

I. INTRODUCTION

Globally, the broiler industry consumes approximately 417-million parent stock (PS) per year, the equivalent of an estimated 58 billion broilers. The US industry alone now generates over US$44 billion per year in retail sales (USDA).

Over the years, selection for improved efficiency has been extremely successful. The cost of producing a pound of live chicken dropped from US$2.32 in 1934 to US$1.08 in 1960 down to US$0.45 in 2004 in today's money (USDA poultry yearbook, 2006).

At the same time, within a flock, improvements in veterinary medicine, environmental control, nutrition and other management factors, have undoubtedly had dramatic impacts on bottom line performance, Havenstein (2003), stated that his results "indicate that genetic selection brought about by commercial breeding companies has brought about 85 to 90% of the change that has occurred in broiler growth rate over the past 45 years. Nutrition has provided 10 to 15% of the change." The challenge for this century will be to continue these improvements so that, as an industry, we make high quality animal protein available to all, but to do this in a way that is "sustainable".

II. SUSTAINABILITY – WHAT IS IT?

Sustainability has become the watch word of the early twenty-first century. However there are many definitions as to what sustainability is and many of these definitions are unclear or open to interpretation. Often the definition "to meet the needs of the present without compromising the ability of future generations to meet their own needs" (World Commission on Environment and Development, 1987) is used. However, as commonsense as this may sound, it provides no benchmarks for us to measure ourselves against. The International Reporting Initiative, an organization that provides a framework for reporting "sustainability", looks at 3 "pillars" for sustainability; economic, environment and social. These 3 pillars are

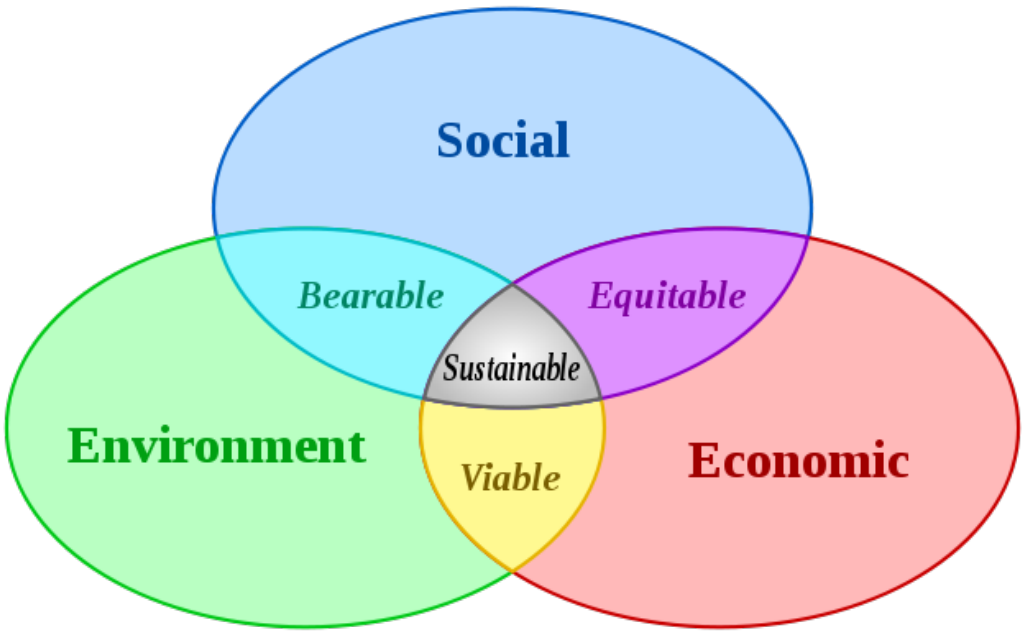
[1] Aviagen International

used in a number of models, but one of the most frequently referred to is that of the interlocking and overlapping circles of influence as shown in Figure 1.

Sustainability, to a breeding company, must encompass these three circles of influence. As a privately held company in a competitive market place, we must ensure that our products fit the variety of markets that exist world-wide, in order that we are able to have a business that continues to be profitable for the future. At the IUCN Renowned Thinkers Meeting (Adams 2006), it was noted that "Different strategies [for sustainability] will be needed in different contexts: no holistic 'one size fits all' plan will be effective. Los Angeles (L.A.) and Liberia are different places, with different challenges." This is certainly true in the poultry industry. What is a key requirement for poultry in L.A. (high breast meat yield, with pathogen "freedom") would be totally different to Lagos, where dark meat may be a preference but price is the primary driver.

In order for a breeding company to remain successful, the social and ethical issues associated with animal production have become increasing important. These are not just "welfare" issues, although accumulations of leg health issues, poor immune responses, high feed intake or high outputs of waste product are today not considered suitable for a long term sustainable breeding program, nor for the individual birds that it produces. These are important ethical factors in consumer and central government thinking in many parts of the world but, as an industry we also face issues of human health (pathogen freedom and the nutritional factors in the meat we produce) and the availability of animal protein for human nutrition on a global scale.

Figure 1 Sustainability Model (Adams 2006)



III. ENVIRONMENTAL THINKING

Environmental traits will be increasing considered in breeding goals for the future, but these interlink with social issues across many parts of the globe (Table 1). Free or cheap sources of

non-vegetable protein are in decline. For example, the loss of capture fisheries is reducing an inexpensive source of protein in developing countries. Per capita fish consumption in developing countries, excluding China, declined between 1985 and 1997. As wild populations of fish and animals shrink and human populations become increasingly urbanized, the provision of low cost animal protein will be increasingly desired by consumers world-wide. Poultry, with its scalability, high throughput and excellent FCR, is an opportunity for people in both established and emerging markets.

Table 1 Millennium Ecosystem Assessment - Poverty

1.1 billion people survive on less than $1 per day. 70 percent live in rural areas where they are highly dependent on ecosystem services
Inequality has increased over the past decade. During the 1990s, 21 countries experienced declines in their rankings in the Human Development Index
Over 850 million people were undernourished in 2000–02, up 37 million from the period 1997–99
Per capita food production has declined in sub-Saharan Africa
Some 1.1 billion people still lack access to improved water supply, and more than 2.6 billion lack access to improved sanitation
Water scarcity affects roughly 1–2 billion people worldwide.
Global improvements in levels of poverty are skewed by rapid economic growth in India and China; poverty elsewhere (especially in sub-Saharan Africa) is profound and persistent.

Less than 1% of the world's fresh water is accessible for direct human use. If just the base flows of rivers and streams are considered as renewable and accessible, it has been estimated that this leaves as little as 11100km3 to supply 3829 km3 of demand (World Water Assessment Program 2009). Whilst this might be underestimating the supply, when you consider that demand is expected to increase to 6900 km3 by 2030, you can see why water availability is both a local and global issue. It has been hypothesised that the 2030 demand may be over 50% of what is theoretically sustainable. Although it may appear that there should still be sufficient water available, a major issue is its distribution with 15% of the world's available water being in the Amazon, available to only 0.4% of the population.

Land is becoming more expensive if not scarce. Peri-urban land previously used for agriculture is being utilized for urban expansion and industry. This will increase the distance our food supplies have to travel to reach us, with its knock on effects of greenhouse gas emissions, congestion and so forth. This has become such an issue that Dr. Dickson Despommier (Despommier, 2010) of Columbia University has proposed vertical urban farms. Prototypes of these are already under construction in Tokyo and South Korea. This model precludes extensive forage systems for animal production, but is well suited to the production of chickens, which require little floor space and can utilize (to some extent) waste products from crop production, whilst providing valuable fertilizers and a rapid feed efficient turnover of "crops".

The publication of a report in the UK (DEFRA, 2008) indicated that of all the meat producing livestock species, commercially raised broilers have the lowest green house gas (GHG) emissions per kg of meat produced. Genetic selection of broilers over the last 20 years has shown a reduction of GHG emissions of around 25%, with this reduction predicted by the authors to continue for the next 20 years or so. Commercial geneticists feel that this is a very conservative estimate given the enhanced technologies available today and in the near future. Selection targets for efficiency, especially in feed conversion ratio and meat yield have

primarily driven this reduction, at the same time as reducing the wholesale price of high quality, healthy animal protein to the customer.

IV. DEVELOPMENT OF BREEDING STRATEGY

Breeding strategies have never been developed in isolation. The breeding industry, as with all corporate entities, is required to produce products that are acceptable and desired by the end user. Therefore, feedback and consultation with direct customers, end users and consumers has always been a key feature in developing breeding goals. Due to the structure and nature of the breeding business, there is approximately 5 years from a breeding decision being made in the breeding program, to the first effects of these changes being experienced in the broiler generation. Unlike a car manufacturer the industry does not have the ability to make wholesale changes in direction overnight. Genetic change is not sudden and is often not greatly noticeable until some years of advancement accrue, much like compound interest. It is therefore crucial to the future success of the business to liaise effectively with policy makers, scientists and lobby groups to best predict consumer issues in the future and to identify emerging issues from the field. Within Aviagen, a major part of our global success has been the emphasis that has been placed on balance, not just in terms of performance traits, ensuring that the products are successful at all stages of the breeding pyramid, but also by ensuring that we focus on the support traits for our birds, with this strategy now broadening to capture environmental and emerging ethical concerns. Although Aviagen is a global company, much of the cultural heritage stems from the UK. Europe in general and the UK in particular has always been a region that is a sentinel for future global feeling on animal welfare and environmental concerns.

V. PERFORMANCE EFFICIENCIES

Aviagen has long been a world leader in the use of technology to improve the feed efficiency of broiler chickens. For over 30 years we have used highly controlled individual pen tests to enhance FCR in our birds. This technique has almost halved the amount of feed required to generate a unit of poultry meat since the 1970's. Individual pens, whilst cost effective and of high welfare standards do not address the behavioural aspects of feed efficiency. In 2005, Aviagen started selecting pedigree chickens using performance testing stations in their breeding program. These stations allow birds to be group housed and demonstrate the behavioural aspects of feed intake and efficiency. This technology will allow Aviagen to make faster improvements in FCR, with improvement rates around 2.5% per annum. This improvement rate is close to the predicted increases in world chicken meat output. This being the case, the industry will be close to truly sustainable with inputs reducing at around the same rate as growth.

Improving meat yields also play a part in ensuring long tem sustainability. Using traditional conformation scoring along with ultrasound technology and information on the actual yield of siblings, we are able to increase yields at over 0.30% per annum. The use of the brothers and sisters of pedigree birds for processing is invaluable in ensuring that muscle or skeletal deformities can be indentified in pedigree families quickly and those families selected against.

VI. GLOBAL FOCUS

As a global supplier it is important to understand that our customers and their consumers have different requirements and standards of nutrition, hygiene and management around the world. For many years Aviagen felt it was appropriate to grow our pedigree birds to their maximum potential under essentially un-limiting conditions, in order to expose any underlying physiological issues. This allows selection from families that show no significant undesirable traits. We still believe that this is appropriate, however we understand that around the world there are people unwilling or unable to achieve the levels of nutrition, biosecurity and management that are recommended. Therefore, almost 10 years ago we set up our sibling test, where brothers and sisters of pedigree birds are exposed to management and feeding practices in line with the bottom quartile of the industry. This exposes our chickens to lower input type scenarios, again potentially improving the sustainability, and ability of our stock to thrive under such conditions. Increasingly robust chickens, in the face of disease challenge or management mis-step, has no negative implications for those with the finances or management skills to follow high input advice. However, with the high prices of corn and soy, along with the increasing cost and scarcity of other raw materials such as shavings in many parts of the world, a number of companies worldwide are choosing to reduce inputs in order to maintain wholesale chicken prices at recent levels. Associated with this moves towards lower energy systems, primarily from a cost control point of view, are becoming increasingly common. This does have the impact of potentially reducing Green House Gas emissions although it will be important for breeding companies to breed increasingly robust lines to ameliorate any negative impacts on performance.

Improving physiological strength has no negatives for those choosing a lower input direction for their farming base, but safeguards skeletal integrity and cardio-vascular function, under all bar the most challenging of management or nutritional situations.

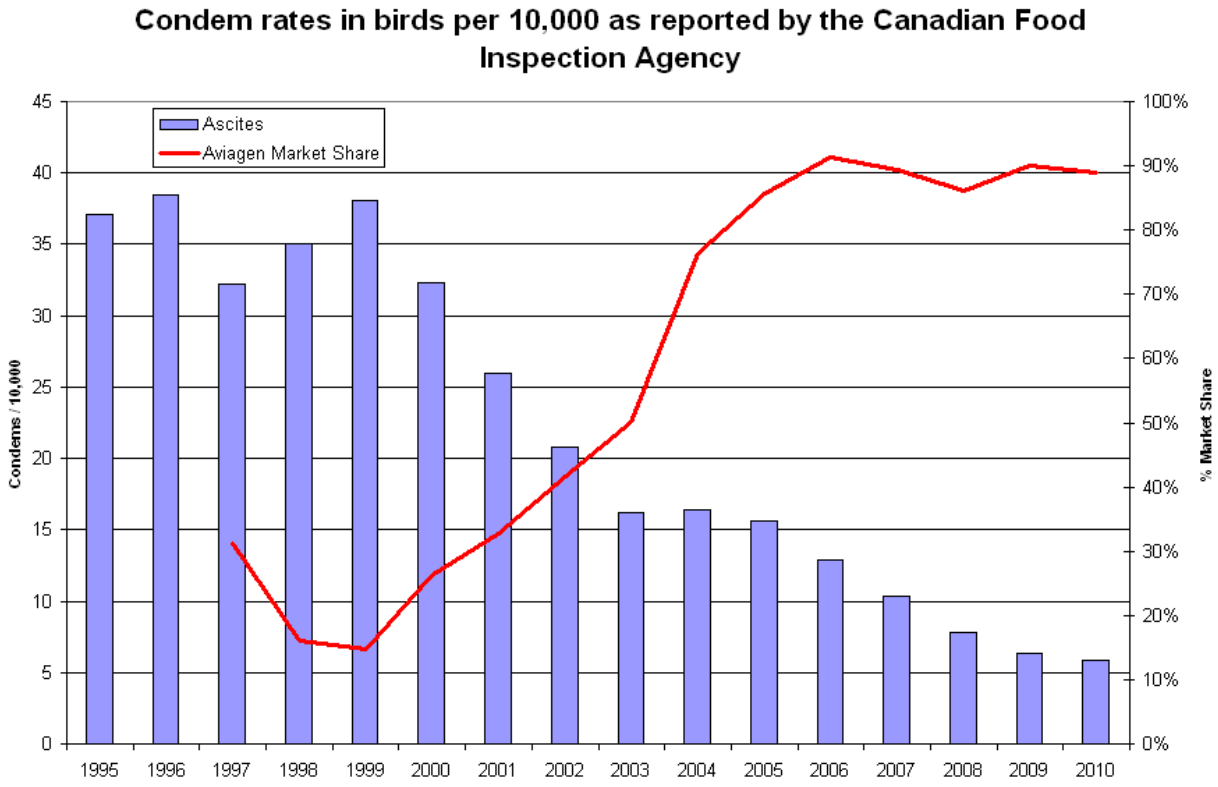
Since the late 1990's we have not utilized prophylactic antibiotics or coccidiostats in our breeding programs, as we believe that our stock should be sufficiently hardy to perform in their absence.

VII. PHYSIOLOGICAL FUNCTION

Many campaign groups and welfare orientated scientists have made claims as to the negative physiological health status of broiler chickens caused by commercial selection practices, discussing issues of leg defects, especially tibial dyschondroplasia (TD), amongst other syndromes (Cooper and Wrathall 2010). Some groups have gone as far as to recommend or campaign for the end of intensive broiler farming due to these issues (CIWF, 2010). These claims may have been valid in the 1980s, when most breeding companies were using mass selection or selection index technologies in their programs. However, twenty years of continued focus on these areas, using traditional inspection methods, medical technologies such as X-Rays, as well as genetic technologies that better understand the relationship between traits, has reduced these issues to very low incidences on effectively run commercial production facilities. Whilst effective measurement of the reduction of these concerns is difficult, large datasets like those available from the Canadian Meat Inspection Service, indicate dramatic improvements in both the underlying genetic susceptibility of modern broilers to these issues, and an increased awareness of the management requirements of these improved individuals. Today, most incidences of TD are associated with incorrect formulation of diets. One study that is often shown by welfare activist groups, carried out in Denmark (Sanotra et al, 1999) showed a 57% incidence of TD in commercial broilers in 1999. Six years later in a repeat of this study (Pedersen et al, 2005) the incidence had dropped

to 0.7%. In the first study it was later reported that the diet had been deficient in calcium, which was most likely to have caused the TD issues (Laughlin, 2006). Even reports from the UK scientific community are now indicating that welfare researchers are acknowledging that significant progress has been made in a range of welfare traits, although there remain other issues. (Whitehead, 2010).

Figure 2 Condemnation rates in the Canada from Ascites. Agriculture and AgriFood Canada (2010)



Further research and development into new issues associated with welfare and sustainability are being implemented globally. Breeders respond to emerging trends as swiftly as they can. However, due to the multiplication structure of the industry, there is a lag from when the issue is first selected upon until the first effects of these selections can be seen. This is sometimes perceived as a lack of concern, especially as when by its nature the industry is competitive and intellectual property needs to be maintained. For example, foot pad condition is currently talked of as one of the primary metrics of welfare in the newly enacted European Broiler Directive. (Council Directive 2007/43/EC). Selection to both improve foot pad quality and reduce the incidence of the conditions that can lead to this condition, have been underway for some years. However, due to genetic lag, there remains a requirement to manage birds effectively to improve 'paw' condition whilst the genetic improvements make their way down the generational pyramid.

IX. PRODUCT DIVERSIFICATION / BIODIVERSITY

Statements has been made that commercial breeding populations lack genetic diversity (Muir et al, 2008), and that the formation of commercial breeding companies and their subsequent selection strategies have directly caused this. All of the large commercial companies keep substantial populations within each "line" to maintain variation and control inbreeding. There

has also been a move for many of the breeders to acquire more varied stock types to give more depth to their programs and provide greater choice to consumers. In some, primarily, developed countries, there is a move away from standard commercially farmed chickens, to something that is perceived as higher "quality". This is done by nutritional restrictions on ingredients; exposure to the range; organic nutrition; restriction on growth rates; etc. The demand stems from a small but significant percentage of consumers, to which producers are responding. All of these strategies tend to reduce "sustainability", through usage of greater amounts of feed and increased GHG production. They also increase cost. For the majority of labelling systems, Aviagen has a diversified product portfolio that can meet the criteria of the system and supply traceable, guaranteed health status stock for these production systems. Care should be taken that lower income consumers are not legislated out of eating chicken, as this remains one of the healthiest animal proteins available.

X. FUTURE POTENTIAL

With the publication of the full chicken genome (Wong, et al, 2004) the inclusion of genomic technologies into commercial poultry breeding programs has come a step closer to reality. All the current primary breeding groups are investing heavily into these areas. It is unlikely that the use of transgenic technologies (artificially moving genes from one individual or species to another, or removing genes) will be acceptable to the majority of consumers world-wide at present, so the focus is very much on the understanding of the function and effect of genes already present in the breeding populations. This knowledge will be used to select more efficiently and effectively for traits of importance. The traits that will benefit the most from these technologies are likely to be the ones for sustainability, environmental impact and robustness, as these are traits often difficult or destructive to measure in a traditional breeding structure. Currently research programs looking at further resistance to ascites, more effective utilization of water, Avian Influenza resistance, Campylobacter exclusion, amongst many others, are candidates for genomic research.

XI. CONCLUSIONS

In developed nations, the percentage of house hold income spent on food can be as low as 7.4% (USA); however in less developed countries this can be over 50% (India and Philippines). The improvement of chicken production efficiency over the last 50 years, coupled with many other agricultural advances, has helped bring high quality, low fat animal protein within reach of all but the very poorest of individuals worldwide. Continued advances in genetics as well as the other associated improvements in optimisation of broiler production will bring chicken within the budget of all.

There is good evidence to suggest that the concerted efforts by Aviagen and other breeding companies have reduced the levels of physiological issues associated with modern fast growing types of broilers. At the same time improvements in absolute performance have greatly reduced the carbon footprint of the industry and will continue to do so. Undoubtedly there will be emerging issues of welfare, sustainability and consumer ethics that will come to the forefront over time, which Aviagen is well placed to understand and react to. Genetic lag will continue to be an issue in these circumstances. It is important to understand that whilst selection for sustainability and welfare traits will continue, with additional power coming through genomics, management, nutrition, biosecurity and so on can have much more dramatic impacts on individual flocks in the field than genetics can.

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ALTERNATIVE GENETICS TO IMPROVE EGG LAYER EFFICIENCY

G.B. PARKINSON[1] and W. STANHOPE[2]

Summary

This paper reports on the development of a bantam gene pool that has commercial egg production characteristics that can be easily hybridised with elite commercial stocks without compromising commercial performance. The usefulness of these genes has been illustrated by conducting cross breeding experiments and undertaking progressive introgression of the bantam genes into commercial lines. The bantam genes provide a mechanism to reduce metabolic body size without compromising egg mass output and hence improve overall efficiency.

To validate the use of these bantam genes, an experiment was undertaken in which a bantam White Leghorn male (Bantam Line (1) male) with commercial egg production traits was crossed with a normal sized commercial female White Leghorn (WL) line. The bantamised experimental cross was then compared to offspring of a conventional commercial White Leghorn (WL), and this study follows on from earlier experimentation between 2000-2006.

The average body weight of the bantam cross was 12.8% less than the body weight of the commercial White Leghorn (1575 g versus 1806 g at 45 weeks of age), and egg mass output of the bantam cross was not significantly different from the commercial WL. The estimated average feed intake was 10% less in the bantam cross and feed efficiency could be reduced by 9%. The individual bird studies of the bantam cross identified a sub-population of birds with very small body size and high egg mass outputs, that far exceed the bantam cross average performance, and meet contemporary industry standards for egg mass output at low body weights. Overall, the recent results are much more convincing than earlier experimentation and provide the egg industry with an opportunity for a quantum efficiency leap by reducing body weight and maintenance requirements whilst holding egg mass output relatively constant.

I. INTRODUCTION

Genetic selection over the last 40 years has seen substantial improvements in the performance of laying hens. The combined effects of reduced body weight, lower maintenance requirements and increased egg production have seen a significant improvement in feed conversion efficiency. The rates of feed efficiency gain are likely to slow as egg production approaches the threshold of 365 eggs per annum. For further efficiency gains beyond the current genetic equilibrium, additional reductions in body weight and maintenance requirements will be required, whilst holding egg mass output relatively constant.

Considering the global projections for population growth and the dramatic requirement for additional food supply to complement this growth, strategies that maintain or increase output of dietary protein (meat) for less input of grain resources are vitally important. The bantamisation of commercial egg laying stocks offers a new biological efficiency strategy for the global egg industry (Yoshida and Saito, 1983, Stanhope and Parkinson, 1989, Parkinson and Cransberg, 2000) that will assist in ensuring global food security.

[1] Livorno Consulting, 86 Wilson Street, Brunswick VIC 3056 Australia
[2] Stanhope Poultry Breeders, 172-176 Old Dandenong Road, Heatherton, VIC 3202

Promising results have been achieved by introgressing special bantam genes into elite commercial egg laying stocks. The pure line bantam White Leghorns owned by Stanhope Poultry Breeders have a range of mature body weights from 1000 to 1300 g and these lines have been used to produce first cross bantam White Leghorns varying in female mature body weight from 1350 g to 1650 g. Research has been investigating the biological outcomes in three different experimental crosses designed to produce either extreme small body size, large eggs or high rates of egg production in first cross bantam lines.

The pure line Stanhope bantams described have been studied in controlled environment shedding between 2000 and 2006, and large improvements in peak production, persistency of production and egg weight have been achieved by slightly increasing 18 week old body weights from 1000 to 1100 g and delaying photo-stimulation from 16 weeks to 18 weeks of age (Figure 1.). Average egg size for these pure bantam lines was recorded between 57 to 59 g and the maximum egg weight to body weight ratio has been recorded at 5.4% with mature body weights of 1100-1200 g live weight.

The experiment reported in this paper is part of a series of long term studies designed to evaluate the production performance characteristics and general viability in single-bird cages of a cross between a extremely small bantamised WL male (Bantam line 1) and a commercial WL female, relative to the performance of a commercial WL cross that uses the identical commercial female line

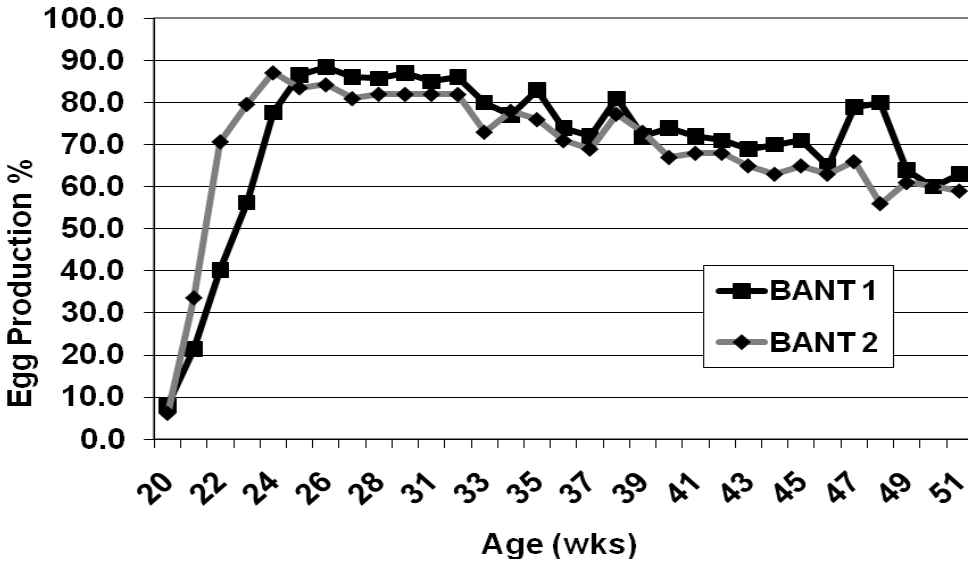

Figure 1 Hen day egg production performance of two pure Stanhope Bantams lines (1) and (2) between 20 to 52 weeks of age. Mature Body weight approximately 1100-1200 g with average egg weight of 57.5 g at 45 weeks of age (n=20-30).

II. MATERIALS AND METHODS

The strains used in these experiments were a commercial WL (mature female body weight 1,800 grams), and a cross between a bantam WL male (Bantam Line 1) (mature body weight of bantam male approximately 1,300 grams) and the commercial WL female. Some 97 bantam cross birds and 44 commercial WL were reared in cages in a controlled environment shed, fed commercial diets and were exposed to controlled day-length with a light intensity of approximately 5 to 10 lux to 14 weeks of age. At 14 weeks of age, all birds were placed into

single bird cages in an environmentally controlled shed with temperatures ranging from 18-28^{o}C with an average of 21-23^{o}C.

All birds were fed a commercial grower ration (12.1 MJ ME/kg and crude protein 160 g/kg) between 9-18 weeks of age, followed by a commercial layer diet (11.61 MJ ME/kg, crude protein of 18.5 % and 3.75% calcium) for the remainder of the experimental period. Feed and water were available *ad libitum* and light stimulation was provided at 18 weeks of age by increasing daylength from 11 to 16 hours between 18 to 20 weeks of age. Light was constant at 16 hours per day, with a light intensity of 5-10 lux.

Birds were weighed at 16 and 45 weeks of age. Egg production was recorded daily and accumulated to provide weekly figures. Egg weight was recorded for all individual birds at 28 and 45 weeks of age, (all eggs from a particular day were weighed) while feed consumption was calculated from a sub-sample of ten individual birds from each strain between 35 to 40 weeks of age. Average feed intakes calculated between 35-40 weeks of age was used to estimate overall cumulative feed intake between 18 and 50 weeks of age (224 days). Total egg mass was estimated by multiplying the total recorded egg numbers by the estimated average egg weight between 18 and 50 weeks of age

III. RESULTS

a) Comparison summary of Bantam cross with Commercial WL

Table 1 Summary of production parameters of the Bantam cross (97 birds) and the Commercial WL (44 birds) to 50 weeks of age. Standard error is in parentheses. *Different superscript* **a** *or* **b** *illustrate significant differences with a probability of p<0.05.*

Parameter	Layer strain	
	Commercial WL	Bantam cross
Ave. body weight @ 16 weeks of age (g)	1200 (12) **a**	1084(10) **b**
Ave. body weight @ 45 weeks of age (g)	1806 (34) **a**	1575 (15) **b**
Ave. feed consumption (g/bird/day) 35-40 weeks	108.8 (5.0) **a**	97.7(5.0) **b**
Ave. egg production from 18-50 weeks	183 (2) **a**	183 (2) **a**
Ave. egg weight (g) 18-50 weeks	60.1(0.4) **a**	59.6(0.4) **a**
Ave. egg weight (g) @ 28 weeks	56.4(0.6) **a**	55.4(0.4) **a**
Ave egg weight (g) @ 45 weeks	63.4(0.7) **a**	61.6 (0.4) **b**
Estimated Average egg mass (kg) 18-50 weeks	11.0(0.2) **a**	10.9(.0.5) **a**
Estimated Cumulative feed intake (kg) 18-50 weeks	24.37(1.12) **a**	21.88(1.12) **b**
Estimated Feed Conversion kg feed consumed per kg of eggs	2.22	2.00
Egg weight: body weight ratio (%) @ 45 weeks	3.5(0.04) **a**	3.9(0.08) **b**
Mortality & culling 18-50 weeks %	10%	5%

b) Body weight

At 16 and 45 weeks of age, the bantam cross had an average body weight of 1084 and 1575 g, respectively, whilst the commercial WL was 1200 and 1806 g at the same ages. The bantam cross was significantly smaller ($P<0.05$) than the commercial WL from 16-45 weeks (Table 1.) with 12.8% lower mature body weight.

(c) Egg production

The bantam cross had high levels of egg production, peaking at 94% at 22-23 weeks of age (Figure 2), whilst the commercial WL line peaked at 97% at 25 weeks of age. Average egg production from 18 to 50 weeks of age was 81.7% in the bantam cross and 81.7% in the commercial WL. Total egg production to 50 weeks of age was identical for both genetic lines at 183 eggs to 50 weeks of age. The bantam cross commenced egg production earlier and there is a trend toward lower persistency of production by 45 weeks of age, compared to the commercial WL.

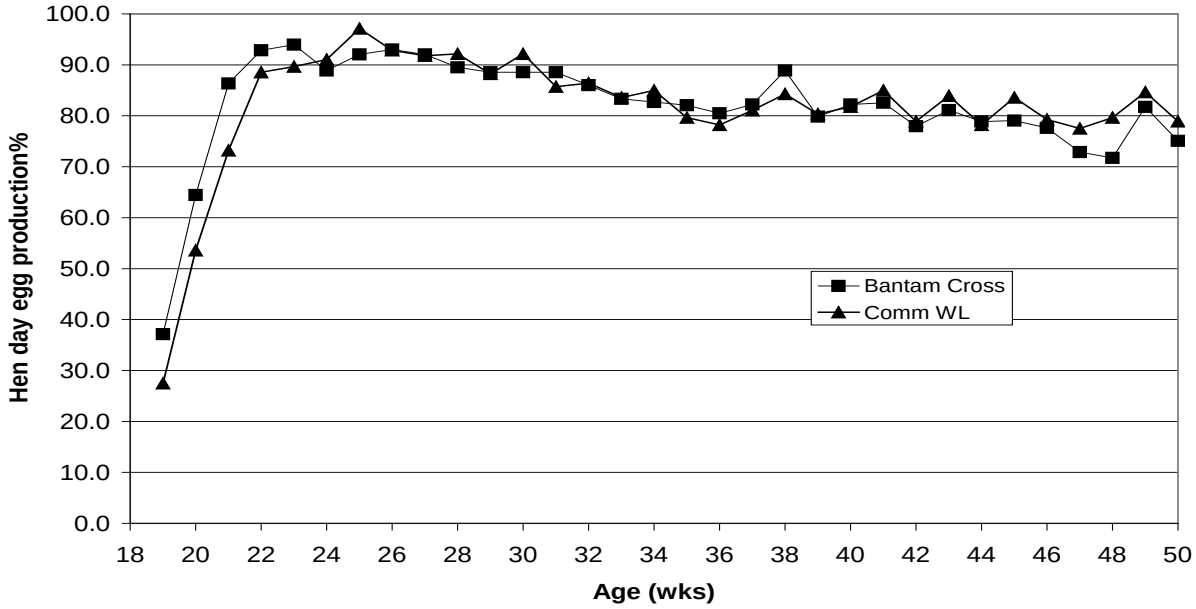

Figure 2 Average hen day egg production of the Bantam cross (■) (n=97 birds) and Commercial WL (▲) (n=44 birds) between 18 to 50 weeks of age. *Birds housed in single bird cages.*

d) Egg weight

The average egg weight of the commercial WL tended to be 1 to 2 g greater than the bantam cross in the later period of the experiment (45 weeks), although the overall difference was not significantly different between 18 and 50 weeks of age (Table 1.). Average egg weight from week 18 to 50 was 59.6 g in the bantam cross and 60.1 g in the commercial WL. The egg weight to body weight ratio at 45 weeks was significantly higher in the bantam cross (3.9%) than the commercial WL(3.5%) (*$p<0.05$).* (Table 1.).

e) Feed Intake, Egg Mass and Feed conversion

Estimated daily feed intake for the bantam cross was 10% lower than the commercial WL, whilst egg mass was similar for the bantam cross. As a consequence of the significantly lower feed intake combined with similar egg mass output, the feed conversion efficiency was approximately 9% improved in the bantam cross (Table 1.).

IV. DISCUSSION

The performance in this study of both the pure bantams and bantam cross birds in comparison to the commercial WL birds is extremely encouraging.

a) Pure line Bantam Performance

The pure line bantams can achieve peak production of almost 90%, with average egg weights of 56-59 grams (Figure 1.), given particular attention to pullet weights and age of photo-stimulation. Within the pure line bantam population, there are individual birds with mature body weights as low as 900 g that are capable of producing 55 g eggs. These birds have the potential to provide genes that can be introgressed into elite stock, resulting in very substantial increases in egg weight to body weight ratios possibly approaching 6%.

b) Bantam Cross Performance

For the experimental bantam cross birds, average egg weight was only 1-2 g or 2.4% lower than compared to the commercial WL birds at 45 weeks of age, but egg production performance was identical despite the large reduction in body weight (12.8% at 45 weeks of age) in the bantam cross birds.

A close examination of the individual bird egg production performance (Figure 3.) illustrates that approximately ten percent of the bantam cross birds laid more than 202 eggs over 224 days (equates to 90% hen day production) between 18-50 weeks, with an egg weight well in excess of 60 g at 45 weeks of age, and mature body weights ranging from 1300-1500 g. Furthermore, there were a substantial number of extremely small birds with body weights less than 1500 g producing at commercial rates of egg production with eggs ranging from 60-65 g by 45 weeks of age (Figure 3.)

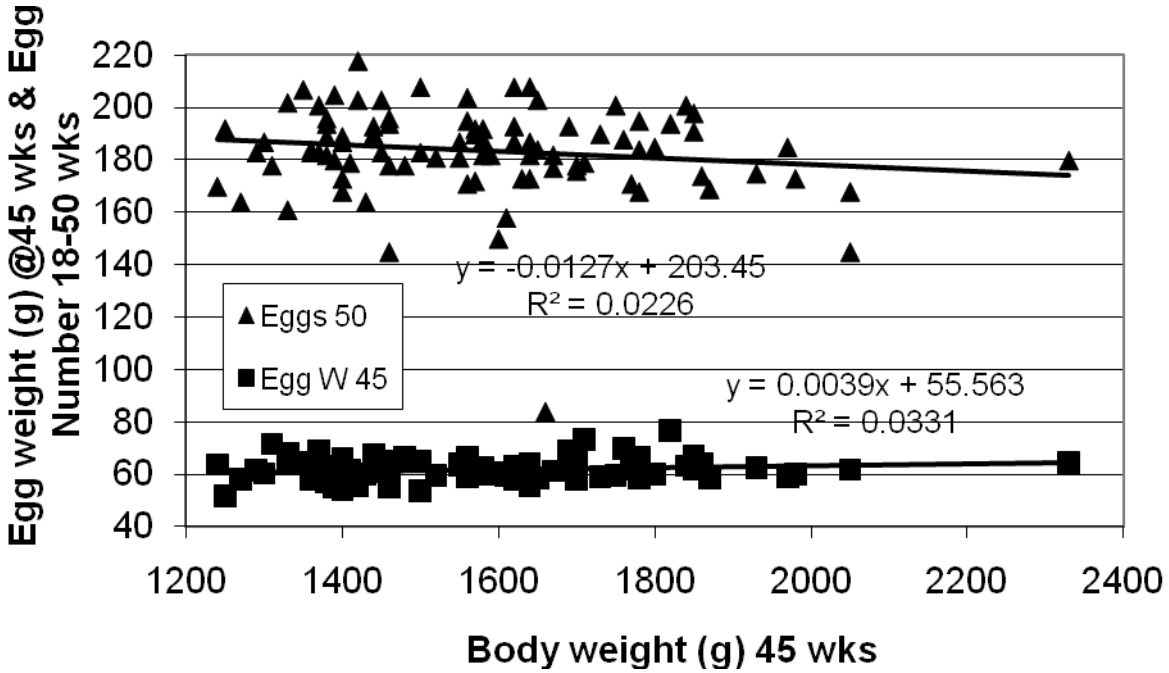

Figure 3 Relationship between body weight (g) at 45 weeks of age with egg numbers(Eggs 50) 18 to 50 weeks(▲) and egg weight (Egg W 45) at 45 weeks of age (■) for the Bantam cross WL (n=97) *Ninety percent egg production equates to 202 eggs in 224 days between 18-50 weeks of age.*

c) Commercial WL Performance

A similar examination of the commercial WL strain performance indicates that approximately ten percent of the birds also achieved egg production performances greater than 90% between 18 to 50 weeks of age (Figure 4.). For the commercial WL the correlation between body weight at 45 weeks of age and egg weight at 45 weeks was stronger than for the bantam cross (R^2= 0.112 verses 0.0331)(Figures 3, 4.)

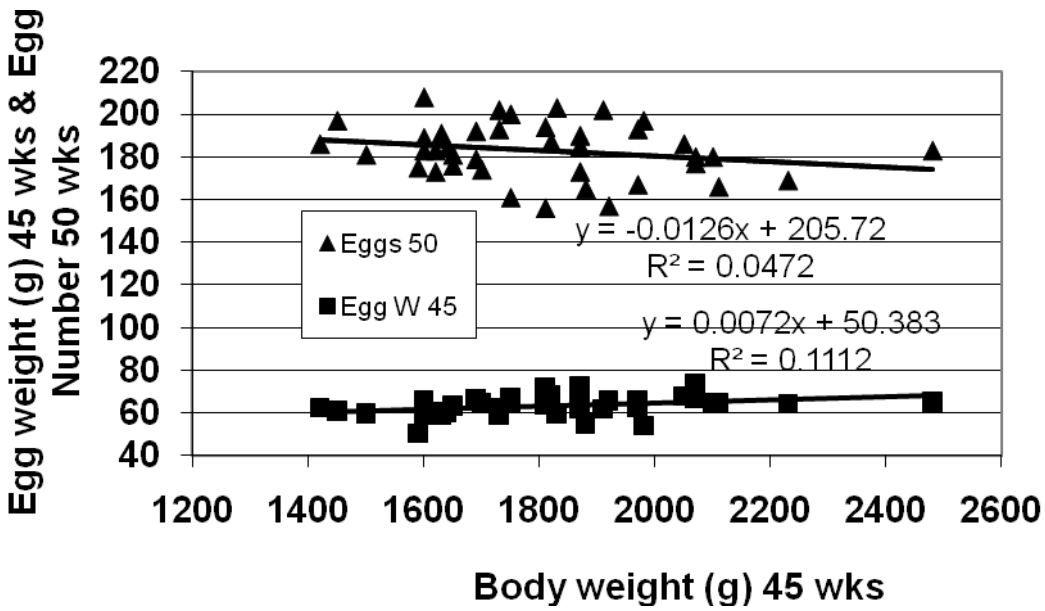

Figure 4 Relationship between body weight (g) at 45 weeks of age with egg numbers (Eggs 50) 18 to 50 weeks (▲) and egg weight (Egg W 45) at 45 weeks of age (■) for the Commercial WL (n=44) *Ninety percent egg production equates to 202 eggs in 224 days between 18-50 Weeks of age*.

d) Elite Bantam Cross Performance

A selection of the best 30% of the bantam cross birds (Elite bantam cross exceeded 190 eggs in 224 days, and averaged 88.9% production) were maintained from 18 to 72 weeks of age (Figure 5.). An aggregation of the production performance of these birds illustrated a peak production of 98% at 24 weeks of age and hen day production levels averaging 308 eggs to 72 weeks of age. Average body weight and egg weight of these selected birds (Elite bantam cross) was no different from the flock average (Bantam Cross) of 1575 and 61.6 g, despite the superior egg production performance. Egg production to 72 weeks of age was negatively correlated with body weight (-0.0526) (Figure 5.), and suggests higher egg production in the small birds.

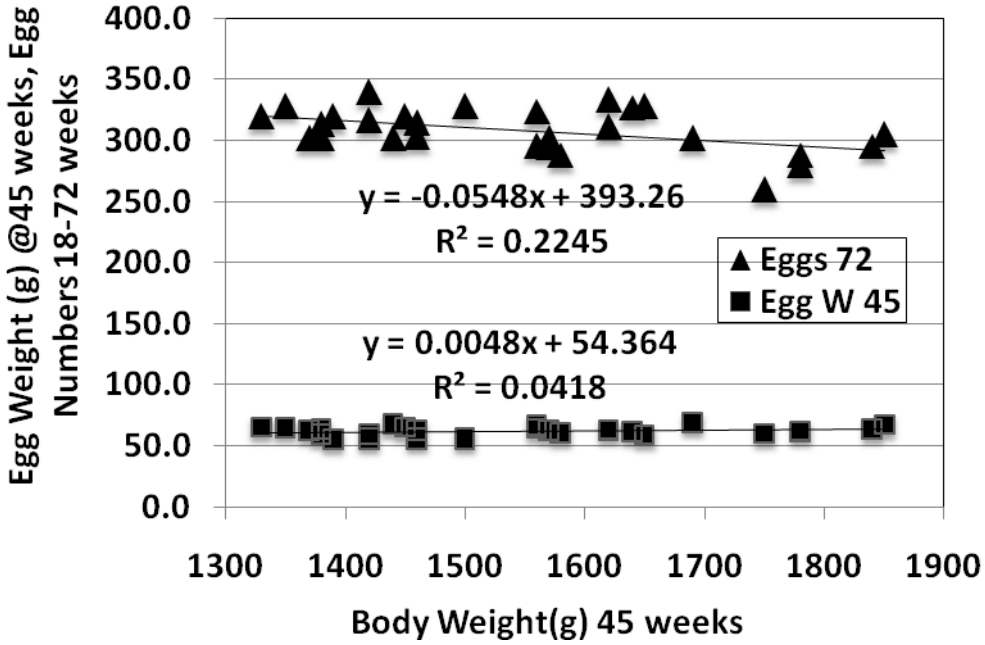

Figure 5 Relationship between body weight (g) at 45 weeks of age with egg numbers (Eggs 72) 18 to 72 weeks (▲) and egg weight (Egg W 45) at 45 weeks of age (■) for the Elite Bantam cross WL (n=28) *Ninety percent egg production equates to 329 eggs in 365 days between 18-72 Weeks of age*.

It is anticipated that the production performance and egg mass output can be further improved by crossing the bantam line with a superior female parent line, further selection of the pure bantam line, and/or a selection program that identifies the populations of pure bantams and commercials that produce progeny with a superior genetic recombination of small bantam body size but high egg mass outputs. As anticipated, the ratio of egg weight to body weight in the bantam cross was considerably higher than in the commercial WL birds and illustrates the potential of bantam genes to influence this ratio. A few individual bantam cross birds had egg weight to body weight ratios as high as 5%.

e) Introgression of Bantam Genes

Additional research has recently been undertaken to introgress bantam genes into the commercial WL (results not presented in this paper). This process has involved selection of superior first cross (F1) bantams and mating these females to small first cross (F1) males. In the next generation (F2), approximately 30% of the population was again selected for small body size and high egg mass output. These selected F2 females had a mature body weight of approximately 1500 g, but high egg production characteristics. In a subsequent F3 generation, a bantamised bird has been produced that has a mature body weight of 1400 g with egg production characteristics similar to the original commercial WL.

Two lines of normal sized commercial WL's (1800-1900 g) (Line L and Line C) have been bantamised in this fashion, and have been called the L50 and C75. Both lines have a mature body weight of approximately 1400 g with commercial egg production characteristics. The cross breeding of these two lines(L50 and C75) has produced a hybrid with a body size of 1430 g, an average egg size of 60-61 g and a rate of production of 87% between 20-50 weeks of age. This hybrid would be commercially competitive, and is likely to exhibit 5%

higher feed efficiency than the smallest commercial White Leghorn currently available in the world.

The global trend towards out-of-shell egg products will also increase the emphasis on feed conversion efficiency at the expense of shell colour and probably very large eggs, and lends strong support to the development of the sorts of commercial lines described. Additional research will be directed at evaluating both bantam cross WL's with superior egg production performance and bantam crosses generated from brown egg layers.

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THE RELEVANCE OF RAPID GROWTH IN BROILERS TO MANAGEMENT AND GENETIC ASPECTS OF THE ASCITES SYNDROME

A. CAHANER[1]

Summary

Modern broilers are characterized by high growth rate (GR), high rate of feed intake and metabolism and increased oxygen demand. Cold stress increases oxygen demand and those broilers that fail to fully supply it develop the ascites syndrome (AS), which is induced also at high altitudes where low oxygen availability is the stressor. The development of AS can be avoided by reducing GR, but with longer rearing period and often poorer feed conversion. The susceptibility of broilers to AS is a genetic 'defect' which is not genetically correlated with GR, hence selection against AS susceptibility is recommended. Such selection will be enhanced by the expected use of genomic markers linked to AS-controlling genes.

I. INTRODUCTION

Rapid growth, due to the consequent shorter rearing time to marketing and favorable feed conversion ratio (FCR), is the main factor contributing to economically efficient broiler production. Accordingly, commercial breeding programs have been continuously selecting for high growth rate (GR) and achieved outstanding progress in developing fast-growing meat-type broiler chickens. These achievements were clearly demonstrated in two similar trials, conducted by Havenstein and co-workers in the years 1991 and 2001, where the contemporary broilers reach market body weight (BW) in fewer days, with a superior FCR (Havenstein et al., 1994, 2003).

The higher GR of contemporary broilers is driven by a higher feed intake per time-unit and higher metabolic rate, and consequently a higher demand for oxygen, beginning from the embryonic stage (Tona et al., 2004). Higher metabolic rate, especially when coupled with exposure to low ambient temperatures or lower availability of oxygen (high-altitude rearing), leads to reduced capability of some broilers to regulate oxygen supply and energy balance (Wideman et al., 1999), leading to development of the ascites syndrome (AS) that results in eventual mortality (Julian, 1993; 2000, Wideman, 1998).

The AS is a cardiovascular metabolic disorder characterized by accumulation of ascitic fluid in the abdominal cavity and around the heart. It is a common cause of economic losses due to mortality and downgrades in fast-growing broiler strains. In a survey during the 1990's, the average incidence of ascites was 4.7% (Maxwell and Robertson; 1997). In a recent study conducted at a slaughterhouse in the Netherlands, Nijdam et al. (2006) found that 42.4% of dead-on-arrival birds showed pathological signs related to cardiovascular disorders, including AS. Generally, the development of AS is associated with fast growth and high metabolic rate, which represent high oxygen demand (Decuypere et al., 2000; Julian, 2000; Wideman, 2000; Balog, 2003).

II. GENETIC AND BREEDING ASPECTS OF THE ASCITES SYNDROME (AS)

a) Management approaches to reduce the incidence of AS

In the 1970's, AS was observed among broilers reared at the Andes high altitudes (Cueva et al., 1974), and later in South Africa (Huchzermeyer et al., 1988). Since the 1990's, following the continuous genetic improvement in broilers' GR, AS had been found also at low altitudes

[1] The Hebrew University, Faculty of Agriculture, P.O. Box 12, Rehovot 76100, Israel

(Albers and Frankenhuis, 1990), mainly among broilers reared at low ambient temperatures and/or fed high-energy pelleted feed. Management schemes have been applied since the 1990's to avoid or minimize AS mortality: (1) increasing broiler-house temperature by costly heating and insulation, (2) slowing actual GR, and consequently reducing metabolic rate and demand for oxygen. The latter can be achieved by feed restriction (FR) – either by restricting the daily feed ration or by providing less hours of light to reduce actual daily feed consumption, or by low-energy mash feeds to reduce intake of dietary energy (Balog 2003; Julian 2000).

The relative effects of heating and FR on the development of AS were investigated in broilers at high altitude, reared at cold vs. normal ambient temperature (Ta), and 3 feeding regimes (Ozkan et al., 2010). In the cold conditions, Ta ranged between 16 to 17ºC in the 4th week, 17 to 19ºC in the 5th week and 19 to 21ºC thereafter. In the normal conditions, Ta was 24ºC in the 4th week and ranged between 22 to 24ºC thereafter. Broilers in each condition were divided into 3 groups: FR from 7 to 14 days (FR7-14); FR from 7 to 21 days (FR7-21); and *ad libitum* (AL). Mortality due to AS and related parameters were recorded. Under normal Ta, AS mortality was lower in females (8.6%) than in males (13.8%) and was not affected by the feeding regime. Under cold Ta, there was higher AS mortality, but only in males; it was 44.2% among AL-fed males and only about 26% under the FR regimens, suggesting that FR helped some males to better acclimatize to the cold Ta and avoid AS. However, mortality was only 13.3% in AL-fed males at normal Ta and FR did not further reduce the incidence of ascites under these conditions. Thus, avoiding cold Ta in the poultry house by slight heating was more effective than FR in reducing ascites mortality.

However, all management approaches to reduce the incidence of AS compromise the efficacy of broiler production. If FR is applied to reduce GR, the broilers do not fully express their genetic potential for rapid growth, and consequently production costs are increased due to longer period of rearing to marketing BW, poorer feed conversion, and less efficient use of labour and facilities. Extra heating and insulation also increase production costs. Therefore the following parts of this paper deal with the genetic approach to overcome AS susceptibility of high-GR broilers. Compared to the management approaches, breeding provides a sustainable solution the AS problem, but it is feasible only if there is an inherent susceptibility to AS, and if effective selection against it can be performed.

b) <u>High GR and the incidence of AS are associated phenotypically</u>

Contemporary commercial broilers were compared in 1991 with a control population representing commercial broilers of 1957 (Havenstein et al., 1994). Average daily BW gain of the 1957 and 1991 broilers were 10 vs. 31 g/d from hatch to 3 wk of age, respectively, and 19 vs. 68 g/d from 3 to 6 wk. The tremendous increase in GR coincided with a cumulative mortality of 14.1% in the 1991 broilers, mainly due to AS, whereas the 1957 broilers had a cumulative mortality of only 2.8%, with no cases of AS. Based on these findings, the authors suggested that AS developed due to the selection for higher GR, because it occurred without concomitant development in efficacy of the cardiovascular and respiratory systems.

In a pedigree population of commercial broiler line with a wide genetic variation for GR, %AS per sire family among progeny exposed to ascites-inducing conditions (AIC) was positively correlated with GR of their sibs under normal conditions (Deeb et al., 2002). Also Moghadam et al. (2001) found a positive genetic correlation between the tendency of broilers to develop AS and their BW under normal climatic conditions. Several earlier publications stating that AS develops in individuals with more rapid early growth were reviewed by Julian (1998, 2000). These findings led to the suggestion that further enhancement of broilers' GR, either by selective breeding or by advanced management, should be avoided as it will increase the incidence of AS in contemporary broiler flocks.

