EVALUATION OF TECHNIQUES FOR IMPROVING PHOSPHORUS UTILISATION IN MEAT POULTRY

COLIN OLAWOLE SANNI

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ABSTRACT

Phosphorus plays an important role in bone development and mineralisation, but approximately two-thirds of the phosphorus contained within the plant-based ingredients of poultry feed occurs in the form of phytate which is poorly available to poultry. Consequently, the low availability of phytate phosphorus necessitates the inclusion of supplementary dietary phytase enzyme or inorganic phosphorus (a finite global resource). However, inorganic phosphorus is often added at levels that exceed requirement to ensure dietary sufficiency, as there are concerns that the current phosphorus requirement guidelines (NRC, 1994) may not accurately reflect the current physiological needs of the modern broiler strain. To this end, the accurate evaluation of the effect of dietary phosphorus supply on the skeleton of modern broilers is required. The overarching aim of this project was twofold: to characterise the bone and whole body phosphorus content of commercial broilers, and to examine possible approaches for improving phosphorus utilisation in broilers.

Before accurate evaluation of broiler phosphorus requirements or response to interventions could be assessed, it was necessary to establish the optimum method for determining bone ash content. Four investigations, conducted using two bird trials were initially carried out to examine the effect of four common divergences in the bone ash methodology: the effect of fat extraction, the inclusion of cartilage caps, the minimum fat extraction time required and the effect of autoclaving prior to fat extraction. Sensitivity in elucidating differences in treatment means improved for tibia ash % when fat was extracted, and when cartilage caps were removed from the bones prior to ash determination. However, increase in ash % have been reported when cartilage caps are affected by disease, and it may be advantageous to include the cartilage caps for better comparison of data, particularly when the health status of a bird flock is unknown. A minimum fat extraction time of 6h using the Soxhlet procedure

was adequate in extracting fat from bones while autoclaving the bones prior to fat extraction did not have any significant effect on bone ash or ash %.

There is a current lack of bone ash and phosphorus content reference dataset for commercial broilers. A normal range of age and sex-related normal bone morphology and mineralisation values of healthy commercial broilers were sequentially sampled across 6 commercial farms from 24 flocks of birds and data presented. The whole body phosphorus content determined showed that, despite the improvements in growth rate and muscle mass observed in modern broiler strains, when broilers are fed nutritionally adequate diets whole body phosphorus content retained (5g/kg) has remained constant. The partitioning of calcium in relation to phosphorus in the whole body of broilers has also remained the same.

A 4-day transient reduction in available dietary phosphorus during early life not only confirmed previous findings that nutritional imprinting improves phosphorus utilisation in later life, but also leads to improved bone strength. The nutritionally imprinted group of birds had significantly stronger bones ($P < 0.012$) than the control group of birds.

Finally, the potential use of a high phytase wheat (HIGHPHY) variety with high purple acid phosphatases content was examined in this project by feeding broilers with standard wheat diets, which were replaced with graded levels of HIGHPHY (33%, 67% and 100%). A 100% substitution of standard wheat with HIGHPHY resulted in the best ileal digestibility coefficients for P and Ca (34.6% and 22.9 % greater than control respectively). This project demonstrates that HIGHPHY has a promising potential for improving phosphorus digestibility in animal feed, and provides fundamental initial data into the use of acid phosphatase phytase in broilers through plant breeding to improve the phytase activity of grains.

To conclude, a minimum Soxhlet fat extraction time of 6h with cartilage caps included is proposed to be adopted by researchers in order to improve accuracy when comparing bone ash data from unrelated studies. The dataset of bone measurements reported in this thesis provides a significant contribution to the knowledge gap of the current range of bone data applicable to commercial broiler production. Nutritional imprinting for better phosphorus utilisation and the use of high phytase wheat varieties are useful techniques that can be employed to improve phosphorus utilisation in broilers.

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GLOSSARY

Ca - Calcium

HIGHPHY - High phytase wheat

ICP-OES - Inductively coupled plasma optical emission spectrometry

MGPA - Mature grain phytase activity

NaPi-IIb - Sodium-phosphate cotransporter type IIb

nPP – Non-phytate phosphorus

P – Phosphorus

PAPhy - Purple acid phosphatase

WBCa – Whole body calcium

WBP – Whole body phosphorus

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

The world population currently estimated at 7.3 billion is predicted to increase by a third to 9.7 billion in 2050 (UN, 2015). Based on the projected growth in income and changes in food consumption pattern, this will require increasing the overall food production by 70% and this includes a projected increase in the annual meat production from 200 million tonnes to 470 million tonnes (FAO, 2009).

Poultry production plays an important part in the food chain. It accounted for 36.2% of the total 320.7 million tonnes of meat produced in 2016 (Poultry Trends, 2016). Broiler production is the fastest growing (Cherian, 2013) and most successful animal industry (Leeson, 2008) and accounts for about 85% of the total poultry meat produced worldwide (Huyghebaert *et al.,* 2009). According to the poultry production farm survey report of Hoyle *et al.* (2016), 125 million birds accounted for the total English poultry flock in 2015, and of these, 83 million were broilers. Broilers accounted for 84% of the 1.67 million tonnes of poultry meat produced.