Results from a study with 6 high-GR broiler crosses and 2 lines of "Label"-type slow-growing broilers indicated that AS develops only in fast-growing broiler lines, but not at the same incidence in all of them (Gonzales et al., 1998). It was suggested that AS develops in broilers in which GR exceeds the rate at which their pulmonary vascular capacity increases, but they do not necessarily have to be the fastest growing birds in a flock. This was supported by Decuypere and Buyse (2005) who stated that AS is caused by an impaired oxygen supply that cannot sustain the rapid growth, rather than by increased oxygen requirement *per se.*

c) High GR and the incidence of AS are not correlated genetically

Due to the association between high GR, oxygen demand, and AS, it has been suggested that AS is induced by high GR. If true, further GR enhancement should be avoided as it will increase the proportion of AS-susceptible individuals in contemporary stocks. An alternative hypothesis claims that AS is associated with high actual GR only because the latter increases oxygen demand and that there are genetically AS-resistant broilers that do not develop AS even when exhibiting high GR. These two hypotheses were tested in trials in the years 2002 and 2006, with contemporary fast-growing commercial broiler lines and an experimental line derived from commercial broilers in the year 1986 (Druyan et al., 2008). A protocol of high-challenge ascites-inducing conditions (AIC) from d 19 was used to distinguish between AS-susceptible and AS-resistant individuals and to determine their GR to this age.

In the high-GR broiler lines, AS incidence was 31% and 47% in 2002 and 2006, respectively, and 32% in the 1986 slow-growing line. Most broilers that remained healthy under the high-challenge AIC exhibited the same early GR and BW as those that later developed AS. These results, and the relatively high incidence of AS in the slow-growing line, indicate that there is very little if any direct genetic association between AS and genetic differences in potential GR, and suggest that AS-resistant broilers can be selected for higher GR and remain healthy even under AIC.

In another study with contemporary commercial broilers, all the chicks were reared under standard conditions to d 19, and thereafter exposed to AIC that effectively induced AS in all the susceptible individuals (Druyan et al., 2007a). This experimental procedure revealed that the GR up to d 19 of the broilers that later developed ascites was similar to the GR of their counterparts that remained healthy, suggesting that within that study's population, high early GR was not associated with susceptibility to AS. In another trial by Druyan et al. (2007b), about 250 broilers that later developed AS, and about 650 broilers that remained healthy to day 49, all reared together under moderate-challenge AIC, exhibited similar GR until day 17.

Similar results were obtained in two recent trials in Ecuador; broilers from two commercial breeds were reared at high altitude (2400 m) under management that allows maximal GR (pelleted feed and 22 h/d of light). The incidence of AS ranged among the breed-by-sex groups, from 8% to 57%. However, in all groups, the range and mean of BW on day 21 (before AS effects on BW were apparent) were similar in the broilers that later died due to AS and their counterparts that remained healthy to the end of the trial at the 7th week (Romo and Kalinowski, unpublished data). Based on these results, it appears that the genetic variation in susceptibility to AS is not associated with GR variation in modern commercial broiler breeds. Accordingly, the continuous genetic enhancement of GR has not increased the proportion of AS-susceptible individuals in modern broiler stocks; it is the higher GR and the consequent higher oxygen demand that trigger AS development in a larger proportion of the AS-susceptible individuals in these stocks. This conclusion was supported by a study where genetically slow-growing broilers were exposed to high-challenge AS inducing conditions for 54 days, and eventually 30% of them developed AS (Druyan et al., 2008).

d) The genetic control of susceptibility to AS

Several studies found a genetic component in broilers' tendency to develop AS, with heritability estimates from 0.11 to 0.44 (Lubritz and McPherson, 1994; Lubritz et al., 1995; Moghadam et al., 2001; Pakdel et al., 2005; Druyan et al., 2007a,b; Pavlidis et al., 2007). Wideman and French (2000) suggested that gene or genes were involved in the response to their successful 2-cycle selection against AS susceptibility. Single gene inheritance was suggested also by Navarro et al. (2005). These authors performed a complex segregation analysis of data on oxygen saturation of the hemoglobin in arterial blood (SaO_2), a trait known to be closely related to AS (Druyan et al., 2007a). Data on SaO_2 from 12,000 males in fully pedigreed populations of an elite male line that has been closed for about 35 generations were available for that study. The results suggested that a single di-allelic dominant locus was responsible for 90% of the genetic variation in SaO_2, with high levels of SaO_2 indicating AS resistance, whereas low levels indicated AS susceptibility. Druyan et al. (2007b) noted that the extremely rapid divergence between their selected AS-S and AS-R lines may suggest the involvement of one or several major genes. Moreover, analysis of AS segregation within families in the selected lines suggested dominance of AS resistance.

Most studies considered AS susceptibility as a polygenic trait (e.g., Moghadam et al., 2001; Pakdel et al. 2005) but they were conducted under low-challenge AIC, where birds with relatively low GR, hence low oxygen demand, do not develop AS even if they are genetically susceptible. Therefore under such conditions, genes that affect GR are indirectly affecting the development of AS in the genetically susceptible birds. This situation, which apparently was common in many studies on the genetics of AS, complicated the efforts to select against AS susceptibility and to identify the genes with direct effect on AS. The studies of Wideman and French (1999, 2000), Pavlidis et al. (2007), and ours (Druyan and Cahaner, 2007; Druyan et al., 2007a,b; 2008;) were conducted under high-challenge AIC protocol. Under such conditions, all AS susceptible birds develop AS, even those with lower growth rate, thus facilitating the conclusion that only a few major genes are directly responsible for AS susceptibility.

e) Direct selection against susceptibility to AS

Successful selection against AS susceptibility was conducted by Wideman and French (1999, 2000) in a full-pedigreed elite commercial broiler breeder line. They used for reproduction only males and females that did not develop AS following AS-inducing surgery (unilateral pulmonary artery occlusion). After 2 cycles of such selection, %AS among males that were exposed to cool temperatures (14ºC) from 17 to 49 d of age, was reduced to 4%, compare to 15% after 1 cycle of this selection, and 31% in the base population. That study demonstrated the feasibility of selection based on mortality of AS-susceptible individuals under a protocol of high-challenge AIC. Divergent selection for AS mortality was conducted by Anthony and co-workers (Pavlidis et al., 2007). The AS was induced in a hypobaric chamber where oxygen content was reduced to the level of 2900 m above sea level. After 10 generations of divergent sire-family selection, %AS increased to about 90% in the AS-susceptible line and decreased to about 20% in the AS-resistant line, thus reaching a divergence of about 70% (Pavlidis et al., 2007).

Similarly successful divergent selection was applied by Druyan et al. (2007b). The 1st selection cycle was based on progeny testing for AS mortality under low-challenge AIC. Two additional cycles of full-pedigree progeny testing were conducted under high-challenge AIC protocol (Druyan et al., 2007a,b). Two divergent lines were established, AS-susceptible (AS-S) and AS-resistant (AS-R), with respectively 95% and 5% of AS (a divergence of 90%) when reared together under the same high-challenge AIC (Druyan et al, 2007b).

f) Indirect selection against susceptibility to AS

In order to conduct effective selection on AS mortality, the candidate birds must be exposed to extreme AIC up to about 6 wks. This has been done in all the experimental selection projects mentioned in Section e, in order to assure that all the genetically susceptible birds develop AS, because under standard broiler conditions only few individuals – is at all – develop AS. Keeping the candidate birds under extreme AIC precludes the possibility to select them for normal broiler performance traits. Therefore, it is important to find indicators of broilers' susceptibility to AS, that can be measured on birds under standard broiler conditions; such indicators allow the integration of indirect selection against AS susceptibility into the standard breeding programs of commercial broiler stocks. Many studies (reviewed by Druyan et al., 2007a) found significant physiological differences between broilers with AS vs. healthy ones. However, in all these studies, the physiological variables were measured at the phase when the susceptible broilers had already started to develop AS. Hence, these variables cannot serve as indicators of AS-susceptibility under normal conditions at early ages (at hatching or during the brooding period).

The exact initial biochemical and physiological factors related to the genetic propensity to develop AS are still not known. It is often difficult to prove that a particular change is primary in nature, and so determinative, or is a subsequent secondary manifestation in the development of AS. If specific parameters to predict AS susceptibility or resistance are sought, it is of paramount importance that the primary changes be determined and evaluated. Moreover, in order to assess their significance as criteria for selection, it is necessary to estimate the heritability of these parameters, and their genetic correlation to consequent AS development under AIC.

g) Genomic selection against susceptibility to AS

Data from test-crosses between our fully-divergent AS-S and AS-R lines suggested that only 2 major genes control this genetic divergence (Druyan and Cahaner, 2007). If indeed only few genes are involved in the genetic control of susceptibility to AS, and given the current rapid advancement of genomic tools, the AS genes should be detected and mapped in the near future (if not mapped already by the breeding companies). Once mapped, with the help of current and future genomic methodologies, the causative SNPs (or closely linked ones, as markers) in these genes will be identified. High-throughput genomic asseys may soon facilitate efficient genotyping of these marker SNPs, and their routine utilization in commercial breeding programs. With such markers, high-challenge AIC is not needed to effectively select against susceptibility to AS, because breeders will be able to easily detect and cull individual birds, within the elite lines, that carry the alleles for AS susceptibility. All major broiler breeding companies have been heavily involved in R&D efforts aimed at achieving this goal.

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THE RELATIONSHIP BETWEEN SHED CLEANLINESS AND HEN PRODUCTIVITY

L. E. EDWARDS[1]

Summary

The relationships between the average concentration of total airborne dust in 33 caged laying sheds, the duration and frequency of cleaning methods used by stockpeople, and the productivity of the hens was studied. Regression analyses indicated that the average dust concentration was lower when stockpeople spent more time cleaning in the sheds. In comparison, hen day production was lower when stockpeople used a high proportion of noisy cleaning methods, and the peak in egg production was higher when the concentration of airborne dust was higher. Mortality rates were higher in sheds where stockpeople used a greater variety of cleaning methods. Larger sheds, as indicated by the number of tiers of cages, had lower average dust concentrations and mortality rates, and higher hen day production. The results of these regression analyses indicate that while an increase in the use of noisy cleaning methods may pose a stressor for laying hens, exposure to higher concentrations of airborne dust do not appear to impair productivity.

I. INTRODUCTION

Recent observations on cage-egg farms examining between-farm relationships have found that laying hens that were exposed to high levels of man-made noise in the laying shed not only displayed high fear of humans, but also produced more eggs and had lower concentrations of corticosterone (a stress hormone) in their egg albumen (Edwards, 2009). One explanation for the unexpected relationship between noise and egg production may relate to the cleaning routines used by stockpeople in the laying sheds. The majority of noise that occurs in laying sheds is related to motorised cleaning procedures, and it is plausible to suggest that sheds that experience the most man-made noise are also the sheds that receive the most cleaning. Potentially, the flocks studied by Edwards (2009) that were exposed to a lot of noise may have been producing well because they were living in sheds that were cleaned more often, resulting in a cleaner living environment. It was hypothesised that the frequency of cleaning in laying sheds and the amount of noise made during cleaning are related to the productivity of the laying hens in those sheds. To investigate this hypothesis, the present study assessed the cleanliness (airborne dust concentration) of commercial laying sheds and compared this to the cleaning routines employed by the stockpeople and the productivity of the hens.

II. MATERIALS AND METHODS

Thirty-three laying sheds on eight egg farms in Victoria were visited between 11th November and 23rd December, 2009. Data were collected on the following variables: the average concentration of airborne dust in each laying shed; the physical features of each laying shed that may affect air flow and dust concentrations; an interview with

[1] Animal Welfare Science Centre, University of Melbourne, Parkville, VIC 3011

stockpeople regarding the cleaning routines in each shed, and the productivity records for each shed (where possible). The methods used for collecting these data are described below.

Sampling airborne dust concentrations: The average total airborne dust concentration (mg/m^3) in each laying shed was assessed using a handheld air sampler (DustTrack ^{TM} Aerosol Monitor, Model 8532). The researcher sampled each shed by walking down each aisle once with the air sampler held at chest height, and these data were used to calculate an average value for the shed. After the first reading was taken at the start of the day, the researcher then sampled the shed on an hourly basis for eight hours (usually from 8am to 4pm). Thus the average airborne dust concentration at each hour could be used to calculate an overall daily average for each shed (Mean Dust, mg/m^3).

Shed details: For each laying shed the following variables were recorded: the number of fans present (No of Fans); the temperature that the shed was maintained at (Shed Temp); the age of the birds (Flock Age); the number of birds in the shed (Flock Size); the average number of birds in each cage (Birds per Cage); the area of the shed in m^2 (Shed Area); the number of rows of cages (No of Rows), and the number of tiers of cages (No of Tiers).

Interview with stockpeople: Ethics approval to conduct research involving humans was obtained from the Human Research Ethics Committee at the University of Melbourne (Ethics ID # 0932600.1). All stockpeople who conducted cleaning inside the sheds were asked to participate in an interview regarding the frequency and method of their cleaning routines. The results of these interviews were analysed and the following variables were calculated: the total number of different types of cleaning methods used in the shed (No of Methods); the total number of hours spent cleaning in the shed per week per 1000 birds (Hrs / 1000 Birds); the total number of hours per week per 1000 birds spent cleaning using loud cleaning methods such as a leaf blower or air hose (Noise Hrs / 1000 Birds); the proportion of total cleaning time that consisted of loud cleaning procedures (Prop Noise); the number of days since the shed was last cleaned using any method (Days Since Last Clean), and if the shed was blown out with a leaf blower or air hose as part of the cleaning routine, the number of days between blowouts (Days Between Blowouts).

Productivity records: From the productivity records that were available and adequate, the following variables were obtained: the cumulative mortality rate on the day of sampling (Mortality) (n = 24); the hen day production on the day of sampling (HDP) (n = 29), and the peak hen day production (Peak HDP) (n = 21). The value from the appropriate breed standard (ISA, 2006; Hyline, 2009) was subtracted from the actual productivity value to give a + or – value, indicating how well the flock was performing in comparison to the breed standard. It was these comparisons to the breed standards that were used in the analyses.

The data were checked for normality and transformed where necessary. The mean dust concentration and productivity data were then subjected to a forward linear regression analysis.

III. RESULTS

The results of the linear regression analysis are presented in Table 1. The average concentration (mg/m^3) of airborne dust inside caged laying sheds was greater when stockpeople spent less time cleaning in the shed (P = 0.003), when the sheds were maintained at a warmer temperature (P = 0.000) and when there were less tiers of

cages in the shed (P = 0.001). These three variables explained 71% of the variation in average dust concentration.

Flock productivity was associated with cleaning routines, shed parameters and airborne dust concentrations. Hen day production (HDP) was greater when stockpeople made less noise while cleaning (P = 0.013) and when there were more tiers in the shed (P = 0.031), with 33% of the variation in HDP explained by these two variables. The Peak HDP was greater when the average airborne dust concentration was greater (P = 0.000), and this relationship explained 61% of the variation in peak egg production. Mortality rates were greater when stockpeople used a greater variety of cleaning methods (P = 0.000) and when there were less tiers of cages in the shed (P = 0.023), and these two variables explained 47% of the variation in mortality rates.

Table 1 Forward regression analyses for the relationships between shed parameters, cleaning routines, airborne dust concentration and productivity measures.

Variable	Predictors	B	sed	t	P	Adj R^2
Mean Dust SqRt	Hrs/1000 birds	- 0.45	0.14	-3.30	.003	0.71
	Shed Temp	0.12	0.01	8.86	.000	
	No of tiers	- 0.06	0.02	-3.88	.001	
HDP	Prop noise	-21.91	8.23	-2.66	.013	0.33
	No of tiers	1.80	0.79	2.28	.031	
Peak HDP	Mean dust SqRt	13.41	2.35	5.72	.000	0.61
Mortality	No of methods	0.46	0.10	4.54	.000	0.47
	No of tiers	- 0.49	0.20	-2.48	.023	

IV. DISCUSSION

The mean dust concentrations observed in this sample of fully-enclosed, environmentally controlled laying sheds in Victoria were all below 1.27 mg/m^3, which is comparable to those reported in the literature (< 2 mg/m^3) for caged laying sheds (Ellen et al., 2000). The results of this study found that both average airborne dust concentration and flock productivity on caged egg farms were related to the cleaning routines used by stockpeople. In addition, one factor associated with the size of the shed, the number of tiers of cages, was also associated with dust concentration and productivity.

The relationships between dust concentrations, time spent cleaning and shed temperature are as expected. If the amount of time spent cleaning by stockpeople is effective in reducing airborne dust concentrations then a negative relationship between these two variables is expected. In addition, it is not surprising to find a positive relationship between shed temperature and dust concentration, as warmer sheds are more likely to have lower ventilation rates, which would presumably reduce the removal of airborne dust. The negative relationship between dust concentration and the number of tiers can not be readily explained, as it would be expected that more tiers would be associated with a larger flock and thus a larger source of airborne dust. The number of tiers in the shed was also positively associated with egg

production and negatively associated with mortality rates, and it is plausible to suggest that the larger sheds may be representative of more modern sheds, in which improvements to cage design, ventilation and automatic systems (e.g. feed distribution) have been implemented. In this sense, a larger shed may be representative of an improved living environment for laying hens when compared to a smaller shed with fewer tiers. However, as no measurements were made of the quality or age of the sheds, this is conjecture.

The lower hen day production associated with the use of a high proportion of noisy cleaning methods suggests that these cleaning methods may be a stressor for the hens. Laying hens will avoid exposure to loud noise when given the opportunity (MacKenzie et al., 1993), and exposure to loud noise (90 dB) has been associated with increased fear and stress in laying hens (Campo et al., 2005). Thus, a stress response resulting from exposure to loud noise during cleaning may result in compromised productivity, and could be responsible for the observed relationship between egg production and the use of noisy cleaning methods. The positive relationship between peak egg production and average dust concentration suggests that the observed dust concentration does not limit egg production.

The positive relationship between mortality rates and the number of cleaning methods used by stockpeople suggests that this is not a measure of shed cleanliness. Again, there is no obvious explanation for these results, however it is interesting to note that sheds that used a motorised form of cleaning (such as blowing out dust with leaf blowers or air hoses) also used fewer different types of cleaning methods (6.6 Motorised vs 14.3 Non-motorised, $t = -8.36$, $P = 0.000$). Thus, hens that were exposed to a low number of cleaning methods were also exposed to more noise and experienced a lower mortality rate. If motorised cleaning methods are more effective than non-motorised methods, these methods may provide a cleaner living environment for the hens that could be reflected in lower mortality rates.

In conclusion, these results indicate that despite not knowing the particle size or toxicity of the airborne dust, the total concentration of dust in the air was related to the peak productivity of the hens and the amount of time spent cleaning by stockpeople in the sheds. It appears that while an increase in the use of noisy cleaning methods may pose a stressor for laying hens, exposure to higher concentrations of airborne dust may not limit egg production, as was expected.

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MINIMISING WEIGHT LOSS IN NEW BROILER HATCHLINGS THROUGH EARLY FEEDING OF TREHALOSE

M. M. BHUIYAN[1], F. GAO[2], S. H. CHEE[3] and P. A. IJI[1]

Summary

Two experiments were conducted to assess the effect of delayed access to feed post-hatch and the potential of two carbohydrate oligomers, trehalose and palatinose, as supplements for early feeding of broiler chickens. Delayed access to feed and water was found to reduce starting weight at placement while early access to feed generally improved it. Supplementation with trehalose, in particular, led to higher ($P<0.001$) 21d weights than in the other groups, although FCR was not improved. The benefit of early feeding was demonstrated and trehalose may have some potential as an early feed supplement. However, further studies are required into how it can be applied at economic levels to keep production costs down.

I. INTRODUCTION

In the commercial hatchery, broiler chicks within a set batch of eggs hatch within several hours of one another and chicks need to be sorted before being delivered to various farms. Therefore, some chicks may be without food or water for up to 36 hours post-hatch (Noy and Sklan, 1999). This results in weight loss, but the most negative effect is on the development of the gastrointestinal tract (GIT). The GIT develops most rapidly within the first 4-7 days of life (Uni *et al*., 1995; Iji *et al*., 2001). Access to feed within this period aids the development of the GIT. The science of early feeding in the poultry industry is still nascent but equipment for the *in ovo* delivery of nutrients to the developing embryo has been developed by Uni and her research collaborators (Uni and Ferket, 2004). Along with the development of appropriate delivery technology is the concerted search for suitable products that can be delivered *in ovo* or in a manner that can be ingested by the chick at hatch.

The overall objective of the current study was to determine the potential of low-molecular weight carbohydrates in the diet of newly hatched birds in terms of GIT development, nutrient digestibility and growth.

II. MATERIALS AND METHODS

Eggs were obtained from a commercial hatchery at 18 days of incubation and hatched on site at the University of New England (UNE). The chicks were either provided access to feed and water within 8 hours of hatch or held for 36 hours, to simulate the industry standards where chicks hatch over this time window. In experiment 1, 360 chicks were assigned to 3 broad dietary treatments; a commercial diet or the same diet supplemented with 10 g palatinose or trehalose per kg diet. The commercial diet was formulated to UNE specification (wheat-sorghum-soybean basal diet) and supplied by Ridley Agriproducts Pty Ltd (Tamworth NSW, Australia). Each diet was fed within 8 hours of hatch or introduced to the chicks after 36 hours of holding, making a total of 6 groups. The experiment was therefore a 3 x 2 factorial design. Each group was replicated six times, with 10 chicks (6 males and 4 females) per replicate. In experiment 2, 252 chicks were randomly assigned to the same commercial diet

[1]University of New England, School of Environmental and Rural Science, Armidale, NSW 2351
[2]College of Animal Science & Technology, Nanjing Agricultural University, Nanjing, 210095, China
[3]AB Vista, Asia Pacific, Singapore.

as-is or supplemented with Zn-Bacitracin (ZnB), palatinose (10 or 20 g/kg) or trehalose (10 or 20 g/kg). Each diet was replicated 6 times, with 6 chicks (3 each of male and female) per replicate. One group on the unsupplemented diet was held for 36h while another group on the unsupplemented diet and all chicks on the supplemented diets were fed within 8h of hatch. In both experiments, the supplements were fed for the duration of the feeding trial. Weight loss or gain over the first 36h was determined, and feed consumption and growth were measured up to 7, 14 and 21 days of age. The ileal digesta were collected at day 21. All diets were supplemented with celite, a source of acid-insoluble ash, to enable assessment of nutrient digestibility.

Data from each experiment were analysed according to the design, GLM for experiment 1 and one-way ANOVA for experiment 2. Data were analysed with Minitab version 12 (Minitab Inc., 1998). Mean values were considered significantly different if P level was ≤ 0.05.

III. RESULTS

In the first experiment, holding chicks for up to 36h post-hatch significantly reduced ($P < 0.001$) their body weight (up to 11 %), with the held group on the unsupplemented diet being lower ($P<0.05$) than groups that were supplemented and fed at hatch (Table 1). Diet supplementation with trehalose resulted in higher ($P < 0.001$) body weight at 21d, and this was largely supported by a higher ($P < 0.001$) feed intake. Supplementation with palatinose or trehalose had no effect on FCR and birds that were held from feed also had a similar FCR to those that were given early access to feed.

Table 1 Feed intake (g/bird), body weight (g/bird) and FCR of birds at different stages of growth, in response to immediate or delayed feeding and different supplements in experiment 1

	Control[1]		Palatinose[2]		Trehalose[2]		
Parameters	Held[3]	Fed[4]	Held[3]	Fed[4]	Held[3]	Fed[4]	SEM
1-7 days							
36h-weight	44.7^{b}	50.9^{a}	44.6^{b}	50.6^{a}	44.7^{b}	50.6^{a}	0.51***
Feed intake	98.1	107.2	107.7	107.5	108.4	112.8	1.48
7d-weight	129.7^{b}	141.8^{ab}	139.3^{ab}	142.9^{a}	135.6^{ab}	143.9^{a}	1.46*
FCR	1.16	1.18	1.14	1.16	1.19	1.21	0.01
1-14 days							
Feed intake	317.5^{b}	325.3^{b}	323.2^{b}	332.6^{ab}	347.3^{ab}	369.3^{a}	4.73**
14d-weight	343.8	341.2	337.3	355.2	344.5	371.2	4.79
FCR (1-14d)	1.06	1.13	1.11	1.09	1.16	1.16	0.01
1-21 days							
Feed intake	819.3^{b}	851.1^{b}	869.8^{b}	853.5^{b}	1012.2^{a}	1053.0^{a}	16.96***
21d-weight	619.2^{b}	670.1^{b}	665.8^{b}	668.2^{b}	735.7^{a}	784.8^{a}	10.77***
FCR (1-21d)	1.43	1.38	1.40	1.38	1.46	1.44	0.01

[1] Commercial diet; [2] Fed at 1 % of diet; [3] Feed was delayed for 36h; [4] Fed within 8 hours of hatch; [a, b] values with unlike superscripts within each row are significantly different; * P<0.05; ** P<0.01; *** P<0.001.

The digestibility of energy at 21d of age varied between 0.69 and 0.73 while that of starch ranged from 0.86 to 0.90 but this was not affected by access to feed or supplementation with the sugars (Table 2).

Table 2 Nutrient digestibility at the ileum of 21d old chicks in response to immediate or delayed feeding and different supplements in experiment 1

	Control[1]		Palatinose[2]		Trehalose[2]		
Parameters	Held[3]	Fed[4]	Held	Fed	Held	Fed	SEM
Gross energy	0.69	0.69	0.70	0.73	0.69	0.71	0.006
Protein	0.81	0.80	0.83	0.83	0.82	0.83	0.004
Starch	0.86	0.87	0.88	0.88	0.90	0.90	0.007

[1] Commercial diet; [2] Fed at 1 % of diet; [3] Feed was delayed for 36h; [4] Fed within 8 hours of hatch.

In experiment 2, there was up to 5.5 % loss ($P < 0.001$) in body weight as a result of holding without feed and water for 36 hours (Table 3). Feed intake to 7d was significantly higher ($P < 0.001$) on the ZnB-supplemented diet than on the control diet, regardless of access to feed. Birds on the high trehalose diet were heavier ($P < 0.01$) than those on the ZnB-supplemented diet, the two groups on commercial diet and high-palatinose diet. Up to this age, birds on the ZnB-supplemented diet were the least ($P < 0.001$) efficient in feed conversion. Over 21d of feeding, feed intake on the negative (commercial) control diets was only significantly lower ($P < 0.01$) than that on the high trehalose and ZnB diets. Birds on the ZnB-supplemented diet were also less ($P< 0.001$) efficient in feed conversion than the other groups. There were no differences between the groups in terms of body weight and FCR. The ileal digestibility of gross energy, protein and starch were unaffected by the dietary treatment at 21 days of age.

Table 3 Feed intake (g/bird), body weight (g/bird) and FCR of birds at different stages of growth, in response to immediate or delayed feeding and different supplements in experiment 2

	Control[1]		Palatinose		Trehalose			
Parameters	Held[2]	Fed[3]	Low[4]	High[5]	Low[4]	High[5]	ZnB[6]	SEM
Hatch – 7d								
36h-weight	44.7^{b}	47.2^{a}	47.5^{a}	47.3^{a}	47.0^{a}	47.5^{a}	47.4^{a}	0.16***
Feed intake	126.7^{b}	131.7^{b}	137.7^{ab}	133.5^{b}	141.6^{ab}	140.5^{ab}	145.3^{a}	1.34***
7d-weight	150.2^{b}	151.7^{b}	156.1^{ab}	149.5^{b}	158.5^{ab}	161.4^{a}	147.7^{b}	1.14**
FCR	1.26^{bc}	1.20^{c}	1.27^{bc}	1.31^{b}	1.27^{bc}	1.23^{bc}	1.46^{a}	0.02***
1 – 14d								
Feed intake	439.9^{b}	450.3^{ab}	472.0^{ab}	466.0^{ab}	471.9^{ab}	476.1^{a}	488.4^{a}	4.29*
14d-weight	369.0	372.1	377.6	377.1	379.0	388.4	379.0	2.79
FCR(1-14d)	1.39^{ab}	1.34^{b}	1.43^{ab}	1.41^{ab}	1.42^{ab}	1.40^{ab}	1.48^{a}	0.01*
1 – 21d								
Feed intake	1003.8^{b}	1036.5^{ab}	1051.5^{ab}	1101.2^{ab}	1085.0^{ab}	1133.7^{a}	1132.9^{a}	11.56**
21d-weight	741.1	708.5	720.7	737.1	748.2	801.7	779.5	10.35
FCR(1-21d)	1.45	1.57	1.57	1.61	1.56	1.51	1.55	0.018

[1]Commercial diet; [2] Feed was delayed for 36h; [3] Fed within 8 hours of hatch; [4]10 g supplement /kg diet and fed within 8 hours of hatch; [5] 20 g supplement /kg diet and fed within 8 hours of hatch; [6]supplemented with zinc bacitracin at 50 ppm and fed within 8 hours of hatch; [a, b, c] values with unlike superscripts within each row are significantly different; * P<0.05; ** P<0.01; *** P<0.001.

IV. DISCUSSION

The weight loss associated with post-hatch holding may be due to faster depletion of the yolk sac, lack of feed in the gut, depletion of muscle tissue and failure in tissue growth. The first 4-7 days of life is the period of most rapid development of the GIT (Uni *et al*., 1995; Iji *et al*., 2001). During this phase of development, the GIT grows more rapidly than the rest of the body, and prepares the bird for more efficient use of nutrients. There is evidence that lack of access to feed adversely affects mucosal development and, given the very short production

cycle of the modern broiler chick, the bird may be unable to recover from such retardation, in terms of growth or health.

The results obtained in the present studies clearly indicate that early access to feed had no significant effect on early weight gain in birds fed any diet. However, the results of this study also indicate that trehalose supplementation of the commercial diet significantly improved 21-d weight on both feeding treatments. The birds tended to respond positively to the supplements, particularly trehalose, even after initial holding. This has ramifications for the industry, as it may be possible to continue with the current simpler hatchery practice of hatching the entire batch of set eggs, processing them and then delivering them to farms, on to pre-supplemented feed.

In the second experiment, best responses in body weight were again obtained with trehalose although the differences in 21-d body weights were not significant. The response to the supplements was comparable to the response to zinc bacitracin, the antibiotic supplement that is commonly used by the Australian poultry industry. It is recognised that antibiotics in the diet do not greatly improve growth but maintain good health and conceivably improve flock uniformity.

The test supplements do not appear to be as beneficial at the lowest levels, 0.5 %, tested in the current studies. The fact that no further gain is achieved by feeding trehalose for longer than one week would, however, mean that savings can be made through shorter-term feeding of the supplement.

V. CONCLUSION

Of the two test supplements, trehalose holds some potential for inclusion in early feed for broiler chickens. More studies are required into the *in ovo* application of this supplement, in order to include it at a more economical level.

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FURTHER INVESTIGATION OF NON-INVASIVE MEASURES OF STRESS IN LAYING HENS

J. ENGEL[1], T. WIDOWSKI[2], A. TILBROOK[3] and P. HEMSWORTH[1]

Summary

Chronic stress in animals is often monitored by measuring baseline concentrations of plasma corticosteroids. The most common procedure for blood collection in laying hens is venipuncture of the brachial vein. However, this procedure is invasive and causes a rapid corticosteroid response which can affect the measure of baseline concentration if the sample is not collected quickly enough. Corticosterone (CORT) can also be measured non-invasively in both the egg and faeces. Most studies aimed at validating these non-invasive measures of stress have studied the relationship between egg or faecal corticosterone and plasma corticosterone following an intra-muscular injection of adrenocorticotropic hormone (ACTH) or application of acute stressors to induce a maximal response of corticosterone. We therefore examined the relationships between non-invasive measures of corticosterone and plasma corticosterone in conditions in which modest differences in baseline corticosterone concentrations were expected, using samples collected from hens in peak and late production. HyLine brown laying hens (n=154) of two ages (34 and 47 weeks) were housed in cages in two commercial poultry sheds. Plasma was collected from each bird on the 1st day of the study, and eggs and faeces were collected prior to blood sampling on this 1st day as well as the 3rd and 4th days following blood sampling. Plasma, egg and faecal samples were pooled from each cage on each day and analysed for corticosterone concentration. Older hens (47 weeks) had higher mean yolk CORT than younger hens (4.12 vs. 3.61 ng/g, respectively. $P < 0.01$) and a trend for higher plasma CORT (1.38 vs. 1.03 ng/ml, respectively, $P < 0.06$) However, there were few significant correlations between non-invasive measures of physiological stress and plasma corticosterone. Clearly, the lack of relationships between plasma corticosterone and the various non-invasive measures of stress in laying hens warrant further investigation. In particular, these relationships should be established under conditions in which differences in baseline concentrations are expected, rather than under conditions in which large corticosterone changes are expected such as following an ACTH challenge.

I. INTRODUCTION

Within animal welfare research, there is increasing interest in using non-invasive methods for measuring physiological stress. To date, the most common method for measuring circulating concentrations of corticosteroids has been to use plasma or serum which involves blood sampling the animals. Blood sampling in itself is invasive because it involves handling and drawing blood from the animal, which can cause a stress response. For most species, glucocorticoids are released in approximately 2 minutes following application of an acute stressor (Broom and Johnson, 1993). If a blood sample is not taken within a short period of time, the corticosterone (CORT) concentration measured in the plasma could be elevated due

[1] Animal Welfare Science Centre, Melbourne School of Land and Environment, University of Melbourne, Parkville, Vic, 3010, Australia
[2] Department of Animal and Poultry Science, Ontario Agricultural College, University of Guelph, Guelph, Ontario, N1G 2W1, Canada
[3] Department of Physiology, Faculty of Medicine, Nursing, and Health Sciences, Monash University, Clayton, Vic 3800, Australia

to a stress response caused by the procedure itself, and therefore may not be a valid measure of the animal's general physiological state.

In the laying hen, it is possible to measure corticosterone non-invasively through the egg or the excreta (Rettenbacher et al., 2005). The egg can also be divided into its two components, the yolk and the albumen, and corticosterone can be measured in both. Corticosterone is secreted by the adrenal gland into the blood where the free (active) component can then be transferred into other tissues. The relationships between plasma, faecal and egg corticosterone concentrations have been established following administration of either adrenocorticotropic hormone (ACTH), which causes the release of corticosterone or by administering radio-labeled corticosterone, giving scientists a way to compare the distribution of CORT or its metabolites in the various components (plasma, egg, faeces, etc.) over time. Rettenbacher et al. (2005) reported that after administration of radio-labeled CORT, peak levels were measured in the albumen and outermost layer of the yolk after one day, while the highest levels of CORT appeared in the innermost layers of the yolk four to six days later. Similarly, Downing and Bryden (2008) reported a significant positive correlation between plasma and albumen CORT concentrations one day after hens were given injections of varying concentrations of CORT. The temporal pattern of CORT metabolite excretion in faeces has also been established. Dehnhard et al. (2003) reported a similar, but less pronounced, pattern of CORT metabolite secretion in faeces to plasma CORT concentrations after ACTH administration, which occurred two to six hours after the response in plasma.

While previous studies have examined the relationships between plasma corticosterone and faeces or plasma corticosterone and components of the egg, there have been no experiments that compared all 3 non-invasive samples with a corresponding plasma sample. Additionally, there have been few comparisons of baseline values between plasma and non-invasive CORT concentrations Therefore, this experiment proposed to examine relationships between plasma and non-invasive methods of measuring CORT concentrations under baseline conditions, taking into account the observed differences in plasma CORT concentration noted between hens of different ages (Davis et al., 2000).

II. OBJECTIVES

The objective of this experiment was to investigate the relationship between plasma corticosterone concentrations and the concentrations of corticosterone in the egg yolk, albumen and faeces, in peak and late production laying hens.

III. METHODOLOGY

a) Animals and housing

Laying hens (n = 154) of the HyLine Brown variety were housed in cages in groups of 7-8 birds with space allowances from 550-625 cm^2 per bird within two commercial poultry sheds. The hens were 34 and 47 weeks of age. Within each shed (age group), ten cages were randomly selected. Two cages from each aisle within the shed were allocated with one being toward the middle (M) of the shed and the other toward the end (E) of the shed. The cage was the experimental unit. Hens were exposed to 14.5 and 16 hours of light for 34 and 37 weeks of age, respectively. They were fed a balanced diet and feed was topped up 4 times daily.

b) Sample collection, preparation and analysis

Blood sampling occurred on the first day with corresponding 24 hour pooled faecal and egg samples. Sampling then ceased for one day to remove any effect of blood sampling. Egg and

faeces were then collected and pooled for each cage over 24 hours for the following two days (days three and four of collection).

Birds were blood sampled on the first day of collection. Via the wing vein, 1.5 mL of whole blood was taken using a 3 mL heparinized syringe with a 23 gauge needle. Hens were then placed in a holding crate until all birds from the cage had been sampled. Blood was kept on ice until it was centrifuged, and the plasma was poured into vials and stored at -20°C until analysed. Plasma was not extracted before analysis.

After collection, faeces were weighed and dried at 60°C for 48 hours. Once dried, the samples were weighed again and ground before being stored at -20°C until analysed. For extraction, 0.1 g of ground faeces was extracted with 1 mL of 80% methanol. The samples were then vortexed for 30 minutes and centrifuged. The supernatant was taken and dried down. The samples were then resuspended in Phosphate Buffered Saline (PBS) and analysed.

Eggs were collected to correspond with faecal samples. Eggs and their components were weighed and separated. The albumen and yolk were stored individually at -20°C until analysed for corticosterone concentration. For extraction of egg yolk, 0.5 g of egg yolk was taken and 1 mL of distilled water was added and vortexed until mixed. The mixture was extracted with 3 mL hexane:diether (30:70 ratio), vortexed and left to settle before snap freezing with an ethanol/dry ice bath. The supernatant was collected and dried. 1 mL of ethanol was added to the samples which were then frozen -20°C overnight. The samples were centrifuged the next day and the supernatant taken and dried once more before being resuspended in PBS and analysed. Egg albumen was extracted following the methodology of Downing and Bryden (2008) and then analysed.

All samples were analysed for corticosterone using a Corticosterone HS Enzyme Immunoassay (EIA) (IDS Ltd., Boldon, UK).

c) Statistical analysis

Statistical analysis was carried out using SPSS 18 (SPSS Inc., Chicago, IL, USA). The cage was the experimental unit. Analysis of variance was conducted to test for age effects. Relationships between plasma corticosterone and yolk, albumen and faecal corticosterone were examined using Spearman correlations.

IV. RESULTS

The effect of age on plasma, egg albumen and yolk and faecal CORT concentrations are shown in Table 1. The values presented are the means of the concentrations over the three sampling days.

Table 1 The effect of age on plasma, egg albumen and yolk, and faecal corticosterone concentrations.

	Age (Weeks)			
Sample	34	47	S.E.M.	P-value
Plasma CORT (ng/ml)	1.03	1.38	0.13	0.06
Mean egg albumen CORT	19.02	19.82	0.37	ns
Mean egg yolk CORT (ng/g)	3.61^{b}	4.12^{a}	0.11	0.01
Mean faecal CORT (ng/g)	30.09	31.81	0.95	ns

Means with different superscripts are significantly different.

While there was a trend for plasma CORT to be positively correlated with yolk CORT on the first collection day ($r = 0.39$, $P < 0.091$) and negatively correlated with faecal CORT on the

fourth day ($r = -0.42$, $P < 0.067$), there were no significant correlations between plasma CORT and any of the non-invasive measures of CORT. The only significant relationship between the different non-invasive measures of stress was a relationship in the negative direction between faecal CORT on the fourth day and yolk CORT on the first collection day ($r = -0.47$, $P < 0.035$). Age was confounded by shed. When the data were run for each shed individually, there was a significant relationship between plasma CORT and albumen CORT on day 1 ($r = 0.72$ and $r = -0.67$, $P < 0.05$; for 34 and 47 week-old hens, respectively).

V. DISCUSSION

This experiment showed a tendency for plasma CORT to be higher in older laying hens, which is opposite to that previously reported by Davis et al. (2000). Any number of factors could have resulted in this difference, such as environmental or genetic differences.

There was a clear lack of relationships between plasma and non-invasive measures of CORT in laying hens under baseline conditions. However, some non-significant relationships did appear to exist. It is possible that under conditions in which particular eggs and faecal samples could be matched to individual birds, a more clear relationship could be defined, though this was not practical under the commercial conditions studied.

Furthermore, the samples collected, while assumed to be representing corresponding 24 hour periods, probably did not represent the same periods within the 24 hours. The faeces would have represented the full 24 hours, however, each component of the egg is not formed over a whole 24 hours. The yolk may take days to form as indicated by the results of Rettenbacher et al. (2005) in which the radio-labelled CORT was still being measured in the yolk of eggs laid four to five days after administration of the CORT. So the yolk sample corresponding to the single plasma sample may not be collected until days later. However, albumen is laid down just prior to the shell being formed and would likely represent the time only during which the albumen is being formed. According to the same report by Rettenbacher et al. (2005) any albumen sample corresponding to a physiological event may only be relevant in the egg laid the day following the event.

The plasma sample only represented a single sample during the day. CORT concentrations in the laying hen are known to be variable throughout the day (Beuving and Vonder, 1977). Therefore, it is likely that a single sample of plasma may not correlate with samples that represent a larger window of time. It is likely that multiple plasma samples should be taken to best represent the diurnal variation in CORT that is present in birds.

Clearly, further experiments utilising differences in CORT concentrations under baseline conditions need to be studied, as well as, more intensive experiments on the topic. It is important to attempt to get samples representing the same window of time.

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INDIVIDUAL VARIATION IN HOW HENS INTERACT WITH A DUST SUBSTRATE

S.M. LAINE[1], G.M. CRONIN[2], J.C. PETHERICK[3] and P.H. HEMSWORTH[1]

This paper re-examines in further detail the findings of an experiment reported previously by Laine *et al.* (2009). Briefly, Laine *et al.* (2009) preference tested laying hens (n = 15) for their choice between social contact (a familiar, subordinate hen) and a dust substrate (a tray of peat moss) in a Y-maze apparatus. Although the dust substrate was much preferred to social contact, it was observed that hens differed in how they interacted with the dust substrate in the Y-maze when they chose it. To examine individual variation further, each hen was classified as one of three types, based on the proportion of Y-maze trials in which the hen chose the Y-maze arm containing dust and commenced a dustbathing bout. A dustbathing bout was defined as commencing when the hen first rolled onto her side. Hens which commenced a dustbathing bout on >85% of dust-chosen trials were defined as "dustbathers", while hens which commenced a dustbathing bout on <15% of dust-chosen trials were defined as "non dustbathers". When dust was chosen but dustbathing was not performed, hens generally used the dust substrate for foraging, specifically pecking and scratching at the dust. Of the 15 hens, 4 hens were defined as "dustbathers", 6 were defined as "non dustbathers" while the remaining 5 hens commenced a dustbathing bout on 15-85% of dust-chosen trials. The behaviour of the hens had been video recorded for an 11-day period in the home cage prior to Y-maze testing and their dustbathing behaviour during the hour following the daily filling of the home cage dustbath was recorded. The dustbathing behaviour of "dustbathers" and "non dustbathers" was compared using a t-test. During the 11-day home cage observation period, hens defined as "dustbathers" dustbathed on a higher proportion of days ($P = 0.0135$) and had a shorter latency to commence dustbathing ($P = 0.0043$) compared to hens defined as "non dustbathers". Moreover, "dustbathers" performed more real dustbathing bouts (i.e. dustbathing bouts incorporating the dust substrate) compared to "non dustbathers" ($P = 0.0032$). These results imply that individual hens differed in how they interacted with the dust substrate, but these differences were consistent between the home cage and Y-maze environments. Similar results were described in Petherick *et al.* (1990) who suggested that peat moss may "switch on" dustbathing for only some hens. This variation in how hens interact with a dust substrate implies that dustbathing and foraging opportunities may differ in their importance to different individuals. If this is the case, it raises the question of the relative importance of a dust substrate for these two types of hens. Potentially, the presence or absence of a dust substrate may have a differential effect on the welfare of individuals. For example, do "dustbathers" suffer more in the absence of a dustbathing substrate compared to "non dustbathers"? It also raises the question about the genetic and/or experiential basis of preferences. Further research is warranted to determine whether this effect applies to a larger population.

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[1] Animal Welfare Science Centre, Melbourne School of Land and Environment, University of Melbourne. Parkville Vic 3010.

[2] Faculty of Veterinary Science, University of Sydney, Camden NSW 2570.

[3] Agri-science Queensland, Department of Employment, Economic Development and Innovation, Rockhampton Qld 4702

EFFECTS OF DEPRIVATION OF A PREFERRED RESOURCE, SOCIAL CONTACT OR DUSTBATHING SUBSTRATE, ON THE BIOLOGICAL FUNCTIONING OF LAYING HENS

B.H. STEVENS[1], A.J. TILBROOK[2] and P.H. HEMSWORTH[1]

Summary

Two common approaches used to assess animal welfare are the animal preferences approach and the biological functioning approach. This study compared these two approaches by testing the hypothesis that, when animals are deprived of a preferred resource, they will display disrupted biological functioning. The study consisted of two parts: preference testing (Part 1), and restriction (Part 2). In Part 1, the choice behaviour of 48 Hy-line brown laying hens was determined using a Y maze testing apparatus which offered the birds a choice between accessing sawdust as a dust bathing substrate or social contact with an unfamiliar bird. For Part 2, 24 hens were selected based on their choice behaviour; 12 were labeled "social preferred" and 12 were labeled "dust preferred". They were subsequently deprived of either social contact or dustbathing substrate in a factorial arrangement with preference. Stress physiology data collected at the conclusion of 6 weeks of restriction showed no main effects of restriction (P = 0.179) or preference (P = 0.535) on average daytime corticosterone, nor any significant interactions between main effects (P = 0.184). There was a significant main effect of restriction on maximum corticosterone response to adrendocorticotropic hormone (ACTH) stimulation (P = 0.034) and heterophil:lymphocyte (H:L) ratio (P = 0.011) with birds restricted of social contact showing an elevated response to ACTH and higher H:L ratio. This suggests, based on the biological functioning approach, that deprivation of social contact, regardless of the birds' preference, may be detrimental to bird welfare.

I. INTRODUCTION

Although there is a wide acceptance of the scientific method in problem solving, its ability to contribute to the welfare debate has been limited to some extent by a lack of consensus on the scientific approach to studying animal welfare (Barnett and Hemsworth, 2009). For many scientists, animal welfare is defined and measured on the basis of how well the animal is performing from a biological functioning perspective. That is, measures of how much has to be done in order to cope with the environment and the extent to which these coping attempts are succeeding (Broom 1986) including biological responses such as the functioning of body repair systems, immunological defenses, physiological stress responses and a variety of behavioural responses. For others, animal welfare concerns affective states, such as suffering, pain, and other feelings or emotions, and thus animal welfare can be studied by measuring animal preference on the basis that preferences are influenced by the animal's emotions, which have evolved to motivate behaviour in order to avoid harm and facilitate survival, growth, and reproduction (Duncan, 2004). An important question in addressing this scientific uncertainty is how do animal preferences relate to biological functioning? This experiment is part of a series of experiments with pigs

[1] Animal Welfare Science Centre, Melbourne School of Land and Environment, The University of Melbourne, Parkville Vic 3010.
[2] Animal Welfare Science Centre, Department of Physiology, Monash University, ClaytonVic 3800.

and poultry which sought to test the hypothesis that deprivation of a preferred resource results in disrupted biological function.

II. MATERIALS AND METHODS

The experiment was conducted in a temperature controlled shed located at Werribee, Victoria (38^{o}S). Birds were housed individually in cages (0.57m x 0.50m x 0.48m) and fed a commercial layer ration at maintenance level. Birds were kept in commercial conditions: at a constant environmental temperature of 21^{o}C and a light:dark cycle of 16:8 hours.

The experiment consisted of two parts. Part 1 investigated the choice behaviour of 48, 40 week-old laying hens, for a dust-bathing substrate (sawdust) vs. social contact (visual and tactile contact with another bird) in a series of Y-maze trials when they either had access to social contact or dust in the home cage (trials 1-7) or were deprived of social contact and dust (trials 8-14). The side of each resource in the Y-maze was randomized between birds but remained consistent for each individual over the 14 trials.

From the 48 birds studied in the first part of the experiment, 24 were selected for Part 2: 12 birds that chose social contact in the majority of Y maze trials (labeled "more social preferred" group of birds) and 12 birds that chose dust in the majority of Y maze trials (labeled "more dust preferred" group of birds). Birds from each preference group were allocated to a pair of adjacent individual cages separated from other pairs by a solid dividing wall. These pairs were then allocated to one of two restriction treatments: *Social restriction* which consisted of no tactile or visual contact with other birds but access to sawdust as a dustbathing substrate; or *Dust restriction* where the birds did not have access to dustbathing substrate but had visual and tactile contact with the neighboring bird (other member of the pair) through the wire-mesh sidewall.

The restriction treatments were imposed for 6 weeks. Catheterization of the jugular vein in week 6 allowed for the collection of serial blood samples (1.2 ml samples collected at 1-h intervals between 0800 and 1700 h using lithium heparin coated tubes to measure the average daytime corticosterone concentrations. To prevent hemorrhagic shock, red blood cells and saline were returned to the birds every 3 hours. An additional blood sample (1.2 ml) was collected at the 0800 h bleed using EDTA coated tubes and analyzed heterophil and lymphocyte counts from which we calculated the ratio between heterophils and lymphocytes. Two days later, an ACTH challenge was conducted by administering a single intra-muscular injection of synthetic ACTH (12.5 IU Synacthen, Ciba Geigy). Blood samples were collected prior to injection and again at 15, 30, 45, 60, and 90 min post injection. Blood samples were centrifuged and the plasma was stored frozen at –18°C until assayed for total corticosterone using a high sensitivity enzyme-immunoassay kit (Immunodiagnostic Systems Ltd. Boldon, UK)

The GLM function in PASW statistics 18 was used to analyse the stress physiology data for interactions between the main effects of restricted resource and preferred resource. Where required, data were log transformed.

III. RESULTS

Part 1: Overall, birds chose social contact in 54% of Y maze trials. However there was considerable variation between birds in their choice behaviour (Figure 1). The 12 birds with the highest choice of social contact in the 14 trials were selected (labeled

"more social preferred" and chose social contact on average in 83.3% of trials) and the 12 hens with the highest choice of dust were selected (labeled "more dust preferred" and chose dust on average in 81.0% of trials).

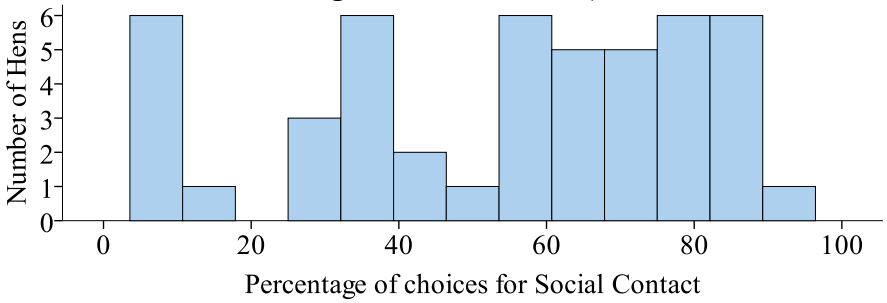

Figure 1 Histogram of the percentage of choices for social contact

Part 2: There were no main effects of restriction (P = 0.179) or preference (P = 0.535) on the average daytime corticosterone concentrations (Table 1). Nor were there any significant interactions observed between the main effects of restriction and preference when analyzing the average daytime corticosterone concentrations (P = 0.184) or the maximum corticosterone response to ACTH stimulation (P = 0.608). However, restriction of social contact regardless of preference did significantly increase the maximum corticosterone response to ACTH stimulation (P = 0.034) suggesting heightened sensitivity of the adrenal gland in socially restricted animals. White blood cell counts, in particular the heterophils and lymphocytes, were also affected by restriction treatment with the H:L ratio being significantly higher in birds restricted of social contact (P = 0.011). There were no interactions between the main effects on the H:L ratio (P = 0.196).

Table 1 Main effects of restriction and preference (mean ± s.e.) on stress physiology

Variable	Restriction		Preference	
	Dust	Social	Dust	Social
Average Daytime Corticosterone [1]	0.20 ± 0.03 (0.59)	0.26 ± 0.03 (0.82)	0.22 ± 0.03 (0.66)	0.24 ± 0.04 (0.74)
Peak Corticosterone response to ACTH	27.32 [a] ± 1.25	32.32 [b] ± 1.69	30.29 ± 1.46	29.35 ± 1.51
H: L ratio	7.81 [a] ± 3.13	21.32 [b] ± 3.43	11.93 ± 2.80	17.21 ± 3.70

[1] log +1 transformed, back transformed means in parentheses
Values with different superscripts within rows differ a,b: P < 0.05

IV. DISCUSSION

While further research is clearly required, the apparent difference between individual birds in their preferences for social contact and sawdust is unexpected. If this difference in choice behaviour is a real effect, it also raises some interesting questions about its genetic and/or experiential basis. Furthermore, if this is a real effect, these results may have important implications for animal welfare. One interpretation, for example, is that individual laying hens may differ in their long-term choice behaviour for social contact or dust and thus may also differ in their welfare requirements in relation to these two resources, which is the focus of this present research.

The results of this research do not support the hypothesis that deprivation of a highly preferred resource disrupts biological functioning. Evidence to support this hypothesis would be the finding of a significant interaction between the main effects of restricted resource and preferred resource. That is, deprivation of the more preferred resource rather than the less preferred resource disrupts biological functioning. A limitation in this study in testing this hypothesis is that the absolute strength of preference of individual birds for the more preferred resource is unknown: it is possible that birds in either group may have had at the most only a moderate preference for their more preferred resource. Previous work using a similar design in pigs has demonstrated that growing pigs deprived of a highly preferred resource (social contact or feed) gained significantly less weight and tended to have a higher average daytime free cortisol concentration than those deprived of a non preferred resources (Stevens et al 2009). In addition, other studies in poultry indicate there is a relationship between choice behaviour and physiological indices of welfare (Nicol et al 2009). The present study did elucidate some interesting results. Both heightened corticosterone response to ACTH (Dantzer and Mormède 1983) and elevated H:L ratio (Gross and Siegel 1983), as were seen in socially isolated birds, have been shown to be indicators of stress. This suggests, based on the biological functioning approach, that the welfare of birds in the present study may be challenged when socially isolated, regardless of the preferences they displayed in the Y maze. Further analysis of behavioural data collected during the restriction phase may expand our understanding of the results in light of considering the relationship between these two welfare methodologies.

ACKNOWLEDGEMENTS

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A RETROSPECTIVE STUDY OF THE IMPACT OF INJURIOUS PECKING ON STRESS RESPONSE IN HENS, MEASURED VIA EGG CORTICOSTERONE

G.M. CRONIN[1,2], J.A. DOWNING[1], T.H. STOREY[3], S.S. BORG[2], B.N. SCHIRMER[2] and J.L. BARNETT[3]

Summary

This paper describes the temporal change in egg corticosterone concentrations around a minor outbreak of injurious pecking by laying hens. The event occurred during an experiment in which we were measuring corticosterone concentrations in egg albumen at weekly intervals, as an indicator of physiological stress response. For the experiment, a total of 96 Hy-Line Brown birds were housed in two adjacent controlled environment rooms, enabling the imposition of different photoperiod treatments. Six identical 8-bird cages were used per room. Injurious pecking occurred in one of the two rooms, in two cages (situated back-to-back) containing birds aged 18-23 weeks. Affected birds were treated by swabbing the injured area(s) with Stockholm tar. Corticosterone concentrations were elevated in eggs from these cages, as well as the two abutting cages, even though those birds were neither handled nor treated with Stockholm tar. The findings suggest that the elevated corticosterone was elicited by aversive stimuli (stressors) experienced by the hens. It is also possible that disturbance caused by the stockpeople catching and treating birds in the affected cages, induced a stress response in birds in the abutting cages. In the more-distant cages in this room, and in the other room, corticosterone concentrations were not elevated.

I. INTRODUCTION

Recently, Downing and Bryden (2008) reported that corticosterone concentrations measured in egg albumen could be used as a convenient, non-invasive method of measuring physiological stress response in laying hens. Consequently, in a series of experiments investigating the importance of nest boxes for the welfare of laying hens in cages, we measured the physiological stress response of hens via egg albumen corticosterone assay, to assess bird welfare (Barnett et al., 2009; Cronin et al., 2007, 2010). During one experiment (Cronin et al. 2010), injurious pecking occurred in two cages situated back-to-back. Stockholm tar (Pharmachem, Australia) was applied to the wound(s) of injured birds as a deterrent to further pecking. All birds were monitored daily and any husbandry procedures applied to the birds were recorded in a diary, as required under the approved animal ethics protocol. As part of the experiment, we had also collected all eggs laid on one day per week (Friday), to assay for corticosterone. The objective of the present paper is to provide a retrospective description of the temporal change in egg corticosterone concentrations that occurred around the outbreak of injurious pecking and its subsequent treatment.

II. MATERIALS AND METHODS

The observations reported here occurred within an experiment involving a total of 96 Hy-Line Brown laying hens. The birds had been reared from day-old to 13 weeks in group cages in a controlled environment rearing shed, using conventional commercial lighting programs and nutrition. The birds were not beak trimmed. At 13 weeks the birds were placed at random

[1] Faculty of Veterinary Science, The University of Sydney, Camden NSW 2570 (present address)
[2] Animal Welfare Science Centre, Department of Primary Industries, Werribee VIC 3030
[3] Animal Welfare Science Centre, The University of Melbourne, Parkville VIC 3010

in groups in Victorsson 8-bird cages measuring 1.2m x 0.5m, in one of two controlled environment rooms. The experiment investigated the effects of two photoperiod treatments on egg laying patterns and stress response in laying hens (see Cronin et al., 2010). Each room contained a bank of cages with ten cages per tier. The middle six cages in the top tier were used for the experiment and non-experimental birds were located in the end cages of the tier. Although photoperiod was manipulated, birds were exposed to the same duration of light per day in both rooms, increasing from 12 h at 15 weeks to 16 h at 23 weeks in 30 min increments weekly. The experiment involved exposing birds to a period of light during the night, inserted either gradually from 18 weeks (Room 1) or abruptly at 23 weeks (Room 2). The two treatments had been assigned at random to the rooms, and lighting was independently controlled in each room, providing ~20 lux at the level of the top tier.

All eggs laid each Friday were collected, numbered and taken to the laboratory where each egg was weighed whole before being broken to separate the albumen from the yolk. The albumen was weighed then frozen for later analysis of corticosterone concentrations using the method developed by Downing and Bryden (2008). Total corticosterone concentrations were assayed using a commercial diagnostic kit (ICN ImmuChem Double antibody RIA, Seven Hills, NSW). Analysis of variance (GenStat 10.1, Lawes Agricultural Trust) was used to examine differences due to room (i.e. the light introduction treatments) on hen day egg production per week and egg albumen corticosterone concentrations. The experimental unit was the cage of birds and the analyses were blocked on cage.

III. RESULTS and DISCUSSION

Maximum hen day egg production was reached by the birds in the two rooms by 20 weeks of age (Figure 1) and there was no effect of room on this variable. Egg albumen corticosterone concentrations however, differed between the rooms in weeks 20 and 21 (Figure 1). At 20 and 21 weeks of age, eggs collected in Room 1 had higher corticosterone concentrations than Room 2 (20 weeks: 1.134 and 0.907 ng/g, respectively, $P<0.001$, sed 0.0456; 21 weeks: 0.751 and 0.670 ng/g, respectively, $P=0.015$, sed 0.0277). In other weeks there were no differences.

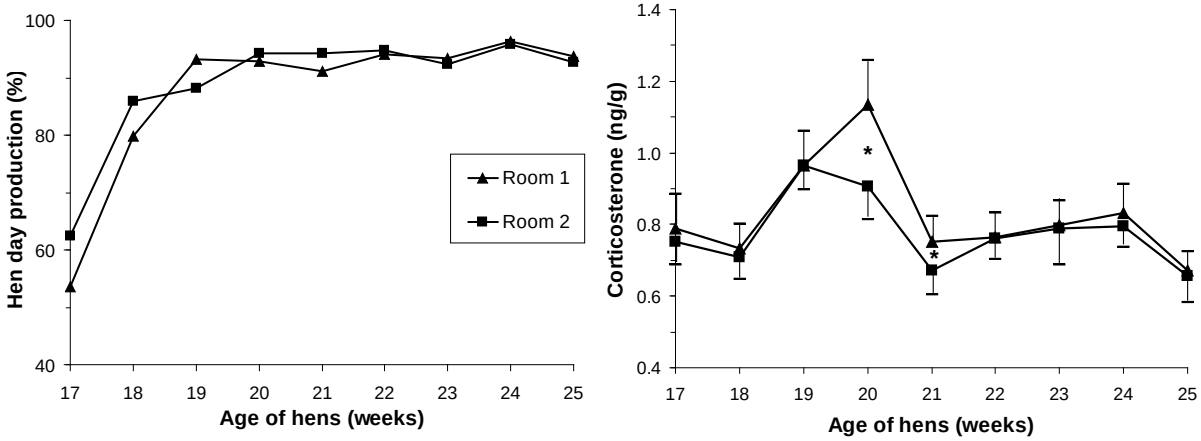

Figure 1 Changes in hen day egg production (left) and mean corticosterone concentrations in egg albumen (right) from cages in Rooms 1 and 2. There were 6 cages per room and 8 birds per cage. * indicates significant difference within weeks ($P<0.05$).

A retrospective investigation was conducted to identify events that may have elevated corticosterone concentrations in the hens in Room 1 compared to Room 2. Each room had a

daily diary and the records showed that injurious pecking had occurred in two cages in Room 1, *viz*. cages 1 and 6 (located back-to-back). Affected birds were treated with Stockholm tar applied to wounds. The Room 1 diary indicated that at 18 weeks, one bird in cage 1 had been pecked and was treated. At 19 weeks, pecking occurred in cage 6, and treatment with Stockholm tar was required on eight occasions over the subsequent 3 weeks. On the fifth treatment day, all birds in cage 6 had been pecked and thereafter all birds in the cage were treated. At 22 weeks, pecking occurred again in cage 1, and Stockholm tar was applied on two days, coinciding with the final two dates of treatment for cage 6.

Figure 2 shows the changes in the mean, maximum and minimum egg corticosterone concentrations for two cages in different rooms, recorded weekly around the time of the injurious pecking event. Cages 6 and 7 were in identical locations relative to the position of the entry door and within the bank of cages, in the two rooms.

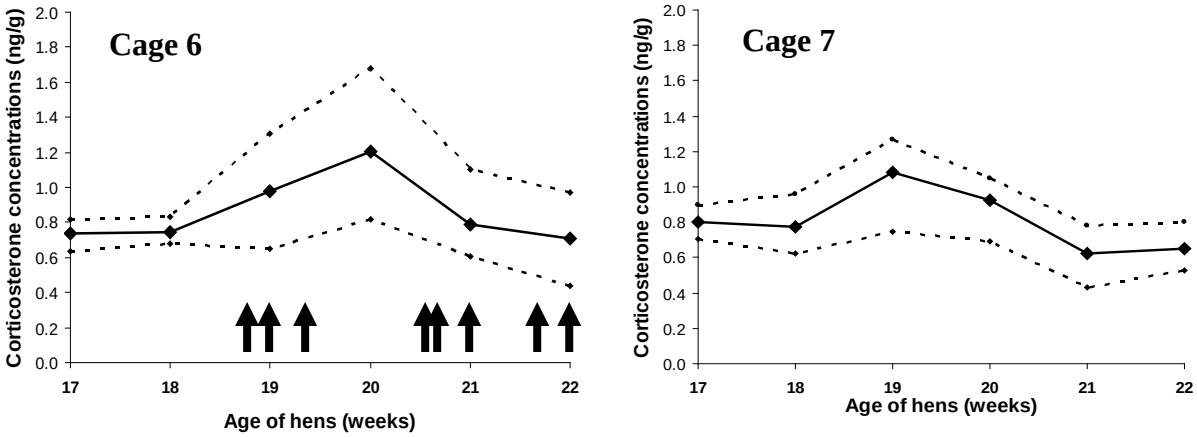

Figure 2 Change in corticosterone concentrations in eggs from cage 6 (Room 1) and cage 7 (Room 2). The solid lines represent the cage mean and the upper and lower dashed lines, respectively, represent the maximum and minimum values. Arrows indicate when birds in cage 6 were treated with Stockholm tar. From the fifth treatment onwards, all eight birds in cage 6 were treated.

Mean corticosterone concentrations in eggs collected from each cage in week 20 are shown in Figure 3. The column positions represent cage location in the rooms. Corticosterone concentrations appear higher from eggs in cages 6, 5, 1 and 2 than cages 3 and 4. In addition, corticosterone concentrations were lower for all cages in Room 2 than Room 1.

Injurious pecking is assumed to be painful in hens and the threat of being pecked may also be fear-provoking. Both situations are likely to elicit a physiological stress response. For example, Campo et al. (2008) reported that 20-week old pullets that were vent pecked showed signs of stress compared to birds that were not vent pecked. In the present retrospective investigation, the elevated corticosterone concentrations recorded in eggs from birds aged 20-21 weeks in Room 1, appear to have been associated with the injurious pecking event. There were no other records in the shed diaries that would lead us to suggest there were other contributory factors. Interestingly, although the injurious pecking and its treatment only occurred in two cages (1 and 6) in Room 1, elevated corticosterone responses were also detected in the eggs of hens from the abutting cages (cages 5 and 2). It is likely that the presence of stockpeople catching and treating hens in cages 1 and 6 also contributed to the elevated stress response in hens in the abutting cages. Human behaviour in the vicinity of caged laying hens is known to affect both behavioural (fear) and physiological (corticosterone) responses to stress. For example, Barnett et al. (1994) found that hens exposed to 'additional' visual contact with stockpeople performing slow and predictable

movements in front of their cages had reduced fear of humans and lower corticosterone. However, in the present retrospective study, the combination of extraordinary visual, auditory and olfactory stimuli associated with capture of birds, their removal from the cage and treatment with Stockholm tar, appears to have been sufficiently aversive to elicit elevated corticosterone responses, even in neighbouring cages in which birds were not treated.

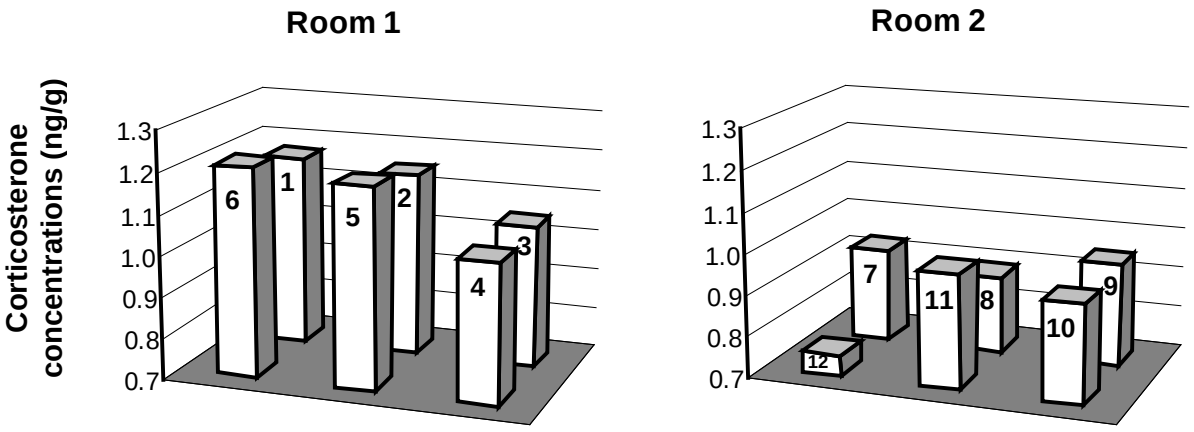

Figure 3 Mean corticosterone concentrations (ng/g) in eggs collected from each cage, when the hens were 20 weeks old. The position of the columns represents the location of cages within rooms, with cage numbers identified on the respective columns.

IV. CONCLUSIONS

Corticosterone concentrations were elevated in eggs from two cages in which injurious pecking and associated treatment occurred. In addition, responses were measured in neighbouring cages, even though those birds were not injuriously pecked, handled or treated with Stockholm tar. The findings thus suggest that aversive stimuli (stressors) experienced by laying hens elicited elevated corticosterone concentrations in eggs. It is also likely that disturbance caused by the stockpeople catching and treating birds in the affected cages, induced a stress response in birds in the abutting cages, even though the birds were not handled or treated. In the more-distant cages in this room, and in the other room, corticosterone concentrations were not elevated. As reported by Downing and Bryden (2008), egg albumen corticosterone provides a practical, non-invasive means to measure stress hormone concentrations in birds. The short term elevation in corticosterone detected in response to the injurious pecking event suggests that the technique is sensitive enough to detect changes in stress physiology relevant to welfare assessment in birds.

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SORGHUM GRAIN STARCH DIGESTIBILITY: EFFECTS OF PARTICLE SIZE AND ENZYME TREATMENT

M. J. GIDLEY[1], A. M. PLUSCHKE[1], P.A. SOPADE[1], G.J.S. AL-RABADI[1], A. SULTAN[2], C.Y. GAN[2], X. LI[2], D. ZHANG[1,2] and W.L. BRYDEN[1,2]

Summary

Sorghum has many attractions as a feed ingredient for broilers, including a high energy content and a competitive price compared with other grains available in Australia. However, these advantages are not always realised due to limitations in starch (and protein) digestibility and the presence of anti-nutrients such as phytate and tannins. In this review of recent work, we show how these two features can be partly overcome by (1) control of particle size of milled grains, and (2) addition of enzymes. A combination of *in vitro* starch digestibility and *in vivo* growth performance can be used to delineate the roles of specific factors in controlling (particularly) starch digestion rates.

I. INTRODUCTION

The utilisation of sorghum as a feed for broilers presents a number of challenges based on both variability between harvests and inherent properties of the grain (Black et al, 2005; Selle et al., 2010). These limitations can be traced back to the fact that sorghum grains lack the husk characteristic of most other grains. Biological requirements for seed survival in the absence of a protective husk or other coating include limiting attractiveness to insects, fungi etc prior to germination. In order to accomplish this, the macronutrients (starch and protein) are very tightly packed together within a robust cellular structure bounded by cell walls containing cellulose and glucuronoarabinoxylans, and the grain contains potential anti-nutrient components such as phytate and tannins.

Compared to other cereal grains, sorghum is considered to be relatively slowly digested in monogastric animals, an effect that has been variously ascribed to the influence of a tight packing of starch granules with kafirin protein bodies, and the presence of anti-nutrients (Black et al, 2005; Ezeogu et al, 2005; Wong et al., 2009; Correia et al., 2010; Selle et al., 2010). In order to enhance the digestibility of sorghum, two main approaches have been taken. One involves investigation of different processing conditions, particularly heat / moisture processing such as conditioning (Abdollahi et al., 2010) or extrusion (Mahasukhonthachat et al., 2010a). Whilst these have the benefit of opening up the grain structure to enhance access of digestive enzymes, there is a risk that protein digestibility may decrease due to disulphide bond rearrangements mediated by heat and moisture (Taylor, 2005). However, low moisture processes can minimise these rearrangements (Mahasukhonthachat et al., 2010a). Current evidence also suggests that increasing starch digestibility may not always be beneficial for broiler performance as it can lead to nutrient asynchrony if protein digestion lags behind starch digestion (Enting et al, 2005), particularly as specific amino acids such as arginine have been implicated as being growth limiting (Black et al, 2005). On the other hand, if starch (and protein) digestion is slow or incomplete this will affect the efficiency of weight gain. In terms of starch digestibility, it is of interest to understand the mechanisms driving the rate of starch digestion in order to design rational strategies for manipulation or selection of both grains and processing conditions.

[1] Centre for Nutrition and Food Sciences, The University of Queensland, St. Lucia, QLD 4072
[2] School of Animal Studies, The Unviersity of Queensland, Gatton QLD 4343

II. PHYSICAL FACTORS AFFECTING SORGHUM GRAIN STARCH DIGESTION

Isolated sorghum starch granules are similar to maize (corn) and other cereal starch granules in having a so-called slow-digesting property (Zhang et al, 2006). This is reported to be due to the restrictions of amylase action on a solid substrate, counter-balanced by a porous granule structure that allows access of amylase to the interior of the granule (Benmousa et al, 2006). 'Slow'-digesting is a relative term compared with e.g. soluble starches which are 'rapidly'-digested. In pigs and humans, cereal starch granules (excluding high amylose types) are essentially completely digested in the small intestine i.e. they have negligible resistant starch contents (Bird et al, 2009). However, in broiler feeds, sorghum starch is fed in the form of processed grain fragments in which the starch is tightly packed with protein bodies within endosperm cells. Therefore two factors that could control starch digestion rates are the granular vs gelatinised form of starch and the presence of physical barriers to amylase access caused by proteins and/or cell walls. Amylose contents in sorghum starches are typically less than 30%; higher amylose contents may affect digestibility after moist heat treatment.

In vitro analysis of starch digestion rates typically shows a major increase after disruption of grain structure and gelatinisation of starch by e.g. extrusion of sorghum (Mahasukhonthachat et al., 2010a). However, *in vivo* response to thermal processing of sorghum appears to be more complex with probable trade-offs between digestion rates and pellet quality parameters (Abdollahi et al., 2010). More detailed studies are needed to define the materials and molecular properties of thermally processed sorghum from different regions of the digestive tract in order to better understand the interaction between processed grain properties and broiler nutrition.

The effect of particle size of milled grains (sorghum and barley) on amylase digestion rates has recently been studied *in vitro* (Al-Rabadi et al., 2009). Single pass hammer milling through a 4 mm screen was shown to lead to a wide range of constituent particle sizes (Fig 1).

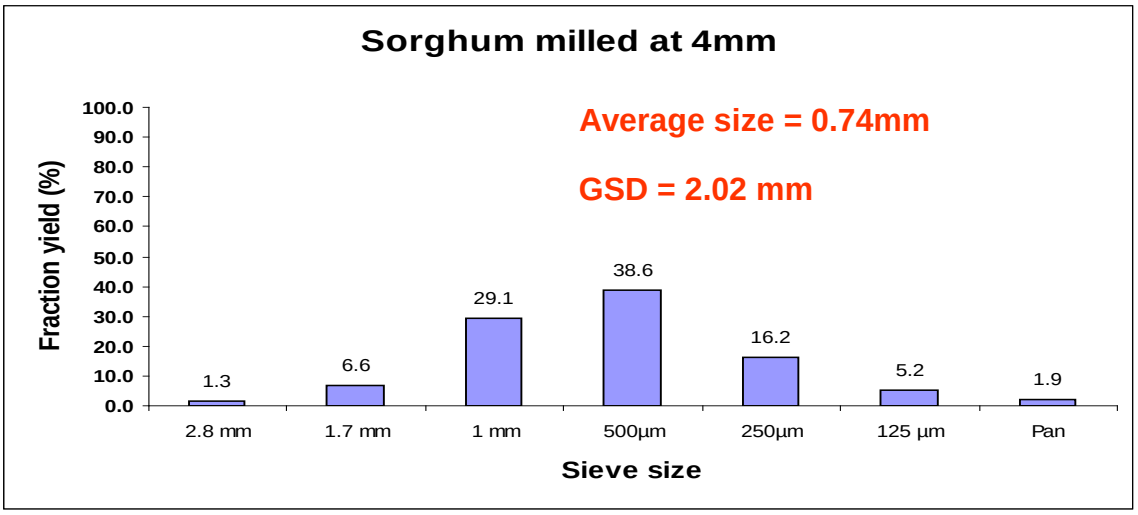


Figure 1 Particle size distributions for a typical sorghum grain (cv Buster) hammer milled though a 4 mm screen. GSD = geometric standard deviation.

The state of each of the particle size fractions is shown in Figure 2, which illustrates that a single pass through a hammer mill results in particles ranging from essentially whole grains to a fine powder.

Starch digestion profiles for each of these size fractions compared with the non-fractionated hammer milled sample are shown in Figure 3, demonstrating a major effect of

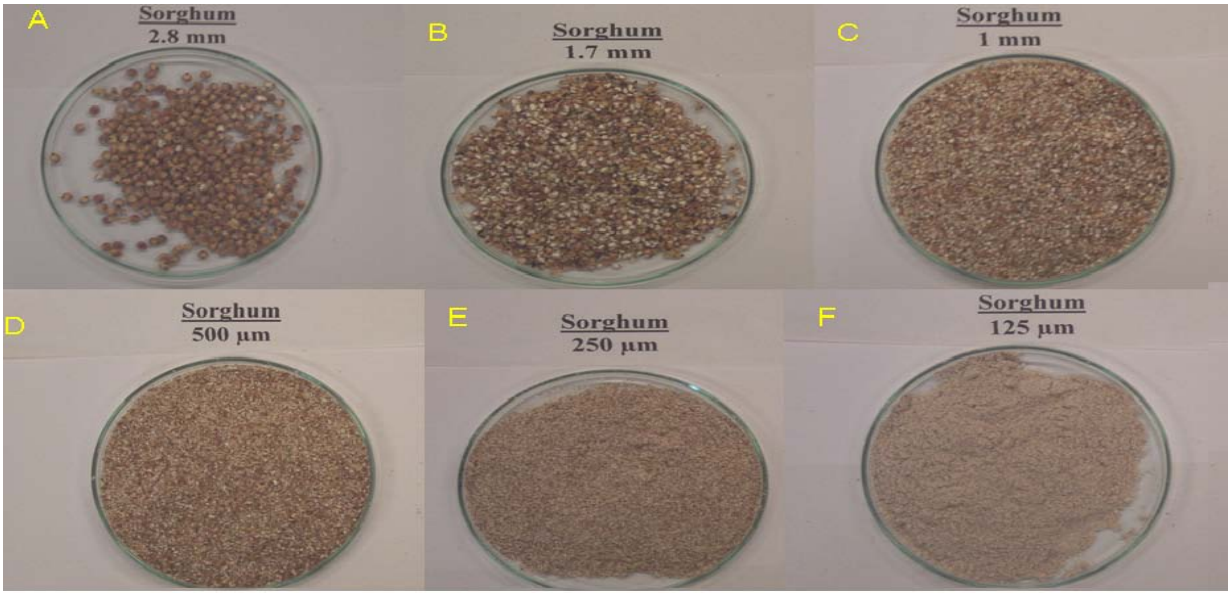


Figure 2 Major fractions separated from sorghum grains (cv Buster) hammer milled through a 4 mm screen

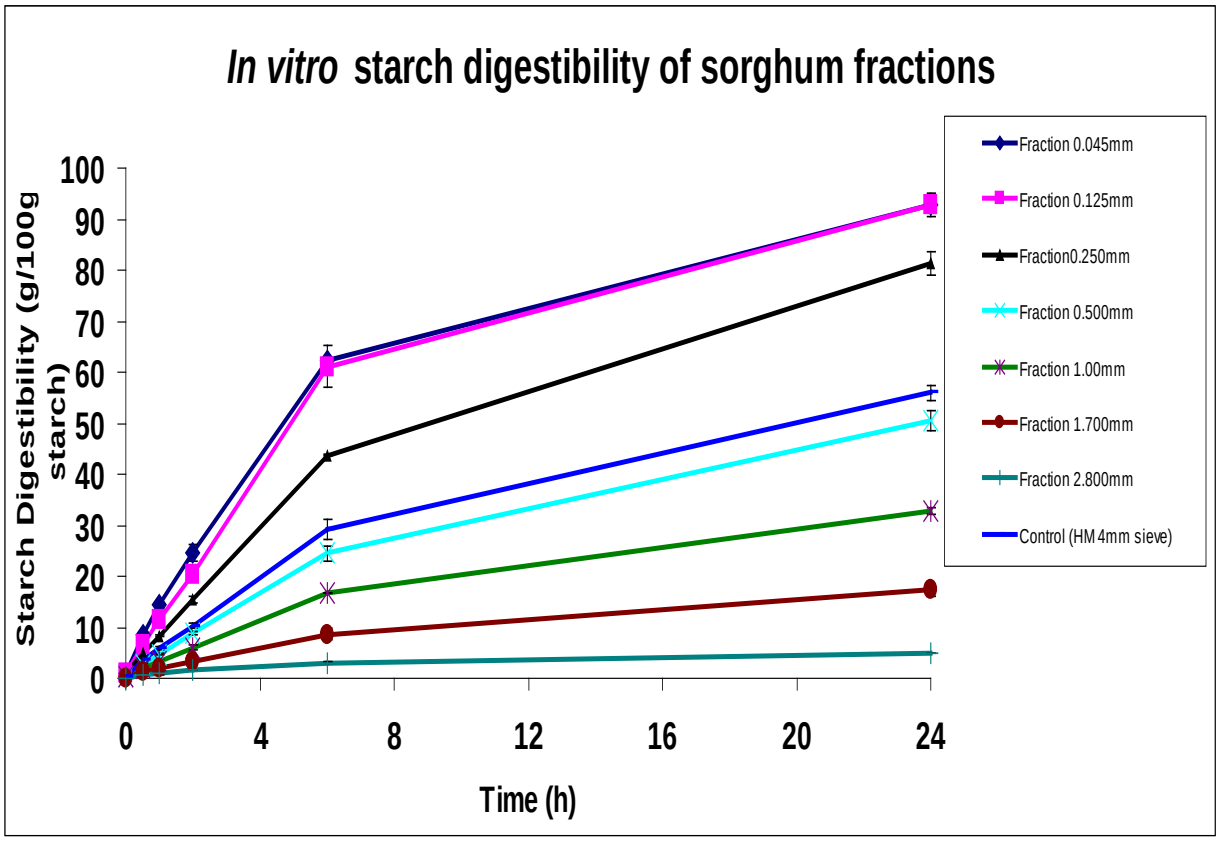


Figure 3 Time course of glucose release from size fractions of hammer-milled sorghum grain (cv Buster) show increasing digestion rates for fractions captured on smaller sieves with non-fractionated material having similar kinetics to the 0.5 mm fraction.

particle size. Similar effects of average particle size for variably-milled (non-fractionated) samples on these digestion profiles (or digestograms) were found and shown to conform to first order kinetics, and not to the Michaelis-Menten kinetics usually associated with enzyme-catalysed reactions (Mahasukhonthachat et al, 2010b). The first order nature of the digestion kinetics is important for two reasons. First, it suggests that access of enzyme to substrate is rate-limiting as Michaelis-Menten kinetics assumes that enzyme-substrate complexes are formed rapidly. Secondly, rate coefficients can be calculated, e.g. from the data shown in Figure 3, and used to show that these increase with the inverse square of particle size (i.e. doubling particle size quarters the digestion rate) for fractionated samples (Al-Rabadi et al, 2009) with a similar relationship inferred for average particle sizes from non-fractionated milled sorghum grain (Mahasukhonthachat et al, 2010b). An inverse square relationship could suggest surface area control in which reaction rates are dependent on the breaking of a surface 'skin'. However, there is no physical reason for proposing this mechanism as micrographs show surfaces including exposed starch granules which should be readily attacked by amylase. The alternative explanation for the inverse square dependence is that enzyme diffusion is rate-limiting. This can be tested by calculating an apparent diffusion coefficient from the enzyme kinetics which is about 13 times slower than that expected for the enzyme in aqueous solution (Al-Rabadi et al, 2009). This is an entirely reasonable quantitative result and provides strong support for enzyme diffusion being rate-limiting in the digestion of starch in milled (sorghum) grains. The calculated enzyme diffusion rate in milled sorghum grains is about 50% slower than in milled barley grains, confirming that the dense packing of starch and protein within sorghum endosperm cells is a restrictive environment for enzyme diffusion.

Extrapolation of these findings to starch digestion in broilers is complicated by the efficient grinding of milled grains in the gizzard of the bird. Thus, unlike other monogastric animals such as humans or pigs, the particle size distribution of the constituent particles in feed may be altered significantly prior to digestion. Whilst the kinetic analysis outlined above should be expected to apply, the challenge is to measure the actual particle sizes in the digestive tract after grinding by the bird. Studies of feed particle size effects (Amerah et al, 2007) have shown inconsistent results, partly because of the difficulty in tracking diverse particle sizes through the digestive tract. Whereas fine particles are expected to result in more rapid energy availability, the active grinding of grains in the digestive tract means that larger particles may be reduced in size. There is also evidence that active grinding of larger particles may help to develop the digestive system. Current evidence suggests that there is an optimum average particle size for gizzard development of between 600 µm and 900 µm which subsequently impacts on grinding activity, gut motility and nutrient digestion (Amerah et al, 2007). In the future, measurement/reporting of particle size fraction distributions rather than an average value may help to identify the effects of specific particle size fractions on digestible energy availability.

III. ENZYME TREATMENTS TO ENHANCE STARCH DIGESTION

Based on the discussion above, it can be hypothesised that starch digestibility in milled sorghum grains could be enhanced by enzyme treatments that reduce the physical barriers to access of amylase. Thus enzymes that help to degrade cell walls and protein bodies such as xylanases and proteases respectively might be expected to aid starch digestion. In addition, the role of anti-nutrients such as phytate and tannins may be important. These grain components have the potential to bind with digestive enzymes or co-factors thereby limiting starch (and protein) digestion *in vivo*. Commercial phytases are available to reduce phytate levels by hydrolysing phosphate substituents and have the added benefit of making more

phosphorous and other minerals such as calcium, iron and zinc available to the bird and thereby reducing phosphate excretion and enhancing the utilisation of other minerals (Selle et al, 2000). Tannin-degrading enzymes are less widely applied, but as the tannin level in Australian sorghums is relatively low (Selle et al, 2010) may not be as critical.

Most enzyme trials report positive effects on starch / protein digestion and broiler performance of xylanase, protease and phytase enzymes both in isolation and in combination. For example, ileal digestibility coefficients for starch of 0.75 are significantly improved by each of xylanase, protease and phytase treatments (Table 1), and this correlates with fractional starch digestion rates, determined in separate experiments by analysis of residual starch contents at different points in the digestive tract (Sultan et al, 2011a,b).

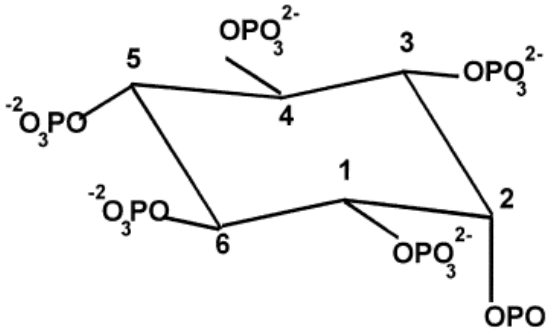
Treatment	Ileal starch digestibility coefficient	Fractional starch digestion rate (h^{-1})
Control	0.75^{c}	2.85^{c}
Xylanase	0.83^{b}	3.08^{bc}
Phytase	0.86^{ab}	3.63^{ab}
Protease	0.83^{b}	3.66^{a}
Xyl + Phytase	0.89^{a}	
Xyl + Protease	0.86^{ab}	
Phyt + Prot	0.88^{a}	
Xyl +Phyt +Prot	0.87^{a}	
Pool SEM	0.01	0.183

Table 1 Effect of single and combination enzyme treatments on starch ileal digestibility coefficient and of single enzymes on fractional starch digestion rate in broilers. Superscripts denote statistical significance ($p<0.05$). Data from Sultan et al, 2011ab. Xyl = xylanase; Phyt = phytase; Prot = protease.

Evidence for the mechanism behind the effective action of cell wall degrading enzymes (e.g. xylanase) and protease comes from the work of Perez-Carilloa and Serna-Saldivarb (2006) who showed that extractability of starch from sorghum was improved by prior action of these enzymes (Perez-Carilloa and Serna-Saldivarb, 2006). If starch extraction is enhanced by enzyme action, then amylase action would also be expected to be enhanced as the amylase would be able to more rapidly diffuse to the starch and therefore speed up digestion rates.

Positive effects of phytase on starch digestion are possibly due to complexation of phytate with either amylase or calcium (an amylase co-factor), thereby limiting enzymic starch digestion. Another proposed mechanism is the direct interaction of phytate with starch thereby limiting enzyme digestion (Rickard and Thompson, 1997). These possible mechanisms are supported by early work which showed that phytate added directly to *in vitro* starch digestion mixtures caused a decrease in digestion rates (Knuckles et al, 1987;

Thompson et al, 1987). However, phytate is located primarily in the aleurone layer and germ of the sorghum grain whereas the starch (and protein) is located primarily in the endosperm. Therefore, in order for phytate to exert an effect on starch digestibility in milled sorghum grains, either the phytate needs to be released from its cellular environment, or the amylase or calcium need to diffuse into cells containing phytate and be complexed there. In order to test these possible mechanisms, milled sorghum grains have been subjected to phytase treatment, with the level of phosphate released used as a measure of the phytase reaction which proceeds by stepwise removal of phosphate groups from the original hexaphosphate (Figure 4). Reactions were carried out such that between 20 and 70% of starting phosphate



Inositol hexakisphosphate (IP_6)

$$\text{Phytic Acid} + H_2O \xrightarrow{\text{Phytase}} \text{D - myo - inositol 1,2,4,5,6 - PKP} + P_i$$

Figure 4 Structure of phytic acid (inositol hexakisphosphate) and initial phytase reaction

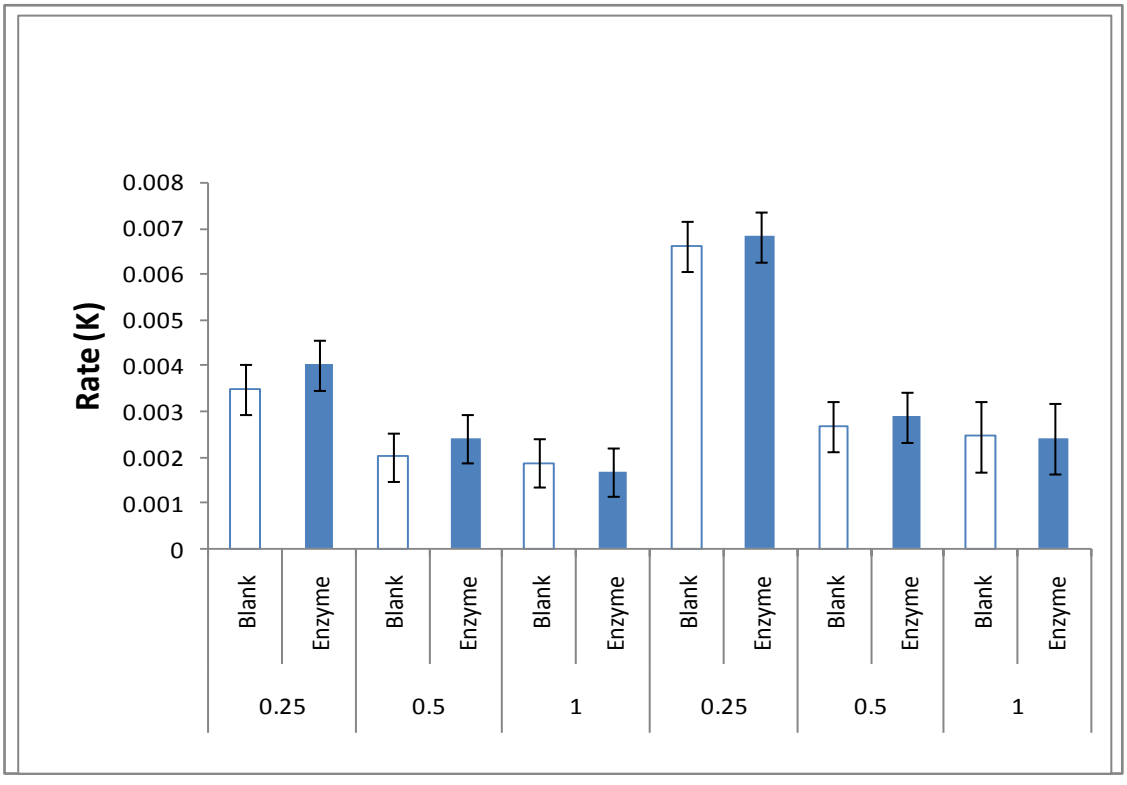


Figure 5 Starch digestion rates after phytase reaction (enzyme) compared to no phytase action (blank) for different particle size fractions – 0.25, 0.5, 1 mm for each of two sorghum cultivars, Buster and Liberty (Pluschke et al, in preparation).

levels were removed by phytase using a newly-developed *in-vitro* phytate digestion procedure. Enzyme-treated milled grains were then subjected to *in vitro* starch digestion. The results are shown in Figure 5 and demonstrate that prior reduction of phytate levels in milled grains does not affect the subsequent rate of amylase digestion. This is presumably because the phytate does not have a chance to interact with the amylase, starch or calcium as it is located within cellular compartments that do not contain starch. In the light of these results, it is interesting that phytase addition to sorghum-containing broiler feeds enhances starch digestion (Table 1; Selle et al., 2010). One possibility is that grinding of feed by the bird leads to sufficient disruption of aleurone cells that phytate is solubilised and able to complex with amylase or starch. Another possibility is that complexes of phytate with amylase, starch or calcium are not involved, and that effects on bird performance and digestion are due to a changed metabolic status from the greater availability of phosphorous and/or minerals.

IV. CONCLUSIONS

A high energy content coupled with the normally competitive cost compared with other grains makes sorghum an attractive feed component for broilers. Recent work has shown that control of particle size of milled sorghum grain can be used to alter starch digestion rates in a quantitatively predictable way *in vitro*, with the rate determining step being the diffusion of amylase through the densely-packed grain fragment. Treatments such as heat / moisture conditioning or extrusion processing may open up the grain and thereby enhance starch digestion, but run the risk of reducing protein digestibility through disulphide bond rearrangements that are dependent on process moisture. Enzyme treatments to hydrolyse cell wall polymers, proteins, and/or phytate are sufficiently mild to avoid protein rearrangement, and show positive effects in animal trials. There is scope for further work to quantify the effects of these enzyme treatments on their substrates *in vivo*, and to understand the opportunities for further enhancing sorghum usage as a broiler feed grain through control of grain cultivar and feed process conditions.

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THE PROTEIN QUALITY OF SORGHUM

P. H. SELLE[1]

Summary

Both concentrations and ileal digestibilities of amino acids in sorghum for poultry are highly variable. This "fragility" of protein quality in sorghum is recognised but it is probable that all contributing factors have not been properly identified. Kafirin (protein bodies) and glutelin (protein matrix) are the dominant protein fractions in sorghum endosperm and their relative proportions influence amino acid composition because of their divergent amino acid profiles. Kafirin constitutes the majority of sorghum protein but contains relatively low levels of lysine and, with the exception of leucine, essential amino acids. Moreover, kafirin is a poor source of digestible amino acids due to its hydrophobicity and the structure of protein bodies. Both kafirin and glutelin may impede enzymic access to starch granules in sorghum endosperm, thus any improvements in sorghum protein quality may also enhance energy utilisation.

I. INTRODUCTION

The review of grain sorghum protein by Duodu et al. (2003) is pivotal while Salunkhe et al. (1977) concluded earlier that sorghum has a greater range of protein/amino acid digestibility values than maize or wheat. Sorghum frequently constitutes part, or all, of the cereal grain base for chicken-meat diets in Australia, despite the perception that the growth performance of broilers offered sorghum-based diets is sub-optimal and usually inferior to xylanase-supplemented, wheat-based diets. Approximately 30% of protein in a complete diet is derived from sorghum and the likelihood is that the 'quality' of protein/amino acids in sorghum contributes to inconsistent growth performance. Both concentrations and digestibilities of amino acids in sorghum are variable, probably to a greater extent than in other cereal grains. The dominant sorghum protein fractions are kafirin and glutelin, which are both located in the endosperm; however, kafirin is not an ideal source of amino acids due to its hydrophobicity, disulphide linkages and low lysine content. There is the suggestion that exogenous proteases, with the capacity to degrade kafirin, may enhance amino acid digestibility in sorghum-based diets. The likelihood is that, as sorghum protein concentrations increase, the kafirin proportion of protein is elevated at the expense of glutelin, which alters amino acid concentrations including reductions in lysine. The *in vitro* pepsin digestibility of sorghum protein is uniquely vulnerable to 'moist-heat', which raises the possibility that steam-pelleting sorghum-based broiler diets at high temperatures may be deleterious. Sorghum invariably contains phytate, which depresses amino acid availability; however, this can be addressed, at least partially, by the dietary inclusion of phytate-degrading enzymes. If present, condensed tannin has the capacity to compromise the digestibility of protein/amino acids but the extent to which tannin is present in local, sorghum crops remains an issue. The intent of this review is to consider sorghum as a feedstuff for broiler chickens from the standpoint of protein with emphasis on the kafirin and glutelin fractions and their contribution to the nutritive status of poultry diets.

[1] Poultry Research Foundation, The University of Sydney, 425 Werombi Road, Camden, NSW 2570

II. STARCH AND PROTEIN

Any consideration of starch digestibility and energy utilisation of sorghum is peripheral to this paper; nevertheless, kafirin and glutelin are intimately associated with starch in sorghum endosperm. Broiler chickens have been reported to utilise energy more efficiently on wheat- than sorghum-based diets (Black et al., 2005) and this may be a crucial difference between the two cereal grains. Kafirin may depress energy utilisation in poultry as significant negative correlations between kafirin, as a proportion of protein, with apparent metabolisable and nitrogen-corrected true metabolisable energy have been reported in caecectomised roosters (Salinas et al., 2006). These findings draw attention to the likelihood, as discussed by Wong et al. (2009), that kafirin and/or glutelin can impede the digestibility of starch with the implication that poor protein quality depresses energy utilisation in sorghum.

III. SORGHUM PROTEIN

Of the dominant fractions, kafirin is located in protein bodies and glutelin comprises the protein matrix of sorghum endosperm (Seckinger and Wolf, 1973). Virupaksha and Sastry (1968) found that kafirin constituted 54.1%, glutelin 33.4%, globulin 7.0% and albumin 5.6% of endosperm protein in five sorghum samples. Subsequently, Taylor et al. (1984) reported average concentrations of 54.0 g/kg kafirin and 27.1 g/kg glutelin in 41 samples with an average content of 111 g/kg protein; thus kafirin and glutelin comprised 48.0% and 27.7% of sorghum protein, respectively. However, kafirin, as a proportion of protein, was positively correlated ($r = 0.886$; $P < 0.005$) with sorghum protein concentrations; whereas, the proportion of glutelin was negatively correlated ($r = -0.401$; $P < 0.01$) as shown Figure 1. Conflicting data have been recorded, but it appears that the proportion of kafirin increases at the expense of glutelin with increasing sorghum protein levels. The amino acid profiles of kafirin and glutelin are dissimilar, thus their reciprocal relationship impacts on the amino acid composition of sorghum.

IV. KAFIRIN AND GLUTELIN

Kafirin concentrations are usually determined by the Landry and Moreaux (1970) method. From a review of six studies involving 88 sorghum samples, the average kafirin proportion of protein ranged from 37.5 to 72.9% around a mean of 49.3% (Selle et al., 2010a). Hamaker et al. (1995) reported a range of kafirin proportions from 49.0 to 54.7% in five sorghums using the classical method but a higher range of 68.1 to 72.9% using the alternative method of Wallace et al. (1990). Quantifying the variable concentration of kafirin in sorghum is not straightforward and this is reflected in the inconsistent outcomes that have been reported.

There are three kafirin classifications and, structurally in protein bodies, α-kafirin occupies the central core, while β- and γ-kafirin are located in the periphery. As reviewed by De Mesa-Stonestreet et al. (2010), α-kafirin is dominant (66-84%) relative to β- kafirin (8-13%) and γ-kafirin (9-21%); however, quite different proportions have been reported (Selle et al., 2010a). The amino acid composition of the three kafirin categories as determined by Watterson et al. (1990) and Shull et al. (1992) are shown in Table 1, where lysine levels are uniformly low, leucine is highest in α-kafirin and cystine levels are noticeably higher in β- and γ-kafirin.

Glutelin has received less attention than kafirin; however, Beckwith (1972) found that glutelin accounted for more than half the total protein in three sorghum hybrids where glutelin contained more cystine and basic amino acids than kafirin. It was considered that glutelin consisted of small protein units linked by disulphide bridges and its solubility was substantially increased from 23 to 87% by the reducing agent β-mercaptoethanol.

Both kafirn:glutelin ratios and the constituency of kafirin are determinants of the amino acid composition of sorghum. Importantly, Mosse et al. (1988) reported divergent amino acid profiles for kafirin and glutelin as shown in Table 2. Glutelin contains more essential amino acids (42.6 versus 37.1%) as kafirin is relatively deficient in arginine, histidine and lysine in particular but contains more leucine. If leucine is overlooked, glutelin contains substantially more essential amino acids (32.2 versus 19.7%) than kafirin. In the Mosse et al. (1988) study, the average protein content of 12 sorghum samples was 133.6 g/kg with a lysine content of 2.82 g/kg or 2.15% of protein. While absolute lysine and protein concentrations were positively correlated ($r = 0.887$; $P < 0.001$), when expressed as a proportion of protein, lysine was negatively correlated ($r = -0.880$; $P < 0.001$) with sorghum protein (Figure 2). This is entirely consistent with an increase in proportionate amounts of kafirin with increasing protein levels. The high leucine content in sorghum is noteworthy as excess leucine has been shown to compromise isoleucine and valine availability in poultry and leucine may also have the capacity to depress voluntary feed intakes (Morrison et al., 2007).

V. AMINO ACIDS IN AUSTRALIAN SORGUMS

Initially, Ravindran et al. (1998) surveyed the concentration and digestibility of amino acids in six local sorghums and subsequently Bryden et al. (2009a) analysed a further eleven samples; the combined results are summarised in Table 3. There are tangible variations in both concentration and digestibility of amino acids and it is noteworthy that arginine, lysine, methionine and tyrosine contents are not significantly correlated with protein concentrations. As a consequence, it is questionable if intended dietary amino acid specifications are being accurately met in the formulation of sorghum-based diets where amino acid concentrations are derived from sorghum protein contents. Presumably, this problem would be compounded when sorghum-based diets are being formulated on the basis of ileal digestible rather than dietary amino acids.

It is possible to compare data from the two surveys where the mean protein content of the six “1995” sorghums (98.7 g/kg) and the eleven “2005” sorghums (103.6 g/kg) were similar and there were no significant differences in apparent ileal digestibility (AID) coefficients between the two sub-sets. However, the 2005 sorghums contained significantly less lysine (1.89 versus 2.40 g/kg; $P < 0.015$) and arginine (3.32 versus 4.47 g/kg; $P < 0.005$) than the 1995 sorghums. Given that kafirin contains a paucity of lysine and arginine these reduced concentrations indicate that kafirin, as a proportion of total sorghum protein, was greater in 2005 than in 1995. Similarly, Horn and Schwartz (1961) determined the amino acid profiles of three sorghums, which may be compared with the “2005” sorghums. As percentages of protein, arginine, histidine and lysine contents were higher in the “1961” sorghums such that total basic amino acids were significantly greater (10.8 versus 7.1%; $P < 0.001$). Both comparisons, albeit limited, suggest that kafirin:glutelin ratios are higher in contemporary sorghums than in the past and lend weight to the opinion that “sorghum has changed”.

Perez-Maldonado and Rodrigues (2009) generated additional local sorghum amino acid digestibility data where there was a remarkable difference between consecutive harvests. The mean AID coefficient in the 2004 harvest (17 samples) of 12 amino acids was only 0.651; whereas, in the 2005 harvest (14 samples), the mean AID coefficient was 0.768. Thus the 2005 amino acid digestibility was 18.7% higher than 2004 (Table 4). The average crude protein contents in both harvests were effectively identical, there are curious variations in the concentrations of the amino acids and a minority of 2004 sorghums samples had extremely low digestibilities. Nevertheless, the average AID coefficients from both sorghum harvests (31 samples) were significantly correlated with breast meat yields ($r = 0.672$; $P < 0.001$), which suggests that the marked discrepancy in amino acid digestibilities was valid. An explanation for this marked difference may stem from my understanding that the 2005 harvest received substantially more rainfall than the previous harvest.

Water deprivation has been shown to decrease sorghum yields but increase their protein content (Eppendorfer et al., 1985) and decrease the solubility of amino acids and impact on amino acid profiles (Oliveira Neto et al., 2009). It has not been established if *in vitro* pepsin digestibility assays of sorghum are indicative of protein/amino acid quality in poultry. However, substantial variations in pepsin digestibility of raw and wet-cooked sorghums were reported by Buckner (1997) both between genotypes and, in a particular sorghum, across six harvests. Low pepsin digestibilities were associated with the extraction of a specific γ-kafirin fraction and higher average maximum temperatures during the growing seasons. There is a tenuous suggestion that high temperatures were associated with greater concentrations of β- and γ-kafirin and lower pepsin digestibilities. This raises the possibility that the very poor amino acid digestibilities in 2004-harvested sorghums may have been due to the negative impacts of both extreme temperatures and water deprivation on sorghum protein quality.