The increase in demand for poultry meat has stimulated the growth of the industry towards industrial farming, characterised by concentrated poultry operations (FAO, 2007). However, the generation of large quantities of poultry manure, often in quantities which exceeds the producing farm's capacity to use it as a nutrient source for crops (Foy *et al.,* 2014), is a direct consequence of increased poultry production (Vadas *et al.,* 2004). Despite the benefits of manure as a soil amendment, and as a potential source of animal feed and fuel highlighted in the review of Bolan *et al.* (2010), the responsible land application of the poultry litter has been of concern (Miles *et al.,* 2003). Its disposal is one of the challenges faced by the poultry industry (Leeson, 2008).

Phosphorus naturally contained within the plant-based ingredients of poultry feed occurs in the form of phytate. This molecule renders the phosphorus contained in phytate poorly available to poultry (Simons *et al.,* 1990) as chickens lack sufficient endogenous phytase (myoinositol 1, 2, 3, 4, 5, 6,-hexakisphosphate phosphohydrolase), the enzyme required to break the covalent molecular bonds and release phosphorus for absorption from phytate (Sebastian *et al.,* 1998; Waldroup *et al.,* 2000; Applegate *et al.,* 2003). Hence, poultry diets often contain highly available mineral forms of phosphorus in addition to the phytate phosphorus. Typically, less than one- third of plant-derived feed phosphorus is utilised by poultry, and the remainder excreted and applied on land as manure (Patterson *et al.,* 2005).

Poultry manure is a potential source of water contamination (Chapman, 1996). Poultry contributes around 16% of the total phosphorus in livestock manure in the EU (IFP, 2009), and there are environmental concerns that excreted phosphorus accumulates in the soil, leaches to water bodies thereby causing wide spread eutrophication (Schindler, 1977; Carpenter *et al.,* 1998; Bennett *et al.,* 2001). Improving phosphorus utilisation in poultry through its efficient use will not only help minimise wastage and reduce the negative effect on the environment but will also help in preserving the world phosphorus reserves.

This literature review considers the role of phosphorus in poultry nutrition, and the importance of preserving the dwindling global phosphate reserves through improved utilisation and management practices.

1.2. Phosphorus

1.2.1. Phosphorus: an essential nutrient

Phosphorus is an essential element required by all living organisms. It plays a crucial role in maintaining cellular osmotic pressure and acid balance, energy metabolism and transfer through the activity of adenosine triphosphate (ATP) and creatine phosphate, and a variety of other major physiological functions including the transfer of genetic information and protein regulation via deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

In plants, it is required for seed germination and growth (Tirado and Allsop, 2012), and it is routinely added in cropping systems where soils are naturally low in phosphorus to maximise crop yield. Although phosphorus is naturally found in soils, it is often found in a form unavailable for immediate uptake by plants, necessitating the need for land application of phosphate fertilisers derived from phosphate rocks to support high yields in non-organic agriculture.

In animals, phosphorus is the second most abundant element in the body next to calcium, and plays a major role in bone development and mineralisation (Veum, 2010). It is mainly stored in the skeleton where it combines with calcium to form hydroxyapatite [$(Ca_{10}(PO4)_6(OH)_2)$]. The skeleton of growing birds contains about 80% of the total phosphorus found in the body (De Groote and Huyghebaret, 1997), and the rest is present in body fluids and soft tissues (Underwood and Suttle, 1999). Phosphorus is also important for improved feed utilisation and feed conversion efficiency. Significant health and welfare issues such as poor growth, poor bone mineralisation and leg problems such as lameness and tibia dyschondroplasia have been reported when phosphorus deficient diets are fed to broilers. Despite the relatively high cost (Singh, 2008), supplementation with inorganic phosphorus is considered necessary to meet the requirements of growing poultry (Beck *et al.,* 2014).

1.2.2. Phosphorus in agriculture: current reserves, global supply and demand

Historically, plant production was supported by the addition of organic matter such as manure and human excreta (Matsui, 1977). By the middle of the 19th century, guano (bird droppings) deposited from previous millennia discovered on the islands off the Peruvian coasts and the South Pacific were mined to replace the use of organic matter. This was in response to an increase in the demand for food due to rapid population growth (Cordell *et al.,* 2009). However, the decline in the world trade of guano led to the growth of mined mineral fertilisers from phosphate rocks, which was then viewed as an unlimited source of phosphorus.

According to the United States Geological Survey, the current estimates of the world reserves of phosphate rock is 68 billion tonnes (USGS, 2017). This refers to the resources of rock phosphates, which are easily accessible and can be economically mined. In 2016, China, Morocco and Western Sahara, and the United States accounted for over two-thirds of the total 261 million tonnes mined globally (53%, 11.5%, and 10.7 % respectively). 80% of the global rock phosphate mined is used for the manufacture of agricultural fertiliser, 15% for industrial applications including detergents and metal treatment, and 5% for animal feed (Smit *et al.,* 2009).

Between 2002 and 2009, India and China showed a strong increase in phosphate fertiliser usage (80% and 28% respectively), while a reduction in usage was observed in Europe and America (29% and 10% respectively) due to market price increases and environmental restrictions (Tirado and Allsopp, 2012). Globally, an increase in the demand for phosphate fertilisers is expected, considering growth forecasts in population, increased preference for diets rich with meat, and demand for alternative energy sources such as ethanol which has stimulated growth for bioenergy crops (Tirado and Allsopp, 2012; Smit *et al*., 2009). In 2014, India, America, and China consumed about 73% of the global phosphate fertiliser (FAOSTAT, 2017). The world consumption of phosphate (P_2O_5) contained in fertilisers and

industrial uses was projected to increase from 44.5 million tonnes in 2015 to 48.9 million tonnes in 2020 (USGS, 2017).

1.2.3. Phosphorus: a finite resource

The increasing scarcity of mineral forms of phosphorus is of concern to global food security (Tirado and Allsopp, 2012). It has been reported that phosphorus obtained from phosphate rock is being mined faster than it is being discovered, and is therefore subject to eventual depletion (Vaccari and Strigul, 2011). The report of Cordell *et al.* (2009) which predicted a global peak in phosphorus production by 2030, published in the wake of a 700% increase in the price of phosphate rock between 2007 - 2008 led to increased work in estimating how much phosphorus is left (Elser and Bennett, 2011). The price increase was attributed to China's decision to impose a 135% export tax on its rock phosphates, and an anticipated increase in global demand with a concomitant decrease in supply (Ulrich *et al.*, 2009). This was further exacerbated by increases in the price of oil, which increased the costs of the energy required for mining phosphorus (Tomlinson, 2010).

Van Kauwenbergh (2010) in a report issued by the International Fertilizer Development Centre reassessed the world phosphorus reserves by incorporating previously overlooked geological reports and refuted the claim that phosphorus would peak by 2030. That report estimated the global phosphate rock concentrate reserves would be available for 300 – 400 years based on an estimate of 60 million tonnes of global phosphate reserves, and 290 – 460 billion tonnes of global phosphate resources. This consequently led the US Geological Survey to review its global phosphate reserve from 15 million tonnes to 65 million tonnes (Elser and Bennett, 2011). Cordell *et al.* (2011) reanalysed peak phosphorus using Bayesian statistical methods and predicted it would occur between 2051 and 2092. Despite the wide range (100 - 300 years) of the current estimate of the remaining phosphate reserve, it is accepted that peak

phosphorus is imminent and that phosphate rocks are becoming scarcer and more expensive to mine (Tirado and Allsopp, 2012; Childers *et al.,* 2011).

1.2.4. Environmental effect of phosphorus contained in poultry manure

Eutrophication of water bodies has been directly linked to over-enrichment with phosphorus from surface water derived from the over application of poultry manure (Bougouin *et al.*, 2014). The water-soluble fraction of manure (Maguire *et al.,* 2005), which is the form most available to aquatic life (Moore *et al.,* 1999) constitutes the greatest eutrophication threat to waterways. In order to understand this better, an understanding of the phosphorus cycle, which involves complex chemical and microbiological processes is required.

Phosphorus is deposited in soils through the weathering of minerals and additions from applied fertilisers and organic residues. It reacts with iron and aluminium to form insoluble iron and aluminium phosphates in acidic soils; or with calcium to form insoluble calcium phosphates in alkaline soils. Phosphorus is then released in soils as the minerals dissociates and as soil organic matter decomposes. Phosphorus is subsequently removed from soils through plant uptake, water runoff and erosion, and leaching. The phosphorus absorbed by plants is transformed into organic compounds. When animals consume these plants, it is either incorporated into their tissues or excreted. In a series of processes which involves the use of intrinsic enzyme complexes, microbes act on excreta, dead plants, and animals by decomposing them and releasing phosphorus back into the soil in the soluble or particulate form.

Poultry litter contains about 3% P_2O_5 (Spiehs, 2005) but the actual concentration may vary with age of birds, diet, management, and type of manure storage. It has a beneficial use as plant fertiliser, particularly when applied to phosphorus deficient soils. However, the continual long-term application of poultry manure results in the accumulation of soil phosphorus content (Sharpley, 1999; Sims *et al.,* 2000) which increases the risk of phosphorus runoff into

water bodies. Phosphorus runoffs have been linked to harmful incidences of algal blooms and production of trihalomethane in drinking waters (Sharpley and Moyer, 2000).

1.3. Phosphorus requirement in poultry

Phosphorus derived from plant materials is mainly obtained from phytic acid (myoinositol 1,2,3,4,5,6, -hexakis (dihydrogen phosphate) or $InsP_6$ (Sandberg, 2002; Zeller *et al.*, 2015a). It is also referred to as phytate when it is mineral-bound within a seed. The dissociation of protons from the phytic acid molecule during intestinal transit leaves it with several negative charges. This renders it highly susceptible to chelation with multivalent cations such as calcium, iron, zinc, and magnesium (Morris, 1986), and proteins depending on the prevailing pH and concentration (Adeola and Sand, 2003). The resulting mineral-phytate complexes formed have been associated with reduced mineral bioavailability and absorption (Maenz *et al.,* 1999; Selle *et al.,* 2000). Phytate has also been shown to reduce amino acid digestibility (Cowieson *et al.,* 2006; Onyango *et al.,* 2009).

The current non-phytate phosphorus (nPP) recommendations for broilers stipulated by the National Research Council (NRC, 1994) are 0.45% (0-3 weeks), 0.35% (3-6 weeks) and 0.30% (6-8 weeks). These are based on peer-reviewed research published between 1952 and 1983. However, it has been reported that the current fast growing strains of broilers are more efficient in utilising nutrients due to genetic selection (Havenstein *et al.,* 2003a,b) and this continues to influence the use of nutrients. Consequently, these levels of dietary supply have been questioned due to major concerns over the cost of inorganic forms of phosphorus and the potential for environmental pollution when phosphorus is oversupplied to poultry (Rama Rao *et al.,* 1999).