VI. SORGHUM GRAIN TEXTURE

The texture of grain sorghum is governed by the ratio of 'hard' (corneous/vitreous) to 'soft' (floury/opaque) fractions of the endosperm. Any consideration of sorghum texture is complicated by the various methods used to determine hardness. One method is the particle size index (PSI), which was originally developed to determine the hardness of wheat (Symes 1961, 1965). Under this method, a PSI of 8-12 is classified as "very hard" and a PSI of 20-30 as "very soft". De Alencar Figueiredo at al. (2006) determined the texture of 117 global samples of sorghum. The mean PSI was 13.6, which ranged from 8.2 to 25.7, but the dominant categories were 10 and 11. Recently, the Poultry Research Foundation completed a survey of 32 sorghums sourced from Queensland and New South Wales. The average PSI was 10.0 with a range of 8 to 14 but only one sample fell outside the "very hard" category. PSI values were negatively correlated ($r = -0.438$; $P < 0.02$) with protein contents which ranged from 83 to 116 g/kg, so increasing protein, and by implication kafirin, levels were associated with increasing grain hardness. This is not surprising, several studies have shown that increasing protein and kafirin concentrations are associated with harder sorghum textures and Watterson et al. (1993) found the kafirin proportion of protein is higher in the corneous (67%) than in the floury endosperm (35%).

Cabrera (1994) determined the impact of particle size of "hard" and "soft" sorghums prior to steam-pelleting (65ºC) sorghum-based diets on broiler growth performance from 7 to 28 days of age. Reductions in the mean particle size of hard

sorghum from 1000 to 400 µm enhanced weight gain (5.5%), feed intake (7.7%) and feed conversion (5.1%); interestingly, the reverse was true for broilers offered soft sorghum as their best performance was observed with the coarsest particle size. Given that the majority of local sorghums are "very hard" the Cabrera (1994) data indicates that fine grinding to a geometric mean diameter in the order of 400 µm would be appropriate. In this context, Mikkelsen et al. (2008) reported that finely-ground (and presumably very hard) sorghum supported marked improvements in growth performance in comparison to the same coarsely-ground sorghum; whereas, in contrast, the particle size of wheat was of little consequence.

VII. STEAM-PELLETING TEMPERATURES

The method of Mertz et al. (1984) is frequently used to determine pepsin digestibilities in sorghum and considerable weight is given to these assays. The *in vitro* pepsin digestibility of sorghum protein is notoriously vulnerable to 'moist-heat' and this phenomenon has been confirmed in poultry. Mitaru et al. (1985) boiled a 3 to 1 slurry of distilled water and sorghum for 50 minutes and this extreme exposure of sorghum to 'moist-heat' reduced the mean true ileal digestibility coefficients of 15 amino acids by 30.7% (0.629 versus 0.908). Also, Selle et al. (2010c) mixed equal parts of distilled water and finely-ground sorghum for 2 hours at 45ºC, which was then dried at 60 ºC for 70 hours and blended into sorghum-casein diets. In comparison to unprocessed, 'raw' sorghum, exposure to 'moist-heat' significantly reduced weight gain (20.0%), feed intake (21.4%), feed efficiency (4.5%) and nitrogen (N) retention by 8.5% (0.574 versus 0.627) but not AME in broilers. The reduction in N retention implies that 'moist-heat' compromised protein digestibility and perhaps, in addition, differences in grain texture contributed to reductions in feed intake and both factors drove the depression in weight gain.

The vulnerability of sorghum protein digestibility to 'moist-heat' is mainly attributed to the formation of disulphide cross-linkages in β- and γ-kafirin in the periphery of protein bodies and this "case-hardening" impedes the digestion of the centrally located α-kafirin fraction. Interestingly, Emmambux and Taylor (2009) reported that wet-cooking reduced kafirin digestibility by 24% (0.576 versus 0.760) but wet-cooking reduced the digestibility of sorghum protein by a similar magnitude of 27%, (0.599 versus 0.826). This implies that the balance of sorghum protein, which is mainly glutelin, may be as equally vulnerable as kafirin to moist-heat.

Increasing attention is being paid to the possibility that elevated steam-pelleting temperatures of broiler diets have a deleterious influence broiler performance. As suggested by Taylor (2005), there is the possibility that steam-pelleting sorghum-based diets at high temperatures may constitute sufficient 'moist-heat' to compromise the nutritive properties of sorghum. It is relevant that increasing wet-extrusion temperatures for sorghum from 105 to 132.5ºC has been shown to depress broiler performance (Zhuge et al., 1990). However, there appears to be an intriguing conflict, on one hand, high steam-pelleting temperatures may have the potential to reduce protein solubility and digestibility but, alternatively, high steam-pelleting temperatures are associated with better quality and harder textured pellets. The merits of pellets with higher breaking forces have been demonstrated by Parsons et al. (2006). It may be possible to produce hard pellets at low temperatures with the use of binding agents but this approach needs to be economically viable and hard-textured pellets may be of lesser importance under whole grain feeding regimes.

VIII. PHYTATE

Sorghum invariably contains phytate (*myo*-inositol hexaphosphate) and it is generally accepted that phytate negatively influences protein utilisation in poultry (Selle et al., 2000; Selle and Ravindran, 2007). Indeed, sorghum contains somewhat higher phytate concentrations than other cereal grains (Doherty et al., 1982; Selle et al., 2003) and this results in comparatively low protein:phytate ratios. That phytate negatively influences protein availability is reflected in the Ravindran et al. (1999) study where 1200 FTU/kg *Aspergillus niger* phytase increased mean AID coefficients of 14 amino acids by 6.46% (0.791 versus 0.743) in a low protein (73.3 g/kg) sorghum. The low protein content of the sorghum used in this study probably indicates a relatively small kafirin proportion of total protein. The adverse impact of phytate on protein quality in sorghum may be addressed partially by the dietary inclusion of phytate-degrading enzymes, which is an increasingly common practice. There is the impression, however, that the 'extra-phosphoric' responses to phytase in sorghum are muted in comparison to other grains (Wu et al., 2004). In another study (Selle et al., 1999), growth performance and nutrient utilisation responses to phytase in sorghum-based broiler diets were confined to diets with reduced specifications rather than standard diets. If electrostatic attractions between arginine, histidine, lysine residues and polyanionic phytate molecules are crucial to protein-phytate complex formation it follows that phytate would not bind kafirin readily due to its paucity of basic amino acids. This may in turn limit 'extra-phosphoric' responses to exogenous phytases in sorghum-based diets.

IX. "NON-TANNIN PHENOLIC COMPOUNDS"

Sorghum, unlike maize and wheat, contains an abundance of polyphenolic compounds and concentrations in excess of 100 g/kg have been reported (Bravo, 1998). "Bird-proof" sorghums contain condensed tannin, which is distinguished from other polyphenolic compounds by its capacity to bind and precipitate protein (Spencer et al., 1988). That condensed tannin depresses broiler performance is established (Nyachoti et al., 1997) as is the capacity of sorghum tannin to reduce protein/amino acid digestibilities in poultry (Rostagno et al., 1973; Mitaru et al., 1985).

Perez-Maldonado and Rodrigues (2009) reported the presence of condensed tannin in 2004-2005 sorghums in Australia using the butanol-hydrochloric acid analytical method of Dalzell and Kerven (1998). However, the complexities of tannin analysis should not be overlooked (Hagerman and Butler, 1989) and the butanol-hydrochloric acid assay has recognised limitations (Schofield et al., 2001). One contention is that the genotypes of contemporary Australian sorghums preclude the presence of condensed tannin, which is reflected in the absence of a pigmented testa (Bryden et al., 2009b) and earlier, Walker (1999) argued that locally grown sorghums are now tannin-free. Nevertheless, it is noteworthy that "tannin" concentrations in 2005-harvested sorghums were negatively correlated with live weight gain ($r = -0.723$; $P < 0.005$) and feed efficiency ($r = 0.733$; $P < 0.005$) in broilers at 21 days post-hatch (Rodrigues at al., 2007). If the sorghums were in fact tannin-free, then one interpretation of this data is that 'non-tannin' polyphenols, rather than condensed tannin, were exerting anti-nutritive effects in young birds.

Low molecular weight polyphenolic fractions are absorbed by chickens (Jimenez-Ramsay et al., 1994) and Butler and Rogler (1992) have contended that

these derivatives have negative systemic effects. This may be a rationale for the deleterious effects of non-tannin polyphenols, although the underlying mechanisms have not been clarified. Alternatively, chlorogenic acid is commonly found in plant-sourced feedstuffs and has been described as a 'non-flavanoid phenolic compound' (Yamanaka et al., 1997). Chlorogenic acid has been shown to interact with and reduce the pepsin digestibility of lysozyme (Rawel et al., 2000), moderately decrease whey protein utilisation in rats (Petzke et al., 2005) and delay intestinal uptakes of glucose in humans (Johnston et al., 2003). Also, it may be relevant that Reed (1987) recommended that lignin should be included in studies on the nutritive value of sorghum grain. Thus, while condensed tannin may not be present in contemporary crops, there is the possibility that other phenolic compounds inherent in sorghum are negatively impacting on broiler growth performance and this area would appear to merit closer attention.

X. FEED ADDITIVES

Various exogenous enzymes have been evaluated in sorghum-based diets including multi-component 'cocktails'; however, Madacsi et al. (1988) reported equivocal outcomes some time ago and essentially this trend has continued. An enzyme preparation containing subtilisin protease, xylanase and β-glucanase activities increased 42-day weight gains in broilers offered sorghum-based diets by 6.7% (2675 versus 2507 g/bird; $P < 0.06$) with a numerical advantage in feed efficiency (Selle et al., 2010b). Consideration was given to the possibility that the protease component was responsible for the weigh gain response. Taylor (2005) indicated that protease inclusions in sorghum-based diets to target kafirin may be beneficial with the caveat that kafirin may not be readily degraded by exogenous enzymes. Sultan et al. (2010) evaluated 4,000 U/kg protease activity derived from *Bacillus subtilis* in "all-sorghum" (918 g/kg) diets offered to broilers at 36 days of age. This protease significantly enhanced ileal protein digestibility by 4.5% (0.815 versus 0.780) and apparent metabolisable energy by 0.74 MJ (14.81 versus 14.07 MJ/kg DM). These responses were numerically increased when protease and phytase were added in tandem. Assessments of protease enzymes *per se* in sorghum-based broiler diets remain limited but at least on the basis of the Sultan et al. (2010) report thy would appear to hold promise.

Several reducing agents have been shown to ameliorate the negative impact of sorghum exposure to moist-heat on the basis of pepsin digestibility assays. These include sodium sulphite (Oria et al., 1995) sodium bisulphite (Arbab and El Tinay, 1997) and sodium metabisulphite (Elkhalifa et al., 1999), which reduce disulphide linkages and polymerisation of sorghum protein. Consequently, the evaluation of reducing agents such as sodium bisulphite in sorghum-based broiler diets, individually and in combination with exogenous proteases, would appear to be justified as this approach may enhance the quality of sorghum protein.

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Table 1 Amino acid profiles (% total amino acids) of sorghum kafirins (adapted from Watterson et al., 1990; Shull et al., 1992).

Amino acid	α-kafirin	β-kafirin	γ-kafirin
Arginine	1.12	3.80	2.84
Histidine	1.50	1.13	8.71
Isoleucine	4.64	2.44	2.78
Leucine	15.92	12.73	9.61
Lysine	0.47	0.59	0.48
Methionine	0.72	6.88	1.21
Phenylalanine	5.31	2.54	2.42
Threonine	3.07	4.43	4.07
Valine	4.52	4.93	5.53
Alanine	10.89	9.66	4.57
Aspartic acid	6.00	3.55	0.65
Cystine	1.07	4.80	6.80
Glutamic acid	25.80	21.06	16.53
Glycine	2.48	4.13	5.26
Proline	8.24	9.03	21.18
Serine	4.90	3.91	4.28
Tyrosine	3.35	4.40	3.10

Table 2 The amino acid profiles (g amino acid /16 g N) of sorghum protein and kafirin and glutelin fractions of sorghum protein [adapted from Jambunathan and Mertz, (1973)[1]; Bryden et al., (2009a)[2]; Perez-Maldonado and Rodrigues, (2009)[3]; Mosse et al., (1988)[4]]

Amino acid	Sorghum			Kafirin[4]	Glutelin[4]
	A[1]	B[2]	C[3]		
Arginine	3.48	3.69	3.68	1.06	4.91
Histidine	1.95	2.26	1.62	0.70	3.60
Isoleucine	3.96	4.04	3.48	4.00	4.40
Leucine	13.85	13.32	11.99	17.37	10.40
Lysine	2.08	2.06	2.15	0.23	2.94
Methionine	1.00	1.55	1.29	0.89	1.30
Phenylalanine	5.04	5.14	3.96	5.63	4.57
Threonine	3.16	3.12	2.50	1.87	2.40
Tryptophan	-	-	1.17	0.18	1.07
Valine	5.19	4.95	4.31	5.14	7.04
Alanine	9.60	8.93	-	9.60	9.01
Aspartic acid	7.60	6.37	-	5.08	5.57
Cystine	0.89	-	1.49	0.54	1.16
Glutamic acid	22.54	20.47	-	27.33	21.47
Glycine	2.98	3.02	-	1.67	3.43
Proline	7.92	-	-	10.71	8.96
Serine	4.39	4.44	-	3.56	5.06
Tyrosine	4.37	3.21	2.87	4.50	3.06

Table 3 Concentrations and apparent ileal digestibility (AID) coefficients of amino acids in 17 sorghum samples with crude protein contents ranging from 71 to 118 g/kg (mean: 101.9 ± 14.35 g/kg) and linear regressions between crude protein and amino acid concentrations (adapted from Bryden et al., 2009a)

Amino acid	Concentration (g/kg)			AID coefficient		Regression	
	Mean	Range	C of V (%)	Mean	Range	r =	P =
Arginine	3.72	2.5-5.1	21.4	0.795	0.71-0.86	0.390	0.122
Histidine	2.29	1.7-3.1	17.2	0.717	0.62-0.80	0.649	0.005
Isoleucine	4.12	2.7-5.4	23.2	0.818	0.74-0.87	0.639	0.006
Leucine	13.57	9.2-17.0	19.9	0.855	0.79-0.92	0.721	0.001
Lysine	2.07	1.4-2.6	20.3	0.743	0.67-0.83	0.329	0.197
Methionine	1.57	1.1-2.1	24.0	0.818	0.75-0.88	0.399	0.113
Phenylalanine	5.23	3.4-6.7	21.9	0.835	0.75-0.90	0.630	0.007
Threonine	3.16	2.4-4.3	17.0	0.678	0.58-0.76	0.725	0.001
Valine	5.04	3.4-6.5	21.3	0.788	0.71-0.85	0.614	0.009
Alanine	9.11	6.1-11.9	21.8	0.856	0.79-0.92	0.698	0.002
Aspartic acid	6.49	4.3-8.5	21.4	0.798	0.72-0.87	0.671	0.003
Glutamic acid	20.85	14.6-26.3	19.6	0.846	0.73-0.90	0.735	0.001
Glycine	3.06	2.1-4.1	19.9	0.721	0.64-0.92	0.599	0.020
Serine	4.51	3.5-6.7	20.2	0.771	0.70-0.84	0.665	0.004
Tyrosine	3.25	1.9-5.4	30.5	0.775	0.71-0.84	0.322	0.208

Table 4 Concentrations (g/kg) and apparent ileal digestibility (AID) coefficients of amino acids in sorghum samples harvested in 2004 and 2005 with percentage differences in AID between harvests (adapted from Perez-Maldonado and Rodrigues, 2009)

Amino acid	2004 harvest (n = 17)		2005 harvest (n = 14)		% difference
	g/kg	AID	g/kg	AID	2005 vs 2004
Crude protein	116.5		115.9		
Arginine	4.36	0.695	4.15	0.805	15.8
Histidine	1.64	0.581	2.18	0.727	25.1
Isoleucine	4.16	0.683	3.93	0.805	17.9
Leucine	14.26	0.739	13.64	0.849	14.9
Lysine	2.97	0.707	1.87	0.769	8.8
Methionine	1.39	0.759	1.62	0.853	12.4
Phenylalanine	4.14	0.728	5.21	0.833	14.4
Threonine	2.91	0.527	3.19	0.681	29.2
Tryptophan	1.41	0.584	1.29	0.777	33.0
Valine	5.18	0.662	4.80	0.784	18.4
Cystine	1.58	0.452	1.93	0.531	17.5
Tyrosine	2.98	0.690	3.82	0.806	16.8
Sum/Mean	46.98	0.651	47.63	0.768	18.7

Figure 1 Relationships between sorghum protein concentrations and kafirin (r = 0.886; P < 0.005) and glutelin (r = -0.401; P < 0.01) as proportions (%) of protein (adapted from Taylor et al. 1984)

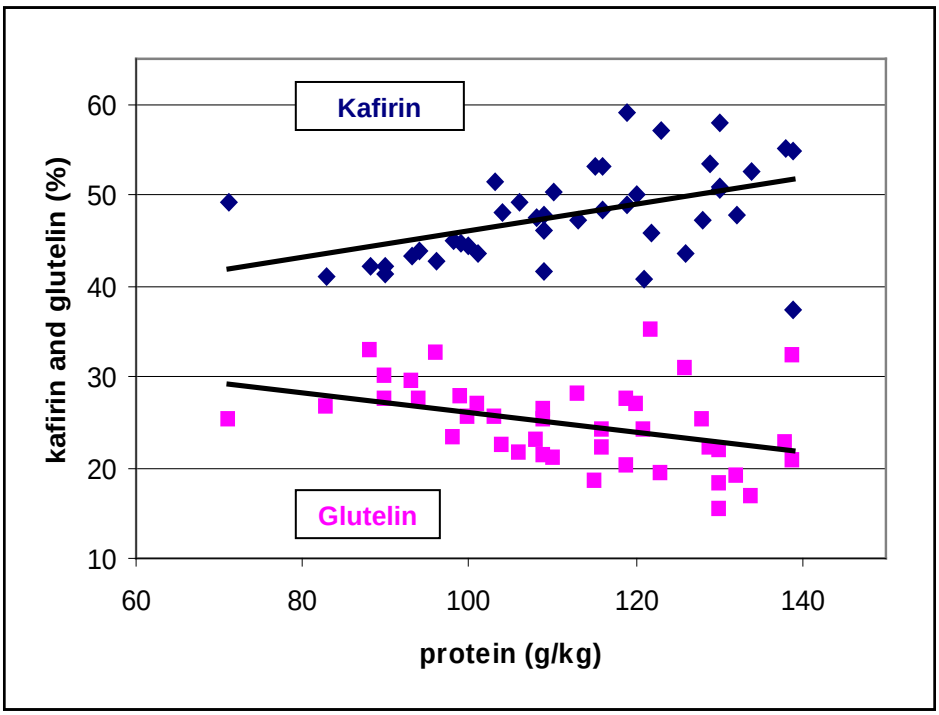
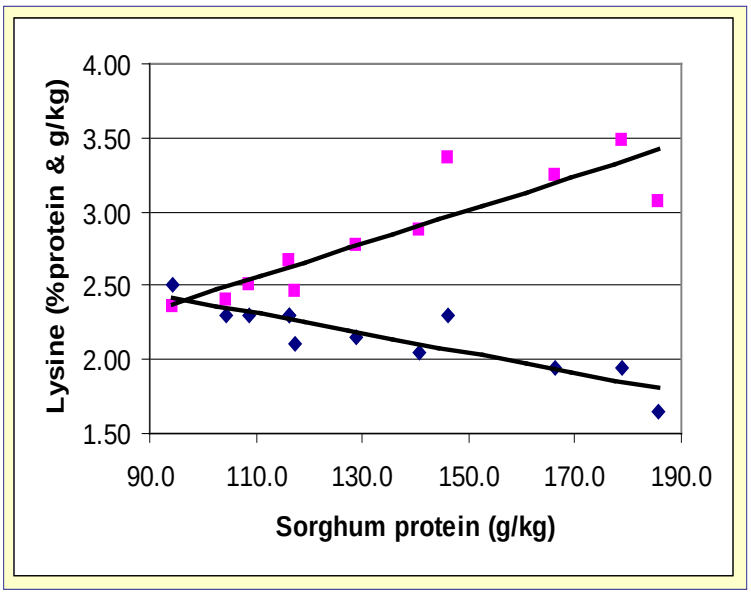


Figure 2 The significant (P < 0.001) linear relationships between sorghum protein and lysine in absolute (g/kg) and relative (% protein) terms, where r = 0.887 and r = -0.880, respectively (adapted from Mosse *et al.* 1988)



A REAPPRAISAL OF THE POTENTIAL OF DIETARY FATTY ACIDS TO AMELIORATE HEAT STRESS

P. B. CRONJÉ[1]

Summary

Although the concept of manipulating dietary fat content to ameliorate the effects of heat stress in poultry, pigs and cattle seems reasonable on theoretical grounds, it has yielded mixed results in all species. However, little attention was given to the fatty acid composition of the fat sources used, presumably because the premise on which this strategy is based was that all lipid sources have lower heat increments than the dietary carbohydrates that they replace. Nevertheless, there is evidence that dietary supplementation with long-chain fatty acids such as palmitic, linoleic and oleic acid can ameliorate the adverse effects of high temperatures in poultry (Njoku and Nwazota, 1989; Balnave, 1998; Mujahid et al., 2009). Recent advances in the elucidation of the mechanism by which hyperthermia exerts its effects strongly suggest that upregulation of avUCP expression using specific fatty acids may prevent the cascade of events that results in decreased production and tissue damage during heat stress. Furthermore, recent studies on the pathophysiology of heat stress strongly suggests that the strategic use of new oil seed varieties high in oleic acid may ameliorate the adverse effects of heat stress in poultry.

I. INTRODUCTION

Dietary fat is metabolised with greater efficiency than dietary carbohydrate or protein. Thus, less heat is generated during the metabolism of dietary fat than during the metabolism of dietary carbohydrate or protein. A logical application of this concept to the nutrition of livestock is replacement of a portion of the diet with fat to decrease dietary heat increment under heat stress conditions. Despite many studies on the inclusion of various sources of fat in the diets of dairy cows exposed to hot environmental conditions, several reviews on this topic concluded that the literature on the benefits of fat supplementation during heat stress is inconclusive (Beede and Collier, 1986; Huber et al., 1994; West, 1999). Similarly, the NRC (1981) reviewed the literature on the addition of fat to poultry diets fed during heat stress and concluded that this practice has not been consistently successful.

More recently, Balnave (2004) noted that as the type of fat affects nutrient partitioning to adipose tissue in broilers, interactions between environmental temperature and fat source may be worth exploring. Explication of the disparities between studies on the use of high fat diets during heat stress is difficult because sources of fat differ and their fatty acid composition is often not defined. Although no systematic study of the effects of different dietary fatty acids on animal responses to heat stress has been conducted to date, a remarkable series of studies conducted by Toyomizu's group at Tohoku University in Japan on the pathophysiology of heat stress in poultry strongly suggests that the adverse effects of heat stress could be alleviated by strategic supplementation with specific fatty acids (Mujahid et al., 2005,2006,2007a,2007b,2007c,2009).

The aim of this review is to discuss recent advances in our understanding of pathology of heat stress in poultry and to determine whether specific dietary fatty acids could play a role in ameliorating heat stress in poultry.

[1] Cronjé Consulting and Editing, 3 Quambi Pl, Buderim, QLD 4557

II. FATTY ACIDS ARE INVOLVED IN THE PATHOLOGY OF HEAT STRESS

Although the reduction in feed intake that accompanies heat stress undoubtedly contributes to a decrease in production under hot conditions, it has been demonstrated that it is only responsible for half the reduction in growth rate in broilers (Geraert et al., 1996a). In contrast to the expected effect of decreased feed intake on adipose tissue, heat stress increases the mass of certain fat deposits by 33–64% (Geraert, 1998). Furthermore, the fatty acid composition of adipose tissue is altered by heat stress (Geraert, 1998). Cells of the heart, kidney and liver of heat-stressed broilers exhibit an abnormally high accumulation of lipid droplets in the cytoplasm and massive fatty degeneration (Aengwanich and Simaraks, 2004). A similar pathology was described by Butler (1976) for fatty liver hemorrhagic syndrome, a condition that occurs when layers are exposed to hot weather: the liver is putty coloured and grossly enlarged because of excessive fat infiltration, which accumulates as globules within the cell to the extent that the nucleus is displaced and some cells are ruptured.

Heat stress increases levels of plasma fatty acids (Mujahid et al., 2007b), triglycerides (Sahin et al., 2006), cholesterol (Sahin et al., 2006) and enzymes involved in the transport and oxidation of fatty acids (Mujahid et al., 2007b). The respiratory quotient of heat-stressed birds is decreased (Mckee et al., 1997) indicating that hyperthermia promotes oxidation of fatty acids. It is thought that fatty acid oxidation is increased to meet the energy requirements of birds exposed to heat stress (Mckee et al., 1997). However, the pathology of heat stress is indicative of an imbalance between mobilisation of fatty acids and the ability to oxidise them. Excessive fatty acid oxidation and accumulation of fatty acids in mitochondria is conducive to oxidative stress, a condition that causes significant tissue damage.

III. HYPERTHERMIA CAUSES OXIDATIVE STRESS

Oxidative stress is characterised by excessive production of reactive oxygen species (ROS) such as superoxide. ROS remove electrons from fatty acids, mainly polyunsaturated fatty acids, creating fatty acid radicals that in turn attack other fatty acids. This process is called lipid peroxidation. If left unchecked, such chain-reactions damage cell membranes, which consist mainly of lipids, resulting in impaired control of cellular ion homeostasis and eventually, cell death. ROS also damage proteins and DNA. Prolonged heat-induced oxidative stress initiates a cascade of events involving systemic elevation of levels of inflammatory cytokines, widely disseminated intravascular blood coagulation and ultimately, multiple organ failure and death (for review, see Cronje, 2005). In broilers, exposure to 5 h of heat stress per day (33 °C and 60–70% relative humidity) for 21 days resulted in symptoms consistent with excessive oxidative stress: congestion, oedema and haemorrhage of the lungs, oedema and haemorrhage of the kidneys and necrosis of the liver (Aengwanich et al., 2003; Aengwanich and Simaraks, 2004). Heat stress also causes haemorrhages in muscle tissue (Sandercock et al., 2001) and damage to the intestinal mucosa (Quinteiro-Filho et al., 2010) in poultry.

There is ample evidence showing that heat stress results in oxidative stress in poultry (Altan et al., 2003; Sahin et al., 2006; Feng et al., 2008) and that it causes extensive damage to lipids, proteins (Mujahid et al., 2007a) and muscle membranes (Sandercock et al., 2001; Petracci et al. 2009). Oxidative stress arises when the body's natural antioxidant defences are unable to cope with ROS generated during oxidative phosphorylation in the mitochondria. Several studies have shown that vitamins and minerals involved in antioxidant defence are depleted by heat stress (Sahin et al., 2003; Mahmoud and Edens, 2005). That supplementation of heat-stressed birds with antioxidants such as vitamin C (Mckee et al., 1997; Sahin et al., 2003; Mahmoud et al., 2004), vitamin E (Bollenger-Lee et al., 1998) and

lycopene (Sahin et al., 2006) has been shown to ameliorate heat-induced oxidative stress is a strong indication that heat stress induces overproduction of ROS.

In 2005, Mujahid et al. demonstrated for the first time that heat stress induces the production of superoxide in the skeletal muscle mitochondria of broilers and showed that oxidative stress inhibits growth independently of feed intake during heat stress. Therefore, nutritional strategies against heat stress such as increased dietary energy density or protein content only address half the problem (decreased feed intake) and strategies such as supplementation with antioxidants only address the symptoms of the other half of the problem (oxidative stress). A strategy that targets the cause of oxidative stress is lacking. However, the cause of oxidative stress in poultry remained a matter of conjecture until the discovery of avian uncoupling protein by Raimbault et al. in 2001.

IV. MITOCHONDRIAL UNCOUPLING PROTEINS DECREASE OXIDATIVE STRESS

Hydrolysis of ATP to ADP releases energy, which is used to drive metabolic reactions. An active cell can hydrolyse more than two million ATP molecules per second, but the energy stored in the form of ATP in the human body is equivalent to the energy stored in an AA battery, and therefore only sufficient to satisfy the body's energy needs for a few seconds. This necessitates rapid regeneration of ATP from ADP using energy from ingested nutrients or endogenous reserves. Thus, although the human body contains only 250 g of ATP, it turns over its own weight in ATP each day. The task of ATP turnover is accomplished by mitochondria, of which there are 100 to 1000 per cell. Most ATP is produced in mitochondria by oxidative phosphorylation.

A schematic illustration of mitochondrial oxidative phosphorylation is presented in Fig. 1. The mitochondrion contains an inner and an outer membrane, which are separated by an inter-membrane space. In the matrix of the mitochondrion, oxidation of glucose and fat yields the 'hydrogen carriers' $NADH+H^+$ and $FADH_2$. During oxidative phosphorylation, electrons are removed from $NADH+H^+$ and $FADH_2$ and are transported through the respiratory chain until they are donated to molecular oxygen, which is then reduced to water. The transport of electrons drives proton pumps that transfer hydrogen ions from the matrix to the inter-membrane space, creating an electrochemical potential difference across the inner membrane. Protons may re-enter the mitochondrial matrix through the ATP synthase proton channel, which uses this proton-motive force to generate ATP from ADP.

Proton re-entry via ATP synthase is normally regulated by the availability of ADP, but protons may also re-enter through uncoupling proteins (UCPs), which act as a type of "pressure-relief valve" to prevent excessive accumulation of protons in the inter-membrane space. During the reduction of molecular oxygen to water, leakage of electrons from the respiratory chain results in the formation of superoxide radicals, which can be converted into other ROS. These ROS attack the phospholipids and polyunsaturated fatty acids (PUFA) of the inner membrane. Thus, activation of UCPs, which enables protons to leak back into the matrix, reduces ROS production (Azzu and Brand, 2009). As mitochondria account for more than 80% of cellular oxygen consumption, they are the main site of ROS production (Manoli et al., 2007). When the level of ROS exceeds the capacity of cellular antioxidants to remove them, the cell experiences oxidative stress. If left unchecked, DNA and enzymes are damaged and the respiratory chain malfunctions.

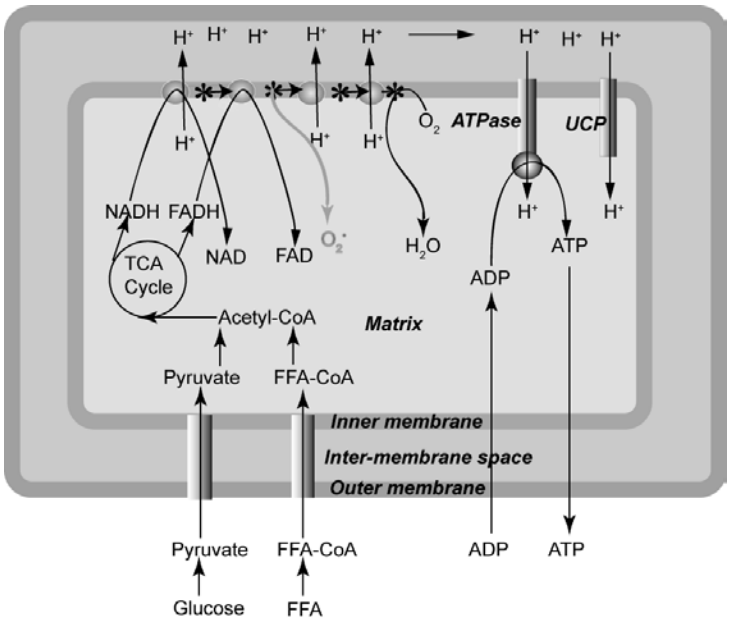


Figure 1 Mitochondrial oxidative phosphorylation. Glucose and free fatty acids (FFA) enter the tricarboxylic acid (TCA) cycle as acetyl-CoA, producing NADH and FADH, which donate electrons to the electron transport chain. Movement of electrons down this chain provides energy to transport protons (H^+) from the matrix to the inter-membrane space, creating a proton electrochemical gradient. Re-entry of protons to the matrix via ATP synthase drives the conversion of ADP to ATP. Electrons that reach the end of the electron transfer chain are accepted by molecular oxygen (O_2) in the formation of H_2O. However, some electrons leak from the chain and form superoxide ($O_2\cdot$).

In addition to its role in decreasing ROS by increasing proton leak, it has been proposed that UCP3 exports fatty acids from the mitochondrial matrix when fatty acid supply exceeds fat oxidation capacity (Hoeks et al., 2003). As fatty acid anions in the mitochondrial matrix are prone to peroxidation, prevention of their accumulation could reduce ROS production. Although there is debate about the relative importance of the various mechanisms by which UCP3 exerts its effects (Azzu and Brand, 2009), there appears to be general consensus that UCP3 plays a key role in decreasing ROS production and protecting against cellular damage. Therefore, heat-induced downregulation of UCP activity could provide an explanation for the oxidative stress observed in birds subjected to heat stress.

V. AVIAN UNCOUPLING PROTEIN IS DOWNREGULATED BY HEAT STRESS

Only one type of UCP has been detected in birds, whereas five isoforms are present in mammals. Avian uncoupling protein (avUCP) was first cloned in 2001 by Raimbault et al. from the skeletal muscle of chickens. The amino acid sequence of avUCP is 70% identical with those of mammalian UCP2 and UCP3, but its tissue distribution is restricted mainly to skeletal muscle, which is similar to the distribution of UCP3.

Mujahid et al. (2006, 2007b,c) showed that heat stress decreases the level of avUCP by up to 50% and proposed that the associated inability to regulate proton motive force caused oxidative stress. They also observed that plasma fatty acid levels increased three-fold and that levels of enzymes involved in the transport and oxidation of fatty acids and those involved in the Krebs cycle were elevated during the early stages of heat stress (Mujahid et al., 2007b). The same group recently showed that heat stress enhances substrate oxidation via the electron transport chain, resulting in an increase in mitochondrial membrane potential and

ROS production (Kikusato et al., 2010). Mujahid et al. (2007b) concluded that a sudden surge in mitochondrial substrate oxidation combined with downregulation of avUCP may be responsible for the increase in superoxide production during heat stress. This hypothesis is supported by further evidence of downregulation of avUCP by heat stress in chickens (Taouis et al., 2002) and a very strong linear correlation (R^2 = 0.92) between ROS production and avUCP-dependant mitochondrial proton leak (Rey et al., 2010). However, the mechanism by which heat stress downregulates avUCP is as yet unclear.

The recent identification of a binding site for thyroid hormone in the promoter sequence of the avUCP gene by Joubert et al. (2010) indicates that thyroid hormone may play a role in downregulating avUCP expression during heat stress.

VI. CHANGES IN THYROID HORMONE LEVEL MAY DOWNREGULATE AVIAN UNCOUPLING PROTEIN EXPRESSION DURING HEAT STRESS

During heat stress, circulating levels of thyroid hormone are decreased (Geraert et al., 1996b; Tao et al., 2006; Lin et al., 2008), presumably because thyroid hormone increases metabolic rate, and thus metabolic heat production. Although it has been known for many years that thyroid hormone stimulates metabolic rate and decreases metabolic efficiency, the mechanism by which thyroid hormone affects energy homeostasis is poorly understood. In 2001, De Lange et al. provided the first in vivo evidence that thyroid hormone increases muscle UCP3 expression. More recently, Rey et al. (2010) showed that skeletal muscle avUCP abundance in ducklings was upregulated by administration of thyroid hormone and decreased by pharmacological blockade of thyroid hormone synthesis. Furthermore, the production of ROS per unit of oxygen consumed by muscle mitochondria was elevated in the hypothyroid state and was attenuated by thyroid hormone administration. In rats, thyroxine level is linearly correlated with muscle UCP3 expression (Sprague et al., 2007). Therefore, a heat-induced decrease in thyroid hormone level may downregulate avUCP expression, resulting in oxidative stress and ROS-mediated tissue damage in birds exposed to heat stress. There is evidence that polyunsaturated fatty acids upregulate UCP expression and that they compete with thyroid hormone for the retinoid receptor X, which is required for binding to some of their target genes (Clarke et al., 1999). Thus, it is possible that dietary mono- and poly-unsaturated fatty acids could be exploited to increase avUCP expression and ameliorate heat-induced tissue damage in poultry.

VII. FATTY ACIDS UPREGULATE UNCOUPLING PROTEIN EXPRESSION

Muscle UCP3 upregulation appears to be specific for long-chain fatty acids, as Hoeks et al. (2003) observed no response in muscle UCP3 level when rats were fed a high-fat diet consisting of medium-chain fatty acids, but observed substantial increases when a diet containing long-chain fatty acids was fed. Thompson et al. (2004) reviewed in vitro studies in which specific fatty acids had been added to cultured cell models or primary isolated cells. None of the cell lines showed a response in UCP3 expression to saturated fatty acids. In muscle cells, the monounsaturated fatty acid, oleic acid (18:1 n – 9) and the polyunsaturated fatty acids, linoleic acid (18:2 n – 6) and linolenic acid (18:3 n –3) increased the expression of UCP3.

Rodriguez et al. (2002) fed rats diets containing 40% of dietary energy in the form of oils rich in saturated fatty acids (palm oil or beef tallow), polyunsaturated fatty acids (sunflower oil) or monounsaturated fatty acid (olive oil). The level of UCP3 in muscle was 33% greater in rats fed the olive oil diet than in those fed the other sources of fatty acids. Based on this evidence, Mujahid et al. (2009) fed olive oil to broilers to determine whether it

could prevent mitochondrial ROS production and oxidative damage during heat stress. In their trial, birds were fed a basal diet (a commercial broiler diet) or the basal diet plus 6.7% olive oil for 8 d before exposure to thermoneutral conditions or 34 °C for 12 h. The addition of olive oil to the basal diet prevented the decrease in avUCP level and the increase in lipid peroxidation observed in birds fed the control diet during heat stress. Birds fed the basal diet lost weight during heat stress, whereas those supplemented with olive oil gained weight. The feed intake of the olive oil-supplemented birds also decreased to a lesser extent than that of birds fed the basal diet during the 12 h heat stress period. Although the practical implications of these results are difficult to interpret because the two diets were not isoenergetic, it establishes a mechanism by which specific fatty acids could alleviate heat stress. As oleic acid, a monounsaturated fatty acid, constitutes 70–80% of the fatty acids in olive oil (Tripoli et al., 2005), it is likely that the upregulation of avUCP observed by Rodriguez et al. (2002) and Mujahid et al. (2009) was mediated by oleic acid.

The specificity of UCPs for certain types of fatty acid may explain why the practice of feeding high-fat diets to poultry exposed to heat stress has been successful in some instances and has failed in others. Furthermore, Hoeks et al. (2003) noted that rats fed a high-fat diet containing medium-chain fatty acids (C8:0 and C10:0; caprylic and capric acid, respectively) gained less weight than rats consuming an equal amount of net energy from a high-fat diet containing long-chain fatty acids (C16:0, palmitic acid), indicating that medium chain fatty acids have a thermogenic effect. Therefore, it is possible that supplementation of poultry diets with certain types of fatty acids could exacerbate heat stress. The long-chain fatty acid diet – but not the medium chain fatty acid diet – increased UCP3 level in muscle. It is noteworthy that the aforementioned thermogenic effect of medium-chain fatty acids occurred in the absence of upregulation of UCP3, supporting the contention of Hoeks et al. (2003) that UCP3 does not increase heat production but protects mitochondria against fatty-acid-induced mitochondrial damage. Baillie et al. (1999) observed that fish oil, which contains long-chain omega-3 C20 and C22 polyunsaturated fatty acids, resulted in less fat deposition in rats fed equicaloric amounts of a diet containing corn oil (rich in C18:2 [n-6]; omega-6 linoleic acid), showing that long-chain fatty acids can have thermogenic effects. In this instance, the thermogenic diet (fish oil) also increased muscle UCP3 level to a greater extent than the corn oil diet. The differential effects of various fatty acids on thermogenesis and UCP expression may be mediated by their affinity for peroxisome proliferator-activated receptors (PPARs).

The PPARs were originally identified in frogs as receptors that induce the proliferation of peroxisomes, organelles that are involved in the breakdown of very-long-chain fatty acids (>18 carbon atoms in length) to medium-chain fatty acids, which are then shuttled to the mitochondrion for further oxidation. Peroxisomal fatty acid oxidation generates 30% more heat than mitochondrial fatty acid oxidation (Baillie et al., 1999). The PPARs are members of the nuclear hormone receptor family, so called because unlike classical hormone receptors, which are located in the cytoplasm and translocate to the nucleus after binding to their ligands, PPARs reside in the nucleus and bind to DNA response elements. The avUCP gene contains a binding site for PPARs in its promoter sequence (Joubert et al., 2010). There are three members of the PPAR subfamily, PPARα, PPARγ and PPARδ, all of which are activated by fatty acids or their derivatives (Clarke et al., 1999). Gene knockout experiments in rodents have verified that UCP3 and UCP2 are not thermogenic whereas UCP1 induces non-shivering thermogenesis (Azzu and Brand, 2009). avUCP does not appear to play a role in thermogenesis in the chicken (Walter and Seebacher, 2009). The distribution of these PPAR isoforms in mammals differs between tissues, and the affinities of activating ligands differs between them (Guri et al., 2006). This may explain why certain fatty acids induce thermogenesis but not UCP expression, why some fatty acids

induce thermogenesis but also increase UCP expression and why some fatty acids do not induce thermogenesis but increase UCP expression.

In addition to their effects on UCPs, PPARs also affect the expression of genes for key enzymes in fat and glucose metabolism, which represents another avenue by which cellular responses to heat stress could be manipulated. For instance, heat stress is associated with fatty degeneration of most tissues and the secretion of inflammatory cytokines. Nagasawa et al. (2006) induced hepatic fat accumulation and inflammation in mice by dietary means and showed that pharmacological overexpression of PPARδ reduced lipid accumulation and the expression of inflammatory cytokines.

VIII. NEW OIL SEED VARIETIES CONTAIN BENEFICIAL FATTY ACIDS

Changes within the oilseed industry brought about by concern about the harmful effects of saturated fatty acids and *trans* fatty acids have resulted in the development of plant varieties that produce oils high in oleic acid. Saturated fatty acids are converted to the *trans* configuration by heat during frying and by hydrogenation, used by the industry to improve heat stability for deep-frying or to increase the solidity of oils used for the production of margarine. *Trans* fatty acids increase cholesterol levels in humans, adding to the incidence of heart disease. As a result of these factors, there is a growing trend away from the use of oils rich in palmitic acid (C16:0) and hydrogenated oils in favour of oils that can provide the required functionality without hydrogenation. Oils low in palmitic acid and rich in oleic acid, a *cis* fatty acid, or stearic acid (C18:0) meet these requirements. Oilseed crops such as soybean, rapeseed (canola), peanut, sunflower and cottonseed have now been bred or engineered to produce oil high in oleic acid (Liu et al., 2002). The widespread availability of oils and oil meals derived from high-oleic-acid plants and their increasing incorporation into livestock feeds calls for re-evaluation of the results of trials conducted before the advent of these plants on the use of high-fat diets for heat-stressed poultry.

VIII. CONCLUSION

Recent advances in the pathophysiology of heat stress strongly suggest that the strategic use of new oil seed varieties may ameliorate the adverse effects of heat stress in poultry. A systematic study of the effects of different dietary fatty acids on avUCP expression, PPAR activity and the responses of poultry to heat stress is warranted.

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USE OF ELECTROLYTES FOR BIRDS - THE PRACTICE OF THEORY

S. A. BORGES[1], J.P. DE OLIVEIRA[2], A.V. FISHER DA SILVA[2] and T. T. DOS SANTOS[3]

Summary

Electrolyte balance is defined as the balance between intake, consumption and loss of essential monovalent ions such as sodium (Na^+), potassium (K^+), and chloride (Cl^-), derived from salts which are dissociated into their ionic components, and the main physiological function of which is maintaining acid-base balance and osmotic pressure of the body. Adjustment of the electrolyte balance of feeds is necessary at all stages of animal growth, even if the improvements in performance are more evident in the finishing stages of growth and under conditions of heat stress. The high metabolic level required by animals with rapid growth demands small adjustments in feed formulation, which can improve animal performance.

I. INTRODUCTION

Modern broilers are characterized by high growth rate, making them sensitive and susceptible to several metabolic problems such as acid-base imbalance. One of the main triggers of acid-base imbalance is heat stress, which is caused when relative air humidity and environmental temperature exceed the comfort zone, preventing heat dissipation, increasing bird body temperature and affecting performance.

Some measures such as the use of ventilators and nebulizers, manipulation of protein and energy intake, use of antipyretics, ascorbic acid or electrolytes, management of feeding and management of drinking water can be taken to minimize losses from heat stress.

The change in the bird's acid-base balance due to exposure to high temperatures is called respiratory alkalosis. One of the methods used to control heat stress is the chemical manipulation of acid-base balance by adding compounds such as sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), calcium chloride ($CaCl_2$), potassium carbonate (K_2CO_3), and ammonium chloride (NH_4Cl) to water and/or feed. Several studies have shown that supplementing electrolytes to birds is more efficient when the balance among them ($Na^+ + K^+ - Cl^-$ in mEq) is taken into account, without neglecting their individual importance.

II. PHYSIOLOGY OF HEAT STRESS IN BIRDS

As birds are homoeothermic animals, they have a thermo regulatory centre located in the hypothalamus capable of controlling body temperature through physiological mechanisms and behavioural responses, producing and releasing heat, thereby maintaining normal body temperature (Macari et al., 2002).

Birds exposed to heat demonstrate compensatory physiological responses which result in an increase in non-evaporative heat loss. Birds are able to increase their surface area by spreading the wings, ruffling feathers, and also increasing peripheral circulation. Urine production can also promote non-evaporative heat loss provided the lost water in the urine is compensated by higher water consumption. The final physiological response observed is an increase in respiratory rate. This causes excessive carbon dioxide (CO_2) losses, leading to lower CO_2 partial pressure (pCO2) and consequently lower blood carbonic acid (H_2CO_3) and

[1] Faculty of Life Sciences and Health University of Tuiuti do Parana, Brazil
[2] UFPR
[3] AB Vista Feed Ingredients

hydrogen (H^+) concentrations. The kidneys respond by increasing HCO_3^- excretion and reducing H^+ excretion in an attempt to maintain the bird's acid-base balance (Fig. 1). This alteration is called respiratory alkalosis.

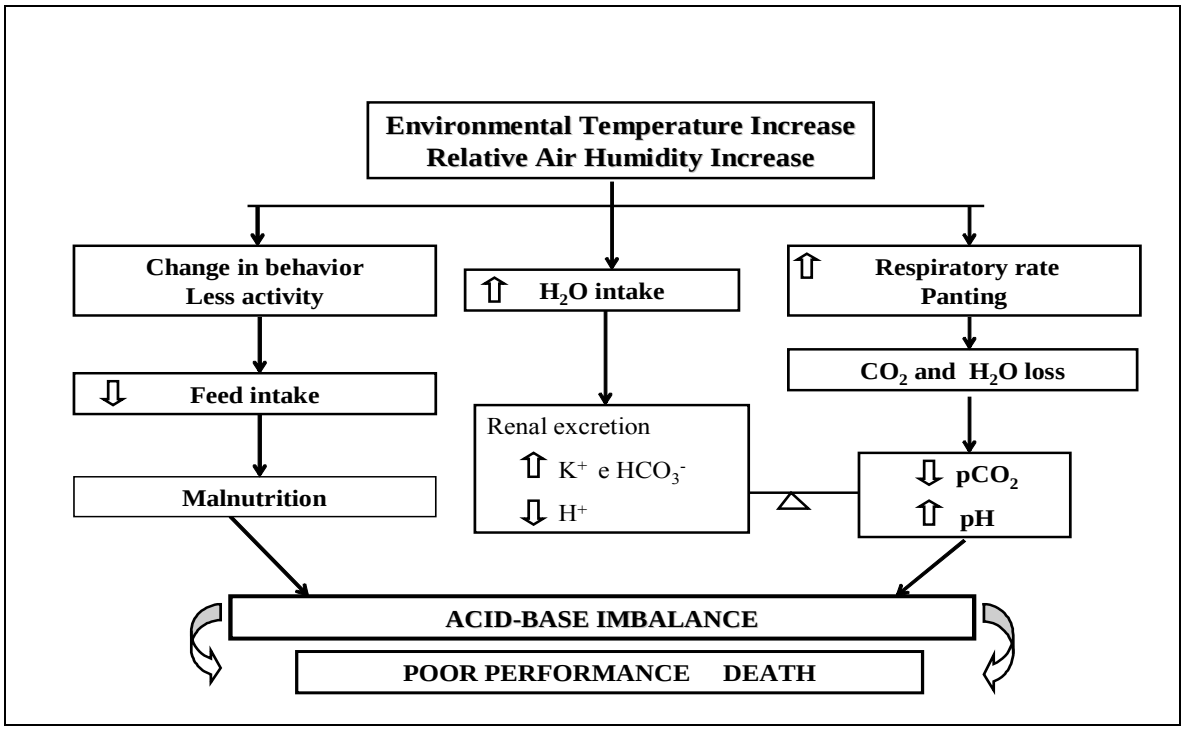

Figure 1 Bird response to high temperature (adapted from Borges *et al*. 2003a) .

The cardiovascular system is particularly sensitive to temperature changes, and an important indicator of the bird's physiological responses to stressors. Changes in blood cells are associated with heat stress, namely changes in blood volume, in the number of circulating leukocytes and erythrocytes, and haemoglobin content in the erythrocytes.

During heat stress, blood volume increases due to an increase in the number of erythrocytes. In addition, heterophil to lymphocyte ratio changes as a consequence of an increase in heterophils and a decrease in lymphocytes. This ratio has been proposed as a sensitive indication of chronic stress in broilers. Adrenaline, noradrenaline and glucocorticoid secretion also increases the glycemia.

III. THE IMPORTANCE OF ELECTROLYTES

An electrolyte can be defined as a chemical substance that is dissociated into its ionic components, the main physiological function of which is maintaining acid-base balance and osmotic pressure of the body. Essential to birds, the monovalent ions sodium (Na^+), potassium (K^+), and chloride (Cl^-) are key minerals in this process, and the effects of the dietary ionic balance on bird performance may be related to variations in the acid-base balance (Mongin, 1981).

Potassium is the main cation of the intracellular fluid, whereas Na^+ and Cl^- are the main ions of the extra cellular fluid. Osmoregulation is a result of the homeostasis between these intra- and extracellular ions. Under optimal conditions, water and electrolyte content are maintained within narrow limits, but when electrolytes are lost and water content does not change the osmolality of those fluids is reduced.

Changes in K^+ homeostasis may affect cell function. Studies on dehydration followed by rehydration in humans have shown that the degree of water deficit in intracellular fluid

was associated with loss of intracellular K^+ and extracellular fluid deficit has been linked to loss of Na^+ in plasma. The degree of rehydration was determined by restoring intracellular K^+ (Nose et al., 1988). Respiratory alkalosis causes a reduction in competition between H^+ and K^+ for urinary excretion and increases the loss of K^+ in urine. Excess K^+ ions compete with the anions of buffers in renal tubular fluid, preventing the removal of H^+, which is reabsorbed and can lead to an acidosis to compensate for the metabolic condition. This mechanism may increase the need for K^+ during heat stress. There is recent evidence that the intercalated cells of the collecting duct, secrete acid and H^+ and this process is increased by hypokalemia (low K^+ in the plasma) and appears to be an important contributor to renal acidification.

Plasma Na^+, K^+ and Cl^- levels are affected by heat stress: K^+ and Na^+ levels decrease as temperature increases, whereas Cl^- increases (Borges *et al.* 2004b; Nassem *et al.* 2005). The reduction in blood K^+ level is attributed to the excretion of this ion during chronic stress, whereas intracellular K^+ usually increases during acute stress. Hyperkalemia (high concentration of K^+ in the plasma) may result in metabolic acidosis, both due to uric acid reduced productionas to carbonate reabsorption by kidneys (Ait-Boulahsen *et al.*, 1995).