The urgent need to review the current phosphorus requirements in broilers has been highlighted (Adedokun and Adeola, 2013; Applegate and Angel, 2014) because of recent changes in the physiology of current bird strains used, and earlier concerns over whether the

NRC phosphorus recommendations (NRC, 1994) represent optimum levels. Nonetheless, supplemental inorganic phosphorus is still often added in excessive amounts to poultry diets to provide a safety margin (Waldroup *et al*., 2000), as the consequences of phosphorus deficiency in broilers are profound (Leske and Coon, 2002; Dhandu and Angel, 2003).

1.3.1. Definition of terms

There is a lack of consistency in the terms used in defining phosphorus availability in poultry (Rama Rao *et al.,* 1999). Various terms have been used to describe phosphorus requirement and availability in poultry, posing a potential source of confusion (Applegate and Angel, 2008; Mutucumarana *et al.,* 2014). The definition of phosphorus availability and response criteria used in evaluating phosphorus availability varies across evaluation systems and research groups (Shastak and Rodehutscord, 2013). For example, Coon *et al.* (2002) observed that non-phytate phosphorus and available phosphorus are erroneously used to mean the same thing. This is partly related to the historical assumption that chicks are unable to utilise phytate-bound phosphorus, and that all other animal and inorganic phosphorus sources, including remaining plant phosphorus, are available (Waldroup, 1999). However, the report of Coon *et al.* (2002), and more recently Morgan *et al.* (2015) shows that non-phytate phosphorus is not 100% available, and that broilers are probably able to utilise some phytate-bound phosphorus.

Total phosphorus refers to the total amount of phosphorus present in a material, whether or not it is bound to other compounds or available for absorption by the animal. Phytate phosphorus describes the portion of phosphorus that is bound to the phytate mineral complex and is largely unavailable due to lack of effective endogenous phytase in poultry. It accounts for 60% and 72% of the total phosphorus found in soybean meal and corn respectively (Ravindran *et al.,* 1995).

Non-phytate phosphorus (nPP) is that portion of phosphorus that is not bound to phytate molecules within a feed material.

Available phosphorus (avP) was originally defined as the portion of phosphorus in a diet or ingredient that can be absorbed from the intestine (distal ileum) when ingested (Weremko *et al.*, 1997). Confusingly, more recently Rodehutscord (2009) defined available phosphorus as the portion of total dietary phosphorus at marginal levels of phosphorus supply, which can be utilised to cover the phosphorus requirements of an animal.

Retainable phosphorus refers to the phosphorus that is retained by the body after correcting for what is lost in excreta and urine, i.e. feed phosphorus minus excreta and urinary phosphorus at a particular phosphorus intake and stage of bird development (Leske and Coon, 2002).

1.3.2. Phytases and phosphorus utilisation

Phytases (myoinositol hexakisphosphate phosphohydrolases) are enzymes that catalyse the hydrolytic cleavage of phosphate groups from phytic acid via several phosphorylated intermediary products (myoinositol pentakis-, tetrakis-, tris-, bis- and monophosphate) up to myoinositol (Zeller *et al.*, 2015a). Phytases are produced from either microorganisms (e.g. fungi, yeast, and bacteria) or plants (e.g. wheat, rye and barley). They are identified by the initial site of removal of the phosphate group on the inositol ring during de-phosphorylation (Selle and Ravindran, 2007). 3- phytases, for example, are groups of phytases whose site of initial de-phosphorylation of the phosphate group occurs at the C_3 position of the inositol ring.

3 classes of phytases have been identified by the International Union of Pure and Applied Chemistry, and International Union of Biochemistry: 3-phytases (*EC* 3.1.3.8), 4-/6-phytases (*EC* 3.1.3.26) and 5-phytases (*EC* 3.1.3.72). Commercial phytases derived from microorganisms are commonly 3-phytases with the exception of some phytases such as the fungal *Basidiomycete* (Lassen *et al.,* 2001) and bacterial *Escherichia coli* (Greiner *et al.,* 1993) which

are classed as 6-phytases. Plant-derived phytases on the other hand are classed as 6-phytases (Schlemmer *et al.,* 2001).

Plant-derived phytases have traditionally been shown not to be as effective as microbial-derived phytases as they lack the key characteristics required for efficiently releasing phytate phosphorus in pig or poultry. These include stability under acidic conditions, an acidic pH optimum, and resistance to pepsin activity (Greiner and Koinetzny, 2006). This was demonstrated in the study of Rapp *et al.* (2001) who reported a 17% higher phytic acid hydrolysis in diets supplemented with microbial phytases (*Aspergillus niger*) compared with intrinsic plant (wheat) phytase.

Ravindran (2013) reported an average pH of 5.5 (for the crop) and 2.5 - 3.5 (for the gizzard and proventriculus) in broilers, and this corroborates the previous finding of Jiménez-Moreno *et al.* (2009) who reported a similar range of digesta pH. These organs have been reported to be the major activity sites for microbial phytase digestion, unlike the lower gastrointestinal tract where microbial phytases are more susceptible to proteolysis (Simon and Igbasan, 2002). Plant-derived phytases have been reported to have a narrower optimum pH range of 5 - 6 compared to a much wider optimum pH range of 2.5 – 6.5 for microbial derived phytases (Woyengo and Nyachoti, 2011); which is much closer to the physiological range of pH 2 -5 in the proventriculus and ventriculus in poultry (Simon and Igbasan, 2002). Microbial-derived phytases are thus able to work over a broad pH range with some being active and stable below pH of 3.5 (Greiner and Koinetzny, 2006).

It has been noted that the ideal phytase incorporated in poultry feed should be cost-effective, tolerant to high feed pelleting temperatures (65 - 80^{o}C), and resistant to low pH and protease enzymes in order to avoid degradation in the digestive tract (Woyengo and Nyachoti, 2011). However, these attributes are difficult to achieve with native microbial phytases (Lei and Stahal, 2001) and therefore techniques such as genetic transformation and thermo-protective

coating have been employed to enhance these qualities in microbial phytases (Garrett *et al.*, 2004).

1.4. Evaluating phosphorus requirements and availability

A list of the calcium and phosphorus content of commonly used plant feedstuff is presented in Table 1.1. There is a growing scientific interest in the evaluation of phosphorus availability, and the determination of optimal dietary inclusions in poultry diets. This is due to the effect on performance and welfare when inadequate levels are supplied, the high cost of inorganic phosphates, and the detrimental effect on the environment when phosphorus is oversupplied.

The mineral content of a feed ingredient or diet has little significance to an animal unless its biological availability is quantified (Peeler, 1972). Consequently, the variability of the phytate content of feed materials alongside intrinsic phytase activity (Eeckhout and de Paepe, 1994); and variability in non-phytate phosphorus concentration (Shastak and Rodehutscord, 2013) is known to affect the phosphorus availability of a feed material. Other known factors include the phosphorus source, dietary levels of other nutrients (e.g. calcium and vitamin D) and their relationship with phosphorus. The effects of the environment, age, sex, strain, and health status of the animal have also been identified as factors that can affect phosphorus availability of a feed material (Applegate and Angel, 2008).

Shastak and Rodehutscord (2013) recently published a comprehensive review of the historical development of estimating phosphorus availability, and outlined the following types of measurements groups:

I. Qualitative measurements
II. Quantitative measurements
III. *In vitro* measurements

Table 1.1. Calcium and phosphorus content of common plant feedstuffs (NRC, 1994)

Feedstuff	Calcium content (%)	Total phosphorus content (%)	Non-phytate phosphorus content (%)	Non-phytate phosphorus (% of total phosphorus)
Lucerne meal, 17% CP	1.44	0.22	0.22	100.0
Barley	0.03	0.36	0.17	47.2
Buckwheat	0.09	0.32	0.12	37.5
Canola meal, 38% CP	0.68	1.17	0.30	25.6
Maize gluten meal, 60% CP	-	0.50	0.14	28.0
Maize, grain	0.02	0.28	0.08	28.5
Cottonseed meal, 41% CP	0.15	0.97	0.22	22.6
Distillers dried grains	0.10	0.40	0.39	97.5
Distillers dried solubles	0.35	1.27	1.17	92.1
Oat, grain	0.06	0.27	0.05	18.5
Groundnut meal	0.20	0.63	0.13	20.6
Pearl millet	0.05	0.32	0.12	37.5
Rice bran	0.07	1.50	0.22	14.7
Rice polishings	0.05	1.31	0.14	10.7
Rye, grain	0.06	0.32	0.06	18.8
Safflower meal, 43% CP	0.35	1.29	0.39	30.2
Sesame meal, 43% CP	1.99	1.37	0.34	24.8
Soybean meal, 44% CP	0.29	0.65	0.27	41.5
Soybean meal, 48% CP	0.27	0.62	0.22	35.4
Soy protein concentrate	0.02	0.80	0.32	40.0
Sunflower meal, 45% CP	0.37	1.00	0.16	16.0
Wheat bran	0.14	1.15	0.20	17.4
Wheat middlings	0.12	0.85	0.30	35.3
Wheat, hard winter	0.05	0.37	0.13	32.0

1.4.1. Qualitative measurements

Qualitative measurements are based on methods that determine the biological value of a phosphorus source. These are usually determined by feeding chicks varying amounts of test phosphates in a phosphorus deficient bioassay diet for 2-3 weeks (Coon *et al.,* 2007); although a range of experimental period (10 - 28 days) have been reported by other investigators (Shastak and Rodehutscord, 2013). Usually, maize - soybean meal diets, which are known to have low phosphorus availability and intrinsic phytase activity, are used (Kornegay *et al.,* 1996). Dietary phosphorus concentration could be further reduced by using feed ingredients with low phosphorus content, e.g. potato protein (Rodehutscord and Dieckmann, 2005).

Qualitative measurements are conducted within a set of pre-qualifying conditions listed in the report of Nelson and Peeler (1961): a sensitive bird response criterion is measured using a suitable phosphate standard to compare the dose response of the test phosphate in animals fed a phosphorus deficient diet, and ensuring the added levels of phosphorus does not exceed the phosphorus requirement. A reference phosphate standard (e.g. from any of the phosphate sources I – III listed in Table 1.2) is assigned a biological value of 100, i.e. 100% available.

Based on the response to growth performance parameters or body measurements (e.g. FCR, body weight gain or bone ash), the test phosphate is assigned a biological value relative to the standard phosphate. Phosphorus availability is then calculated by difference or regression. Phosphorus bioavailability assays thus measures the chosen response criteria specific to a chosen phosphorus standard. It is a relative value and does not provide actual phosphorus availability. This was further buttressed in the review of Sullivan and Douglas (1990) who identified various factors affecting phosphorus bioavailability assays: selection of response criteria, a reference standard of phosphate selected, diet composition, calcium and phosphorus nutrient ratio, animal species type and length of the bioassay.