Increases in Cl^- plasma levels depress H^+ excretion and bicarbonate (HCO_3^-) reabsorption by the kidneys, thereby contributing to blood acidification, which seems to be an adequate response to alkalosis. However, stress duration must be taken into account, as Salvador *et al.* (1999) observed reduced Cl^- serum levels when broilers were submitted to chronic stress for a week (42 to 49 days of age).

In addition to increased heat, K^+ excretion is also influenced by hormonal factors (aldosterone, ADH – anti-diuretic hormone, and deoxycorticosterone), acid-base balance, and cation balance. The rate of K^+ excretion in the urine is variable and linked to blood Na^+ concentration and hydration status. Loss may be caused by an increase in water intake, as the osmotic gradient allows the movement of water from the intracellular fluid to the urine, carrying K^+. Also, an increase in K^+ intake results in higher urinary loss, as the bird has low capacity to store K^+ in the body.

As for K^+, bicarbonate (HCO_3^-) serum levels also decrease when birds are exposed to heat stress for more than two weeks (Nassem, *et al*. 2005). This is possibly caused by increased panting by the birds, which causes hyperventilation, reducing HCO_3^- regeneration. Normal plasma HCO_3^- levels depend on its daily replacement by respiratory regeneration and reabsorption of all HCO_3^- filtered by the kidney glomerular capillaries (Kumar and Clark, 2002).

IV. THE USE OF SALTS IN FEED

The supplementation of feed with salts is a practice adopted by nutritionists who aim to minimize the harmful effects of heat stress on poultry. The most commonly used salts are KCl and $NaHCO_3$.

Potassium (K^+) is abundant in most feed ingredients; on the other hand, Na^+ is usually deficient. Sodium (Na^+) supplementation requires alternative sources, as the use of common salt (sodium chloride – NaCl) is often limited by the presence of Cl^-. Interestingly, an interaction occurs between the levels Na^+ and Cl^- present in the diet. Mushtaq et al. (2005) observed increased breast yield and reduction of abdominal fat in broilers receiving 0.30% Na; however, with increasing Cl^- level (0.30% 0.40% and 0.50%), abdominal fat increased significantly ($P < 0.003$) and breast yield declined, ie, there was a negative correlation between the levels Cl^- and carcass parameters.

Many studies have been carried out to determine which sodium sources promote the best bird performance. Ahmad *et al.* (2006), evaluated the effect of three different Na^+ sources – $NaHCO_3$, sodium carbonate (Na_2CO_3), and sodium sulfate (Na_2SO_4) on the performance of broilers exposed to heat stress, and observed that salt supplementation

increased both water consumption and water to feed intake ratio. The highest water intake resulted in the lowest body temperature, thereby helping to control heat stress. These effects were more pronounced when $NaHCO_3$ was supplemented. Teeter et al. (1985) demonstrated the impact on weight gain, comparing birds under thermal comfort and heat stress conditions. In the same study, the addition of 0.5% $NaHCO_3$ in diets of birds that were in heat stress improved the weight gain of 9%, although not statistically significantly. Fischer da Silva et al. (1994) showed that broilers exposed to temperature ranges of 39 to 41ºC and 34 to 36ºC, and supplemented with 0.5 and 1.0% $NaHCO_3$ in feed, improved feed intake, weight gain, and feed conversion ratio. The supplementation of 0.5, 1.0 and 1.5% $NaHCO_3$ in the feed of 21 to 49d broilers during the summer promoted better feed conversion (Table 1).

Table 1 Performance of broilers supplemented with sodium bicarbonate ($NaHCO_3$) in feed after 21 days of age.

Treatments	Weight gain (g)	Feed intake (g)	FCR
Control	2,005	3,781	1.899 b
0.5% $NaHCO_3$	2,031	3,794	1.870 ab
1.0% $NaHCO_3$	2,066	3,808	1.851 ab
1.5% $NaHCO_3$	2,090	3,838	1.841 a
P	0.32	0.89	0.05

Means followed by different letters in the same column as statistically different ($P \leq 0.05$ by Tukey test). – Adapted from Borges, 2006

Potassium chloride (KCl) supplementation in the diet and / or in the drinking water of birds has been proposed as a way to minimize the consequences of high temperatures (Smith and Teeter, 1993; Borges, 1999). Souza et al. (2004) supplemented KCl (0, 0.4, 0.8, 1.2, 1.6, and 2.0%) in the feed of broilers during the summer and observed a positive linear effect on potassium intake, excretion, and retention. In the same paper, water consumption increased linearly as feed KCl levels increased, and excreta moisture increased 28% between the birds fed 2.0% KCl and those in the control treatment. Nassem et al. (2005) evaluated the individual or combined use of 1.5% KCl and 0.5% $NaHCO_3$ in the feed of broilers raised in temperatures above thermal comfort, and concluded that the individual use of both salts improved weight gain at 35 days of age but the highest weight gain and K^+ and HCO_3^- serum levels were replenished only when KCl and $NaHCO_3$ were combined. Starting the supplementation of 0.5 or 1.0% KCl at 21 or 35 days, for broilers raised up to 49 days of age in the summer (Borges, 1997), the most pronounced weight gain differences were obtained when 1.0% KCl supplementation started when broilers were 21 days old Table (2).

Table 2 Performance of broilers supplemented with potassium chloride (KCl) in feed.

Treatments	Weight gain (g)	Feed intake (g)	FCR
Control	1,988 b	3,744	1.879 a
21 days + 0.5% KCl	2,080 ab	3,877	1.872 ab
21 days + 1.0% KCl	2,103 a	3,856	1.828 b
35 days + 0.5% KCl	1,998 b	3,803	1.898 a
35 days + 1.0% KCl	2,070 ab	3,890	1.884 a
P	0.01	0.24	0.05

Means followed by different letters in the same column as statistically different ($P \leq 0.05$ by Tukey test). Adapted from Borges, 2006

V. THE USE OF SALTS THROUGH DRINKING WATER

Climate change and the difficult logistics of delivery of chicks and chickens for slaughter can trigger stress in birds and feeding management can be a good alternative in these situations. As the use of salt in feeds requires prior programming at the mill, the use in drinking water is a simple and effective practice that can be adopted at any time by the poultry farmer to minimize economic losses resulting from stress.

Different daily circumstances may stress broilers; the most common are day-old chicks transported long distances from the hatchery to the farm, atypical high temperature and high humidity days during the year, pre-slaughter handling, and transport. The objective is to increase water consumption, improving heat dissipation and bring electrolyte blood concentration to normal levels (Kidd, 2001), consequently re-establishing acid-base and hydrolytic balance. Krabbe (2000) observed a positive linear effect on water consumption of broilers fed different Na^+ levels (0.10, 0.22, 0.35 and 0.48%) in the drinking water. In the same study, feed intake, weight gain, and feed conversion were also significantly affected by water Na^+ content, with estimated maximum response of 0.33, 0.37, and 0.39% respectively. This improvement may be explained by the relationship between water and feed intake. Whiting et al. (1991) found that broilers submitted to high temperatures (35-38°C) and supplemented with 0.5% KCl in the drinking water increased water consumption by 46%. Belay and Teeter (1993) exposed 42d broilers to 35°C for a period of 4 hours, supplied 0.75% KCl in the drinking water, and verified higher water intake, higher evaporative water loss, and better apparent respiratory efficiency.

Pre-slaughter fasting is a practice adopted to reduce carcass contamination caused by leakage of gastrointestinal contents at evisceration. There is an animal welfare concern in relation to the pre-slaughter handling and especially regarding the stress caused to birds during this period (Bressan and Beraquet, 2002), since birds subjected to conditions of stress alter the acid-base balance (Borges et al., 2007). Most industries recommend working with a maximum fasting period of 12h max., including the withdrawal from the farm to the slaughter, the transportation and resting time before starting the slaughtering process (Gomes, 2007). Broiler slaughter age and weight, environmental temperature and humidity, catching process, density and place inside the truck, transport duration, and environmental conditions are directly related to bird susceptibility to stress. Barbosa Filho et al. (2006) evaluated the effect of transport time and temperature and relative humidity of the environment on rectal temperature and weight loss of 250 birds at 40 days old. The birds were placed in shipping boxes and subjected to three different temperatures (27, 30 and 35° C) and humidity (70, 75 and 85%). The authors concluded that 30 minutes is long enough for birds subjected to temperatures of 30 and / or 35 ° C to show signs of stress. Weight loss increased significantly as the temperature and humidity were increased.

The fasting period may be directly related to meat quality as, during loading and transport, dehydration becomes evident. In addition, considering that broiler breast meat contains 72% moisture, fasting time and dehydration may affect its organoleptic characteristics and tenderness. In this context, water retention is important to determine breast meat quality (Mendes, 2001). Bressan & Beraquet (2002) investigated the influence of travel time, rest and ambient temperature in the pre-slaughter stage on meat quality, concluding that, at temperatures above the comfort zone, the onset of rigor mortis is accelerated. The shear force of the pectoral muscles is lower when the birds were transported for shorter distances and consequently shorter time, so this measure is important in determining the comfort conditions of the birds pre-slaughter. The use of salt in drinking water can be an alternative to stimulate the consumption of water in the pre-slaughter stage, thereby reducing losses due to dehydration and stress. Gomes (2007), applied increasing KCl or $NaHCO_3$

(0.15, 0.30 and 0.45%) levels in the drinking water of broilers 24 hours before slaughter, and observed a linear increase in water consumption, independent of the salt. That author stressed the importance of water intake during the pre-slaughter period for gastrointestinal emptying and to reduce weight loss during fasting. Interestingly, no differences in carcass or part yields were found.

The response of birds to salt supplementation in the drinking water seems to be linked to stress time (hours), duration (days), and intensity (temperature). The required concentration of salts may depend on stress intensity, and therefore each situation must be individually assessed.

VI. APPLYING THE THEORY OF ELECTROLYTIC BALANCE

The feed, as the environment, can affect acid-base homeostasis in poultry. There are several reports that demonstrate the effect of dietary electrolytic balance on bird performance. The maintenance of this balance is important to improve the performance of birds raised under high temperatures and to overcome the damaging effects of respiratory alkalosis resulting from heat stress. Feeds with high contents of Cl^- (NH_4Cl, HCl and $CaCl_2$) decrease pH in chicken blood, impairing their growth in neutral conditions.

Heat stress, besides increasing the waste of a large amount of organic acid, may be associated to electrolyte losses through cell membranes (Fischer da Silva et al., 1994). Electrolytic imbalance can be prevented by adding cations and anions – usually expressed in mEq/kg – to the feed (Mongin, 1981). However, the availability of the electrolytes can be influenced by intestinal and renal homeostatic regulation, with higher absorption of monovalent ions.

Some authors have described equations to explain the relationship between cations and anions and acid-base balance. Melliere and Forbes (1966) described this interrelation can be by the equation:

$$\text{Relative level} = \frac{\text{mEq cations}}{\text{mEq anions}} = \frac{Ca + Mg + Na + K}{PO_4 + Cl + SO_4}$$

However, their ratio must be optimal to maintain acid-base homeostasis and to obtain optimal performance. In order to maintain its acid-base balance, the bird needs to regulate acid intake and excretion, as there are differences in dietary cation and anion ingestion and excretion. Also, acids produced by metabolism ($H^+_{endogenous}$) also contribute to acid-base balance.

According to Mongin (1981) , the result of the acid power when ingesting $Na^+ + K^+ – Cl^-$, is equal to the difference between excreted cations and anions ((cations - anions)$_{excreted}$), plus the production of endogenous acid ($H^+_{endogenous}$), plus exceeding alkalis (BEecf) or alkaline reserves. Optimal electrolyte ingestion, in terms of acid-base balance, should minimize the presence of BEecf. The optimal electrolyte balance requirement is defined as mEq ($Na^+ + K^+ - Cl^-$)/kg feed with recommended values for poultry diets generally being around 250.

$$(Na^+ + K^+ - Cl^-)_{ingested} = (\text{Cations} - \text{Anions})_{excreted} + H^+_{endogenous} + \text{BEecf}$$

$$\text{mEqNa} + \text{mEqK} – \text{mEqCl} = 250$$

Examples: 0.28%Na x 10000 / 23.0 = 122 mEq Na
0.90%K x 10000 / 39.1 = 230 mEq K
0.30%Cl x 10000 / 35.5 = 84 mEq Cl
122 + 230 – 84 = 268 mEq/kg feed

In these equations, some factors should be considered:

a) The equation assumes that the minerals Na^+, K^+ and Cl^- have an impact on acid-base balance without considering the way they are ingested. Supplementation of sodium and potassium increases blood pH and HCO_3^-, whereas the addition of chlorine decreases these parameters (Hurwitz et al., 1973). However, there is evidence that metabolizable anions influence acid-base balance. Gorman and Balnave (1994) concluded that weight gain associated with sodium carbonate and sodium bicarbonate was significantly different on diets with the same electrolyte balance, concluding that heat stress can induce a metabolic requirement for the bicarbonate ion;

b) The equation does not take into account the specific effects of each ion, as well as the individual requirements of these ions, which may limit its use; remembering that these ions are not always quantified in the ingredients and that K^+ is present in abundance in most of the ingredients in diets for birds, while Na^+ is present in small amounts. The nutritional recommendations for these electrolytes broilers are varied, NRC (1994) recommends 0,30 and 0,30; 0,20 and 0,15%, 0,20 and 0,15% for K, Na and Cl 0-3 and 3 to 6 weeks of age, respectively;

c) Although other cations and anions also participate in acid-base balance, these are not considered in this equation due to their secondary importance. The electrolytic potential of the elements can classify them in terms of importance in acid-base balance in the body. For example, K^+, Na^+ and Cl^- have more potential than Mg, S, P and Ca, and the electrolytic potential of these is greater than Fe, Mn, Zn, Cu, Se, Mo, Co and I. Trace elements have capacity to act as electrolytes, but they are present in small amounts in feeds and in low concentrations on tissues, which naturally reduces their impact on acid-base balance and electrolyte balance. Thus, the equation would be complete electrolytes $(Na^+ + K^+ + Ca^{2+} + Mg^{2+}) - (Cl^- + SO_4^{2-} + 2PO_4^{2-} + HPO_4^-)$.

d) For those elements not considered in the summarized equation (Mongin, 1981) is of the opinion that: divalent cations are not as rapidly absorbed as monovalent cations, magnesium (Mg) is not usually included in feed, phosphates are difficult to quantify because they come from various sources, the rate of calcium absorption is controlled by the endocrine system, sulphate is present in small quantities and is related to the prevention of catabolism of methionine.

e) There is a relationship between mineral ions and other nutrients such as Na and Cl and arginine: lysine ratio in heat-stressed broilers (Brake et al., 1998). In chickens between 3 and 7 weeks of age reared under heat stress, the arginine: lysine optimum was 1.34, while Na and Cl were kept within the recommendations of the NRC (1994). However, when Na and Cl were increased, the arginine: lysine ratio was below 1.05, showing that under heat stress adjusting the electrolyte balance for maximum broiler performance can be related to amino acid composition diet.

Hurwitz (1981), as opposed to Mongin (1981), proposed that it is the balance between Na^+ and Cl^- that determines plasma HCO_3^- content and pH; K^+, Ca^{2+}, and Mg^{2+} are the other cations, whereas HCO_3^-, proteins, phosphate, sulphate, lactate and pyruvate are the other plasma anions. HCO_3^- and proteins (including haemoglobin) are buffers, and BEecf are considered as metabolic components of acid-base balance. BEecf expresses the amount of acids or bases that, when added to one litre of blood, bring the pH back to normal. Fig. (2) illustrates the relationship between electrolytes and acid-base balance. There was better growth of birds when the Na: Cl ratio was approximately 1:1, with the use of diets with $(Na^+ + K^+ - Cl^-)$ 200mEq/kg (Hurwitz et al., 1973).

Changes in acid-base balance and imbalances in $(Na^+ + K^+ - Cl^-)$ supplementation cause loss of appetite and therefore lower weight gain, worse feed conversion ratio, lower egg production, and when imbalances are not corrected, increased mortality (Mongin, 1981). In

birds in alkalosis, the blood concentration of electrolytes (Na^+, K^+) is decreased. A reduction in the state of alkalosis occurs when the Na: Cl ratio decreases; the addition of 0,5 and 1,0% $CaCl_2$ results in an 8.0% improvement in poultry performance (Teeter et al., 1985).

Johnson & Karunajeewa (1985), concluded that a dietary electrolyte balance lower than 180 mEq/kg and higher than 300 mEq/kg decreases 42d broiler weight, and optimal dietary electrolyte balance was between 250 and 300 mEq/kg. Hulan et al. (1987), investigated the effect of diets containing different $Na^+ + K^+ - Cl^-$ ratios and different calcium levels, and found that the lowest and the highest weight gains were obtained when the "Mongin number" was 174 and 215 mEq /kg, with 1.38 and 0.95% calcium, respectively.

Milliequivalents per liter (mEq/L)							
Substance	Plasma			interstitial fluid		intracellular fluid	
Sodium	142			147		15	
Calssium	5	+154		4	+154,5	150	+ 194
Potassium	5			2,5		2	
Magnesium	2			1		27	
Chloride	105			114		1	
Bicarbonate	24			30		10	
Phosphate	2			2		100	
Sulfate	1	- 154		1	-154,5	20	- 194
Organic acids	6			7,5		–	
Proteins	16			–		63	

Figure 2 Interrelation of electrolytes and acid-base balance

The impact of the cation/anion ratio on broiler acid-base balance, blood pH, and growth rate was studied by Hurwitz *et al.* (1973). Growth rate was maximum when blood pH was 7.28, whereas it declined when pH values were higher than 7.30 or lower than 7.20, whereas the electrolyte balance for maximum growth was 226 to 260 mEq/kg. However, the authors did not clarify if that response is due only to pH changes or to other electrolytic or metabolic effects. During panting, pH values higher than 7.25 depress growth rate and feed efficiency, and that blood pH increases can be reduced when respiratory rate decreases (Teeter et al.,1985).

Animal feed contains protein and energy sources along with macro and micronutrients to meet the animal requirements. Dietary protein source can affect acid-base balance as certain sources, particularly animal proteins, increase the production of organic acids and reduce the contribution of Na and K, increasing the relative amount of Cl (Portsmouth, 1984). The supply of soybean meal-based diets, which contain low Na and high K levels, had a significant influence on the development of broilers supplemented with 0.5 or 1.0% NaCl (March, 1984). Excess of amino acids, such as methionine and cysteine, may interfere with acid-base balance, causing metabolic acidosis. Research studies have shown that reducing crude protein levels and supplementing amino acids during hot months improve performance (Cheng *et al*. 1997a,b). However, reductions in the level of crude protein in diets based on corn and soybean meal, reduce the levels of K due to reduction in the inclusion of soybean meal and may also increase Cl level from synthetic source of amino acids. This scenario may be a problem for nutritionists to formulate diets for broilers reared at high temperatures (Teeter & Belay, 1996).

Balnave & Gorman (1993) showed the benefits of supplementation of $NaHCO_3$ in broilers raised at high temperatures. Kidd et al. (2003) , evaluated the performance of broilers raised in an controlled environment and submitted from 20 to 40d to 34°C for 12 hours followed by 26°C for 12 hours daily, and did not observe any performance improvement when supplying diets containing 107% of the lysine requirements recommended by the NRC (1994). However, when the electrolyte balance was changed from 187 to 225 mEq/kg by the use of $NaHCO_3$, these authors found a 6% improvement in feed conversion ratio and mortality reduction. In a similar study, Zarate et al. (2003) evaluating amino acid requirements of broilers raised in hot environments – capable of reducing performance, but not causing mortality – did not observe performance or carcass benefits of feeding 110% NRC (1994) amino acid requirements (lysine, methionine, arginine, threonine, and tryptophan), but only an increase in abdominal fat deposition. The interaction between dietary protein level and electrolyte balance is not elucidated yet.

VII. ELECTROLYTE BALANCE DURING PRE-STARTER AND STARTER PHASES

The use of a specific diet for broilers during the first week of age is recommended by several nutritionists. This practice is based on the fact that, at this age, broilers have specific nutritional requirements, which are different from other phases, due to their gastrointestinal tract characteristics. Although there is a minimum requirement for crude protein, the oxidation of amino acids supplied in excess may cause metabolic acidosis (Patience, 1990). Studies have been carried out to analyze the interactions between dietary protein and amino acid levels and electrolyte balance. Borges et al. (2002), evaluated two crude protein levels (21.0 and 23.5%) in pre-starter diets, and three electrolyte balances (166, 260, and 360 mEq/kg) in two experiments and concluded that feeds must be formulated for an electrolyte balance around 260 mEq/kg, independent of protein level. Dall'stella et al. (2007) who evaluated the effects of increasing ratios of methionine + cysteine: lysine in the diet (66, 73, 80 and 87%) with fixed electrolyte balance (240mEq/kg) on performance of broilers from 1 to 7 days, concluded that the different ratios evaluated only affected feed intake, and the best ratio was 76%. Thon et al. (2007a) studied the effect of graded levels of digestible lysine (1.065, 1.215, 1.365, and 1.515 mg/kg) and two electrolyte balance values (250 and 320 mEq/kg) on the performance of broilers in the pre-starter phase (0-7d), and concluded that an electrolyte balance of 250 mEq/kg improved feed conversion, whereas there was no response to different lysine levels. Arginine may be antagonized by lysine, and thus become deficient in the diet (Macari et al., 2002). Thon et al. (2007b) evaluated the effect of graded digestible arginine levels (1.313, 1.443, 1.573, and 1.703 mg/kg) and two electrolyte balance values (250 and 320 mEq/kg) on the performance of broilers in the pre-starter phase and concluded that 1.313 mg digestible arginine/kg feed promotes good live performance.

Borges et al., (1999) added NaCl, $NaHCO_3$, NH_4Cl, and $KHCO_3$ to broiler feed during the first week of age, aiming at determining the best electrolyte balance. Two experiments were conducted: potassium levels remained constant in the first trial, and sodium in the second one. The author concluded that the responses depend on the manipulated electrolyte and that extreme Cl (0.15 and 0.71%), K (0.52 and 1.21%), and Na (0.15 and 0.60%) levels must be avoided. The main response to excessive Cl and K seems to be related to feed intake. In these experiments, optimal electrolyte balance ranged from 199 to 251 mEq/kg. Further studies were then performed by maintaining constant levels of potassium and simultaneously manipulating the levels of potassium and sodium in the diet (Borges et al., 2002). Extreme levels of Cl (0,77%) and K (1,05%) depressed feed intake and should be avoided. The best electrolyte balance in pre-starter (0-7d) ranged between 246 and 277 mEq / kg, Fig. (3).

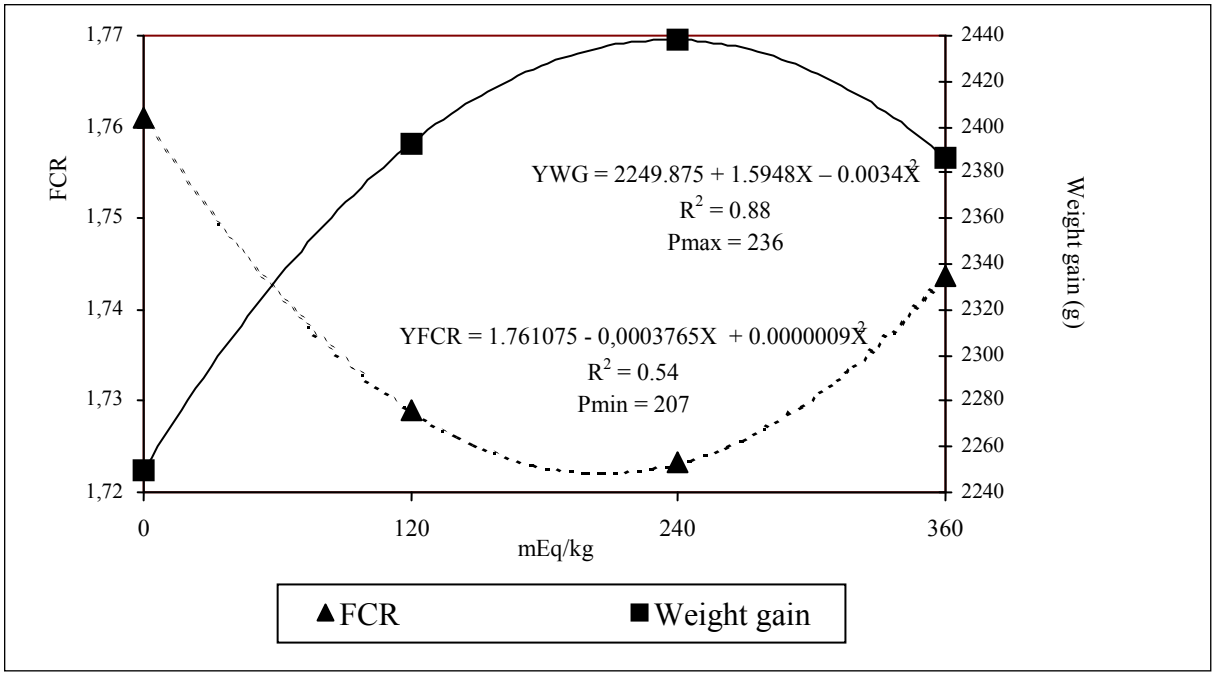

Figure 3 Effect of dietary electrolyte balance on the weight gain and feed conversion. Adapted from Borges et al. (2007).

As for the starter phase (1-21d), literature data are conflicting, and present different estimates for optimum dEB: 250 mEq/kg (Mongin, 1981), 245 to 315 mEq/kg (Rondón, 1999), 186 to 197 mEq/kg (Borges et al., 2003b), and 250 mEq/kg (Borges et al., 2003c). Ugioni *et al.* (2004) evaluated the effect of two protein levels (17 and 19%) in a starter diet formulated on ideal protein concept (Met+Cys 71%, Thr 59%, and Trp 16%) and four electrolyte balances (220, 250, 280, and 310 mEq/kg), and concluded that the feed containing 19% crude protein, formulated on ideal protein, and with 220 mEq/kg promoted the best performance from 1-21d.

VIII. ELECTROLYTE BALANCE DURING THE GROWER PHASE AND OVERALL PRODUCTION

During the grower phase (22-42d), Rondon (1999) suggested a dietary electrolyte balance between 249 and 261 mEq/kg for optimal performance. Oliveira (2002), when adding offal meal or feather meal to feeds, concluded that best performance was obtained with an electrolyte balance between 292 and 300 mEq/kg. Borges et al. (2003b) found best weight gain and feed conversion with 240 mEq/kg by manipulating sodium and chloride levels. Using increasing levels of sodium or sodium and potassium, Borges et al. (2004a) concluded that the best electrolyte balance for this phase is between 202 and 235 mEq/kg.

Considering the entire production (1-42d), broilers raised in a thermoneutral environment presented better performance with an electrolyte balance between 201 and 220 mEq/kg (Borges et al., 2003c); however, under heat stress, the best performance was obtained when the electrolyte balance ranged from 207 to 236 mEq/kg feed. In these studies, electrolyte balance was obtained by manipulating dietary sodium and chloride levels.

Water intake is dependent on bird age, environmental temperature, and the level of salts in the diet. Increasing electrolyte ratio linearly increases water intake and the water intake to feed intake ratio; however, after a certain limit, higher water intake may result in wet litter. Broilers fed diets containing 360 mEq/kg from the first week of age presented

wetter litter compared to those fed 240 mEq/kg, making management more difficult and affecting performance as higher water intake increased passage rate (Borges et al., 2003b). Stimulating water consumption and body water exchange may be beneficial as higher water consumption reduces the mortality of broilers exposed to heat stress (Branton et al. 1986).

Broiler body temperature is also influenced by dietary electrolyte balance (Borges et al., 2003b). During the hot period of the day, rectal temperature decreases linearly as electrolyte balance increases: birds fed diets containing 240 and 360 mEq/kg presented lower body temperature and narrower body temperature range (difference between the lowest and the highest body temperature during the day). This is a direct response to the higher water consumption observed in these birds, reinforcing the hypothesis that stimulating water intake is important to reduce mortality during hot months, as heat dissipation and evaporative loss efficiency increase with water intake. A comparative analysis of broilers raised in thermoneutrality or under chronic heat stress (Borges et al., 2003c) showed the negative impact of heat stress on the performance of broilers already at 21 days of age, and extended to market age. The physiological response to electrolyte balance in these birds shows that those fed between 140 and 240 mEq/kg were less susceptible to heat stress, as shown by less significant changes in the heterophil to lymphocyte ratio, and in blood pH. Alkali concentration in whole blood expressed in mEq/L (BEecf) was also influenced by temperature. Under thermoneutrality, birds tended to exhibit metabolic alkalosis when supplied with feeds containing 140 and 340 mEq/kg. Birds fed 340 mEq/kg always became alkalotic. The authors concluded that an electrolyte balance between 140 and 240 mEq/kg promotes better homeostasis, independent of environmental temperature.

When birds are exposed to acute stress, those fed with electrolyte balance between 140 and 240 mEq/kg had lower body temperatures and blood pH, showing a better acid-base homeostasis, panted less, resulting in lower loss of CO_2. Diets with high $NaHCO_3$ concentration (340 and 360 mEq/kg) caused metabolic alkalosis, independent of the environmental temperature (Borges et al., 2004b). A trial with colostomized broilers raised under thermoneutrality or under chronic heat stress (Borges et al., 2004c) showed that birds are able to regulate the higher volume of water intake by increasing urinary excretion. The amount of excreted electrolytes depends on their concentration in the feed, and on environmental temperature, and the highest electrolytes retention occurred in broilers fed a diet containing 240 mEq/kg.

IX. ELECTROLYTE BALANCE IN BREEDERS

Studies on the application of this concept in commercial layer and broiler breeder are limited. Hamilton and Thompson (1980) and Balnave & Muheereza (1997) found higher egg production in layers as the (Na+K)/Cl ratio increased through the addition of $NaHCO_3$. Eggshell thickness and strength improved (Austic & Keshavarz, 1988), and thin eggshells were reduced (Ernest et al. 1975) when feeds were formulated considering the dietary eletrolytic balance (DEB). Nobakht et al. (2007), working with 24 to 34-week-old layers submitted to different electrolyte balances (0, 120, 240, and 360 mEq/kg), observed significant differences in eggshell quality, weight, thickness, and ashes. Also, specific gravity increased with dietary electrolyte balance. The authors concluded that increasing dietary electrolyte balance up to 360 mEq/kg in layers may improve eggshell quality.

Santos et al. (2005) carried out an experiment with 57,000 broiler breeders to evaluate the impact of the adoption of the electrolyte balance concept (Table 3). Better egg production and feed conversion ratio and lower mortality were observed when the feed was formulated on that concept as compared to the control group. Electrolyte balance concept can be used to

adjust bird acid-base balance, and to improve performance. At least 180 mEq/kg feed is recommended.

Table 3 Effect of feed formulated on electrolyte balance (mEq/kg) on the performance of broiler breeders supplemented with sodium and potassium salts.

Parameter	Control 150 mEq/kg	Electrolyte Balance 180 mEq	CV %*
Egg production (%)	55.75 b	56.81 a	1.13
Yield (%)	98.64	98.61	1.40
Mortality (%)	0.535 a	0.285 b	3.58
Feed intake (g/bird/day)	152	152	--
Eggs/bird/week	3.918 b	3.990 a	1.81
FCR (g/egg)	273 b	268 a	1.82

*Coefficient of variation among treatments. Means followed by different letter is the same row are statistically different (P≤0.05 by Tukey test). Adapted from Santos *et al*. (2005).

X. CURRENT & FUTURE DEVELOPMENTS

Physiological parameters should always be used by nutritionists thinking of adopting new technologies. These parameters should be studied and interpreted as a whole, and put into a production context. Electrolytes may be supplemented both in feed and in the drinking water; however, when using electrolytes, water availability, quality, and temperature should be taken into account as electrolyte supplementation stimulates water consumption. This is a positive response when birds are submitted to heat stress, as it allows better body heat dissipation but care must be taken not to exceed 250 mEq, considering both water and feed, as a high electrolyte balance causes metabolic alkalosis, independent of environmental temperature, resulting in performance losses.

Feed formulation based on the electrolyte balance concept can be implemented to correct acid-base balance distortions; however the adoption of this practice requires a detailed study of the ingredients available for feed formulation, particularly as to Na, K, and Cl contents. Feeds should be formulated to supply an electrolyte balance between 180 and 260 mEq/kg (decreasing as bird ages). This range allows the birds to maintain the acid-base balance required for optimal performance.

The main limiting factor for adopting this concept is the increase in litter moisture, particularly when broilers are 28 days of age or older. It must be highlighted that, although Na, K, and Cl are the most important ions for the electrolyte balance and acid-base balance for monogastric animals, other ions may be considered, making the equation more complex. Therefore, the relationship among acid-base balance, heat stress, and other cations and anions must be further studied.

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GENETIC APPROACHES TO REDUCING HEAT SUSCEPTIBILITY IN BROILERS

A. CAHANER[1]

Summary

Modern broilers are characterized by high growth rate (GR), high rate of feed intake and metabolism, and elevated internal heat production, all acting as internal stressors that enhance the effects of external heat stress. Hot ambient temperature hinders dissipation of excessive internal heat, leading to elevated body temperatures, depressed appetite and growth (which results in poorer feed conversion ratio), and higher mortality. These negative effects can be alleviated by compromising sustainability: costly climate-controlled housing or lower efficiency (low stocking density, low marketing weight). The negative effects of heat can be mitigated by reducing feather coverage thus enhancing heat dissipation. During the 1990's it was shown that the reduced feather coverage in naked neck broilers provides partial heat tolerance; hence it was hypothesized that higher heat tolerance can be achieved by complete elimination of the feather coverage. Experimental featherless broilers were developed by repeated backcrossing of the *Scaleless* mutants (*sc/sc*) to contemporary fast-growing broilers. Featherless broilers and their feathered counterparts were compared in a series of studies under warm and hot conditions ranging from 25ºC to 35ºC without cooling or forced ventilation, on diets ranging in nutrient density, and at stocking densities between 7 to 22 birds/m^2. It was demonstrated that hot conditions had no negative effects on welfare and livability of featherless broilers. The heat tolerance of the featherless broilers was reflected also in their superior performance under hot conditions: higher growth rate, better FCR, and higher yield and quality of carcass and breast meat. Moreover, featherless broilers exhibited superior performance under heat stress without reducing stocking density and on low-density diets, thus maintaining efficient production, similar to that of standard broilers under normal conditions. With their various advantages combined, featherless broilers can markedly improve the sustainability of broiler meat production under hot conditions (Cahaner *et al*., 2010a,b).

I. INTRODUCTION

Remarkable genetic progress has been achieved in broiler growth rate (GR) and meat yield since the 1950s (Havenstein et al., 2003). Greater GR of broilers is driven by greater rate of feed intake and metabolism, and consequently there is an elevation in production of internal heat (Sandercock et al., 1995). However, the continuously increasing potential for rapid growth, and the consequent desirable reduction in time to marketing with its contribution to better feed efficiency, cannot be fully expressed under hot conditions (see Cahaner, 2008, for review). Hot conditions decrease the difference between ambient temperature (AT) and the average temperature of the body surface, reducing the rate at which metabolic heat can be dissipated. The lower rate of sensible heat loss leads to an elevation in body temperature (BT) which may lead to mortality under very high temperature conditions or acute heat waves.. Under less extreme or chronic hot conditions, broilers acclimate by reducing feed intake (Eberhart and Washburn, 1993; Cooper and Washburn, 1998; Deeb and Cahaner, 1999, 2001), but consequently GR is reduced, resulting in lower marketing body weight (BW) and poorer breast meat yield (Cahaner and Leenstra, 1992; Leenstra and Cahaner, 1992; Settar et al., 1999).

[1] The Hebrew University, Faculty of Agriculture, P.O. Box 12, Rehovot 76100, Israel

Hot conditions can be avoided in modern broiler houses equipped with efficient cooling systems. However, the global broiler industry continues to expand to hot-climate developing countries where climatic control of broiler houses is limited due to high installation and operational costs and an unreliable supply of electricity. The use of cooling systems is presently increasing also in temperate-climate countries, because contemporary commercial broilers (CCB) are continuously selected for greater GR and meat yield and reared to higher BW, and consequently generate more metabolic heat (Sandercock et al., 1995). They thus need lower AT in order to maintain normal BT and to fully express their genetic potential for rapid growth (Emmans and Kyriazakis, 2000). With the limited availability and rising cost of energy, and the increasing tendency to minimize the total amount of resources used for human food production, artificial cooling of broiler houses is also becoming an economical and political burden in developed countries. Breeding heat-tolerant broilers may offer a sustainable approach to mitigate the negative effects of heat on broiler production.

Skin temperature in broilers is lower than BT by only about 0.5ºC (Yahav et al., 1997), but due to the insulation of the feathers, the temperature of the feather-covered body surface is close to AT, hence this surface contributes minimally to the overall sensible heat loss (Cangar et al., 2008). It was shown that, in high-GR broilers under hot conditions, feather coverage impedes thermoregulation because it hinders sensible heat loss (Yahav et al., 1998; Deeb and Cahaner, 1999). Already in the 1980's and 1990's, several groups have tested the hypothesis that the negative effects of high ambient temperatures can be alleviated by introducing genes that reduce or eliminate feather coverage into the genetic makeup of CCB stocks (e.g., Somes and Johnson, 1982; Hanzl and Somes, 1983; Merat, 1986).

II. GENETIC AND BREEDING ASPECTS OF HEAT STRESS

a) Heat stress effects on the performance of high-GR broilers

Chickens, like all homeothermic animals, maintain a constant body temperature (BT) over a wide range of AT. In birds, heat loss is limited by feathering and by the lack of sweat glands. The ability of animals to maintain BT within the normal range depends on a balance between internally-produced heat and the rate of heat dissipation. The amount of internal heat produced by broilers depends on their BW and feed intake. The heat dissipation rate depends on environmental factors, mainly AT, and on feather coverage. When the physiological and behavioral responses to high AT are inadequate, an elevation in BT occurs, causing a decrease in appetite and in GR. Consequently, the time needed to reach marketing weight is increased, leading to poorer feed conversion and overall lower efficiency of poultry meat production (Cahaner and Leenstra, 1992; Leenstra and Cahaner, 1992; Settar *et al*., 1999). Moreover, hot conditions depress the yield and quality of broiler meat (Leenstra and Cahaner, 1992; Mitchell and Sandercock, 1995; Sandercock *et al*., 2001), and may lead to PSE (pale, soft, exudative) meat (Barbut, 1997). Therefore high AT has been the main factor hindering broiler meat production in hot climates, especially in developing countries where farmers cannot afford costly artificial control of AT in broiler houses.

b) Selection on GR under hot conditions

Breeding for adaptation to a specific stressful environment is the strategy of choice when GxE interaction affects economically important traits (Mathur and Horst, 1994). Such breeding activity may take place in a particular stressful location (localized breeding) or under artificially induced stress. We are aware of only one published report of experimental selection of broilers under controlled hot conditions (El-Gendy *et al*., 1992). Lack of later reports by these authors, or others, may indicate that this approach was not successful. Commercial localized breeding under

suboptimal hot conditions has been applied successfully in India (Jain, 2000, 2004). When compared under local hot conditions, the imported high-GR broiler stocks were inferior to the locally-bred stock, but in absolute terms the latter's performance was much lower than the genetic potential of contemporary high-GR stocks, i.e. their performance under optimal conditions. Thus, it could be concluded that broilers cannot be bred to exhibit high GR and high BW (in absolute terms) under hot conditions. So far, the latter has not been an important limitation in most hot-climate countries where customers traditionally prefer to buy live broilers with small body size (≈1.5 kg). However, broilers that are produced for mechanical slaughtering and processing must have large BW at marketing and high yield of quality meat – the traits most depressed in high-GR broilers reared under hot conditions. Therefore, with the current trend to increase production of carcass parts and deboned meat in hot-climate countries, either for export or for local consumption, it will no longer be possible to avoid the negative effects of heat by marketing small-body broilers.

c) The effects of the *Naked Neck* gene (*Na*) on feather coverage and heat tolerance

Many studies had been conducted with the co-dominant 'naked-neck' (*Na*) gene, which is common in rural chicken populations in hot regions (Merat, 1986). This gene reduces feather coverage by 20% and 40% in heterozygous (*Na/na*) and homozygous (*Na/Na*) chickens, respectively (Crawford, 1976; Cahaner *et al.,* 1993; Yunis and Cahaner, 1999; Cahaner et al., 2008). Merat (1986) suggested that heat tolerance of chickens can be improved by the *Na* gene. Under hot conditions, naked-neck broilers exhibited greater sensible heat loss (Yahav et al., 1998) and better thermoregulation (Deeb and Cahaner, 1999), resulting in greater actual GR and meat yield than their fully feathered counterparts (Cahaner et al., 1993; Yalcin et al., 1997; Deeb and Cahaner, 2001). However, in these studies, the naked-neck broilers raised at 25°C were superior to their counterparts at hot conditions, suggesting that the 20 or 40% reduction in feather coverage provides only partial heat tolerance. Hence it was hypothesized that complete feather elimination may enhance heat tolerance of genetically fast-growing broilers (Cahaner et al., 2003; Cahaner, 2008).

d) The *Scaleless* gene (*sc*)

Abbott and Asmundson (1957) reported on a recessive mutation called *Scaleless* that blocks feather formation in homozygous (sc/sc) chicken embryos. This spontaneous mutation was found in the New Hampshire breed, which is much lighter and slower-growing than contemporary commercial meat-type chickens. The featherless mutants were thus not considered for practical purposes (Somes, 1990). In the late 1970s, experimental featherless broilers were derived from a cross between the scaleless mutant and commercial broilers of that time. Under hot conditions, the GR and carcass composition of these featherless birds were superior to those of their feathered counterparts (Somes and Johnson, 1982), but the effects were small because the GR of the birds in this study was very low: maximum GR of 30 g/d and average BW of approximately 1200 g at 8 wk (compared with about 100 g/d in today's broilers that reach the same BW in about 4 weeks).

The development of a new line of featherless broilers was initiated in the year 2000 by crossing original scaleless mutants with contemporary high-GR broilers, followed by a series of backcrossing and intensive selection on BW (Cahaner and Deeb, 2004). The birds in this line are either normally feathered (*Sc+/sc*, carriers of the *sc* allele) or featherless (*sc/sc*). Currently, the genetic potential GR of this experimental line is only slightly lower than that of contemporary commercial broilers. When compared to their feathered sibs (brothers and sisters with the same genetic background) the featherless broilers can exhibit the net effects of being featherless on economically-important traits under the trial's conditions.

e) Viability of featherless broilers vs. their feathered counterparts

At an earlier stage of the development of the experimental line of featherless broilers, 27 featherless birds and 49 of their feathered sibs were reared in an AT-controlled chamber (Azoulay *et al*., 2011). On Day 47 the AT was gradually elevated from 30 to 35ºC for 2 days, leading to an increase in BT to 42.8ºC in feathered birds, but only to 41.4ºC in featherless birds. On Day 53 (BW averaged 1900g) AT was elevated to 36ºC. This led to lethal elevation of BT and death of 17 feathered birds (35%), whereas BT of the featherless birds remained at 41.4ºC, and only 1 of the 27 birds died. In a recent trial, after GR of the experimental line had been enhanced by several additional cycles of backcrossing to CCB, featherless broilers and their feathered sibs, as well as feathered commercial broilers, were kept together under constant hot conditions (32±1ºC). When the birds where 41 days old (BW of about 1750 g), AT in one room increased un-intentionally to 38ºC for about 5 hours. Consequently, 20 of 28 commercial broilers died (71%), 30 of 72 feathered sibs died (42%), but only 2 of 100 featherless birds died (unpublished data). This event suggests an association between potential GR and susceptibility to heat in broilers with feathers, and demonstrated the exceptional heat resistance of the featherless birds, regardless their GR.

These two studies indicate that the welfare and livability of featherless broilers are not compromised under acute hot conditions. Maintaining normal BT even under extreme AT is apparently the key to the heat tolerance of the featherless broilers. Elevated BT under heat stress was shown to negatively affect GR, feed consumption and feed conversion in standard broilers (Cooper and Washburn, 1998). The results suggest that the superior GR and meat yield of featherless broilers under high temperature conditions in comparison to high-GR standard (feathered) broilers, are due to their capacity to dissipate all the excessive internally-produced heat and maintain normal BT, and consequently normal (i.e., not-depressed) feed consumption and GR.

f) Comparing slow-growing featherless broilers vs. naked neck and feathered counterparts

A unique study (Cahaner et al., 2008) consisted of 4 experimental genetic groups (fully feathered, heterozygous naked neck, homozygous naked neck, featherless), progeny of the same double-heterozygous parents (*Na/ na +/sc*), as well as commercial broilers as industry reference. Birds from all 5 groups were brooded together until d 21 when one-half of the birds from each group were moved to hot conditions (constant 35°C), whereas the others remained under comfortable conditions (constant 25°C). Individual BW was recorded from hatch to slaughter at d 45 and 52 at 25 and 35°C, respectively, when breast meat, rear part, and heart weights were recorded. Body temperature was recorded weekly from d 14 to 42.

Feather coverage substantially affected the thermoregulatory capacity of the broilers under hot conditions. With reduced feather coverage (naked-neck), and more so without any feathers (featherless), the birds at 35°C were able to minimize the elevation in body temperature. Consequently, only the featherless birds exhibited similar growth and BW under the two temperature treatments. The naked-neck birds at 35°C showed only a marginal advantage over their fully feathered counterparts, indicating that 20 to 40% reduction in feather coverage provided only limited tolerance to the heat stress imposed by hot conditions. Breast meat yield of the featherless birds was much greater (3.5% of BW, an 25% advantage) than that of their partly feathered and fully feathered counterparts and the commercial birds under hot conditions. The high breast meat yield (at both 25 and 35°C) of the featherless broilers suggests that the saved feather-building nutrients and greater oxygen-carrying capacity contribute to their greater breast meat yield. Because of these results, it was concluded that further research on genetically heat-tolerant broilers should focus on the featherless phenotype rather than naked neck ones.

g) Comparing fast-growing featherless broilers vs. feathered counterparts

The experimental birds in the previous study (Cahaner *et al.,* 2008) reached a mean BW of only about 1200 g at 6 weeks of age with maximal GR of about 55 g/d. This GR was higher than that of the birds studied by Somes and Johnson (1982), yet substantially lower than that of contemporary commercial broilers (CCB). Hence the practical relevance of the conclusions regarding the advantages of featherless broilers remained questionable. Therefore the genetic potential of the featherless experimental line was enhanced by additional cycles of backcross to CCB stocks. The backcross progeny were used in several studies, with the objective to compare actual GR and performance-related traits of featherless broilers vs. their normally feathered siblings (sibs) and also a group of CCB as industry reference. The AT treatments, after the brooding period, were either constant 35°C (Hot-AT) or constant 25°C (Control-AT) (Azoulay *et al.*, 2011).

The broilers from all groups were reared intermingled to 46 or 53 days at Control- and Hot-AT, respectively, and the measured traits included body temperature (BT), growth, and weight of whole-body and carcass parts: breast meat, legs, wings, and skin. At Hot-AT, only the featherless broilers maintained normal BT; their mean day-46 body weight (2031g) was significantly higher than at Control-AT, and it increased to 2400g on day-53, much higher than the corresponding means of all feathered broilers (~1700g only). The featherless broilers had significantly higher breast meat yield (~20% in both ATs), lower skin weight and supposedly better wing quality (Azoulay *et al.,* 2011). These results confirmed that being featherless improves the performance of fast-growing broilers at hot conditions and suggest that introduction of the featherless phenotype into commercial broiler stocks facilitates highly-efficient yet low-cost production of broiler meat at hot conditions.

h) The effects of being featherless on meat yield and quality in normal and hot condition

In optimal conditions, the cardiovascular system in contemporary feathered broilers develops simultaneously with the muscles growth allowing adequate levels of oxygen and nutrients supply, and clearing metabolism waste products. Hot conditions reduce the feed intake as well as cardiovascular capacity in these broilers, and consequently reduction in breast meat yield and its quality are commonly observed. The latter may lead to Pale, Soft and Exudative (PSE) meat, possibly due to insufficient capillary support. Accordingly, featherless broilers should have higher meat yield and quality under hot conditions. This hypothesis has been tested in 2 trials with 4 groups of broilers: featherless, their feathered siblings, contemporary commercial (Comm), and experimental line representing commercial broilers of the 1980's. Half of the chicks from each group were reared under moderate ambient temperature (constant 26ºC). The remaining birds were reared under hot condition (constant 32ºC) to 45 d and 48 d of age in trials 1 and 2 respectably (Hadad et al., 2010).

At trial's end (45d and 48d in Trials 1 and 2, respectively) about 100 birds per room, equally representing all genetic groups, were randomly selected for carcass measurements. The breast meat (pectoralis major and pectoralis minor) was removed from each carcass (by a single operator) and weighed. The rear part of each carcass, consisting of pelvic, thighs and drumsticks (bones included) was also weighed. The heart of each bird was also removed and weighed. Color of breast meat was measured by Minolta spectro-colorimeter with the CIELAB (L*, a*, b*) system. The L* (lightness) describes the relationship between light reflection and absorption, which relates to the liquids exudates from the meat. The a* (redness) indicates redness when positive and greenness when negative. The b* indicates yellowness when positive or blueness when negative. These measures are commonly used to evaluate variations in meat quality. Color was measured 24 h post-mortem. Drip loss was determined from the reduction in the weight of pectoralis major during 48 hours (from 24 to 72 hours post-mortem) of storage in plastic bags at 4ºc.

Average BW of the Comm broilers reared under heat (32ºC) was 2050g at 47d, 650g lower than their counterparts under moderate temperature (25ºC) (Fig. 1). The 1980's broilers had a lower growth rate, and the heat reduced their mean BW by only 380g (1420 vs. 1800 g). Heat did not affect significantly the growth rate of the featherless broilers; their BW averaged 2000g and 2150g in under hot and moderate conditions, respectively. In moderate conditions, breast meat yield averaged 23%, 22%, 18% and 13% for the Featherless, Comm, feathered and 1980's broilers, respectively. The hot conditions significantly reduced breast meat yield of Comm broilers, from 22% to 19%, and also negatively affect the quality of their meat, e.g. drip loss was 2-fold higher than under moderate temperature. The featherless broilers had the highest breast meat yield (23 and 22%) and also highest quality, reflected by lower drip loss and lightness (L*): 2.6% and 54 vs. over 4.4% and 58 in the feathered broilers. Significantly larger hearts (%BW) and higher breast meat redness (a*) in the featherless broilers (0.6% and 4.03 vs. about 0.4% and 3 in the other groups) suggest that the superior breast meat yield and quality in the featherless broilers is associated with better capillary support (Hadad et., 2010).

i) Nutrition of featherless vs. feathered broilers under normal and hot condition

Trials on the effects of dietary protein and energy content on performance of feathered vs featherless broilers were conducted under temperate (26ºC) and hot (32ºC) conditions and varying stocking densities. Commercial 3rd (days 17-31) and 4th (days 31-46) diets were used as Control. Experimental diets had lower contents (down to 80% of control diets) of protein or energy or both. Body weight (BW) gain and feed consumption were recorded from day 17 to end of trial, when breast meat yield was determined (Tsur et al., 2010).

Under temperate conditions, lower protein and energy contents in the diluted diets reduced body weight and breast meat yield in the feathered broilers, but not in the featherless broilers. It appears that the featherless broilers have lower protein requirement, as could be expected because they do not need the amino acids used to build the feathers in standard broilers. With the lower requirement for protein and energy, being featherless improves also the economic FCR (lower feed costs), and also reduces the environmental impacts of processing, by avoiding the plucking and dumping of the feathers. The hot conditions reduced the performance of standard broilers to a similar extent in all diets, due to lower feed intake of all diets. The heat did not depress feed intake and performance of the featherless broilers (Tsur et al., 2010).

j) The effects of stocking density on feathered vs. featherless broilers under hot condition

In tropical developing countries (e.g. Indonesia) where broiler producers cannot afford costly cooling and ventilation, production of relatively large and meaty broilers is based on high-GR stocks reared at low stocking density of about 7 to 8 birds/m^2. Trials were conducted to quantify the effects of stocking density under hot conditions (constant 32±1ºC) on GR and meat yield and quality of commercial high-GR broilers vs. featherless broilers (Yadgari *et al*., 2006). Feathered broilers were reared at densities ranging from 7 to 17/m^2 and the featherless birds were reared at densities ranging from 12 to 22/m^2. GR of feathered broilers was depressed by increasing stocking density; BW on Day 44 decreased from 2.4 kg (7/m^2) to 1.8 kg (17/m^2). GR of featherless broilers was only marginally affected by stocking density, with mean BW of 2.4 kg (12/m^2), 2.2 kg (17/m^2), and 2.1 kg (22/m^2); the latter resulting in live-weight production of 46 kg/m^2. The stress of heat and high stocking density reduced breast yield of high-GR broilers to 15%, with pale meat (L*=50, a*=4) and 4% drip loss. In the featherless broilers, breast yield was 19% in all stocking densities, with darker meat (L*=44 and a*=5) and less than 2% drip loss. Thus, in contrast to the negative association between

high GR and meat yield and quality of feathered broilers under heat (e.g., Mitchell and Sandercock, 1995), featherless broilers produced high yield of quality breast meat also in hot conditions and at high stocking density.

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ADDITION OF ENZYMATIC COMPLEX AND DEACTIVATED FULL-FAT SOYBEAN PARTICLE SIZE OPTIMIZE PERFORMANCE OF BROILERS

S.A. BORGES[1], A.V. FISCHER DA SILVA[2], M. OPALINSKI[2], F.B. COSTA[2], A.N. PAIM[2] and E.C.C. OLIVEIRA[2]

Summary

This experiment was carried out to evaluate the effect of the addition of an enzymatic complex and deactivated full-fat soybean particle size on performance of broilers in the period from 1 to 42 days of age. Four hundred and eighty Ross male broiler chicks were used, allocated to a completely randomized factorial scheme 2x2 (enzyme x particle size). The experimental diets were based on corn, soybean meal and (±1.5 mm or ±4.0mm) deactivated full-fat soybean, with or without enzyme. The enzymatic complex (xylanase, b-glucanase, mannase, pectinase and protease) was added at 50g t^{-1} ration. Addition of the enzymatic complex increased (P=0.0531) feed intake by 4% and weight gain by 2.8% in the final period. Feed intake increased by 3% in the final period and 2% overall in poultry that were fed the diet with a fine particle size. In the initial period, the chicks that ingested the diet with a coarse particle size had a 5% greater weight gain and had higher feed intake. The addition of the enzymatic complex in diets with deactivated full-fat soybean improves the performance of chickens. The use of the soy with fine particle size (1.5mm) in the initial phases for chickens is not recommended.

I. INTRODUCTION

Growth in production of broilers has led nutritionists to find solutions to meet the nutritional needs of birds which, due to rapid growth, require better quality food. Some factors such as strain, sex, age, temperature, density, physical form and particle size of food, influence the performance of broilers.

The particle size of the food and physical form of the ration influence their rate of passage in the gastrointestinal tract (Macari et al., 1994). Thus, birds fed diets with finely mash may have reduced their consumption and thus gain less weight gain. However, birds fed diets with coarser grain size have the speed of passage of larger particles reduced, resulting in better weight gain (Nir et al., 1994).

Previous studies that evaluated the effect of temperature, energy levels and particle size on performance of chickens have shown that consuming animal feed with smaller particle size resulted in better performance (Lott et al., 1992). It was also verified by Dahlke (2000) that feed rations with finer grain size resulted in poorer feed conversion and lower weight gain compared to diets with coarse grain size, most likely due to reduced feed intake.

The whole soybean antinutritional factors present such as trypsin inhibitors, lectins and non-starch polysaccharides. However, the industry applies thermal processes such as extrusion and roasting, aiming to disable the antinutritive compounds of raw grain. These processes improve the nutrient digestibility and weight gain of birds, however, there are some anti-nutritional factors and low digestibility of constituents that are not affected, either wholly or in part by these processes.

[1] Faculty of Life Sciences and Health, University of Tuiuti do Parana, Brasil
[2] Faculty of Life Sciences and Health, UFPR, Brasil

The presence of non-starch polysaccharides soluble in the intestinal lumen increases the viscosity of digesta due to the formation of polymers or gels with the water, compromising digestion and absorption of nutrients by inhibiting the action of digestive enzymes and diffusion of substances related to digestion and absorption. The increased viscosity in the gut affects the digestibility of starch, protein and lipid (Nunes et al., 2001).

Research shows positive responses to nutrient digestibility and performance of broilers fed diets based on corn and soybeans, when they were supplemented with enzymes such as carbohydrases, proteases, pectinases, and alpha galactosidases (Brito et al., 2006.; Torres et al, 2003, Costa et al., 2004).

The use of enzyme additives in the diets may induce reductions in levels of energy, protein and amino acid requirements in the formulation of diets for broilers, by a minimum reduction of 2.5%, which reduces the cost of feed, even including the cost of the enzyme (Soto-Salanova et al., 1996). Based on the above, this study aimed to evaluate the effect of particle size of soybean and the addition of exogenous enzyme on performance of broilers.

II. MATERIAL AND METHODS

The experiment was conducted on the premises of the Federal University of Paraná. The birds were housed in conventional housing divided into boxes, with dimensions 1.5 x1.0m with floor of wood shavings. Chicks were brooded using an electric bell equipped with infrared lamp for each box. A total of 480 day old Ross male broiler chicks, with an average weight of 44g, were housed 20 animals per box, with six replications per treatment. The newly hatched chicks were weighed when they reached the aviary and then were housed in cages. The animals received food and water ad libitum throughout the experiment and 24 hours of light. At night the lighting was artificial and during the day, we used natural lighting.

The experimental diets were formulated based on corn, soybean meal and soybean with a particle size of 1.5 mm or 4.0 mm with or without added enzymes. The complex of enzymes (xylanase, beta-glucanase, mannase, pectinase and protease) was added at a dosage of 50 g T-1 feed, considering that this contributed 50kcal IN-1 kg of feed. The inert material used to replace the enzyme was kaolin. Birds and rations were weighed on the 7th, 21st and 42nd days of age for determination of body weight, weight gain and feed intake in the period. The feed conversion ratio was obtained from the relationship between food intake and weight gain during the period. The birds were distributed in a completely randomized design in a 2x2 factorial design (x enzyme particle size). Data on feed intake, weight gain and feed conversion were subjected to analysis of variance.

III. RESULTS AND DISCUSSION

The results presented (Table 1) show that food consumption was significantly increased (P = 0.05), by adding the enzyme complex in the period of 1-42 days. Similar results were found by Costa et al. (2004), who found no difference in consumption between birds supplemented with dietary enzyme complex in the starter phase, however, in the final period, the supplemented birds had higher intake compared to birds not supplemented. This variation in food consumption is probably related to the energy equivalent of the enzyme in the proposed formulas (50kcal IN kg-1 diet).

Table 1 Effect of the enzymatic complex and deactivated full-fat soybean particle size on feed consumption of broilers.

Main effects		Feed consumption broilers (Kg)		
		1-7 days	1-21 days	1-42 days
Enzyme	With enzyme	0.163	1.031	3.788
	Without enzyme	0.163	1.068	3.715
Granulometry (mm)	1,5 mm	0.163	1.046	3.789a
	4,0 mm	0.162	1.053	3.714b
		Probability		
Enzyme (A)		0.983	0.060	0.053
Granulometry (B)		0.545	0.735	0.048
A x B		0.674	0.690	0.288

For the whole period of rearing, the diet with soybean grain size 1.5 mm resulted in a 2% increase in consumption of poultry. The results disagree with Nir et al. (1994), Dahlke (2000) and López & Baião (2002), who found that feed rations with finer grain size resulted in lower food intake when compared to diets with coarser grain size. Whereas the composition of feed corn and other ingredients remained the same size and inclusion, reduction in size of soy particles may have increased the passage rate in the gastrointestinal tract of the broilers.

Over the full trial, the addition of the enzymes increased (2.8%) weight gain compared to diets without supplementation. Zanella (1998) observed no effect of enzyme supplementation in all phases except for 38 to 45 days, and weight gain of birds increased by 2.2%. Brito et al. (2006) found that the addition of a multienzyme complex resulted in a 3.8% increase in the weight gain of birds. This improvement in weight gain with dietary enzyme supplementation may be associated with better digestibility of these diets (Opalinski, 2006).

Table 2 Effect of the enzymatic complex and deactivated full-fat soybean particle size optimise weight gain of broilers.

Main effects		Feed consumption broilers (Kg)		
		1-7 days	1-21 days	1-42 days
Enzyme	With enzyme	0.136	0.787	2.402[a]
	Without enzyme	0.136	0.799	2.334[b]
Granulometry (mm)	1,5 mm	0.133[b]	0.780	2.387
	4,0 mm	0.140[a]	0.806	2.349
		Probability		
Enzyme (A)		0.825	0.403	0.007
Granulometry (B)		0.004	0.066	0.114
A x B		0.086	0.457	0.710

The particle size of soybean influenced weight gain in the period from one to seven days old. In the initial period, the birds that ate feed containing soybean grain size 4.0 mm had a 5% higher weight gain compared to birds that were fed a diet composed of soybean grain size of 1.5 mm. Similar results were observed by Lott et al. (1992), which demonstrated that animals that ate food with smaller particle size showed better performance.

The results presented (Table 3) show that the feed conversion ratio was not affected ($P < 0.05$) by the enzymatic complex in any period. Clementine et al. (2002) found that over a

period of 21 days, broilers fed diets containing normal nutritional levels and enzyme protein levels and reduced energy and 3% in 2 supplemented with 2 and 3% for enzymes, respectively, achieved the best results for feed conversion compared with chickens fed diets with normal levels without enzyme supplementation and diets with low nutrient levels in 1% supplemented with 1% enzyme. This result is similar to those observed by Costa et al. (2004). In other studies, Zanella (1998) and Torres et al. (2003) found no statistical difference in feed conversion among the treatments with enzyme supplementation, with reduced levels of energy relative to treatments without enzyme supplementation with normal levels of energy, highlighting the efficiency of enzymes in energy utilization of diets.

Table 3 Effect of the enzymatic complex and deactivated full-fat soybean particle size optimise feed conversion broilers.

Main effects		Feed consumption broilers (Kg)		
		1-7 days	1-21 days	1-42 days
Enzyme	With enzyme	1,100	1,311	1,577
	Without enzyme	1,196	1,338	1,592
Granulometry (mm)	1,5 mm	1,234[b]	1,343[b]	1,588
	4,0 mm	1,162[a]	1,306[a]	1,581
		Probability		
Enzyme (A)		0,843	0,136	0,188
Granulometry (B)		0,000	0,045	0,533
A x B		0,082	0,651	0,056

Feed conversion was significantly affected by the soybean grain particle size during periods of one to seven and one to 21 days. From 1 to 7 days, the birds fed the diet with soybean grain size of 4.0 mm returned FCR which was 6% better than birds fed diets with soybean meal particle size of 1.5 mm. From 1 to 21 days of age, the soybean grain size of 4.0 mm showed the best feed conversion. These results agree with Baião & López (2004), who found higher feed conversion efficiency with coarser grain size. Murta et al. (2004), working with different particle sizes of sorghum (1.20 mm 4.76 mm 6.35 mm and 9.52 mm) for chickens for eight to 42 days old, found no difference between the treatments.

IV. CONCLUSIONS

From the results presented, it appears that feed intake and weight gain of broiler are improved by the addition of an enzymatic complex containing, xylanase, beta-glucanase, mannanase, pectinase and protease. In diets based on deavtivated full-fat soybean, the addition of the enzyme complex improves performance of broilers.

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IMPACT ON QUALITY OF SOYBEAN OIL AND VITAMIN E ON THE INTESTINAL MUCOSA OF TURKEYS

C. ROCHA[1], A.V. FISCHER DA SILVA[1], L.N.E. BARRILLI[1], S.C. DASSI[1], L. PIERRI[1], F.L.P. VALLE[1] and S. A. BORGES[2]

Summary

The addition of oxidized fat in dietary rations may result in damage to the intestinal mucosa, affecting the development of animals. Because vitamin E has an antioxidant action, it helps to fight against free radicals, preventing the oxidation of lipids. Because of the unfavourable impacts caused by oxidized fat in the diet and the antioxidant properties of vitamin E, the aim of this study was to evaluate the effects of oxidized oil, with or without vitamin E supplementation, on membrane peroxidation and morphometry of the intestinal epithelium of turkeys raised from 1–21 days of age. Five hundred and four male turkeys (B.U.T. 9) were distributed among six treatments, each with six replicates of fourteen birds. Fresh or oxidized oil (0, 110, 250 meq peroxide/kg oil), with or without additional vitamin E (65 or 800 mg/kg fed), was added to the diet. A completely randomized 3x2 factorial design was used. A significant interaction was observed between the oil oxidation level and the vitamin E level added to the diet and the villus height and crypt depth. The addition of oxidized oil damages the intestinal epithelium of turkeys. Supplementary vitamin E promotes maintenance of the integrity of the intestinal mucosa of the turkeys which are fed with oxidized oil.

I. INTRODUCTION

The gastrointestinal tract of birds is the site of entry of dietary ingredients, being responsible for the functions of digestion, nutrient absorption and body protection. Oils of vegetable or animal origin are added to diets, to improve the flavour and texture of food, ensure a higher energy density, improve feed conversion and reduce the heat increment (Braga and Baião, 2001). However, the use of fats and oils in animal feed has limitations, because it is an ingredient highly susceptible to oxidation, particularly the lipid sources rich in polyunsaturated fatty acids. This process promotes the formation of free radicals that give rise to compounds such as peroxides, aldehydes, ketones and alcohols that may be detrimental to animal health and production performance. These substances primarily cause disturbances in the mucosa, damaging cellular structures, altering their integrity, compromising the absorption of nutrients and affecting the immune response (Dibner et al., 1996). To avoid the problem of lipid oxidation, natural antioxidants such as vitamin E (α-tocopherol) can be added to diets. Such compounds can prevent the oxidation process (Batista et al., 2007), minimizing the harmful effects of free radicals to the intestinal mucosa to exert a protective effect against peroxidation of phospholipids of plasma membranes and organelles (Batista et al., 2007). The aim of this study was to evaluate the effects of oil addition with different degrees of oxidation, with or without supplementation of vitamin E on lipid peroxidation and morphology of intestinal mucosa of turkeys.

[1] Universidade Federal do Paraná, Brazil
[2] Universidade Tuiuti do Paraná, Brazil

II. MATERIAL AND METHODS

Five hundred and four day-old male poults (BUT 9) were raised in cages and supplied with food and water ad libitum during the experimental period. The birds were allocated to six treatments with six replicates of 14 animals each, totalling 36 experimental units. The design was a completely randomized 3x2 factorial (three levels of oxidized soybean oil and two levels of vitamin E), as shown in Table 1. The diets were isocaloric and isonutrient, from corn and soybeans, with the inclusion of 3.5g/kg of fresh or oxidized soybean oil and met the nutritional requirements recommended for the strain of turkey used.

Table 1 Experimental Treatments

Treatments	Soybean Oil (Peroxide)	Vitamin E
T1	0 meq/Kg of oil	65 mg/Kg
T2	110 meq/Kg of oil	65 mg/Kg
T3	250 meq/Kg of oil	65 mg/Kg
T4	0 meq/Kg of oil	800 mg/Kg
T5	110 meq/Kg of oil	800 mg/Kg
T6	250 meq/Kg of oil	800 mg/Kg

To study intestinal morphology, five birds per treatment were randomly selected, weighed and euthanized at 19 days old. A 2 cm section of jejunum (2 cm from Meckel's diverticulum) was collected for morphological analysis. All samples were opened longitudinally, rinsed with phosphate buffer (0.1M, pH 7.4), placed onto cardstock paper and fixed in Alfac solution for 24 hours. Samples were sent to the laboratory for preparation of slides. Sections 5μm thick were stained with periodic acid-Schiff (PAS). Villi (30) were measured (from the basal region of the villus to its tip, μm) and 30 crypts (from its base to the transition region of the crypt:villus, μm). Villus heights and crypt depths were measured using an Olympus BH2 microscope (Olympus America Inc., NY, USA) and the image analysis program (Motic Images Plus 2.0). To measure the lipid peroxidation of intestinal mucosa, jejunal tissue samples were processed and analyzed according to the methodology described by Sodergren et al., (1998). Data were subjected to analysis of variance at 5% probability.

III. RESULTS AND DISCUSSION

Supplementation with 800 mg/kg of vitamin E in the diet decreased the level of hydroperoxides in the jejunum of turkeys ($P < 0.05$), indicating a lower rate of lipid peroxidation of the intestinal mucosa of these animals (Table 2). The level of oil oxidation did not influence the amount of hydroperoxides in the jejunum of these birds ($P > 0.05$). Also there was no interaction between the factors and level of oxidation level of vitamin E ($P > 0.05$). There was interaction between the degree of oil oxidation and the level of vitamin E supplementation on villus height and crypt depth of the jejunum ($P < 0.05$; Table 3). In Table 4 are shown the results of the interaction between the degree of oil oxidation and vitamin E supplementation for villus height and crypt depth. Birds fed diets containing oil with a higher level of oxidation had smaller villus heights than the birds in the other treatments. According to Yamauchi and Ishiki (1991) when the intestine responds to any agent that causes an imbalance in cellular loss and renewal, there is a change in villus height. However, the treatments containing oil in good condition or with an intermediate level of oxidation did not differ, indicating that the intestinal mucosa of turkeys presents the possibility of recovery of the villi where the animal received a diet containing oil that was oxidized to a moderate extent (110 meq peroxide/kg oil).

Table 2 Concentration of hydroperoxides in the jejunum of turkeys (nmol/mg protein) fed diets with and without oxidized oil, supplemented with two levels of vitamin E.

Treatments		Hydroperoxide (nmol/mg protein)
Oxidized oil	0 meq/kg	88.489
	110 meq/kg	88.294
	250 meq/kg	62.090
Vitamin E	65 mg /kg	91.459
	800 mg/kg	67.789
Probabilities		
Oxidized oil (A)		0.060
Vitamin E (B)		0.022
A x B		0.398
CV (%)		32.49
SEM		5.587

Table 3 Effect of fresh or oxidized oil and vitamin E supplementation on villus height and crypt depth of jejunum of 19d old turkeys

		Major Effects	
		Jejunum	
		Villus (µm)	Crypts (µm)
Soybean Oil	0 meq/Kg	566	46
	110 meq/Kg	553	45
	250 meq/Kg	489	50
Vitamin E	65 mg/Kg	497	49
	800 mg/Kg	574	45
Probabilities			
Oxidized oil (A)		< 0.001	< 0.001
Vitamin E (B)		< 0.001	< 0.001
A x B		0.022	< 0.001
CV %		12.94	18.85

The supplementation of vitamin E in the diet caused a beneficial effect on villus height. The birds had higher villi were fed diets with extra vitamin E, regardless of the level of oxidation of the oil used. Among the treatments that had the greatest level of vitamin, it was observed that, as the degree of oxidation of the oil increased, the length of villi was lower.

Table 4 Interaction between degree of oxidation and levels of vitamin E supplementation on villus height and crypt depth in the jejunum of turkeys.

	Villus		Crypt	
	65 mg/Kg	800 mg/Kg	65 mg/Kg	800 mg/Kg
Oxidation Level				
0 meq/Kg	520^{Ab}	607^{Aa}	41^{Aa}	51^{Bb}
110 meq/Kg	524^{Ab}	582^{Ba}	44^{Ba}	47^{Ab}
250 meq/Kg	447^{Bb}	532^{Ca}	51^{Ca}	49^{Aba}

Means followed by capital letters in column are statistically different from each other ($P < 0.05$). Means followed by small letters in row are statistically different from each other ($P < 0.05$).

The mucosal damage may increase the maintenance requirement of significantly smaller amounts of available nutrients for animal growth (Dibner and Richards, 2004). As the treatments supplemented with vitamin E showed superior results compared to those without supplementation, it is likely that its antioxidant capacity has neutralized the free radicals and reduced the spread of the chain oxidation process, reducing the damage caused by primary and secondary products of oxidation on the intestinal epithelium.

IV. CONCLUSIONS

The presence of oxidized soybean oil in the diet of turkeys had no effect on lipid peroxidation of intestinal mucosa. The supplementation of vitamin E in the diet decreased the production of hydroperoxides in the membrane of the gut and maintained the height and mucosal integrity.

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ALPHITOBIUS DIAPERINUS: MEASURES OF CONTROL AND IMPACT ON PERFORMANCE ON BROILERS

A.V. FISCHER DA SILVA[1], A.K. JAPP[1], N. PUGSLEY[2], M. HUBER[2], C.L. BICHO[3] and S.A. BORGES[4]

Summary

The mealworm is an insect that is one of the most important pests on poultry farms all around the world, due to health and economic losses. Because it is an exotic species in many countries, and due to the lack of knowledge of its natural enemies, the control of this pest is considered difficult and, to date, there is no efficient and safe method. This study aimed to evaluate weight gain, gastrointestinal tract and excreta of broilers fed larvae and adults of *Alphitobius diaperinus*, and to evaluate the action of three different diatomaceous earth (DE) products on the mortality of adult *A. diaperinus*. The experiment was divided into two stages. The first stage was to assess the weight gain, gastrointestinal tract and excreta of broilers fed larvae and adults of *A. diaperinus*, a total of 120 chickens in three treatments (T) with four replicates of ten birds. Between the 7th and 11th days, the insects were introduced in food as follows: T1 received a diet containing insect adults and larvae, T2 received insects (adults and larvae) and feed, T3 received feed *ad libitum*. After this period, between 12 to 21 days of age, all treatments were fed commercial feed. Birds were weighed at 1, 7, 14 and 21 days of age. The second stage was to evaluate the DEs effects on the mortality of adult *A. diaperinus*, tested on six DE samples taken from three different mines, MI, CO and CM. The experimental design consisted of seven treatments and two different exposure times, with five repetitions. One of the treatments served as the control, in which there was no application. In stage one of the experiment, mortality rate was determined at 48 and 96 h after application of the DEs. There was a body weight loss in T1 and T2, respectively, compared to T3, with no recovery of weight in birds following return to the control diet. In addition, the presence of *A. diaperinus* depressed feed intake. In stage two of the experiment, all DEs provided effective control, especially samples from mine MI. The time of exposure is an important factor that influences mortality in both the larvae and adults.

I. INTRODUCTION

The mealworm is an insect that is one the most important pests on poultry farms around the world, due to the health and economic importance that it represents in such agroecosystems. The insects are associated with the spread of pathogens (bacteria, viruses and protozoa) to birds which, in turn, cause immunosuppression and eventually death. Another damaging factor is the substitution of the balanced feed by larvae and adults of the beetle, which compromises feed conversion and causes weight loss, resulting in a lack of uniformity and economic losses in poultry farming. Because it is an exotic species in many countries, and due to the lack of knowledge of its natural enemies, the control of this pest is considered difficult, and to date, there is no efficient and safe control method. Diatomaceous Earth (DE), an inert powder composed of the fossilized shells of diatoms, is emerging as a promising alternative for the control of the mealworm, because it is non-toxic, does not affect the birds or human health and, moreover, leaves no toxic waste in the environment as do chemical

[1] Universidade Federal do Parana, Brazil
[2] Sanex Comercio e Industria Veterinaria
[3] Universidade Estadual da Paraiba
[4] Univserida de Tuiuti do Parana, Brazil

pesticides commonly used. This study aimed to evaluate weight gain, gastrointestinal tract condition and excreta of broilers fed larvae and adults of *Alphitobius diaperinus*, and evaluate the action of three different sources of diatomaceous earths on the mortality of adult *A. diaperinus*.

II. MATERIAL AND METHODS

The experiment was divided into two stages. The first stage, which was to assess the weight gain, gastrointestinal tract and excreta of broilers fed larvae and adults of *A. diaperinus*, was conducted in an experimental aviary at the Federal University of Parana in cages measuring 0.98 x 0.90 x 0.50 m (lxwxh). A total of 120 chickens in three treatments (T) with four replicates of ten birds were used. Between the 7th and 11th days, insects were introduced in feed as follows: T1 received a diet containing insect adults and larvae, T2 received insects (adults and larvae) and feed, the T3 received feed *ad libitum*. After this period, ie 12 to 21 days of age, birds of all treatments were fed with commercial feed. Birds were weighed at 1, 7, 14 and 21 days of age to assess weight gain. The experimental design was completely randomized. Data were submitted to ANOVA and means compared by Tukey test at 5% probability. At 11 days of age, twelve birds, one from each replicate, were necropsied to assess the condition of the gastrointestinal tract.

The second stage of the experiment, to evaluate the effect of DEs on the mortality of adult *A. diaperinus*, was tested on six samples of DE, with a particle size of 0.5 mm, taken from three different mines, MI, CO and CM. Four samples were taken from the mine MI, which correspond to the layers of the mine, MI 1, MI 2, MI 3, MI 4, and were composed, respectively, by 99%, 99.2%, 86.9%, 99.5% of centric algae and 1%, 0.8%, 13.1%, 0.5% of pennate algae. Only one sample from each of the mines CO and CM was removed from a single layer. These samples were composed respectively of 25.8% and 42.8% of centric algae and 71.2%, 57.2% of pennate algae. The six samples of DEs above were used in a bioassay to assess mortality of mealworms. The bioassay was carried out in a climate-controlled room (25ºC and 60% RH) at Federal University of Parana. Twenty adults (not sexed) and 20 larvae (of the same instar) were acclimated in petri dishes, to each of which was added manually 0.0063 g/cm2 of one of the six samples of DEs. The experimental design consisted of seven treatments and two different exposure times, with five repetitions. One of the treatments served as the control, in which there was no application. Mortality rate was determined at 48 and 96 hours after application of the DEs. Data were submitted to analysis of variance (ANOVA) and the means compared by Tukey's test at the 1% level of significance. The dead adults of *A. diaperinus* from the most effective DE treatment, as well as the control, were dissected and photographed using Leica MZ16 Lupa.

III. RESULTS AND DISCUSSION

Data on weight gain are shown in Table 1. After insertion of mealworms in the diet of T1, within a few hours, birds began eating greedily; the same happened in T2 where they had free choice between mealworms and feed, noting a clear preference by the insects. There was a loss of body weight of 173.04 g and 110.56 g in T1 and T2, respectively, compared to T3, ie, there was no recovery of weight in T1 and T2 birds returned to the control diet. This result shows that there is a significant economic loss when heavy infestations occur, as birds prefer to eat the adults and larvae instead of commercial feed. The excreta collected in the treatments receiving the mealworms (T1 and T2) presented with damp, dark coloration, presence of undigested material and cuticle from the exoskeleton of insects. Despins & Axtell (1995) reported weight loss in broilers fed with larvae of mealworms and, during the examination of excreta, observed characteristics similar to those found in this study.

Table 1 Weight gain (g) of broiler chickens during 1-21 days of age

	Weight gain (g)		
Treatments	7 days	14 days	21 days
T1	124.28 a	286.37 c	686.42 c
T2	118.88 a	404.86 b	748.9 b
T3	125.72 a	428.16 a	859.46 a
CV%	4.9344	17.56	9.9626
P	0.2617	0.0001	0.0001

Means followed by different letters are statistically different.

During the necropsy of birds, it was observed that, in T1, lesions and remains of dead insects were present in the crop and gizzard. There were lesions in the duodenum and jejunum with undigested food and dead insects along the intestine. Birds of T2 had lesions in the gizzard, duodenum, undigested food in the ileum, and gas in the caecum. The presence of the exoskeleton caused lesions in the gastrointestinal tract, which can have significant effects on nutrient absorption. These lesions may result in the presence of microorganisms that compromise the overall condition of the birds. The femur in T1 was lower both in length and in diameter, compared to T3.

The results of DEs on the mortality of adult *A. diaperinus* are shown in Table 2. Treatments differ statistically among themselves, and all provide effective control of adult *A. diaperinus*. The best results were obtained with the sample from mine MI 1, which can be explained mainly by the occurrence of a greater quantity of centric diatoms, which have larger areolae and are capable of absorbing the body moisture of the insect, thereby causing death by desiccation. The pennate algae lack large areolae, and, therefore, do not absorb as much water. Athanassiou *et al*. (2004) reported a direct relationship between mortality of adult beetles *Tribolium confusum* (Du Val), *Tenebrio molitor L.* and *Sitophilus oryzae (L.)* and exposure time to DEs, under laboratory conditions.

Analysis of adult insect bodies showed higher adhesion of the diatomaceous earths to the ventral region, close to the joints and the mouth parts, as well as a reduction of body mass in the abdominal region under the elytra (Figure 1). According to Ebeling *et al*. (1966), inert powders, such as diatomaceous earths, adhere to the epicuticle of the insects by electrostatic charge, and act by abrasion and adsorption of the lipids in the epicuticle. Consequently, insects die of dehydration when 60% of body water or 30% of total body mass is lost.

Table 2 Mortality of *A. diaperinus* adults as a function of DE treatment.

	Exposition time			
	48h		96h	
Sample	Mortality ($\overline{X}$)	Standard deviation	Mortality ($\overline{X}$)	Standard deviation
MI 1	12.2 Ab	± 3.27	19.6 Aa	± 0.89
MI 2	3.8 BCb	± 3.63	16.0 Aa	± 2.35
MI 3	6.2 Bb	± 4. 92	17.4 Aa	± 1.82
MI 4	5.6 BCb	± 5.77	17.4 Aa	± 2.61
CO	1.0 BCb	± 1.41	4.0 Ba	± 2.55
CM	1.0 BCa	± 1.22	1.4 Ba	± 1.34
Contr.	0 Ca	± 0	0 Ba	± 0

Means followed by different capital letters (column) and/or small letter (line) are statistically different ($P < 0.01$, CV plot= 46.06%, CV subplot= 25.10%).

Figure 1 *A. diaperinus* adults: treated with diatomaceous earth from the mine MI1 (right side) and control (left side). 10:1 scale. Photographs taken under stereomicroscope.

IV. CONCLUSIONS

The presence of *A. diaperinus* adults and larvae in the feed of broiler chickens depresses feed intake because birds have a preference for the consumption of insects. The cuticle of the exoskeleton of insects causes lesions in the gastrointestinal tract, which interfere with absorption of nutrients and facilitate the establishment of harmful microorganisms.

Under laboratory conditions, there was a difference in the effectiveness of the control of *A. diaperinus* when exposed directly to diatomaceous earths from different mines, due to the peculiarities in the composition of each of them. Furthermore, the time of exposure is an important factor that influences mortality in both the larvae and adults.

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EFFECTS OF DIFFERENT SUPPLEMENTAL LEVELS OF YEAST SE ON THE GROWTH PERFORMANCE AND STRESS-RELATED PARAMETERS IN BROILER CHICKENS UNDER MILD HEAT STRESS

H. QU[1], M. ZHANG[1], Y.M. GUO[1], Y. BOA[2], K. FILER[2] and A. KOCHER[3]

Summary

A 42-day experiment was conducted to determine the effect of different supplemental levels of yeast–Se on the growth performance and heat-stress related parameters in broiler chickens under mild heat stress. A total of 648, day-old, broilers were assigned to six treatments and supplemental levels of yeast Se were 0, 0.1, 0.2, 0.3, 0.4, 0.5 mg/kg diet, respectively. Birds were fed a starter diet (days 0-21) and a finisher diet (days 22-42) based on corn and soybean meal containing 0.02 mg Se /kg diet. From week 2, birds were exposed to 33°C during the day time and 26° C at night. Broilers fed to diets supplemented with yeast Se showed a quadratic increase ($P < 0.05$) in daily feed intake and body weight gain at day 21. Compared with the control diet, the supplementation of yeast Se tended to increase the daily feed intake ($P=0.059$) and body weight gain ($P=0.093$) at 42 days of age. At 21 days of age, different supplemental levels of yeast Se linearly improved the plasma glutathione peroxidase (GSH-Px) activity ($P<0.01$) and decreased the hepatic heterophil/lymphocyte (H/L) ratio ($P < 0.01$). The hepatic heat shock protein (HSP70) expression was quadratically decreased ($P < 0.01$) during this period. In conclusion, the supplementation of 0.3 mg yeast Se/kg diet might be sufficient to reduce heat stress.

I. INTRODUCTION

Heat stress is one of the most important factors responsible for poultry production losses, leading to a series of physiological and metabolic series of physiological and metabolic changes in broiler chickens such as elevated body temperature, panting and respiratory alkalosis. Biochemical and physiological changes associated with hyperthermia potentially produce reactive oxygen species (ROS) that disturb the balance between the oxidative and anti-oxidative defense systems (Lin *et al.*, 2006).

Selenium is involved in the body's antioxidant defense system as a co-factor in glutathione peroxidase (GSH-Px) to remove hydroperoxide to protect the cell membrane from oxidative demage. Yeast-Se is a highly available organic form of selenium for chickens. It has been demonstrated to alleviate the negative effects of oxidative stress induced by heat stress more efficiently than inorganic Se in quail (Sahin, et al., 2008).

The objective of the present study was to investigate the effect of supplemental yeast-Se on broiler performance and stress-related parameters under mild heat stress condition.

II. MATERIALS AND METHODS

a) Birds and dietary treatments

A total of 648 day-old Arbor Acre male broiler chicks were randomly allocated to 6 treatments. Each treatment had nine replicates and each replicate had 12 birds. Birds were reared in cages under standard management conditions of lighting and vaccine inoculation. The room temperature was maintained at 33- 35 ℃ in the first three days and then reduced to

[1] China Agricultural University, Beijing, China
[2] Alltech Asia-Pacific Biosciences Centre, Thailand.
[3] Alltech Australia, Australia

32-30 ℃ in the following days. From week 2, birds were exposed to 33°C during the day time and 26° C at night. Birds were fed to starter (days 0-21) and finisher (days 22-42) diets based on corn and soybean meal containing 0.02 mg Se/kg diet, and were formulated to meet or exceed the requirements of Chinese Feeding Standard of Chickens. Supplemental levels of yeast-Se were 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/kg diet, with each level of supplemental Se allocated to one treatment group to form the six treatments.

b) Data collection

On d 21 and d 42, all birds were weighed in groups and the feed intake was calculated to obtain feed conversion ratio (FCR). On d 21, the serum and liver samples were collected from 6 chickens per treatment for determining the glutathione peroxidase (GSH-Px), heterophils/lymphocytes ratio (H/L ratio) and hepatic heat shock protein (HSP70) level.

c) Statistic analysis

Data are presented as means ± SD and analyzed by one-way ANOVA procedure of SPSS16.0 program. The effect of supplemental levels of yeast-Se was determined using orthogonal polynomials for linear and quadratic effects. Data were assumed to be statistically significant when $P \leq 0.05$.

III. RESULTS AND DISCUSSION

a) Growth performance

Compared with the control diet, the supplementation of yeast-Se showed quadratically increased ($P < 0.05$) daily feed intake and body weight gain at d 21 and tended to increase daily feed intake ($P = 0.059$) and body weight gain ($P = 0.093$) at 42 days of age but it had no effect on FCR (Table 1). This result confirmed that Se deficiency would depress the body weight gain due to decreased feed intake.

b) GSH-Px activity, H/L ratio and hepatic HSP70 level

The results in Table 2 showed that the GSH-Px activities in plasma and liver on d 21 increased (linear, $P < 0.001$; quadratic, $P < 0.001$) with the supplementation of dietary Se. Considering that higher GSH-Px activity increases peroxide destruction prior to cell damage (Combs and Combs, 1986), the supplementation of yeast-Se could alleviate heat stress in broilers.

It is shown in Table 3 that the H/L ratio in birds receiving 0.40-0.50 mg/kg supplemental yeast was lower ($P < 0.05$) than the control group, indicating that adequate dietary Se may be necessary to mitigate heat stress.

The hepatic HSP70 expression is generally considered to be proportional to heat stress (Lin et al., 2006). However, in the current experiment, Se supplementation increased ($P<0.05$) the HSP70 protein levels at 0.1-0.3 mg/kg but the converse was true at 0.5 mg/kg ($P<0.05$) on d 21 (Table 3). The reason is not clear but it seems that under heat stress, broiler chickens may need more Se to alleviate heat stress. Thus, adding 0.3 mg/kg yeast-Se to a conventional diet containing 0.2 mg Se/kg may be sufficient to mitigate the effect of mild heat stress.

Table 1 Growth performance of Se supplemented male broiler chicks to 42 days of age

Yeast-Se (mg/kg)	BW (g)	ADG (g)	DFI (g)	FCR
		d 0-21		
0.0	601.2±26.0[b]	26.4±1.2[b]	40.3±1.8[b]	1.51±0.04
0.1	633.1±26.3[a]	27.9±1.25[a]	42.8±1.26[a]	1.54±0.04
0.2	634.3±39.8[a]	28.0±1.9[a]	42.2±2.0[a]	1.51±0.03
0.3	640.1±36.3[a]	28.2±1.7[a]	42.3±1.9[a]	1.50±0.07
0.4	643.9±29.6[a]	28.4±1.4[a]	42.6±2.0[a]	1.50±0.02
0.5	631.3±30.8[a]	27.8±1.5[a]	42.2±2.2[a]	1.52±0.02
P-value	<0.05	<0.05	<0.05	0.096
Linear	0.029	0.029	0.069	0.153
Quadratic	0.026	0.026	0.045	0.306
		d 0-42		
0.0	1626.0±115.5	37.6±2.8	71.3±3.2[b]	1.86±0.07
0.1	1720.2±119.5	39.8±2.9	75.7±5.4[a]	1.90±0.10
0.2	1671.4±133.4	38.7±3.2	72.5±6.0[ab]	1.88±0.09
0.3	1656.2±150.5	38.3±3.6	72.3±5.9[ab]	1.88±0.05
0.4	1736.3± 94.1	40.2±2.2	74.4±4.9[ab]	1.85±0.12
0.5	1689.0±115.2	39.0±2.7	74.8±6.2[ab]	1.91±0.13
P-value	0.093	0.093	0.059	0.215
Linear	0.288	0.289	0.125	0.542
Quadratic	0.591	0.591	0.616	0.949

Mean values without common superscript within columns differ significantly ($P < 0.05$).
BW: body weight gain
ADG: average daily weight gain
DFI: daily feed intake
FCR: feed conversion rate

Table 2 GSH-Px activity in plasma and liver of birds at 21 days of age

Yeast-Se mg/kg	GSH-Px in plasma (U)	GSH-Px in liver (U)
0.0	99.40±13.28^{f}	3.43±1.73^{d}
0.1	661.33±150.86^{e}	14.87±3.67^{c}
0.2	1757.30±257.19^{d}	26.31±7.98^{b}
0.3	2119.40±443.98^{c}	45.83±9.10^{a}
0.4	3287.40±346.71^{a}	47.26±6.41^{a}
0.5	2860.60±304.58^{b}	45.20±6.56^{a}
P-value	< 0.05	< 0.05
Linear	< 0.001	<0.000
Quadratic	<0.001	<0.000

Mean values without common superscript within columns differ significantly (P < 0.05).

Table 3 HSP70 protein levels in liver and H/L ratio in blood of birds at 21 days of age

Yeast-Se (mg/kg)	HSP 70 protein level	H/L
0.0	0.586±0.160^{bc}	0.443±0.140^{ab}
0.1	0.751±0.020^{a}	0.406±0.044^{ab}
0.2	0.794±0.087^{a}	0.354±0.140^{bc}
0.3	0.623±0.098^{b}	0.313±0.152^{bc}
0.4	0.478±0.049^{c}	0.221±0.061^{cd}
0.5	0.229±0.211^{d}	0.194±0.044^{cd}
P-value	< 0.05	<0.05
Linear	< 0.001	<0.001
Quadratic	< 0.000	0.852

Mean values without common superscript within columns differ significantly (P < 0.05).

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INFLUENCE OF FEED FORM AND CONDITIONING TEMPERATURE ON THE PERFORMANCE AND NUTRIENT UTILISATION OF BROILER STARTERS FED WHEAT-BASED DIET

M.R. ABDOLLAHI[1], T.J. WESTER[1], D.V. THOMAS[1], G. RAVINDRAN[1] and V. RAVINDRAN[1]

Summary

The present experiment was designed to examine the influence of feed form and conditioning temperature in a wheat-based diet on the performance and nutrient utilisation of broiler starters. The experimental design was a 2 × 4 factorial arrangement of treatments, which included two feed forms (mash or pellet) and four conditioning temperatures (20, 60, 75 and 90 ºC). In mash diets, while steam-conditioning at 75 and 90 ºC had negative effects on weight gain compared to 60 ºC, steam-conditioning at 90 ºC was found to deteriorate feed per gain and decrease ileal starch digestibility of broiler starters. However, deterioration in weight gain (due to steam-conditioning at 75 and 90 ºC) and feed per gain (due to steam-conditioning at 90 ºC) was restored when steam-conditioned mash diets were pelleted.

I. INTRODUCTION

The performance of broiler starters fed diets conditioned at different temperatures reflect a balance between nutrient availability and pellet quality (Abdollahi et al., 2010). While nutrient availability is adversely affected at higher conditioning temperatures, pellet quality improves. If the improvements in pellet quality gained by applying higher conditioning temperatures are sufficient enough to overcome the negative effects of high conditioning temperatures on nutrient availability, then the bird performance will be largely restored; otherwise, bird performance will deteriorate. Moritz et al. (2001) reported that the improvement in productive energy achieved from feeding high quality pellets can offset the lowered nutrient availability. The fact that nutrient availability and pellet quality can overcome the effect of each other is highly relevant in determining the broiler performance. It can therefore be hypothesised that birds fed similar steam-conditioned basal diets but different in feed form (mash vs. pellet) may show different patterns of growth response. To test this hypothesis, the present experiment was designed to examine the influence of feed form and conditioning temperature on the performance and nutrient utilisation of broiler starters. In addition, two more treatments, dry-conditioned (conditioned at 20 ºC) mash and pellet diets, were included to better understand the effects of steam-conditioning or pelleting on the nutrient utilisation and performance of broiler starters.

II. MATERIALS AND METHODS

The experimental design was a 2 × 4 factorial arrangement of treatments, which included two feed forms and four conditioning temperatures. Wheat was ground in a hammer mill (Bisley's Farm Machinery, Auckland, New Zealand) to pass through a screen size of 7.0 mm for coarse grade. A wheat-soy based diet was formulated to meet the Ross 308 strain recommendations for major nutrients for broiler starters (Ross, 2007), and divided into eight equal batches. The first four batches were conditioned at four different temperatures: 20 ºC (dry-conditioning), 60, 75 and 90 ºC (steam-conditioning) by adjusting the steam flow rate. The diets (mash

[1] Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand.

form) were collected at the outlet of the conditioner (before entering the pellet die). The second four batches were similarly conditioned at the four temperatures and pelleted using a pellet mill (Richard Size Limited Engineers, Orbit 15, Kingston-upon-Hull, UK) capable of manufacturing 180 kg of feed/h and equipped with a die ring (3-mm hole and 35-mm thickness). Conditioning time of the mash was 30 seconds and the conditioning temperature was measured at the outlet of the conditioner, as a single-point measure using a digital thermometer (Dick Smith Electronics, China). All diets contained titanium dioxide as an indigestible marker. Each of the eight dietary treatments was offered ad libitum to six replicate cages (eight birds per cage). Body weights and feed intake were recorded at weekly intervals throughout the 21-day trial. From d 17 to 20, feed intake and excreta output were measured quantitatively per cage for the determination of apparent metabolisable energy (AME). On d 21, ileal digesta were collected for determination of apparent ileal digestibility of nitrogen (N) and starch. Pellet durability index (PDI) and pellet hardness were determined using a Holmen Pellet Tester (New Holmen NHP100 Portable Pellet Durability Tester, TekPro Limited, Norfolk, UK) and a Stable Micro Systems Texture Analyser (TA-XT Plus, Godalming, Surrey, UK), respectively.

III. RESULTS AND DISCUSSION

In mash diets, birds fed diet conditioned at 60 ºC had higher ($P < 0.05$) weight gain than those fed diets conditioned at 75 and 90 ºC and similar ($P > 0.05$) to those fed diet conditioned at 20 ºC (Table 1). In pelleted diets, while applying steam during conditioning increased ($P < 0.05$) weight gain compared to those fed diet conditioned at 20 ºC, birds fed steam-conditioned diets at 60, 75 and 90 ºC had similar ($P > 0.05$) weight gains. Birds fed pelleted diets consumed more ($P < 0.001$) feed than those fed mash diets. Birds fed diets conditioned at 60 and 90 ºC had higher ($P < 0.05$) feed intake than those fed diets conditioned at 20 ºC and similar ($P > 0.05$) to those fed diets conditioned at 75 ºC. Similar ($P > 0.05$) feed intake was observed for the birds fed diets conditioned at 20 and 75 ºC.

A significant ($P < 0.01$) feed form x conditioning temperature interaction was observed for feed per gain due to the pattern of changes with increasing conditioning temperatures differing in the two feed forms. In mash diets, increasing conditioning temperatures increased the feed per gain, with birds fed diet conditioned at 90 ºC having similar ($P > 0.05$) feed per gain to those fed diet conditioned at 75 ºC, but higher ($P < 0.05$) than those fed diets conditioned at 20 and 60 ºC. In pelleted diets, while applying steam to the conditioner lowered ($P < 0.05$) the feed per gain compared to conditioning at 20 ºC, birds fed diets steam-conditioned at different temperatures had similar ($P > 0.05$) feed per gain values.

Pellet durability and hardness increased ($P < 0.001$) with steam-conditioning and increasing conditioning temperatures. Steam-conditioning of diets at 60, 75 and 90 ºC prior to pelleting increased the pellet durability about 391, 408 and 441%, respectively, compared to the dry-conditioned diet (conditioned at 20 ºC). The corresponding improvements in pellet hardness were 125, 174 and 330%, respectively.

Based on the performance deterioration of the birds fed mash diets steam-conditioned at temperatures above 60 ºC, it can be speculated that higher conditioning temperatures *per se* negatively affect the performance of broiler starters. However, when the feed form is changed from mash to pellets, the performance of broilers is restored by the better pellet quality achieved at higher conditioning temperatures. Due to these opposing effects, broiler feeding trials using pelleted diets conditioned at different temperatures have not always produced consistent results. Raastad and Skrede (cited in Creswell and Bedford, 2006) reported lower body weights when the conditioning temperature was increased from 69 to 86 ºC. Increasing the conditioning temperature from 69 to 78 ºC had no effect on feed conversion, but feed

conversion was poorer when the temperature was increased from 78 to 86 ºC, especially during the 1-21 d period. In contrast, Cowieson et al. (2005) found that in broilers fed maize-based diets, increasing pelleting temperature from 70 to 85 ºC resulted in higher weight gain.

Pelleting reduced ($P < 0.001$) the apparent ileal N digestibility of the diets (Table 1). Ileal N digestibility of the diets conditioned at 60 and 75 ºC were similar ($P > 0.05$) and higher ($P < 0.05$) than those conditioned at 20 and 90 ºC. There was a significant ($P < 0.05$) feed form x conditioning temperature interaction for apparent ileal starch digestibility. In mash diets, increasing conditioning temperatures decreased ($P < 0.05$) the ileal starch digestibility, with diet conditioned at 90 ºC having lower digestibility than the diet conditioned at 60 ºC, but similar ($P > 0.05$) to those conditioned at 20 and 75 ºC. In pelleted diets, those diets conditioned at 60 and 90 ºC had similar ($P > 0.05$) starch digestibility to the diet conditioned at 75 ºC but higher ($P < 0.05$) than the diet conditioned at 20 ºC.

Pelleting reduced ($P < 0.001$) the AME of the diets. The diets conditioned at 60 ºC had similar AME values to those conditioned at 75 ºC and higher ($P < 0.05$) than the diets conditioned at 20 and 90 ºC.

Overall, the present data showed that, in mash diets, while steam-conditioning at 75 and 90 ºC had negative effects on weight gain compared to 60 ºC, steam-conditioning at 90 ºC was found to deteriorate feed per gain and decrease ileal starch digestibility of broiler starters. However, deterioration in weight gain (due to steam-conditioning at 75 and 90 ºC) and feed per gain (due to steam-conditioning at 90 ºC) was restored when steam-conditioned mash diets were pelleted.

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Table 1 Influence of feed form and conditioning temperature on weight gain (g/bird), feed intake (g/bird), feed per gain (g feed/g gain), PDI (%), pellet hardness (Newton), apparent ileal N and starch digestibility and AME (MJ/kg DM) in broiler starters[1]

	Conditioning temperature, ºC	Weight gain	Feed intake	Feed per gain	PDI[2]	Pellet hardness[3]	Ileal N digestibility	Ileal starch digestibility	AME
Mash	20	873^{bc}	1126	1.289^{d}	-	-	0.847	0.959^{ab}	14.10
	60	908^{b}	1174	1.303^{cd}	-	-	0.869	0.977^{a}	14.18
	75	852^{c}	1119	1.319^{bcd}	-	-	0.855	0.940^{ab}	13.92
	90	869^{c}	1179	1.358^{b}	-	-	0.847	0.913^{b}	13.88
Pellet	20	885^{bc}	1253	1.420^{a}	13.7^{d}	8.76^{d}	0.818	0.756^{d}	13.40
	60	1006^{a}	1348	1.340^{bcd}	67.2^{c}	19.7^{c}	0.836	0.842^{c}	13.75
	75	981^{a}	1327	1.353^{bc}	69.6^{b}	24.0^{b}	0.849	0.805^{cd}	13.71
	90	1014^{a}	1322	1.342^{bcd}	74.1^{a}	37.7^{a}	0.822	0.834^{c}	13.42
SEM[4]		13.3	18.5	0.018	0.58	1.01	0.0067	0.0179	0.096
Main effects									
Feed form									
Mash		875	1150^{b}	1.317	-	-	0.855^{a}	0.947	14.02^{a}
Pellet		965	1308^{a}	1.368	-	-	0.831^{b}	0.809	13.56^{b}
Conditioning temperature, ºC									
20		879	1190^{b}	1.354	-	-	0.833^{b}	0.858	13.75^{b}
60		947	1244^{a}	1.318	-	-	0.854^{a}	0.909	13.96^{a}
75		911	1214^{ab}	1.334	-	-	0.852^{a}	0.872	13.83^{ab}
90		934	1244^{a}	1.351	-	-	0.835^{b}	0.874	13.65^{b}
Probabilities, P ≤									
Feed form		***	***	**	-	-	***	***	***
Conditioning temperature		***	**	NS	***	***	**	*	*
Feed form x Conditioning temperature		***	NS	**	-	-	NS	*	NS

^{a,b,c,d} Means in a column not sharing a common superscript are significantly different (P < 0.05).

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

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

[2] Each value represents the mean of six replicate samples.

[3] Each value represents the mean of 15 replicate samples.

[4] Pooled standard error of mean.

EFFECT OF INCREASED LEVELS OF LYSINE, THREONINE AND METHIONINE ON ANTIBODY PRODUCTION AGAINST NEWCASTLE DISEASE UNDER NORMAL CONDITIONS AND HEAT STRESS IN BROILERS

I.M. ELDAGHAYES[1], B. ELAMARY[2], A. SALEEM[2], S.O. AL-GARIB[3] and M.A. HAMID[3]

The aim of this research was to evaluate antibody production following vaccination when three different types of diet were offered during heat stress and under normal conditions to broilers.

The NRC (1994) lists recommended levels of nutrients for broiler chickens from 1 to 21, 21 to 42, and 42 to 56 days of age. In practice, the NRC (1994) requirement estimates are often ignored by poultry companies because their objectives are based on economic end points that are usually not addressed in refereed publications.

A total number of 432 day-old broiler chicks from Ross 308 parent stock were used in this study. These chicks were divided randomly into 18 equal groups (24 birds each group) at 21 days of age; 9 groups under normal conditions (24-26 ^{o}C) and 9 groups under heat stress (35^{o}C for 9 h/day). Each group from both treatment (normal conditions and heat stressed) was fed different concentration of an amino acid as follows: group 1 = 1.07% Lys, group 2 = 1.25% Lys, group 3 = 1.40% Lys, group 4 = 0.75% Thr, group 5 = 0.90% Thr, group 6 = 1.20% Thr, group 7 = 0.38% Met, group 8 = 0.45% Met and group 9 = 0.55% Met). Concentration of amino acids in groups 1, 4 and 7 was according to the NRC (1994), and for other groups were higher than recommended levels. These birds were vaccinated according to the program provided by the Animal Health Center. Blood samples were collected from all birds at 20 days of age as a control, and from all birds at the end of the experiment (49 days of age). Antibody levels against Newcastle disease virus were measured using Haemagglutination Inhibition (HI) test and ELISA test. The results of HI and ELISA were found to be positively correlated, and the t-test was significant in both experimental and test groups (r = 0.98 and 0.88 respectively) upon statistical evaluation ($P < 0.001$).