Table 1.2. Common sources of calcium and phosphorus (Waldroup, 1996)

Source		% Ca	% P
Limestone		38	-
Oyster shell		38	-
I Calcium phosphate	A. Natural or unprocessed	38	-
	Low fluorine rock phosphate	32 - 35	12 - 15
	Curacao phosphate (guano)	36	13 - 15
	Colloidal phosphate (soft phosphate)	18 - 20	9 - 10
	Bone meal, steamed	23 - 26	8 - 18
	B. Chemically processed		
	1. Dicalcium phosphates		
	Di/mono calcium phosphates	15 - 23	18 - 23
	Mono/dicalcium phosphates	15 - 18	20 - 21
	Precipitated dicalcium phosphates	24 - 26	18 - 22
	2. Defluorinated phosphates	30 - 36	14 - 18
II Sodium phosphates			
	Monosodium phosphate	-	25
	Disodium phosphate	-	21
	Sodium tripolyphosphate	-	25
III Ammonium phosphates	Monoammonium phosphate	-	24
	Diammonium phosphate	-	20
IV Phosphoric acid			23 - 24
Fish meals		2 - 14	2 - 7
Meat and bone meals		4 - 14	2 - 10
Poultry by-product meals		2 - 10	2 - 8

Although the slope-ratio assay is a common procedure for evaluating the bioavailability of phosphorus sources (Muir *et al.,* 1990), analysis of linear slopes may not be appropriate. Potchanakorn and Potter (1987) evaluated the biological values of various phosphorus sources in young turkeys, found exponential increases rather than linear responses and therefore adapted the nonlinear regression model to estimate phosphorus biological value. The use of non-linear regression models was proposed by Potter *et al.* (1995) in order to overcome the assumption of linearity when a wide range of phosphorus levels are tested.

1.4.1.1. Bone measurements

The bone plays a functional role in poultry by providing the framework needed to support muscle mass, and to protect vital organs (Korver *et al.,* 2004). It accounts for 80% the total phosphorus contained in the bones of growing birds, and thus its evaluation is considered an important criterion for estimating phosphorus availability (De Groote and Huyghebaert, 1997). In poultry, it has also been demonstrated that the calcium and phosphorus content in the bone increases in response to increases in dietary phosphorus content (Venäläinen *et al.,* 2006); and that tibia ash percentage is reduced when dietary phosphorus is reduced (Watson *et al*., 2006). Also, significant health and welfare issues have been reported when phosphorus deficient diets were fed to broilers demonstrated by poor bone mineralisation, considerable leg deformities and lameness, and a high incidence of broken bones after processing (Driver *et al.,* 2006a).

The various bone parameters that have been used for evaluating phosphorus availability include bone mineral content (Shang *et al.,* 2015); bone ash concentration (Cheng and Coon, 1990); bone densitometry (Shastak *et al.,* 2012a); bone breaking strength (Kim *et al.,* 2004; Shaw *et al.,* 2010); and bone ash (Atteh and Leeson, 1983; Hall *et al.,* 2003). According to the review of Shastak and Rodehutscord (2013), bone ash, bone phosphorus content, bone strength and bone mineral density are the most useful bone criteria used in poultry to assess phosphorus supply.

1.4.1.1.1. Bone ash

Bone ash content has been widely used to evaluate the skeletal status of poultry (Kim *et al*., 2004; Park *et al.,* 2003), and it is the preferred criterion for estimating phosphorus availability due to its simplicity (Sullivan and Douglas, 1990). The ash content of various poultry bones that have been evaluated include the femur (Dickey *et al.,* 2012; Hemme *et al.,* 2005), tibia (Onyango *et al.,* 2003, Coon *et al.,* 2007; Olukosi and Fru-Nji, 2014), feet (Garcia and Dale,

2006; Shastak *et al.,* 2012a) and toe (Yan *et al.,* 2005a; Karimi *et al.,* 2013). The use of the middle toe was proposed as an alternative assay (Baird and MacMillan; 1942) as it eliminates the time and labour required in traditional bone ash methods. It was used in some early phosphorus bioavailability studies, which also concluded that toe ash was as sensitive as tibia ash (Fritz *et al.,* 1969; Potter, 1988). However Shastak *et al.* (2012a) observed it is not always clear from published literature which particular toe or joint the toes were removed when evaluating phosphorus availability, leading to ambiguity in interpreting results. The authors proposed this limitation could be avoided by using the whole foot, which provides a larger sample volume with similar ease of processing compared to the toe. The whole foot ash assay has been investigated as an alternative to toe or tibia ash (Yan *et al.,* 2005a; Garcia and Dale, 2006) and has been shown to be equally reliable in assessing phosphorus availability. Nonetheless, the tibia ash assay is the most commonly used in evaluating bone mineralisation in poultry research (Hall *et al*., 2003). Despite their long and well-established use (Gillis *et al.,* 1954; Nelson and Walker, 1964), the use of bones in phosphorus availability assays has been criticised due to the lengthy and laborious preparation processes required prior to ash determination.

A review of the available literature (Table 1.3.) shows common divergences in processing methods employed in the tibia bone ash methodology. Variations in processing methods may affect results (Orban *et al.,* 1993) and may have significant consequences, especially when comparing bone ash methods from different studies.