The results showed that lysine (groups 1,2 and 3) had no significant effect on antibody production, whereas the increasing levels of threionine (group 6) and methionine (group 8) had a good effect ($P < 0.05$) on immunity as the titre of antibodies against Newcastle disease was increased significantly. Moreover, the response of heat stressed birds was similar to birds under normal conditions in terms of antibody production, except for birds fed higher concentrations of Methionine in group 8 as the antibody titre for birds under heat stress was lower than that for birds under normal conditions. This experiment was repeated and similar results were obtained. We recommend to increase Met concentration in feed from 0.38% to 0.45% and Thr concentration from 0.75% to 0.90%. As a future plan a challenge experiment would be of a valuable work.

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[1] Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, Alfateh University, Tripoli, Libya
[2] Department of Animal Production, Faculty of Agriculture, Alfateh University, Tripoli, Libya
[3] Department of Poultry Diseases, Faculty of Veterinary Medicine. Alfateh University, Tripoli, Libya

INCUBATION CAN AFFECT BROILER LEG STRENGTH

P. J. GROVES[1] and W. I. MUIR[1]

Summary

Leg weakness in broiler chickens remains one of the major animal welfare concerns for the poultry industry worldwide. Recent research has indicated possible effects of incubation conditions on the skeletal integrity of the growing birds. A serendipitous finding of a field occurrence of leg weakness allowed us to target some incubation condition variations which may have been associated with this. While an attempt to reproduce the same condition experimentally (higher temperature (0.5^{o}C) and lower humidity (3-4% RH)) was not entirely successful, we were able to demonstrate repeatable effects on bone characteristics and leg strength in broiler chickens hatched from eggs incubated under higher (0.5^{o}C) temperature conditions. These conditions fell within the range normally acceptable for commercial broiler egg incubation.

I. INTRODUCTION

The aetiologies of leg weakness and lameness in the modern broiler chicken are complex, including factors relating to genetics, nutrition, infection, management and environment. The consequences for the individual bird affected and also for a considerable proportion of some flocks are serious. Bradshaw *et al.*, 2002 stress that the welfare implications of broiler leg weakness include pain, frustration (inability to walk), reduced ability to eat and drink and consequent risk of dehydration or starvation. Birds which have difficulty in moving are also more at risk of excessive disturbance by other birds (Buijs *et al.*, 2010) which can disrupt their sleep/ rest patterns. Immobile birds are also more prone to skin damage from scratches which may result in cellulitis and death. The underlying genetic basis associated with leg weakness is under investigation (as evidenced by Butterworth *et al*., 2003). Major broiler breeding companies are attempting to address many of the leg weakness issues, but this requires years of genetic selection, the results of which may not be seen in the commercial broiler for many years (Elfick, 2010; Hardiman, 2010). In the meantime, broiler producers can ameliorate the prevalence and severity of leg problems by attention to nutritional, managerial and environmental risk factors. A new area which is emerging as another possible contributor to the incidence of leg weakness problems is variation in egg incubation conditions. Research into fine tuning incubation may provide additional management opportunities to further suppress the incidence of leg weakness and lameness.

a) A short review of leg weakness in broiler chickens

Lameness and leg weakness are considered a serious welfare problem. A plethora of lameness conditions in chickens exist. Bradshaw *et al.* (2002) summarised these into Infectious causes (bacterial chondronecrosis with osteomyelitis (so called Femoral Head Necrosis), tenosynovitis, and Infectious Stunting Syndrome), Developmental issues (varus-valgus deformity, tibial dyschondroplasia, rickets, chondrodystrophies and spondylolisthesis) and Degenerative problems (osteochondrosis, epiphyseolysis, degenerative joint disease, ruptured gastrocnemius tendon and contact dermatitis). Apart from the obvious clinical entities listed above, difficulty with locomotion is observed in birds which lack visible

[1] Faculty of Veterinary Science, The University of Sydney

deformities and it has become conventional to assess the locomotory ability of birds and flocks using a standardised "gait scoring" technique as described by Kestin *et al*. (1992). A wide ranging study using gait scoring as its basis in the UK suggested that 27.6% of broilers had poor locomotory ability and 3.3% were unable to walk at all (Knowles *et al*., 2008). Many studies have not gone any deeper and the underlying pathology is often not identified. Bradshaw *et al.* (2002) suggested that bacterial chondronecrosis, contact dermatitis (pododermatitis) and varus-valgus deformity were the most common conditions involved. In most broiler flocks approaching slaughter age, many or all of the described conditions will be present at varying prevalence.

A detailed description of each of these conditions is beyond the scope of this paper but risk factors believed to be involved with the occurrence of the more commonly seen conditions will be summarised.

Rickets describes a condition of inadequate bone mineralisation classically induced by inadequate nutritional levels of Ca, P or vitamin D_3. While broiler nutrition today is well catered for in provision of a balance of nutrients, the occurrence of conditions which appear rickets-like (soft bendable bones and beaks) is seen commonly in young chicks. Clinical rickets can be seen following occurrences of Infectious Stunting Syndrome (ISS) in flocks, relating to poor absorption of nutrients associated with the maldigestion induced by the group of viruses. ISS immunity is poorly understood and although the flock condition occurs sporadically, the viruses involved should be expected to be widely present in the broiler environment. One wonders about the possibility that subclinical ISS in many flocks may play a part in subsequent skeletal problems on a wide scale.

Tibial Dyschondroplasia (TD) is a disruption of normal ossification as bones grow. An interference with adequate blood supply in the metaphysis of the tibiotarsus results in insufficient nutrients reaching the growth plate and a cartilage plug forms which fails to be ossified. Bones are subsequently weak, may bend and cause considerable pain in weight bearing. Genetics, incorrect electrolyte balance in feed and mycotoxins have been implicated in TD development. It is a commonly seen entity in broilers and is often correlated with an imbalance of the Ca:P ratio in the feed, compounded by the difficulty in predicting real available P levels from available ingredients with and without phytase supplementation.

It is quite feasible that the presence of earlier degenerative conditions, especially rickets-like conditions, may predispose birds to the appearance of other conditions later in the flock's life. In the field, rotated tibia is becoming one of the major leg deformities seen. The aetiology of this condition is not known but early rickets may be a predisposing factor (Crespo & Shivaprasad, 2008). Thorp (2008) also implicated the earlier occurrence of rickets or dyschondroplasia with varus-valgus deformity.

Many of the leg weakness conditions can be modified by management and environmental conditions. Field and laboratory studies, however, are sometimes contradictory in the effects observed. Stocking density has often been implicated with an increased incidence of leg problems (Knowles *et al.,* 2002; Bradshaw *et al.,* 2002; Petek *et al.,* 2010) while other studies have shown leg problems to peak at intermediate levels rather than higher stocking densities (Buijs *et al*., 2009; Hepworth *et al*., 2010), or to not be related to stocking density at all (Dawkins et al., 2004). Lengthy photoperiod has also been incriminated with a higher incidence of leg weakness (Brickett *et al*., 2007; Bradshaw *et al*., 2002; Knowles *et al*., 2008; Petek *et al*., 2010) as has lack of exercise (Cooper and Wrathall, 2010; Sherlock *et al*., 2010) which has a relationship to scotoperiod provision. Many relate the primary risk factors to growth rate (Knowles *et al*., 2008, Bradshaw *et al.,* 2002; Sherlock *et al.,* 2010). Maintenance of dry litter conditions also can have major effects on pododermatitis (Sherlock *et al*., 2010). Modification of these factors can lead to better outcomes for broiler leg health.

More recent work, including that reported herein, has demonstrated associations of variations in incubation conditions and subsequent leg strength and this will be summarised below.

b) Links to incubation conditions

Recent published reviews and research have implicated defects in incubation as possible contributors to some bone irregularities in broiler chickens or turkeys. Spraddle legs in broilers have been associated with high humidity during incubation (Crespo & Shivaprasad, 2008), and Genin *et al.* (2008) implicated cyclic overheating during the first 8 days of incubation in the later incidence of Tibial Dyschondroplasia via an effect on growth plate hypoxia. Oveido-Rondon et al. (2008) showed that pre-heating conditions of eggs prior to incubation could affect bone characteristics of chicks at hatch and the incidence of twisted legs as late as 40 days of age. These authors also described effects on bone development and characteristics following early cool and/or late high temperature profiles and low oxygen tensions used during parts of the incubation process. Soft tissue effects have also been seen. In further experiments, Oveido-Rondon *et al*. (2010) demonstrated an effect of an early low and later high incubation temperature profile in producing thinner gastrocnemius tendon fibres and differing collagen banding patterns during subsequent growth. The temperatures used in these studies though were outside the normal realms of incubation practice (36^{o}C and 39^{o}C).

Our local field observations have suggested a possible effect of incubation differences on subsequent leg strength and these will be discussed below.

Commercial hatcheries run differing incubation profiles depending on their machine type and whether these run as single or multi- stage incubation. Multistage incubators target a single temperature and humidity profile usually between 36.9 and 37.2^{o}C and relative humidity between 51 and 65%. Single stage commercial incubation uses a decreasing temperature profile starting at 38^{o}C and decreasing to 37.2^{o}C by 18 days with relative humidities varying between 50 to 58% and sometimes as wide as 30 to 65%. The experimental profile used in our studies was within these commercially used bounds and basically employed a higher temperature (0.5 ^{o}C) over later incubation, a lower relative humidity (3%) between days 7 to 18, and a pulse reduction in temperature at day 6 of 1^{o}C. The objectives of the research reported here were to determine whether the variation in incubation conditions described generated a higher incidence of early bone weakness in newly hatched chicks and to then evaluate if later skeletal deformities or leg weakness could be associated with the incubation profile.

II. MATERIALS AND METHODS

Experiment 1 utilized 2000 and experiment 2 560 fertile eggs from breeders of a fast feathering dam line. In each experiment, the eggs were randomized between 2 incubators. The incubators were set to operate differently up to 18 days of incubation as shown in Figures 1 to 4. The major intended differences were an approximate drop in temperature of about 1^{o}C for one day at 6 days of incubation, a higher continuous temperature from 7 to 18 days of incubation (0.5^{o}C) and a lower relative humidity (3%) throughout. These settings were based on an observed field situation where chicks with poor bone quality at hatch were produced, compared to an “ideal” incubation profile as the control (Jan Meldrum, pers. comm.). From 18 days of incubation, all eggs were transferred into a common incubator set at 36.9^{o}C and reduced by 0.3^{o}C per day until day 21. Temperature and humidity data loggers (AZ 8829) recording conditions at hourly intervals were placed in each machine amongst the eggs. At hatch, 44 randomly selected chicks from each incubator group were blood sampled for serum calcium and phosphorus levels and then humanely euthanized and both femurs were collected

for bone ash analysis. Remaining chicks were placed in floor pens (240 birds per large pen in experiment 1 and 45 birds per smaller pen in experiment 2) and grown on commercial broiler starter and finisher rations (0-21 days and 22-42 days respectively) supplied by Millmaster Feeds, Enfield, NSW.

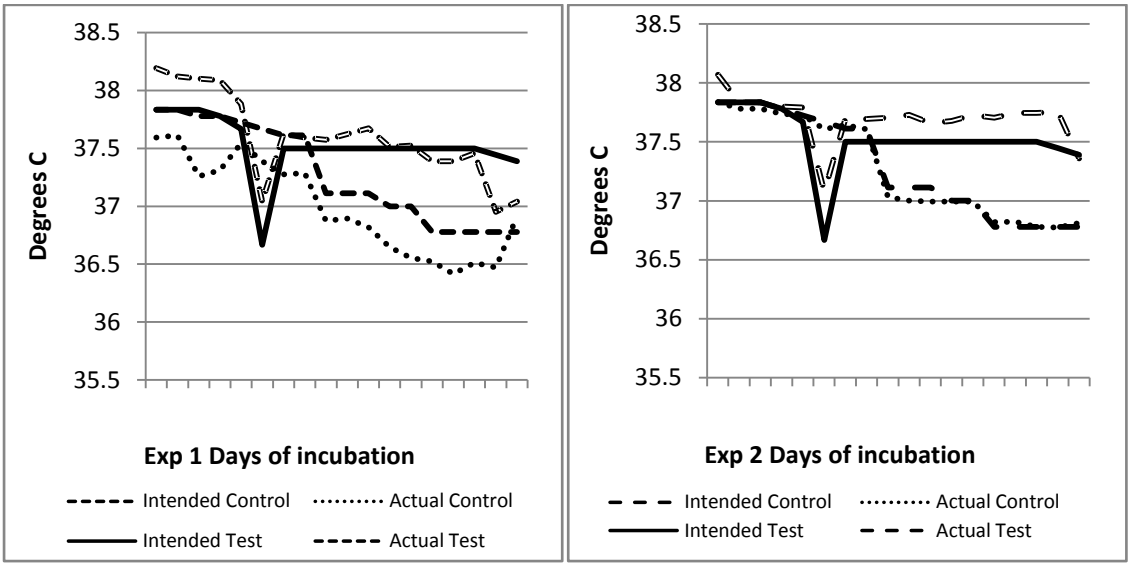
At two weeks of age, 40 or 44 birds were randomly selected from each group, blood sampled for serum calcium and phosphorus levels and humanely euthanized. The proximal ends of their left tibiae were longitudinally sectioned and the epiphyseal growth plate measured at the midpoint of the bone with a digital calliper. Their left femurs were collected for bone ash analysis. At day 28 in experiment 1, 44 birds were randomly selected and euthanized. The proximal end of their left tibiae were sectioned longitudinally and scored for the presence of tibial dyschondroplasia (TD) lesions (on a scale of 0 to 4, where 0 = no lesion and 4 = large lesion spanning the entire growth plate). At six weeks of age, 40 or 50 randomly selected chickens from each group were submitted to a Latency-to-Lie (LTL) test (first described by Weeks et al., 2002 and modified by Berg and Sanotra, 2003) for a maximum of 5 minutes. In experiment 1, a random sample of 30 birds per pen was weighed at 14, 21, 28, 35 and 42 days. In Experiment 2, all birds were weighed on a pen basis at 7, 21, 28, 35 and 42 days.

a) Statistical analysis

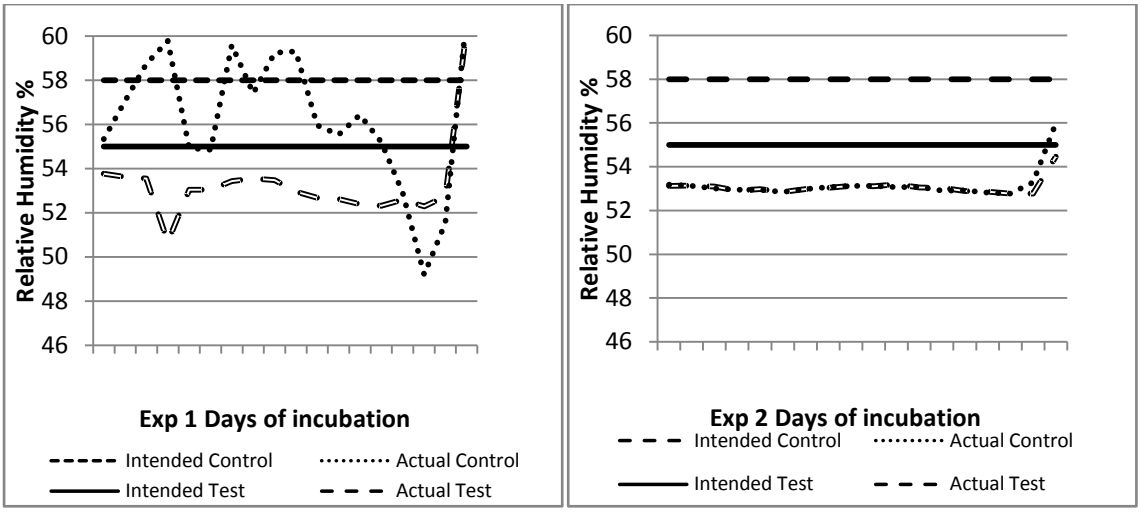
Where data were normally distributed, comparisons were made using Analysis of Variance (ANOVA) where independent variables included incubator and sex and were compared across both experiments. Where data were not normally distributed, the Mann-Whitney U test was used to separate main effect means. LTL tests were compared using Kaplan-Meier Survival Analysis.

III. RESULTS

The incubation temperature and relative humidity profiles recorded by the data loggers in each machine (actual) compared to the intended profiles are shown respectively in Figures 1 to 4. In experiment 1, the control incubator ran slightly cooler than intended and its humidity was not well controlled. In experiment 2, intended temperatures were much better matched but humidity was lower than intended and similar in both incubators.



Figures 1 & 2 Experiments 1 & 2 Temperature profiles



Figures 3 & 4 Experiments 1 & 2 Relative Humidity profiles

At hatch, chicks from the test incubator profile delivered consistently and significantly lower femoral bone ash % and higher serum Ca levels than the control profile (Table 1). Moisture loss from both test treatments was significantly higher (Table 1). In experiment 1, in which the test treatment maintained a 3-4% lower relative humidity, the serum calcium was lower than the serum phosphorus for both control and test treatment (Table 1). At two weeks of age, serum phosphorus exceeded serum calcium in all four groups but this ratio was again consistently higher for experiment 1. At two weeks there was a significant interaction for bone ash % between the two experiments (Table 2), indicating a different response in this parameter under the differing incubation conditions that actually occurred in the incubators.

Growth rate over the first two weeks was significantly greater in the test profile incubated chicks in both experiments, but weights after this age were similar (Table 3).

Table 1 Hatch measurements

Incubator group	Total hatchability (% ± SE)	Egg Weight loss to day 18 of incubation (% ± SE)	Femoral bone ash % ± SE	Serum Ca (mmol/l ± SE)	Serum P (mmol/l ± SE)
Exp 1 Control	67.92 ± 5.74	9.45 ± 0.14	26.9 ± 0.63	1.97 ± 0.04	2.31 ± 1.32
Exp 2 Control	77.8 ± 4.02	10.12 ± 0.26	28.3 ± 0.40	2.17 ± 0.04	1.22 ± 0.03
Control mean	72.86		27.4^{A} ± 0.38	2.01^{B} ± 0.03	1.93 ± 0.85
Exp 1 Test	71.28 ± 1.11	10.28 ± 0.14	25.3± 0.63	2.10 ± 0.04	2.30 ± 1.25
Exp 2 Test	75.1 ± 2.75	11.17 ± 0.27	27.5 ± 0.28	2.28 ± 0.02	1.35 ± 0.03
Test mean	73.19		26.1^{B} ± 0.43	2.16^{A} ± 0.03	1.97 ± 0.82
P=			*0.04*	*0.002*	*0.87*

Table 2 Measurements at two weeks of age

Incubator group	Bird weight (gm ± SE)	Tibial growth plate width (mm ± SE)	Femoral bone ash % ± SE	Serum Ca (mmol/l ± SE)	Serum PO_4 (mmol/l ± SE)
Exp 1 Control	401 ± 5.55	2.05 ± 0.05	44.1 ± 0.24	1.69 ± 0.05	2.03 ± 0.06
Exp 2 Control	393 ± 5.66	2.16 ± 0.08	44.7 + 0.21	2.06 + 0.04	2.30 + 0.05
Control mean	397^{B} ± 3.80	2.10^{B} ± 0.06	44.4 +0.16	1.88 + 0.04	2.17 + 0.04
Exp 1 Test	408 ± 5.98	2.38 ± 0.07	43.0 ± 0.30	1.75 ± 0.07	2.01 ± 0.07
Exp 2 Test	413 ± 5.66	2.36 ± 0.08	45.7 + 0.24	2.12 + 0.05	2.37 + 0.04
Test mean	410^{A} ± 4.00	2.37^{A} ± 0.06	44.3 + 0.24	1.93 + 0.05	2.19 + 0.04
P=	*0.02*	*0.001*	*0.68*	*0.41*	*0.75*

A,B means with different superscripts differ (P < 0.05)

Table 3 Growth rates

Incubator group	Mean body weights (gm ± SE)					
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Exp 1 Control	128 ± 1.91	381 ± 3.97	818 ± 10.1	1398 ± 14.4	2084 ± 9.4	2665 ± 38.4
Exp 2 Control	162 + 1.09	393 +3.24	834 + 9.7	1408 +20.3	2057 + 25.8	2636 + 40.9
Control mean	145^{B} + 6.52	386^{B} + 3.34	825 + 20.1	1403 + 11.7	2071 + 13.6	2650 + 26.6
Exp 1 Test	138 ± 4.50	400 ± 10.9	812 ± 13.2	1363 ± 35.2	2113 ± 27.8	2703 ± 26.9
Exp 2 Test	172 + 2.19	413 + 13.8	863 +15.8	1441 + 43.7	2094 + 27.7	2676 + 38.6
Test mean	155^{A} + 7.00	407^{A} + 6.5	837 + 13.7	1402 + 24.2	2104 + 18.5	2690 + 22.3
P=	*< 0.001*	*0.04*	*0.05*	*0.21*	*0.45*	*0.64*

A,B means with different superscripts differ (P < 0.05)

Survival Analysis for the Latency to Lie test results are shown in Figure 6. Birds from the test incubator groups had significantly shorter LTL time (median 94s compared to 136.5s for the control group, P = 0.0002, Gehan's Wilcoxon test) and had fewer birds that managed to remain standing for the full five minutes.

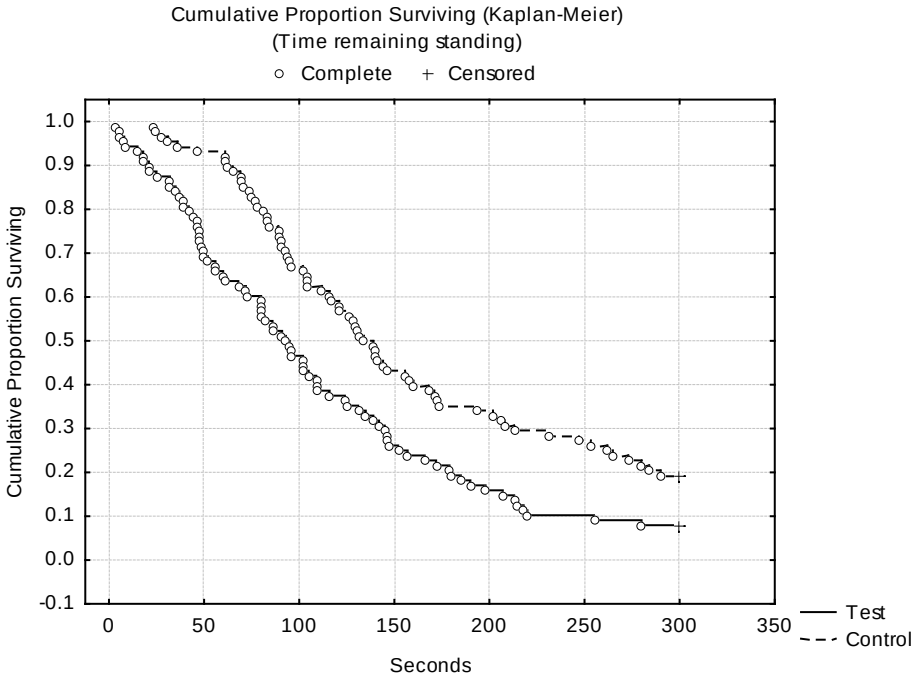


Figure 6 Survival Analysis of Latency To Lie tests

IV. DISCUSSION AND CONCLUSIONS

Although different, the intended temperature profiles used in both incubators fell within acceptable limits for successful incubation (37.1 to 38.2^{o}C – Hill, 2010) Relative humidity was much harder to control with the incubators used. The incubators used in this preliminary work were semi-commercial types, not machines designed to provide fine control necessary for experimental work. Although the incubators did not perform completely as intended, particularly the control machine, significant differences between the chicks from each incubator profile were observed in chick bone ash and serum Ca at hatch and at growth rate to two weeks of age, and this was relatively consistent. The overall higher incubation temperature in the test treatments appears to have increased moisture loss from fertile eggs, and embryonic growth, with commensurate impacts on bone ash and serum calcium.

High early growth rate has been implicated as contributing to leg weakness problems for some time (Bradshaw *et al*., 2002; Brickett *et al*., 2007; Knowles *et al*., 2008) and the overall increased early growth seen associated with the test incubation profile here may have had its effect on LTL when the birds were older. The two experiments show that bone characteristics, serum Ca levels, early growth rate and later leg weakness could be affected by incubation programs within the usually acceptable hatchery range.

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IN VITRO ASSESSMENT OF ANTI-OXIDATIVE ACTIVITIES IN RICE BRAN, PALM KERNEL MEAL, SOYBEAN MEAL AND CORN FEED INGREDIENTS

Y.M. BAO[1], K. FILER[1],
J. SANOMWATTANAWONG[1], N. JANTASILA[1], H. PANDEY[1] and S. CONGSAGUL[1]

Summary

An in vitro study was conducted to determine the total phenolic acid content and DPPH anti-oxidative activity in rice bran, palm kernel meal, soybean meal and corn. The native rice bran, palm kernel meal, soybean meal and corn samples were used as the blank control diets, respectively. The 3×4 factorial dietary treatments were (1) rice bran control; (2) rice bran with SSF enzyme; (3) rice bran with xylanase; (4) palm kernel meal control; (4) palm kernel meal with SSF enzyme; (6) palm kernel meal with xylanase; (7) soybean meal control; (8) Soybean meal with SSF enzyme; (9) Soybean meal with xylanase; (10) corn control; (11) corn with SSF enzyme; (12) corn with xylanase. All samples were processed through a simulated chicken crop, gizzard and duodenum incubation system. The digested samples were then transferred into the dialysis bags of a simulated duodenum incubation period and dialyzed solution was used to analyze DPPH anti-oxidative activity and total phenolics content. It was demonstrated that both SSF enzyme and xylanase significantly improved DPPH anti-oxidative activity ($P < 0.05$) and total phenolics contents ($P < 0.05$) in rice bran and palm kernel meal. However, the supplementation of exogenous enzymes did not increase DPPH anti-oxidative activity and total phenolics in either corn or soybean meal. In conclusion, the improved DPPH anti-oxidative activity in rice bran and palm kernel meal might be due to an increase in release of total phenolics by SSF enzyme and xylanase.

I. INTRODUCTION

Phenolics are a group of natural products commonly found in cereal grains and have been found to be strong anti-oxidants against free radicals (Kim et al., 2008). Recently, these natural anti-oxidants in feed ingredients have been demonstrated to increase anti-oxidant activity in breast and thigh meat of broiler chickens (Goni et al., 2007; Sáyago-Ayerdi et al., 2009). Although most cereals in feed ingredients, in particular high fiber cereal bran, contain high levels of phenolic acids, most of them occur primarily in the bound form and are insoluble. It is assumed that the non-starch polysaccharide (NSP) degrading enzymes will help to release these phenolic acids in feed ingredients. In fact, β-glucosidase and xylanase have been shown to release phenolic acids in a solid state fermentation system (Bhanja et al., 2009). Thus, the supplementation of exogenous enzymes based on solid state fermentation (SSF) may have the potential to release more phenolic acids in feed ingredients and thereby improve the anti-oxidative activity in the chicken meat. Therefore, the objective of this study was to investigate the effect of Allzyme SSF and pure xylanase on the release of total phenolic acids and anti-oxidative activity in rice bran, palm kernel meal, soybean meal and corn feed ingredients.

II. MATERIALS AND METHODS

Allzyme SSF concentrate was provided by Alltech US. The pure xylanase was purchased from Sigma. The full fat rice bran, palm kernel meal, soybean meal and corn were purchased

[1] Alltech Asia-Pacific Biosciences centre, Thailand

from the local market in Thailand and were then ground to pass through a 1 mm sieve for analysis.

(a) Sample preparation and dietary treatments

The native rice bran, palm kernel meal, soybean meal and corn samples were used as the blank control diets, respectively. The 4×3 factorial dietary treatments were (1) rice bran control (without SSF); (2) rice bran with 200 mg SSF concentrate/kg diet; (3) Rice bran with pure xylanase (750U/kg diet); (4) soybean meal control (without SSF); (5) soybean meal with SSF concentrate; (6) soybean meal with pure xylanase; (7) palm kernel meal control (without SSF); (8) Palm kernel meal with SSF concentrate; (9) Palm kernel meal with pure xylanase; (10) Corn control; (11) Corn with SSF concentrate; (12) Corn with pure xylanase.

(b) In vitro assessment

In each treatment, 1 gram (1±0.0001) of sample was weighed in triplicate into 10 mL plastic syringe without Luer-locks. All those syringes were processed through a simulated chicken crop, gizzard and duodenum incubation system. The digested samples were then transferred into the dialyzed bags in the simulated duodenum incubation system and dialyzed solution was used to analyze dialyzed P, DPPH anti-oxidative activity and total phenolics content.

(c) Data analysis

The statistical analysis was performed using STATGRAPHICS software. The results were statistically evaluated using a multifactor ANOVA test. To determine significant differences, the Duncan's test was used at $P < 0.05$.

III. RESULTS AND DISCUSSION

The experimental results are shown in Table 1. Allzyme SSF significantly improved ($P < 0.01$) dialyzed P value in all feed ingredients. There was a significant interaction ($P < 0.01$) between Allzyme SSF and different ingredients in dialyzed P value. Both Allzyme SSF concentrate and pure xylanase improved ($P<0.01$) DPPH anti-oxidative activity and released total phenolics in rice bran and palm kernel meal but had no effect on that in soybean meal and corn.

DPPH, a stable radical, has been generally used to determine anti-oxidative activity or radical scavenging activity in fruit extracts (Thaipong et al., 2006). In the current experiment, in response to improved DPPH anti-oxidative activity, both Allzyme SSF concentrate and pure xylanase also improved release of total phenolics in rice bran and palm kernel meal. Thus, this increased phenolics contents may contribute to improved radical scavenging and anti-oxidative activity. Allzyme SSF is obtained from wheat bran and contains 7 synergistic enzymes including the xylanase, which may contribute to the function of anti-oxidative activity improvement in Allzyme SSF. The fact that both Allzyme SSF and pure xylanase did not have any effect on the anti-oxidative activity and released total phenolics in soybean meal and corn may indicate that the linkage between phenolics and NSP is different from that in rice bran and palm kernel meal.

Table 1 The effect of SSF and pure xylanase on released nutrients and anti-oxidative activity in different feed ingredients

Diet No.	Ingredients	Enzymes	Dialyzed P (g/kg)	Total Phenolics (mg/10g)[5]	Anti-oxidative Activity (μM/10g)[6]
1	Rice bran	–	1.88 [c 4]	115.15 [b]	2709.5 [d]
2	Rice bran	Xylanase	2.09 [c]	131.07 [a]	3367.7 [b]
3	Rice bran	SSF	10.24 [a]	130.23 [a]	3317.1 [bc]
4	PKM[1]	–	1.08 [de]	119.37 [b]	3224.4 [bc]
5	PKM	Xylanase	1.17 [d]	118.72 [b]	3566.4 [a]
6	PKM	SSF	1.64 [c]	135.60 [a]	3517.7 [ab]
7	SBM [2]	–	0.98 [de]	118.28 [b]	1712.6 [e]
8	SBM	Xylanase	1.05 [de]	118.42 [b]	1728.4 [e]
9	SBM	SSF	3.11 [b]	115.20 [b]	1712.6 [e]
10	Corn	–	0.64 [e]	67.90 [c]	1246.1 [f]
11	Corn	Xylanase	0.68 [e]	64.05 [c]	1193.1 [f]
12	Corn	SSF	2.00 [c]	69.30 [c]	1231.2 [f]
Pooled SEM			0.152	2.72	67.6
P value	Ingredient (A)		*** [3]	***	***
	Enzymes (B)		***	***	***
	A×B		***	***	***
R^2			0.13	0.71	

1. PKM: palm kernel meal.
2. SBM: soybean meal.
3. *** Significant difference ($P < 0.01$).
4. Mean values without common superscript within columns differ significantly ($P < 0.01$)
5. Expressed in gallic acid equivalents.
6. Expressed in Trolex equivalents.

It is noteworthy that total phenolics do not indicate the type or the nature of these phenolics. Therefore, no enzyme response on corn and soybean meal may be indicative of the different phenolic types found among plant tissues. Bryden et al. (2009) indicated that the release of phenolics might be detrimental for chicken performance. However, some phenolic acids significantly increased antioxidative enzymes activities in rats (Yeh et al., 2009), suggesting that the type of phenolic may influence anti-oxidative activities in feed ingredients.

It is believed that phytic acid in plant tissues is likely chelating with minerals, making them unavailable for animal nutrition (Cowieson et al., 2006). However, more recently, phytic acid has been demonstrated to have anti-oxidative activity due to its high binding

affinity for Fe^{2+} (Stodolak et al., 2007). Thus, the fact that the addition of Allzyme SSF phytase degrades mineral affinity will limit this anti-oxidative function. However, Ekholm et al. (2003) claimed that phytase has little effect on binding of Fe. In the current experiment, Allzyme SSF significantly released P and also improved the anti-oxidant activity in rice bran and palm kernel meal, suggesting that the release of P by Allzyme SSF did not affect the anti-oxidative activity of phytic acid. As a result, the long term supplementation of Allzyme SSF and xylanase may have the potential to improve meat quality.

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THE ROLE OF DIETARY FIBRE AND LITTER TYPE ON DEVELOPMENT OF NECROTIC ENTERITIS IN BROILER CHICKENS CHALLENGED WITH *CLOSTRIDIUM PERFRINGENS*

S.B. WU[1], L.L. MIKKELSEN[1], V.A. TOROK[2], P.A. IJI[1], A. SETIA[1] and R.J. HUGHES[3]

Summary

Increased structural fibre in the diet and/or ingestion of hardwood litter have shown similar effects to coarse feedstuffs or feedstuffs containing coarse components such as whole grains, which can stimulate gizzard development. It is not clear whether increased dietary fibre and/or ingestion of hardwood litter can provide birds with a degree of protection when exposed to a *Clostridium perfringens* strain known to induce necrotic enteritis (NE). In the present study, it was shown that dietary fibre and litter type had no effects on relative gizzard weight of birds measured at day 14 days of age. On the other hand, there were indications of an interaction between diet and litter type on gizzard weight at day 17. In birds raised on paper, those given a low fibre diet had smaller gizzards than those given a high fibre diet, whereas birds raised on hardwood were unaffected by dietary fibre. Meanwhile, dietary fibre and/or litter type altered the intestinal pH, short chain fatty acids (SCFA), and some microbial groups, but not the whole microbial community. It was concluded that, despite a degree of response to the treatments, no protection of birds from NE by high fibre diet and/or hardwood litter consumption was detected when the birds were subjected to *C. perfringens* infection.

I. INTRODUCTION

Ingestion of even small quantities of litter with large and/or hard particles stimulates gizzard development. These findings are consistent with published studies showing increased gizzard weight and improved gizzard function in broilers given either a coarse feed or feed containing coarse components, such as whole grains (Engberg et al., 2004; Svihus et al., 2004). Another important benefit arising from enhanced gizzard development is the potentially positive role of a functional gizzard in control of bacterial populations. Whole wheat feeding has been reported to significantly reduce the numbers of *C. perfringens* in the intestinal tract of birds (Bjerrum et al., 2005). This indicates that a functional gizzard may act as a barrier organ preventing potential pathogenic bacteria from entering the distal digestive tract. Thus, if access to gizzard stimulating litter materials has a significant impact in broiler chickens, choosing the right litter material may have important health implications in relation to reduced occurrence of NE which is strongly associated with *C. perfringens*. Alternatively, increased structural fibre in feed may have health benefits in terms of reduced enteric bacterial infections. This study aimed to determine whether enhanced gizzard development achieved by either increased dietary fibre concentration or by ingestion of hardwood litter, had a beneficial impact on health and productivity of birds challenged with *C. perfringens*.

II. MATERIALS AND METHODS

One thousand and two hundred day-old Cobb male broiler chickens (42.4±0.1 g) (Baiada hatchery, Kootingal, NSW, Australia) were raised in 48 floor pens. Each pen was assigned to one of 8 treatment groups with variables of paper vs. hardwood litters, high fibre vs. control

[1] School of Environmental and Rural Science, University of New England, Armidale, NSW 2351
[2] SARDI, Plant and Soil Health, Urrbrae, SA 5001
[3] SARDI, Pig and Poultry Production, University of Adelaide, Roseworthy, SA 5371

diets, challenged vs. unchallenged by *C. perfringens* with six replicates per treatment. The pelleted high fibre diet contained 7% of soy hull. The birds were fed starter diets during days 1 to 7, 30% fishmeal was added to the starter diet to induce stress on birds' gastrointestinal tracts during days 8 to 14, and fed finisher diets during days 22 to 28. The procedure for challenge of birds in corresponding groups was as described by Wu et al. (2010). On days 14, 15, and 16, birds to be challenged were inoculated *per os* with 1 ml of *C. perfringens* (type A strain EHE-NE18, CSIRO Livestock Industries, Geelong, Australia) suspension at a concentration of 108–109 CFUs/ml. The strain EHE-NE18 produces a novel toxin NetB which plays a major role in the pathogenesis of NE in the chickens (Keyburn et al., 2008). Birds in the unchallenged group received 1 ml of sterile thioglycollate broth. Feed consumption and live weight of the birds were measured on days 8, 15, 22 and 28 of the experiment. Live and adjusted body weight, body weight gain and feed conversion ratio (FCR) were calculated. FCR within a period was adjusted for mortality. On days 14 and 17, two birds were randomly chosen in each pen and sacrificed for sample collections. Total body weight of each bird was recorded, and the proventriculus, gizzard, small intestine, pancreas, liver, spleen and bursa were removed and weighed individually. The contents of the gizzard, ileum and caeca were pooled for the birds sacrificed in each replicate. pH of the contents was measured, and approximately 1 g of the digesta was collected for microbial culture, and the remaining digesta stored for determination of volatile fatty acid analysis. An approximately 3 cm section of ileum (including digesta) mid-point between the Meckel's diverticulum and caecal tonsils, and one caecal lobe (including contents) per bird were sampled for DNA profiling. Analysis of SCFA, enumeration of individual intestinal bacteria and profiling of total microbial community using T-RFLP analysis were performed. All data except those of T-RFLP were analysed using the statistical package SAS for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA). Significant differences between means were detected by pair-wise t-tests, or data were analysed by the non-parametric one-way analysis of variance procedure when the data were not normally distributed. The T-RFLP data were analysed as described previously (Torok et al., 2008).

III. RESULTS

High dietary fibre provided by soy hulls had no effects on adjusted live weight except for birds being significantly heavier at day 8. Similarly, there was a significant improvement in adjusted FCR in the period 0-8 days of age for birds given soy hulls. However, increased dietary fibre level was disadvantageous for FCR during the periods of 0-15 and 0-22 days of age. Litter type had no effects except for increased live weight on hardwood litter at 28 days of age. Challenge with *C. perfringens* had deleterious effects on live weight at 15 and 28 days of age, with significantly poorer FCR in the periods 0-15, 0-22 and 0-28 days of age. The challenge also resulted in highly significant losses of birds in the periods 15-17 days. Addition of soy hulls to the diet, and hardwood litter resulted in a significant increase of mortality in the periods 9-14 days and 18-21 days of age, respectively, but soy hulls in diets significantly eased mortality rate during the last week of the trial (Table 1).

Dietary fibre and litter type had no effects on any of the organ weights except for an increase in weight of the bursa which approached significance ($P = 0.052$). Challenge with *C. perfringens* resulted in a highly significant increase in weight of the small intestine and a significant decrease in liver weight. Dietary fibre and litter type had no effects on relative weights of proventriculus, small intestine, liver, spleen and bursa, and challenge with *C. perfringens* resulted in significant increases in weights of the proventriculus, small intestine and bursa. The relative weight of the gizzard was significantly affected by interactions between diet and litter type, diet and challenge, and litter type and challenge. Reduction in

gizzard size was significant ($P < 0.05$) in birds housed on paper litter and given a low fibre diet. Birds given the high fibre diet showed a greater decline in gizzard weight when challenged, compared with birds on a low fibre diet. Birds housed on wood litter showed no decline in gizzard size when challenged, whereas birds on paper showed a significant decline in gizzard weight. The 3-way interaction was not significant ($P > 0.05$). The relative weight of the pancreas was affected by interaction between litter type and challenge. Birds housed on paper litter showed a significant decline in pancreas weight when challenged, whereas pancreas weight was unaffected by challenge when birds were housed on wood litter.

Table 1 Main effects of dietary fibre, litter type and challenge with *C. perfringens* on mortality (means ± SE)

	Main effect					
Age in	Dietary fibre		Litter type		Challenge	
days	High	Low	Paper	Wood	Yes	No
0-8	1.2 ± 0.4	1.2 ± 0.4	0.8 ± 0.3	1.5 ± 0.4	0.8 ± 0.3	1.5 ± 0.4
9-14	12.0 ± 1.7*	6.7 ± 1.4	8.5 ± 1.6	10.2 ± 1.6	10.3 ± 1.8	8.3 ± 1.4
15-17	25.7 ± 4.3	21.2 ± 4.2	25.7 ± 4.3	21.2 ± 4.2	41.8 ± 2.5***	5.0 ± 0.9
18-21	1.5 ± 0.5	2.5 ± 0.8	0.7 ± 0.4**	3.3 ± 0.8	2.2 ± 0.8	1.8 ± 0.6
22-28	0.5 ± 0.3*	2.0 ± 0.6	0.8 ± 0.3	1.7 ± 0.6	1.2 ± 0.4	1.3 ± 0.6

Means within a main effect are significantly different if * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

High dietary fibre significantly increased acetic, propionic, isobutyric and butyric acids in caecal contents and depressed formic acid compared with low dietary fibre. There was a tendency for high fibre to increase lactic and succinic acids in ileal contents. Paper litter depressed the concentration of succinic acid in caecal contents and tended to increase acetic acid in caecal contents. The challenge procedure significantly increased lactic and succinic acids in ileal contents, and depressed concentrations of formic and acetic acids in caecal contents. There were tendencies for challenge with *C. perfringens* to raise concentrations of isobutyric acid and lactic acid in caecal contents.

Dietary fibre and litter type had no effects on pH in gizzard, small intestine and caeca at day 14. The challenge procedure significantly raised pH in the small intestine and caeca at day 14. On day 17, high dietary fibre significantly increased pH of the gizzard whereas challenge with *C. perfringens* lowered pH of the caeca.

On day 14, birds on hardwood litter had significantly higher counts of the total anaerobes, lactobacilli and lactic acid bacteria in the ileum, and a significantly lower number of anaerobes in the caeca. Challenge had no effects on numbers of different organisms in the ileum, but significantly raised numbers of enterobacteria and coliform bacteria in the caeca and depressed numbers of lactose-negative enterobacteria. In contrast to day 14, at day 17 litter type had no effects on bacterial counts in the ileum or caeca, whereas challenge with *C. perfringens* significantly increased numbers of lactic acid bacteria, *C. perfringens*, and enterobacteria in the ileum, as well as *C. perfringens*, enterobacteria and lactose-negative enterobacteria in the caecum. Although there was a trend towards a difference ($P = 0.06$) between the NE challenged and unchallenged control groups in the caecal microbial communities of birds aged 17 days which had been raised on the high fibre diet and paper litter, no significant effects of the treatments were detected on the whole microbial community.

IV. DISCUSSION

The hypothesis tested in this study was that enhanced gizzard development through increased dietary fibre and/or ingestion of hardwood litter would provide birds with a degree of protection when exposed to a strain of *C. perfringens* strain known to induce severe NE, with accompanying high mortality, and depression of live weight and FCR.

The NE challenge procedure was highly successful in that birds exposed to *C. perfringens* via oral gavage showed severe symptoms of NE , resulting in depressed live weight gain, poorer FCR and raised mortality. Anticipated protection from enhanced gizzard development was not evident, possibly because dietary fibre and litter type had no effects on relative gizzard weight of birds measured at day 14. In this study, it was observed that an interaction between litter type and challenge tended to produce a lower gizzard weight in birds raised on paper litter and subsequently challenged, which is consistent with the expectation that a bird with a poorly developed gizzard may be disadvantaged when faced with an enteric bacterial challenge, compared with flock mates with larger gizzards.

The general lack of effects of dietary fibre and litter type on relative organ weights at days 14 and 17, with the exception of an enlarged proventriculus in birds on paper litter, and reduced gizzard weight in birds given low dietary fibre and raised on paper, is not consistent with previous findings (Ali et al., 2009; Hetland et al., 2005; Hetland et al., 2003). The underlying causes of these discrepancies warrant further investigation. High dietary fibre resulted in increased concentrations of some SCFA (acetic, propionic, isobutyric and butyric) which have been associated with protection of birds from enteric infections due to their bactericidal properties (Ricke, 2003). Nevertheless, these increases appeared not to protect birds from *C. perfringens* infection; however, the infection was very severe in this study which may have compromised the possible effect of high dietary fibre. In less severe cases, perhaps elevation of SCFA would offset performance and mortality losses.

The hypothesis that enhanced gizzard development through increased dietary fibre and/or ingestion of hardwood litter provides birds with a degree of protection from NE induced by *C. perfringens* was not supported by results obtained in this study.

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VACCINATION AGAINST FOWL CHOLERA WITH LIVE AND INACTIVATED *PASTEURELLA MULTOCIDA* VACCINES IN A MEAT BREEDER FLOCK - A CASE REPORT

C. A.W JACKSON[1]

Summary

A number of field trials of an experimental live *aro*A *Pasteurella multocida* vaccine called Vaxsafe® PM (Bioproperties Pty Ltd) have recently been completed across three states in Australia. Flocks selected for the trials were chosen on the possibility that a prior history of outbreaks of fowl cholera (FC) would offer a location where natural field challenge would generate data on the efficacy of the vaccine. One meat breeder flock experienced FC following challenge with several heterologous and homologous serotypes of *P. multocida* at various times throughout its life. This flock provided a case report of an outbreak of FC in the face of vaccination. This paper details the liveability and productivity of this flock together with an evaluation of the safety and efficacy of the live and inactivated FC vaccines used to provide protection against FC challenge. The productivity data from this flock allowed an overall assessment of the effect of vaccination on hen day per cent egg production and cumulative eggs per hen housed.

I. INTRODUCTION

The primary method of control of FC should be good sanitary practice and effective biosecurity (Rimler and Glisson, 1997). However, in areas where endemic FC is a problem, vaccines are used. Inactivated vaccines, typically based on the prevalent serovars in the target poultry population, and live attenuated vaccines are available overseas (Rimler and Glisson, 1997). To date, only inactivated vaccines have been available for use in Australia. Inactivated vaccines usually require the addition of autogenous components to provide protection against the serovars endemic on the site requiring vaccination. The accepted belief is that the inactivated vaccines protect only against the serovars present in the vaccine, while live vaccines give broad cross-serovar protection (Rimler and Glisson, 1997). In recent years, there have been attempts to develop a live vaccine in Australia. Scott et al. (1999) described experiments in which two auxotrophic *aro*A mutants of *P. multocida,* designated PMP1 (serotype 1) and PMP3 (serotype 3), were tested as vaccine candidates to protect chickens against FC. Those studies and more recent safety and efficacy trials under the Australian Poultry CRC have laid the foundation for choice of dose and vaccination program for field trials that are required by the Australian Pesticides and Veterinary Medicines Authority (APVMA) towards registration of a vaccine in Australia.

II. FIELD TRIAL DESIGN

The field trial described here was designed to evaluate the efficacy and safety of an experimental live *P.multocida* vaccine, Vaxsafe® PM Vaccine (living) [Vaxsafe® PM] (Bioproperties Pty Ltd) administered to two sheds of meat breeder pullets in comparison to an autogenous inactivated PM vaccine administered to two other sheds on the same farm, one of which contained only sires until week 22 (see Table 1). Chickens were vaccinated according to draft labels and leaflets (and Permit in the case of the inactivated PM vaccine) approved by the

[1] Biological Technology Transfer Pty. Ltd, 2 Victory Ave, Camden NSW 2570 Australia

APVMA. Vaxsafe® PM was administered by intra-muscular (IM) injection in a volume of 0.2mL per bird at a commercial release titre of $10^{7.9}$ colony-forming units (cfu) per dose. The likelihood of natural field challenge was anticipated from the previous history of exposure of flocks on this farm. Field challenge was proven by periodic attempts at isolation of *P. multocida* from affected birds showing clinical signs of lameness and swollen wattles typical of FC. Efficacy was evaluated through a comparison of percent cumulative mortality of pullets and sires during rearing and production and by an overall comparison of egg productivity measured by hen day and cumulative eggs per hen housed, of live and inactivated vaccinated sheds. Safety was determined by analysis of data on mortality rates and productivity collected on a shed basis. Statistical analysis was limited to Chi-squared tests of differences in mortality rates as clustering and use of single sheds for different treatments prevented the application of parametric tests.

III. RESULTS

No adverse effects in sires and pullets were noted when Vaxsafe® PM was inoculated into one side of the pectoral muscle whilst another inactivated vaccine was inoculated into the other pectoral muscle. Similarly, a third inactivated vaccine administered SC into the neck of each pullet resulted in no adverse effects. No swelling or other abnormalities were observed around the site of vaccination. No signs of endotoxicity, such as depression after vaccination, were observed.

Evidence of field challenge with FC was found following autopsies of pullets and sires showing lameness and/or swelling of the wattles, principally from sheds 1 and 4 that had been vaccinated with inactivated FC vaccine. A higher than normal standard mortality associated with FC was observed in Shed 1 from eight weeks of age. Mortalities continued to increase in that shed (despite repeated periodic antibiotic in-water medication) reaching 27.0% at the end of rearing at 24 weeks of age. *P. multocida* was isolated from Shed 1 at 11 weeks of age (prior to vaccination) and at 16, 19, 23, 39 and 60 weeks of age. *P. multocida* was also isolated from affected birds from Shed 4 at 43,49 and 60 weeks of age and from lame birds in Shed 3 at 14 weeks of age, one week after vaccination with Vaxsafe® PM. The somatic serovars of *P. multocida* that were isolated belonged to groups 1, 3 and 14 (Table 1). It was apparent that serovar 3 predominated in sheds 1 and 4 where the inactivated PM vaccine was administered. The only isolate obtained in a Vaxsafe® PM vaccinated sheds was a serovar 1 isolated in shed 3, seven days after the first vaccination of that shed.

Table 1 Isolation of *Pasteurella multocida* serovars from field trial sheds in relation to vaccine type and age of birds

Shed No.	Vaccine type	Age in weeks	*P. multocida* serovar
1	Inactivated	11	1
1	Inactivated	16	3
1	Inactivated	19	3
1	Inactivated	23	3
1	Inactivated	39	3,1,14
1	Inactivated	60	3
3	Vaxsafe PM	14	1
4	Inactivated	43	3
4	Inactivated	49	3
4	Inactivated	60	3

The two sheds vaccinated with Vaxsafe® PM at 13 and 19 weeks of age experienced rearing mortalities (and culling) from 6 to 24 weeks of age of 13.5 and 14.2% in Sheds 2 and 3, respectively (Table 2). These mortality rates were significantly lower ($P < 0.001$) than that experienced by birds in Shed 1 with a mortality of 27.0% which was almost twice that of the other sheds. The lower mortality in Shed 3 occurred despite the isolation of *P. multocida* from Shed 3 at 14 weeks of age (one week post the first vaccination with Vaxsafe® PM). No in-water medication of that shed was required. During the laying period (25-59 weeks of age) the cumulative mortality (and culling) of pullets in the Vaxsafe® PM vaccinated sheds was 11.1% (Shed 2) and 11.5% (Shed 3) compared to 13.7% and 14.2% in the inactivated FC vaccinated Sheds 1 and 4, respectively. Sire mortality (and culling) from 25 to 49 weeks of age in the two Vaxsafe® PM vaccinated sheds was 31.6% (Shed 2) and 30.8% (Shed 3) compared to 47.5% and 45.1% in the inactivated FC vaccinated Sheds 1 and 4, respectively. Although all sires were vaccinated with inactivated FC vaccine, the lower mortality in Sheds 2 and 3 was attributed to a lower rate of exposure to FC in the two Vaxsafe® PM sheds.

Table 2 Comparative mortality (and culling) of Vaxsafe® PM vaccinated and inactivated FC vaccinated sheds

Shed No.	No. of birds vaccinated	Vaccination treatment	Pullet rearing mortality (%) 6-24 weeks of age	Pullet mortality (%) 25-59 weeks of age	Sire mortality (%) 25-49 weeks of age#
1	14,309	Inactivated FC	27.0	13.7	47.5
2	9,500	Vaxsafe PM	13.5	11.1	31.6
3	9,500	Vaxsafe PM	14.2	11.5	30.8
4	4,857*	Inactivated FC	NA	14.2	45.1

* Sires only; 5,461 pullets were transferred from shed 1 to shed 4 at 22 weeks of age.
NA = not applicable as there were no pullets placed in this shed.
# All sires were vaccinated with inactivated FC vaccine. Mortality (and culling) figures of sires relate to their shed location after placement for mating with the pullets.

Peak egg production of the two Vaxsafe® PM vaccinated sheds during week 32 was 82.8% (Shed 2) and 78.0% (Shed 3) compared to 81.0% and 80.1% in the inactivated FC vaccinated Sheds 1 and 4, respectively (Table 3). At 59 weeks of age the hen day egg production was 57.4% and 47.7% in the two Vaxsafe® PM Vaccine (living) vaccinated sheds 2 and 3, respectively, compared to 54.6% and 58.4% in the inactivated FC vaccinated Sheds 1 and 4, respectively.

Table 3 Comparative productivity of Vaxsafe® PM vaccinated and inactivated FC vaccinated sheds

Shed No.	No. pullets housed at 22 weeks	Vaccination treatment	Peak lay hen day at 32 weeks (%)	% lay hen day at 59 weeks	Cum. eggs/ hen housed
1	9,686	Inactivated FC	81.0	54.6	147.1
2	6,800	Vaxsafe PM	82.8	57.4	161.8
3	6,754	Vaxsafe PM	78.0	47.7	148.1
4	5,461	Inactivated FC	80.1	58.4	141.4

Productivity in terms of cumulative number of eggs per hen housed was 161.8 and 148.1 in the Vaxsafe® PM vaccinated sheds compared to 147.1 and 141.4 in the inactivated FC vaccinated Sheds 1 and 4, respectively. The mean difference in cumulative eggs per hen housed of 10 eggs in favour of Vaxsafe® PM was considered to reflect the higher mortality in the inactivated FC vaccinated Sheds 1 and 4, either directly or indirectly associated with field challenge by PM.

IV. DISCUSSION AND CONCLUSIONS

This field trial was able to effectively evaluate the safety and efficacy of Vaxsafe® PM following evidence that a natural field challenge with PM had occurred on the farm. Three different serovars were isolated with serovar 3 being the most commonly isolated and only isolated from the birds in the inactivated FC vaccinated sheds. This serovar and one other serovar (serovar 14) are heterologous strains to the vaccine indicating that Vaxsafe® PM was capable of conferring some degree of heterologous protection The only isolate from the Vaxsafe® PM vaccinated sheds was an homologous serovar 1. This isolate was identified as a wild-type and not the vaccine strain and was recovered one week following the first Vaxsafe® PM vaccination. This suggested that it could have infected the birds in the shed prior to vaccination and should not be regarded as an indication of lack of homologous protection. Therefore, it can be argued that a better performance of meat breeder pullets vaccinated with Vaxsafe® PM would be an indication of superior efficacy of the product. Indeed, this was found to be the case where mortality and production parameters of birds vaccinated with Vaxsafe® PM were either equal to or better than those of birds vaccinated with a permitted inactivated FC vaccine. In addition, there was evidence of a further cost saving from the use of Vaxsafe® PM following the need to provided antibiotic medication only to those sheds vaccinated with the inactivated PM vaccine.

Safety of Vaxsafe® PM was demonstrated by the absence of adverse effects immediately following vaccination and when administered at the same time as two other inactivated vaccines. Data collected on mortality (and culling) during rearing and production found that the sheds vaccinated with Vaxsafe® PM had a lower mortality rate than those vaccinated with a permitted inactivated FC vaccine. Further evidence of safety was demonstrated by the superior average cumulative egg production of the layer pullets in the Vaxsafe® PM vaccinated sheds compared to the birds in the inactivated FC vaccinated sheds.

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INFLUENCE OF EGG SHELL TRANSLUCENCY ON EGG SHELL PENETRATION BY BACTERIA

K.K. CHOUSALKAR[1], P. FLYNN[2], M. SUTHERLAND[2], B.F. CHEETHAM and J. R. ROBERTS[2]

Summary

Egg shell translucency, which can be due to changes in the mammillary cores and mamillary layer during the early phases of eggshell formation, has the potential to increase the incidence of microcracks in egg shells, and hence, may facilitate bacterial penetration. There was a significant correlation between egg shell translucency and egg shell penetration by *Salmonella* Infantis. *Salmonella* Infantis was able to penetrate translucent egg shells even at very low doses. The penetration, however, appeared to be hindered in both translucent and non-translucent eggs at 4°C, as compared with room temperature which highlights the importance of storage of eggs at refrigeration temperatures.

I. INTRODUCTION

Eggs produced in Australia, most of which are from cage laying systems, are considered medium to low risk food for food borne illness. The medium risk ranking is mainly because of pathogens like *Salmonella* and some other enteropathogens. The egg industry in Australia is periodically implicated in cases of food poisoning and the egg's contents are an ideal growth medium for microorganisms which are hazardous to humans. It has been observed that the microflora of the eggshell are dominated by Gram-positive bacteria, whereas Gram-negative bacteria are best equipped to overcome the antimicrobial defences of the egg contents (De Reu *et al*., 2008). The Australian poultry industry is considered to be free from *Salmonella* Enteritidis which is of major concern for the food industry all over the world, with Cox *et al*. (2002) reporting that *Salmonella* Infantis (S. Infantis) was the predominant Salmonella serovar in the Australian egg industry. It is difficult for bacteria to move across an intact good quality egg shell. However, small defects in the egg shell may provide means for the predominant bacterial spp. on the egg shell to penetrate and move into the egg contents (De Reu, *et al*., 2006). Translucency is possibly caused by irregular mammillary knobs probably due to the fusion of several mammillary cores during the early phases of eggshell formation (Bain *et al*., 2006) leaving larger spaces among the mammillary cones. It is not clear whether translucency has any role in potentiating the entry of bacteria into the egg contents and the risk that such eggs pose to product safety is uncertain. In the present study, unwashed eggs collected directly from the cage front were scored for egg shell abnormalities such as translucency. The ability of *S.* Infantis to penetrate translucent and non translucent egg shells at different storage temperatures was also investigated.

II. MATERIALS AND METHODS

a) Scanning electron microscopy for studying egg shell translucency.

For studying the ultrastructure of translucent egg shells, eggs were candled and areas of severe translucency were selected and marked with a pencil. Egg shell pieces of approximately 1cm^2 were cut from two non-translucent and four severely translucent eggs.

[1] School of Animal and Veterinary Science, Charles Sturt University, NSW
[2] Animal Science, School of Environmental & Rural Science, University of New England, NSW

After soaking the eggs shells in water, the egg shell inner membranes were removed manually. The outer membrane was removed from the dried shell by plasma ashing, using a Bio Rad RF plasma Barrel Etcher PT 7150, as described by Brackpool (1995).

The mammillary region of the egg shell was examined for ultrastructural characteristics as described by Brackpool (1995). The samples were mounted on aluminium stubs and gold-coated (Polaron E5100). Samples were then observed under a scanning electron microscope (Neoscope, Coherent Scientific, Australia). Observations regarding changes in mammilary cap arrangements, size, early fusion, late fusion depression, erosions, type A and B bodies were made and recorded as described by Solomon (1991).

b) Agar method for assessment of the egg shell penetration

An agar method described earlier by De Reu *et al*. (2006) was adapted to study bacterial penetration across the egg shell. Briefly, 90 fresh eggs were obtained from cage fronts of 40-week old commercial Isa Brown laying hens. Eggs were divided into three groups (n=30) in a 3 x 3 factorial arrangement. Internal contents of eggs obtained from this flock were tested for *Salmonella*. All eggs were candled and translucency was scored as 0 (no translucency), 1 (mild translucency), 2 (moderate translucency) and 3 (severe translucency). Each egg was dipped into 70 % ethanol for 1 min to kill any bacteria on the outside of the shell and was allowed to air dry in a biosafety cabinet. After making a hole in the egg shell, the egg contents were removed using a sterile syringe. Eggs were then washed with sterile phosphate buffered saline (PBS; pH 7.2) to remove all the albumen adhering to the membrane. Each egg was then filled with McConkeys agar. Eggs were sealed with paraffin after the agar solidified. An *S*. Infantis strain obtained from IVMS, Adelaide, Australia was used in this experiment. Bacteria stored at -80°C in 50% glycerol were plated onto horse blood agar and incubated overnight at 37°C. A single colony was then inoculated and grown overnight at 37°C in brain heart infusion broth (BHI; Oxoid, Australia). The broth was then diluted in PBS to a 10^{-6} dilution. Enumeration of viable bacteria was performed by serial dilution, plating 100 µl of each solution onto McConkeys agar plates followed by incubation overnight at 37°C. Ten agar filled eggs were immersed for 1 minute in one of three serial dilutions for *Salmonella* Infantis in approximately 10^7, 10^5 and 10^3 colony forming units (CFU) per ml of PBS. After inoculation, agar filled eggs were kept at 4°C, 20°C and at 37°C. The eggs were aseptically opened in a biosafety cabinet after 72 hrs to inspect for growth of typical visible colonies. Colonies seen nearby the hole in the shell were not recorded as penetration. The shell membranes and agar with colony growth were reinoculated onto triple sugar iron (TSI) agar slopes and incubated at 37°C for confirmation. A one way analysis of variance (ANOVA) (Statview® version 5.0.1 for windows, SAS Institute Inc. Copyright © 1992- 1998) was used to study the effect of egg shell translucency on percentage of egg shells penetrated.

III. RESULTS

a) Scanning electron microscopy for studying egg shell translucency

The ultrastructural appearance of the egg shells from the non translucent egg shells were in agreement with Brackpool (1995) and showed no detrimental changes in the mammillary caps. In the translucent egg shells, mammillary caps were of good quality with extensive attachment with the shell membrane. However, there was alignment of the mammillae, where the mammillae appear to “line up” resulting in long continuous grooves between the cones (Roberts and Brackpool, 1994). Such alignment was not observed in non-translucent egg shells. There was little cuffing (additional calcium around the mammillary cones that appears to contribute to shell strength) in the translucent egg shells and late fusion of the mammillary layer was not recorded. Depression and erosion of mammillae were also observed in the

translucent egg shells. Type B bodies, which are small spherical calcified bodies which have variable contact with membrane fibres, were also found in the translucent egg shells.

b) Effects of egg shell translucency on *S.* Infantis penetration

At 37°C, 70% of eggs inoculated in 10^7 cfu were penetrated by *S.* Infantis and 40% of eggs inoculated in 10^5 and 10^3 cfu/ml, were penetrated by *S.* Infantis. 70% of eggs, which were inoculated at the dose rate of 10^7 cfu and incubated at 20 °C, were positive for bacterial penetration. 40% and 30% of eggs inoculated in 10^5 and 10^3 cfu/ml, respectively, and incubated at 20°C were found positive for bacterial penetration.

Bacterial penetration was recorded in 20% of eggs inoculated with 10^7 cfu and incubated at 4°C. *S.* Infantis penetration was not recorded in any of the egg shells from the remainder of the treatments at 4°C. For the eggs incubated at 37°C, there were significant differences between egg shell translucencies of penetrated and non-penetrated egg shells inoculated in 10^5 and 10^3 cfu/ml (Fig. 1). Bacterial penetration was recorded along the translucent patches of the egg shell. For the eggs incubated at 20°C, there were significant differences between egg shell translucencies of penetrated and non-penetrated egg shells inoculated in 10^7 and 10^5cfu/ml (Fig. 2).

Figure 1 Penetration of *S.* Infantis across translucent and non translucent egg shells at 37°C.

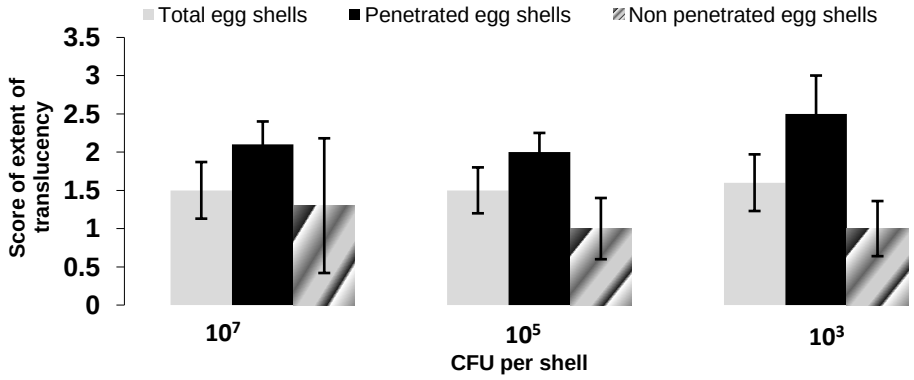
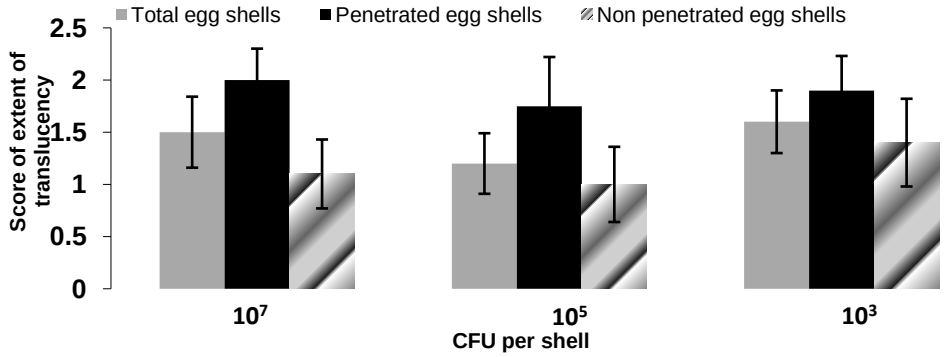


Figure 2 Penetration of *S.* Infantis across translucent and non translucent egg shells at 20°C



IV. DISCUSSION

Egg shell quality can be influenced by various factors like age, strain of hen, temperature and disease (Roberts, 2004). Translucency ultimately has the potential to increase the incidence of microcracks in egg shells (Bain *et al*., 2006). In the present study, translucent egg shells had good quality mammillary caps with extensive attachment with the shell membrane; however

there was alignment of the mammillae resulting in long continuous grooves between the cones which are thought to lower the resistance of egg shells to bacterial penetration (Solomon, 1991). Cuffing is thought to be responsible for increasing bacterial resistance to penetration and, in this study, there was poor cuffing in the translucent egg shells. Pitting can reduce the shell's resistance to bacterial penetration (Nascimento *et al*., 1992). Type B bodies were found in the translucent egg shells. Type B bodies are normally present in avian egg shells, although a large number of type B bodies can disrupt the mammillary layer of the egg shell thereby potentiating the entry of bacteria (Nascimento *et al*., 1992). In the present study, at 4°C, only two egg shells were penetrated at high dose rates of the bacterial inoculums. The current finding highlights the importance of storing eggs appropriately throughout the supply chain. Board and Tranter (1995) reported that the extent of contamination of hatching eggs was in the range from 10^2 to 10^7 CFU per eggshell. In the current experiment, although the dose of bacterial inoculation was very high, it was within the normal contamination range described in earlier studies by Board and Tranter (1995). However, during the present study, eggs were washed in 70% ethanol prior to external inoculation which may have damaged the cuticle, reducing its protective properties. There is still the possibility that pathological lesions in egg shells like cuffing, type B bodies and depressions, seen in the transluscent egg shells, can potentiate the entry of bacteria across the egg shell. Translucent egg shells can increase the likelihood of internal contamination of eggs. In the present study, however, the bacterial contamination of the egg shell was not quantified. It is possible that small, slow-growing colonies of *S.* Infantis were not detected in the present study and further research is needed in this area.

ACKNOWLEDGEMENTS

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PERSISTANCE OF ANTI SALMONELLA ANTIBODY IN EGG YOLK FOLLOWING VACCINATION

S.M. SHARPE[1] and P.J. GROVES[2]

Eggs and egg products contaminated with *Salmonella* are a major health concern, both in reality and in the perception of the Australian consumer and the food safety industry. *Salmonella* are frequently isolated from the environment of poultry farms, sometimes involving the presence of serotypes which are regarded as serious for human health, in particular *Salmonella* Typhimurium. Foodborne salmonellosis in humans can be decreased by preventing *Salmonella* infection during poultry production. The commercial layer industry is under continuous pressure to both improve and to be seen to be improving its approach to public health. It would appear that live attenuated vaccination or inactivated vaccines have given variable results in the fight against Salmonella colonisation of chickens, hence the proposition to use a combination of live and killed vaccines in this research.

In the current study, we used different vaccination regimes with the aim of establishing the best combination for use in Commercial Layers. Day old brown egg laying chicks (650), randomly assigned into 9 groups at a trial facility were reared on the floor, until17 weeks when they were moved into individual cages at The University of Sydney. The birds were vaccinated with a live attenuated *Salmonella* Typhimurium and killed multivalent *Salmonella* vaccines, using different regimes for each group. Vaccination programs were administered to birds in one pen each as follows: **1** No vaccine; **2** Bioproperties Vaxsafe ST (V) orally at day old and 3 weeks of age; **3** V orally at day old, and 3 weeks and Intervet inactivated trivalent *Salmonella* vaccine (N) given IM at 12 weeks of age; **4** V orally at day old, 3 weeks and 6 weeks of age; **5** V orally at day old, 3 weeks and 6 weeks and N at 12 weeks of age; **6** V orally at day old, 3 weeks and 6 weeks and N at 12 weeks of age; **7** N at 6 and 12 weeks of age; **8** V by subcutaneous injection at 4 and 8 weeks of age; and **9** V orally at day old, 4 weeks and 14 weeks of age. The inactivated vaccine was always given by intramuscular injection.

Eggs were collected from five groups 1, 3, 6, 7 and 9 at 24, 34, 40 and 50 weeks. Blood samples were also collected at various ages and all were assayed using the x-Ovo ELISA kits (Guildhay). The antibody levels in the egg yolks correlated well with the blood results, reported previously. The inactivated vaccination Groups 6 and 7 achieved the highest antibody level in eggs, which was decreasing at 50 weeks. The live vaccine given orally showed only short term protection.

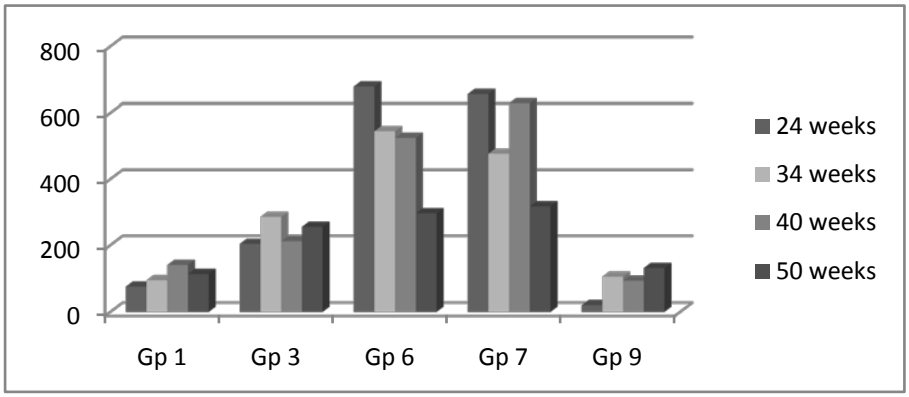

Figure 1 Floor Reared Egg Yolk IgY Antibody Levels

[1] Birling Avian Laboratories, Bringelly NSW
[2] The University of Sydney, Camden NSW

VIRAL LOAD, SHEDDING RATE AND LATERAL TRANSMISSION OF MAREK'S DISEASE VACCINAL VIRUS (RISPENS/CVI988) IN SPF CHICKENS

T. ISLAM[1], K.G. RENZ[1] and S.W. WALKDEN-BROWN[1]

Summary

The Rispens (CVI988) attenuated Serotype 1 Marek's Disease virus vaccine is highly effective and used worldwide. It has been shown that this virus is present in feather tips of vaccinated chickens but the extent to which current commercial Rispens vaccines transmit effectively between chickens is unknown. To determine this, we measured the shedding rate, lateral transmission and changes in viral load over time, of the three commercial Rispens vaccines available in Australia. In each of three climate-controlled rooms, 10 SPF (specific pathogen free) chickens were vaccinated with a commercial Rispens vaccine at day old and left in contact with 10 unvaccinated chickens. A separate room contained unvaccinated control birds. As determined by quantitative real-time polymerase chain reaction (PCR) of room dust and peripheral blood lymphocytes (PBL), vaccine virus was shed from the vaccinated chickens in dander from day 7 and transmitted effectively from vaccinated to in-contact chickens with a lag period of 2-3 weeks.

I. INTRODUCTION

The most effective current Marek's disease vaccine worldwide is the Rispens CVI988 vaccine which is an attenuated serotype 1 Marek's disease virus (MDV1) (Witter et al. 1995; Baigent et al. 2005). As part of a wider investigation into interaction between vaccinal and wild-type MDV, this study was designed to determine the kinetics of Rispens replication at tissue & cell level at different times post vaccination and the spread of Rispens to in-contact chickens following vaccination. Rispens et. al. (1972) showed that CVI988 virus spreads laterally from vaccinated to in-contact chickens at low passage level (35), but working with a high passage clone (65), Witter et al. (1987) found that there was only limited transmission (0/8 and 4/10 for virus isolation and sero-conversion respectively). It is not known whether vaccination with the current Rispens vaccines will result in bird to bird effective transmission or not. In Australia, three different Rispens vaccines are commercially available. We used these vaccines in an experiment designed to test the following hypotheses:

1. Vaccine virus will be shed in dander and this will start from 7 day post-vaccination;
2. Vaccine virus will transmit laterally to in-contact chickens;
3. Viral kinetics will not vary significantly between the three commercial vaccines; and
4. Replication rate and shedding will be lower than published values for pathogenic MDV.

II. MATERIALS AND METHODS

Vaccines: The experiment utilised 3 vaccines (Nobilis®Rismavac, Intervet Australia; Poulvac®CVI Vaccine, Fort Dodge/Pfizer; Vaxsafe®RIS, Bioproperties.

Chickens: SPF white leghorn chickens were used, 20 in each of three vaccine study treatments and 10 in the negative control treatment.

Vaccination Protocol: Ten chickens in each vaccine group were vaccinated subcutaneously at hatch (day 0) with the manufacturer's recommended dose of vaccine {$\geq$1000 $TCID_{50}$ (tissue culture infective dose), $\geq$1000 and $\geq$4000 pfu (plaque forming unit) for the 3 vaccines above

[1] School of Environmental and Rural Science, University of New England, Armidale NSW 2351 Australia

respectively} in 0.2ml of the supplied diluents, and 10 chickens for each group remained unvaccinated but were injected with the respective diluent alone (0.2ml).
The chickens were reared on deep litter in four climate control rooms of the University of New England Animal House. Vaccinated and unvaccinated in-contact chickens were individually identified using wing tags and housed together. Negative control chickens were injected with a mixture of the three vaccine diluents (0.2ml).

Blood samples were collected from the vaccinated and control birds weekly from 7 days of age and from in-contact birds weekly from 21 days of age up to day 56. Blood samples were collected into sodium citrate anticoagulant prior to separation of PBL using ficoll paque^{TM} PREMIUM. For the day 56 sampling, serum was also collected for Enzyme-linked immunosorbent assay (ELISA) to measure antibody levels directed against MDV. Dust collection from an open tray in each room started at day 7 and continued weekly up to day 56.

DNA was extracted from PBL using the X-tractor Gene DNA extraction robot (Corbett, ROBOTICS, Australia). From dust, DNA was extracted using the DNeasy® tissue kit (Qiagen, Clifton Hill, Australia). All DNA was quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA). Taqman real-time qPCR assay for MDV1 was performed using a Rotor Gene 3000 real-time PCR machine (Corbett Research, Australia) using the method of Islam et al., (2006). Indirect ELISA to detect MDV-specific antibody was adapted from the method of Zelnik et al., (2004). Samples were randomized prior to testing.

The rate of which birds become infected was determined by detection of MDV in PBL and treatments effects were investigated using survival analysis (Kaplan-Meier product-limit method). MDV1 load and MDV titer values were log transformed [$\log_{10}$ (x+1)] prior to analysis using ANOVA, fitting the effects of Vaccine (randomly coded A, B, C), Type of infection (vaccinated, in-contact), Day (when required) and their interactions. For repeated measures (PBL, dust) a mixed REML model was used with animal or room fitted as a random effect. Analyses were performed with JMP8 (SAS Institute, NC, USA). Least squares means and standard errors of means are presented for continuous variables. A statistical significance level of $P<0.05$ was used throughout.

III. RESULTS

Control chickens remained negative for MDV in PBL and dust (qPCR), and serum (ELISA) at day 56. Most (93%) vaccinated birds were MDV1-positive in PBL at 7 days of age and 100% by 28 days of age. For the in-contact birds, 38% were positive by the first measurement at 21 days of age, reaching 96% by day 56 (Figure 1). Infection of in-contact birds lagged behind vaccinated birds by 2-3 weeks (Figure 1).

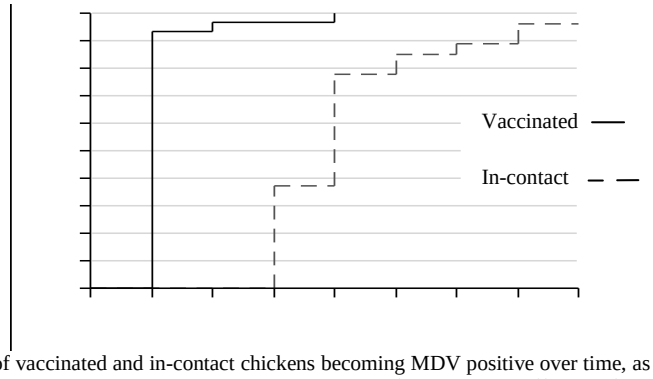

Figure 1 Cumulative proportion of vaccinated and in-contact chickens becoming MDV positive over time, as determined by qPCR of PBL for MDV1 commencing at days 7 (Vaccinated) and 21 (In-contact) respectively (failure curves differ significantly, $P < 0.001$)

There was a significant effect of Type of Infection (P = 0.007, Figure 2) and Vaccine (P<0.001) on $\log_{10}$ MDV copy number per 10^6 PBL with no significant interaction between these effects (P=0.696). In vaccinated birds mean viral load was maximal at day 7 {$10^{3.5}$ VCN (viral copy number)/10^6 PBL} decreasing to $10^{2.5}$ VCN/10^6 PBL at day 21 then plateauing until day 42 before decreasing further to $10^{1.2}$ VCN/10^6 PBL at 56 days of age (Figure 2). In the in-contact birds, mean MDV load in PBL was low ($10^{1.5}$ VCN/10^6 PBL) at 21 days of age (first sampling day for this group) increasing to $10^{3.2}$ VCN/10^6 PBL on day 28 before plateauing at this level until day 42 days before decreasing to 10^2 VCN/10^6 PBL at day 56 (Figure 2).

There was a significant effect of Vaccine (P < 0.045) and Day (P < 0.001) on MDV load in dust with vaccines B and C inducing overall values 0.38 and 0.52 logs higher than vaccine A. Dust from all three vaccine treatments was MDV positive from day 7 onwards with the viral load in dust increasing to day 21 then more slowly and erratically thereafter (Figure 3).

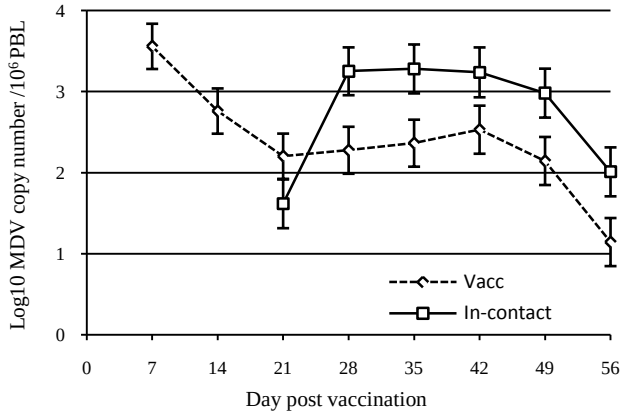

Figure 2 Mean (LSM±SEM) MDV load in PBL in vaccinated and in-contact chickens over time.

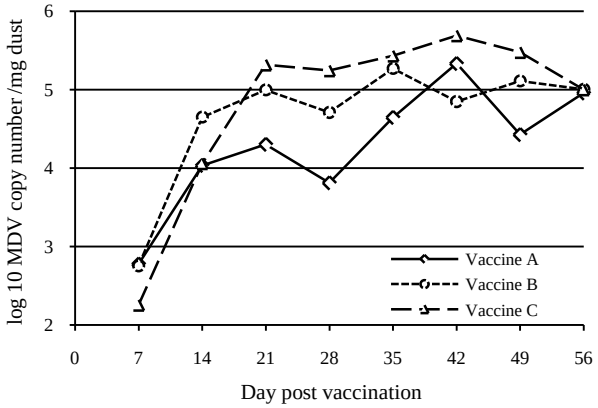

Figure 3 Mean MDV load in dust by vaccine type over time (no SE as only one sample per vaccine/week)

There was no significant effect of Type of Infection (P=0.358) and Vaccine (P=0.189) on MDV antibody titre at day 56, with no interaction between these effects (P=0.714).

IV. DISCUSSION

The study demonstrated that all three serotype 1 MDV vaccines available in Australia induce shedding of vaccinal virus in feather dander as early as day 7 post-vaccination. The experiment also revealed that the vaccine virus transmits very effectively to in-contact chickens, producing higher viral loads in these birds than observed in the vaccinated chickens. There was lag time of 2-3 weeks in infection between vaccinated and in-contact chickens.

Baigent *et. al.* (2005) detected CVI988 in feather tips at 6 days of age but did not extend their findings to measuring shed virus in feather dander or dust. The present study has demonstrated that the current vaccinal strains of CVI988, used in Australia according to the manufacturers' directions, all readily shed from the vaccinated chickens into the environment in large amounts (up to $10^{5.7}$ VCN/mg dust), with shed virus readily detected in dust samples from 7 days of age. Thus the first hypothesis of this experiment is accepted.

When CVI988 was first detected and tested for its properties, Rispens et al., (1972) reported that it spread directly to in-contact chickens using virus isolation and detection of antibody levels from in-contact chickens. However, the Clone C vaccinal strain of CVI988 spread poorly to in-contact chickens, probably due to subsequent passage and attenuation (Witter et al. 1987). Since those two studies, there has been no quantitative data reported on

the transmission of commercial CVI988 vaccine strains. The effective spread of the vaccine virus in the present study is consistent with the earlier results of Rispens et. al. (1972) and thus the second hypothesis of this experiment is accepted.

Regarding the viral load in PBL over time, Baigent et. al. (2005) first detected the CVI 988 genome in PBL at 4 days post vaccination (dpv). Viral load then increased to a peak of 4.7 x 10^3 VCN/10^6 PBL at 14 dpv and steadily decreased to a level of 3.91 x 10^2VCN/10^6 PBL on the last day of experiment at 28 dpv. In the present experiment, very similar viral loads and patterns were observed but the peak in vaccinated birds was earlier, that is at 7 days post vaccination. From day 28 onwards, MDV load in PBL was consistently higher in in-contact birds than in the vaccinated chickens, indicating very effective natural transmission and subsequent replication. Baigent et. al. (2005) reported data on the kinetics of Rispens vaccine manufactured by Ford Dodge UK. Until this present experiment, no other published studies have compared the kinetics or spread of Rispens vaccines from different manufacturers. While differences between vaccine products was not a significant focus of our experiment, it did show that there were significant differences between the three Rispens vaccines in the viral load induced following vaccination, with some trends also evident for rate of spread. However MDV titre at the end of the experiment did not differ significantly between the 3 vaccines. Thus, based on the PBL and dust data, hypothesis 3 is rejected.

Our fourth hypothesis, that replication rate and shedding of CVI988 will be lower than published values for pathogenic MDV is accepted. Mean vaccine virus in PBL never exceeded 10^4 VCN/10^6 PBL while values of 10^5-10^6 VCN/10^6 PBL have been reported for pathogenic MDV up to 35 dpi (day post infection) using the same methods (Islam et al., 2006). Furthermore MDV load in PBL continues to increase up to day 35 with virulent MDV (Islam et al., 2006), whereas with CVI988 vaccine, values peak early (days 7-14) then decline (present study; Baigent et al., 2005). Regarding virus shedding, vaccinal MDV values in dander in the present study were approximately 1-1.5 logs lower than reported values of 10^6-10^7 VCN/mg of dander for virulent wild type virus (Islam and Walkden-Brown 2007).

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USE OF *SERRATIA MARCESCENS* FOR FEED PROCESSING: BROILER PERFORMANCE AND PATHOGENICITY ASSAY

M.E. MAHATA[1], A. DHARMA [2], I. RYANTO[1] and Y. RIZAL[1]

Summary

Serratia marcescens is a bacterium able to produce chitinase for degrading chitin. Broiler chickens cannot digest significant amounts of chitin because they produce very little chitinase in their digestive tract. Therefore, broiler feed containing chitin must be processed first with chitinase. *Serratia marcescens* is an opportunistic pathogenic bacterium, and a pathogenicity test is required before using its chitinase for processing of feed containing chitin. An experiment was conducted with broilers. A split-plot, completely randomized design was used in this experiment. The *Serratia marcescens* dosages (0, 40, 80, 160 and 320 mg/ kg body weight) were the main plot factor, and observation length (2, 4, 8, 16 and 22 days) was the sub plot factor. The rations for different treatments had the same protein (23%) and energy (3000 kcal ME/kg) content. Feed consumption, average daily gain, body temperature and mortality were measured. Results showed that feed consumption and average daily gain were significantly affected ($p<0.05$) by the presence of *Serratia marcescens*. The highest feed consumption (123.10 g/broiler) and average daily gain (76.75 g/broiler/day) were found for the highest bacterium dosage of 320 mg/kg body weight over 22 days of observation. Body temperature ranged from 39.82 $^{\circ}$C to 40.08 $^{\circ}$C, and there was no affect of *Serratia marcescens* on mortality. In conclusion, the *Serratia marcescens* had no negative effect on broiler performance.

I. INTRODUCTION

Serratia marcescens is a gram negative bacterium which produces chitinase for degrading chitin (Carter and John, 1992). Chitinase from *Serratia marcescens* has been tested as a biocontrol agent of microbial pathogens of food crops (Kobayashi, 1995). *Serratia marcescens* belongs to the *Enterobacteriaceae* family, *Serratia* genus, *Serratia marcescens* species (Buchanan and Gibbsons, 1974). *Serratia marcescens* is found naturally in the intestines of humans, animals, and in various environments (Volk & Wheeler, 1993; Guentzel, 2004). *Serratia marcescens* is usually not harmful to human health, but can be pathogenic under circumstances of low immunity . Shrimp waste is a by product from frozen shrimp processing and contains high levels of protein, chitin and calcium carbonate. This waste could be used as an alternative animal protein source for broilers by treating the chitin with chitinase. *Serratia marcescens* could be used as a source of chitinase for hydrolyzing chitin in shrimp waste but it is necessary to know whether this bacterium is an opportunistic pathogen in broiler chickens and, so far there has been no research conducted to determine this.

[1] Feed and Nutrition Departement, Faculty of Animal Science, University of Andalas, Indonesia
[2] Chemistry Departement, Faculty of Life Science, University of Andalas, Indonesia

II. MATERIALS AND METHOD

a) Materials

Bacterium *Serratia marcescens*, one week old unsexed Arbor Acre broilers CP 707- 25 birds per treatment, nutrient agar (NA) media for growing bacteria, and the commercial ration (code 511) produced by PT Charoen Pokphand, Indonesia. The ration contained 23% crude protein 3000 kcal metabolizable energy/kg.

b) Methods

The experiment was designed by using a split plot completely randomized design with two factors. The main plot factor was dosage of the bacterium *Serratia marcescens* (0,40,80,160, and 320 mg/kg body weight) and the subplot factor was observation length (2, 4, 8, 16, and 22 days after force feeding of bacterium) with five replicates. The bacterium *Serratia marcescens* was grown on nutrient agar (NA) and incubated at 30°C for 3 days. The bacterium was force - fed to broilers at 1% of body weight (Thomson,1985) using a funnel inserted into the crop. Feed consumption, average weight gain, body temperature, and mortality were measured. Feed consumption and the average weight gain were measured at 2, 4, 8, 16, and 22 days after bacterium force feeding. Cloacal temperature was measured daily at 08.00 for 22 days. Mortality was monitored daily. Data were analysed using *Duncan's Multiple Range Test*.

III. RESULTS AND DISCUSSION

a) Feed consumption

Based on statistical analysis, there was a significant interaction ($P < 0.05$) between *Serratia marcescens* bacterium dosage and observation length in affecting feed consumption (Graph 1).

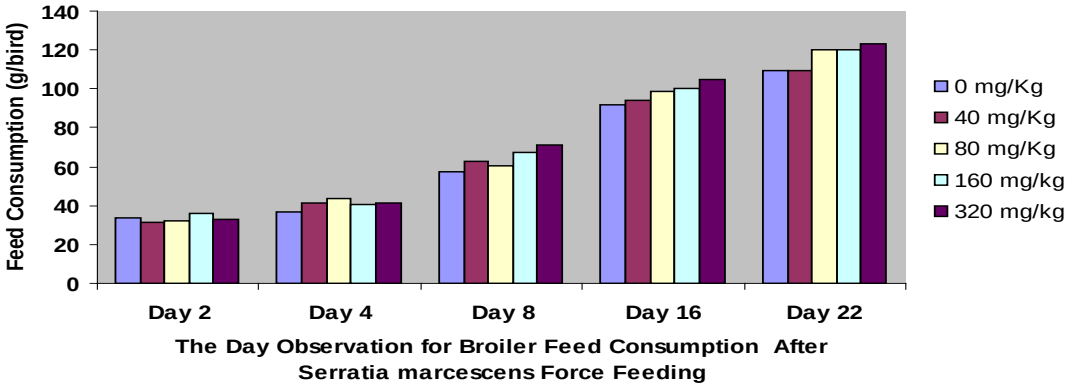

Figure 1 The effect of interaction between *Serratia marcescens* dosage, duration and average broiler feed consumption (g/bird)

For feed consumption, there was a significant interaction of observation on day 8 and day 16 with bacterium dosage of 160 and 320 mg/kg body weight affected feed consumption ($P<0.01$), and on day 22 with bacterium dosage at 80,160, and 320 mg/body weight ($P<0.01$). The highest feed consumption occurred on bacterium dosage 80, 160, and 320 mg/kg body weight at day 22 with total consumption respectively: 119.75 g/bird, 120.46 g/bird, and 123.10 g/bird (at that time broilers were 5 weeks old), respectively. Broiler feed intake obtained in this study is slightly higher than broiler feed intake at 5 weeks old reported by NRC (1994) of 105.7

g/bird/day for male and 92.14 g/bird/day for female birds , and by Scott *et al*. (1982) for male broilers 93 g/bird/day and females 69 g/bird/day. It is possible that *Serratia marcescens* produced enzymes that accelerated the process of digestion of food components into simple compounds in the broilers' digestive tract, so that the nutrients in the diet were absorbed and metabolized more rapidly. These conditions accelerate the emptying of the gastrointestinal tract, so that appetite and feed consumption were increased.

b) Average weight gain

There was a significant interaction between bacterium dosage and observed days on average weight gain ($P<0.05$) (Figure 2). There was no interaction between bacterium dosage (0 to 320 mg/kg body weight) and time for days 2 and 4 after bacterium force feeding, but on day 8, 160 and 320 mg/kg body weigh, bacterium dosage increased the average weight gain ($P<0.01$). The average weight gain was also affected ($P<0.01$) by interaction of bacteria dosage (80,160, and 320 mg/kg body weight) at days 16 and 22.

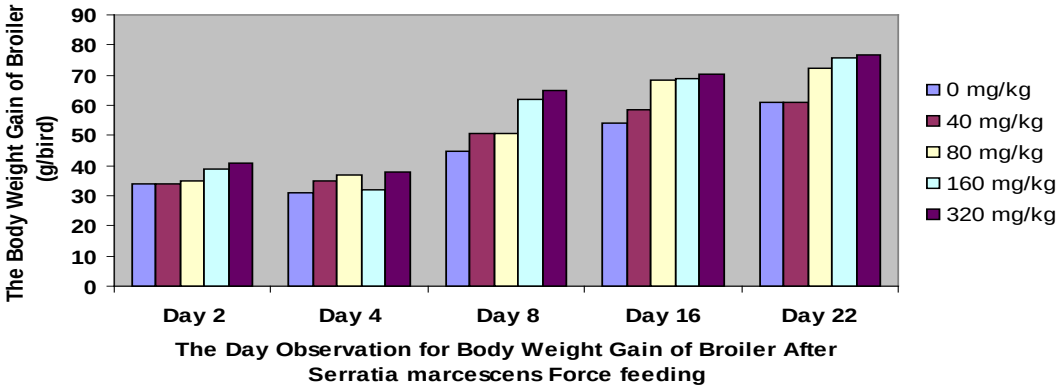

Figure 2 The effect of the interaction between *Serratia marcescens* dosage, time and observation length on average of body weight gain of broilers (g/bird)

The highest body weight gain was 76.75 g/bird, which occurred at a bacterium dosage of 320 mg/kg body weight at day 22 after bacterium forced feeding. Scott *et al*. (1982) report body weight of male broiler chickens at 5 weeks old at 1.445 kg and 0.890 kg for females, and NRC (1994) reported body weight of male broiler chickens aged 5 weeks as 1.250 kg and 1.110 kg for females. Broiler weights found in this study were slightly higher than body weights of male broiler chickens based on NRC standards (1994) and approached the body weight of male broiler chickens reported by Scott *et al*. (1982).

c) Body Temperature

The average body temperatures of broiler chickens on days 2, 4, 8, 16, and 22 after bacterium forced feeding were bewteen 39.82 and 40.08°C as shown in Figure 3. According to Sturkie (1965), normal chicken body temperature ranges from 37.5 to 40°C, and Parkhurst and Mountney (1988) reported 41 to 43°C. In the present study, body temperatures were within the range reported for normal chicken body temperature. The dosage of *Serratia marcescens* up to 320 mg/kg of weight with observation length until day 22 after bacterium force feeding had no negative effect on broiler body temperature. This phenomenon indicates that *the Serratia marcescens* is not an opportunistic pathogenic bacterium in broilers.

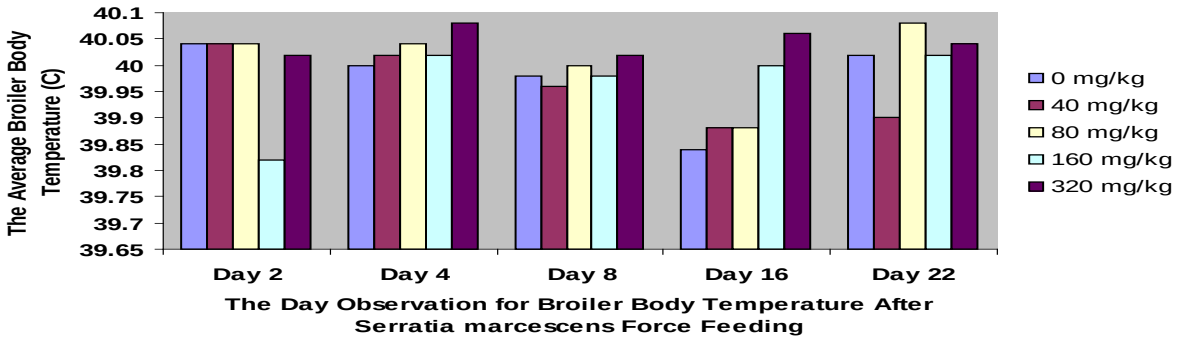

Figure 3 The effect of interaction between *Serratia marcescens* dosage with observation length in average of body temperature of broiler (°C)

d) Mortality

There was no mortality of broiler chickens caused by interaction of the bacterium dosage with observation length (2, 4, 8, 16, and 22 after bacterium forced feeding). This fact indicates that the bacterium *Serratia marcescens* is safe for broilers.

V. CONCLUSIONS

Serratia marcescens is not an opportunistic pathogen in broiler chickens. This bacterium increased feed consumption and weight gain, but did not affect body temperature or mortality.

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SALMONELLA VACCINATION IN LAYERS

P.J. GROVES[1], S.M. SHARPE[2] and W.I. MUIR[1]

Live attenuated vaccines and inactivated bacterins against *Salmonella* serovars are available in Australia. These have had some use in meat breeder chickens but have yet to be considered by the commercial layer industry. Australian commercial flocks are free of *S.* Enteriditis, so the focus here is mainly against *S.* Typhimurium and other less common serovars. A project is underway to evaluate the effectiveness of a live and an inactivated vaccine in preventing or decreasing *Salmonella* intestinal colonisation in layer breeds. A selection of results is presented here.

Seven hundred and seventy-four brown egg layer breed day-old chicks were obtained from a commercial hatchery and placed into 9 floor pens in a trial facility. Vaccination programs were administered to all birds in one pen each as shown in Figure 1. Live vaccine (Bioproperties Vaxsafe ST- designated V) was given by oral gavage in all groups except for the group given this vaccine by subcutaneous injection (s/c) at 4 and 8 weeks. Inactivated vaccine (Intervet trivalent *Salmonella* vaccine - designated N) was given by intramuscular injection. All vaccines were given at one label dose per bird (for the live vaccine at this time, this was 10^8 organisms per bird). At 18 weeks of age, the birds were moved from the floor pens into individual cages at the University of Sydney.

Ten birds per group were bled at 4, 8, 12, 14, 23, 31, 39 and 50 weeks of age and their sera tested for *S.* Typhimurium antibody using the x-Ovo (Guildhay) ELISA (the cut off point for a positive result with this ELISA is >784 units). At 4, 10, 17, 20 24, 32 and 50 weeks, birds from each group were removed to floor pens and challenged with an oral dose of 10^8 cfu of a field strain of *S.* Typhimurium per bird. Twenty-one days later, these birds were euthanized and their caecae cultured for presence of Salmonellae.

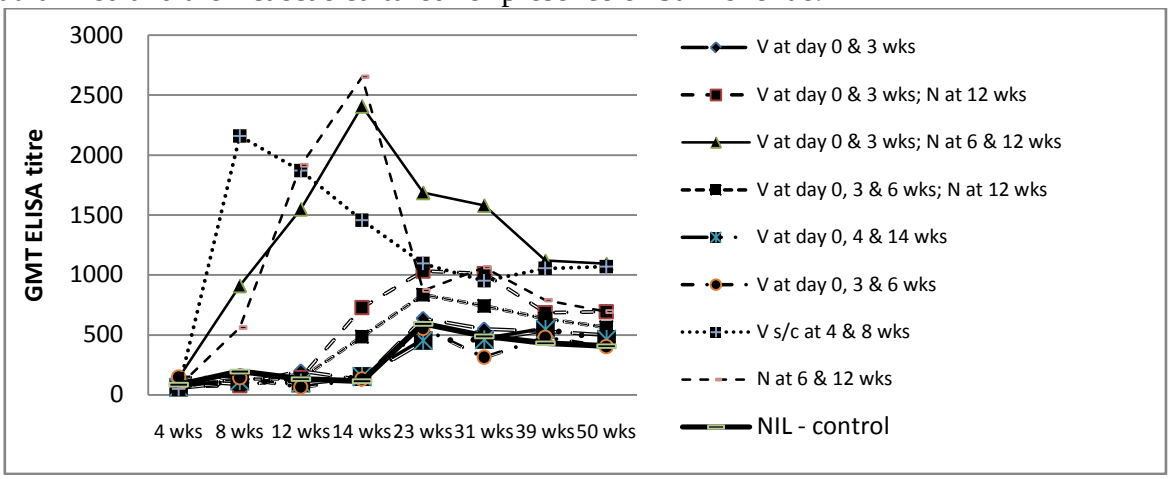

Figure 1 *S.* Typhimurium antibody titres by vaccination group

Seroconversion was only achieved following inactivated vaccine or live vaccine given subcutaneously. Only the dual live vaccine given by injection or two doses of inactivated vaccine following oral live dosing gave serological results lasting to 50 weeks of age.
Challenge and recovery of *S.* Typhimurium proved difficult to achieve, even in unvaccinated birds. Higher serum antibody levels were associated with higher protective index against challenge. The live vaccine given orally only gave short term protection.

[1] Faculty of Veterinary Science, the University of Sydney, Camden NSW
[2] Birling Avian Laboratories, Bringelly NSW

AUTHOR INDEX

Name	Page(s)	Email Address
Abdollahi, M.R	61, 209	
Ahammad, M.U.	69	muslah_ahammad@yahoo.com
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Choct, M.	73	mchoct@poultrycrc.com.au
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Cowieson, A.J.	22, 84	aaron.cowieson@sydney.edu.au
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Cronje, P.B	161	pierre.cronje@bigpond.com
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Eldaghayes, I.M.	213	ibrahim.eldaghayes@vetmed.edu.ly
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Gady, C.	65	Cecile.gady@adisseo.com
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Geier, M.S.	9, 18, 74	geier.mark@sa.gov.au
Geraert, P.	78	Pierre-Andre.geraert@adisseo.com
Gan, C.Y	82, 83, 139	
Gidley, M.J.	139	m.gidley@uq.edu.au
Groves, P.J.	214, 238, 247	peter.groves@sydney.edu.au
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Hemsworth, P.H.	126, 130, 131	phh@unimelb.edu.au
Huang, H.K.	26	khuang@aviagen.com
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Hughes, R.J.	9, 18,74, 226	Bob.Hughes@sa.gov.au
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Iji, P.A.	60, 73, 122, 226	piji@une.edu.au
Islam, T.	239	tislam3@une.edu.au
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Laine, S.M.	130	s.laine@pgrad.unimelb.edu.au
Leary, A.	56	alison.leary@alltech.com
Liu, N.	84	002jinquan@163.com
Liu, Y.G.	65, 78	Kevin.liu@adisseo.com
Li. X.	17, 82, 83, 139	x.li1@uq.eu.au
Mahata, M.	243	mariamahata2002@yahoo.com
Maquire, A.	17	
Mikkelsen, L.L.	226	lmikkels@une.edu.au
Moore, R.J.	9	rob.moore@csiro.au
Moran, E.T.	1	moranet@auburn.edu
Muir, W.I.	214, 247	wendy.muir@sydney.edu.au
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Nolan, J.V.	60	jnolan@une.edu.au
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Parkinson, G.B.	104	livornoconsulting@pacific.net.au
Penz Jr., A.M.	49	mpenz@provimila.com
Peron, A.	92	Alexander.peron@danisco.com
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Ravindran, G.	61, 209	
Ravindran, V.	61, 88, 209	V.Ravindran@massey.ac.nz
Renz, K.G	239	krenz2@une.edu.au
Rizal, Y.	243	
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Roberts, J.R.	234	jrobert2@une.edu.au
Romero, L.F	88	Luis.romero@danisco.com
Ryanto, I.	243	
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Selle, P.H.	147	Peter.selle@sydney.edu.au
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Sharpe, S.M.	238, 247	sue_sharpe@baiada.com.au
Sopade, P.A.	139	
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Stanhope, W.	104	
Stevens, B.H.	131	stevensb@unimelb.edu.au
Storey, T.H.	135	
Sultan. A.	17, 82, 83, 139	asad.sultan@uqconnect.edu.au
Sutherland, M.	234	
Tatemoto, H.	69	
Thomas, D.V.	61, 209	D.V.Thomas@massey.ac.nz
Tilbrook, A.J.	126, 131	Alan.tilbrook@med.monash.edu.au
Torok, V.A.	226	torok.valeria@sa.gov.au
Tredrea, A.M	74	
Valle, F.L.P	192	
Walkden-Brown, S.W.	239	swalkden@pobox.une.edu.au
Webster, T.J.	61, 209	
Widodo, A.	60	awidodo3@une.edu.au
Widowski, T.	126	twidowski@unoguelph.ca

Wu, S.B.	226	shubiao.wu@une.edu.au
Zaefarian, F.	88,	F.Zaefarian@massey.ac.nz
Zhang, D.	82, 83, 139	
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