Table 1.3. A review of methodologies used in determining bone ash content in broilers

Sex and strain	Orientation (left or right)	Storage temperature (°C)	Tissue removal	Drying preparation	Ash preparation	Sampling age (day)	Reference
Unsexed Ross broiler chicks	Right tibia and right foot	-20	Manually and enzymatically removed	30°C for 48h then 105°C for 24h	Ashed at 600°C for 24h	11 and 25	Shastak *et al.*, 2012a
Ross 708 male broiler chicks	Left tibia	-20	[1]NS	100°C for 24h	Defatted in petroleum ether. Ashed at 600°C overnight	21	Walk *et al.*, 2012
Ross 308 broiler chicks	Toes and left tibia	-20	Manually removed	100°C for 24h	Defatted in petroleum ether for 20h. Ashed at 600°C for 24h	21	Tang *et al.*, 2012
Cobb 500 breeder pullets	Tibia bones	-20	[1]NS	Cut length-wise and oven dried	Ashed at 600°C for 16h	315	Ekmay *et al.*, 2012
Lohmann pink-shell hens	Left tibia	[1]NS	Manually removed	Determined on a moisture-free, fat-free basis	Ashed at 600°C for 24h	17 and 20	Lei *et al.*, 2011
Unsexed Hubbard chicks	Toe and tibia ash	[1]NS	[1]NS	Dried to a constant weight at 100°C	Ashed in a muffle furnace at 600°C for 6h	42	Khan *et al.*, 2010
Cobb male broiler chicks	Left tibia	[1]NS	Manually removed, including cartilage caps	55°C for 72h	Ashed at 550°C for 3h	42	Barreiro *et al.*, 2009
Vencob female broiler chicks	Tibia bones	[1]NS	Manually removed	100°C for 3h	Bones were soaked in petroleum ether for 48h and ashed at 600°C for 2h	17 and 35	Rama Rao *et al.*, 2006

[1]Not stated.

1.4.1.1.2 Bone breaking strength

Rath *et al.* (2000) defined bone strength as the ability to endure mechanical stress. The degree to which a bone mineralises is known to affect its strength (Reichmann and Connor, 1977; Boivin and Meunier, 2002). Increase in bone mineralisation is associated with an increase in bone strength and conversely decrease in bone mineralisation is associated with a decrease in bone strength (Shim *et al.,* 2012a).

Breaking strength is the load at break, and it is defined as the total of all forces and moments applied to a bone (Nigg and Grimstone, 1994). It is related to the ultimate load or stress at which bone will break. Rowland *et al.* (1967) examined the relationship between bone breaking strength and dietary calcium and phosphorus content and found a 0.98 correlation coefficient between average tibia ash and average bone breaking strength, leading the authors to conclude that bone breaking strength was as good as tibia ash in indicating phosphorus availability. Bone breaking strength has since been used by various researchers as an indicator of phosphorus availability in poultry with good reliability (Ruff and Hughes, 1985; Sohail and Roland 1999; Coon *et al.,* 2007; Rousseau *et al.,* 2012). This however is in contrast to the findings of other investigators (Onyango *et al.,* 2003; Ravindran *et al.*, 1995; Shastak *et al.,* 2012a) who reported that bone breaking strength was not a sensitive assay. Furthermore, Korver *et al.* (2004) reported bone breaking strength measurements *ex vivo* may not accurately reflect resistance to fracture *in vivo*. Different assay preparation procedures and instruments are known to affect results (Orban *et al.,* 1993) and may explain the differences reported in the literature.

Species differences in bone strength have been reported (Rowland *et al.,* 1972), while other authors (Merkley, 1981; Knowles and Broom, 1990; Fleming *et al.,* 1994) reported significantly

weaker bones in caged birds compared with floor-reared birds suggesting husbandry has an additional influence on bone strength. Knowles *et al.* (1993) found that bone strength increased with bird weight, but the tendency of being broken during transportation and handling also increased with weight, leading the authors to conclude that the increase in bone strength due to weight was not sufficient enough to prevent additional damage suffered by heavier birds.

Poor bone mineralisation can increase the incidence of bone deformity and fractures thereby affecting bird welfare. This comes at a cost as fragile bones are correlated with bone fragments in meat products and discoloured meat which is less appealing to consumers (Rath *et al.,* 2000). The importance of maximising bone mineralisation for improved bone strength and a reduction in leg problems was noted in the study of Cheng and Coon (1990). Factors affecting bone strength in poultry include inherited genetic traits, infectious disease, ingestion of toxins, growth rate, gender, nutrition, physical activity (influenced by housing) and hormonal function, and are further elaborated in the report of Rath *et al.* (2000).

1.4.1.1.3 Bone densitometry

The use of invasive techniques for assessing nutrient bioavailability (e.g. bone ash and strength) requires that animals are sacrificed before such assays are performed. On the other hand, the use of non-invasive techniques allows for the repeated measurements of bone mineralisation *in vivo* over an extended period without necessarily sacrificing animals. This is particularly useful in studies aimed at age-related investigation of bone development, and in breeding programmes for the identification of genetic traits linked to leg health in live birds.

The improved method of measuring mineral content of bones in humans *in vivo* by direct photon absorption techniques (Cameron and Sorenson, 1963) was adapted to measure bone mineral content in chickens (Babcock *et al*., 1965). Using this technique, bone mineral content is evaluated by measuring the transmission of a mono-energetic photon through a bone; and has subsequently been used in both *in vitro* (Cantor *et al.* 1980) and *in vivo* studies (Akpe *et al*., 1987).

A more developed technique, the dual-energy X-ray absorptiometry, has also been used to measure bone mineral density in poultry (Hester *et al.,* 2004; Shang *et al.,* 2015). Using this technique, Onyango *et al.* (2003) fed broilers varying dietary calcium and phosphorus content and reported a high correlation coefficient between bone ash, and bone mineral content or bone mineral density (0.92 and 0.93 respectively). The authors concluded it was faster than the bone ash methodology. A limitation of the dual-energy X-ray absorptiometry method however is that bone mineral density is determined in 2 dimensions (g/cm^2).

Quantitative computer tomography, which measures bone density per unit volume (g/cm^3), has also been used in the study of bone mineral density in poultry (Jendral *et al.,* 2008; Silversides *et al.,* 2012). It provides more precise details on bone mineral density and cross-sectional image compared to dual-energy X-ray absorptiometry measures (Kim *et al.,* 2011). Shastak *et al.* (2012a) used the quantitative computer tomography technique in broilers and reported tibia ash was well correlated with total bone mineral density in 3-week old but not in 5-week old broilers (r^2 = 0.78 and 0.39 respectively), and alluded the observed differences to the higher fat content of bones in the 5-week old broilers.

1.4.1.2. Blood parameters

It has been demonstrated that the plasma concentration of phosphorus in broilers is related to dietary phosphorus concentration (Miles *et al.,* 1982). Linear increases in plasma phosphorus concentration as a result of increases in dietary phosphorus have also been reported (Hurwitz, 1964; Williams *et al.,* 2000a). Shafey and McDonald (1991) reported that high dietary calcium reduced plasma phosphorus but increased total plasma calcium; while Perney *et al.* (1993) and Sebastian *et al.* (1996a) reported an increase in dietary phytase resulted in increased plasma phosphorus concentration. This was corroborated by the findings of Viveros *et al.* (2002) who also reported that increasing phytase supplementation increased plasma phosphorus concentration. Although it has been suggested that plasma phosphorus is a useful measure of relative phosphorus availability (Hurwitz, 1964), Shastak *et al.* (2012a) evaluated phosphorus availability from 2 phosphate sources and reported serum phosphorus was not sensitive in differentiating the phosphate sources compared to the bone ash assay. The authors concluded the blood assay was not very useful for evaluating phosphorus availability due to the complex mechanism involved in the regulation of plasma concentration.

1.4.1.3. Feed utilisation and growth rate

Feeding broilers below their dietary phosphorus requirement negatively impacts on body weight gain and feed utilisation (Fritz *et al.,* 1969; Moran and Todd, 1994). Subsequently, body weight gain and feed efficiency have been used for evaluating the relative availability of phosphorus sources (Sullivan and Douglas, 1990). Potter *et al.* (1995) examined the bioavailability of various phosphorus sources and reported body weight gain and toe ash provide were equally sensitive in assessing phosphorus availability in broilers. This was corroborated by the report of Ravindran *et al.* (1995) who also evaluated various response

criteria used in assessing phosphorus availability in broilers and found body weight gain and toe ash to be equal to, or more sensitive than tibia ash in assessing phosphorus availability. This is however in contrast with the findings of other authors.

Nelson and Walker (1964) in a meta-analysis of 82 experiments concluded growth was less sensitive than tibia ash for evaluating phosphates, and even less accurate at levels near requirement. In agreement, Huyghebaert *et al.* (1980) concluded that neither body weight gain nor feed conversion could be used as indicators of phosphorus availability. Similarly, Shastak *et al.* (2012b) reported that no significant differences in feed intake or growth were found in 3 and 5 weeks old broilers given graded levels of two phosphate mineral sources: anhydrous monosodium phosphate and anhydrous dibasic calcium phosphate. The reports from these authors suggest growth performance alone is not sufficiently sensitive to accurately assess phosphorus requirements.

1.4.2. Quantitative measurements: retained and digestible phosphorus

Peeler (1972) identified that the bone, blood and growth criteria used for measuring phosphorus availability were merely qualitative, as values are relative to the criteria chosen. Coon *et al.* (2002) and Leske and Coon (2002) further elaborated that although relative bioavailability assays provide comparison data specific to various standards, they are of limited value as they fail to determine the amount of phosphorus retained, and therefore do not properly account for the phosphorus excreted. The three quantitative assay types commonly used in quantifying phosphorus availability in poultry are comparative whole body phosphorus analysis, phosphorus retention studies and prececal digestibility.

1.4.2.1. Whole body phosphorus analysis

Phosphorus retention, determined by measuring the phosphorus content in the whole body has been examined by different investigators (Nieß *et al.*, 2005; Shastak *et al.*, 2012c; Van Krimpen *et al.*, 2013). This is done by determining the phosphorus concentration in homogenised samples of the whole body, and calculating the difference between the start and the end of a feeding period. It is a process thought to be precise and has the advantage of not requiring a metabolic cage, although the laboratory efforts for getting representative samples are high (Rodehutscord, 2009).

1.4.2.2. Retained phosphorus

Leske and Coon (2002) used acid insoluble ash as an indigestible marker in a 5-day bioassay and proposed the use of retained phosphorus assay which quantifies ingested and excreted phosphorus. They suggested phosphorus retention values would account for phosphorus derived from phytate sources as well as non-phytate phosphorus sources, and would provide precise information on the amount of phosphorus retained irrespective of the source material. According to their report, retainable phosphorus was defined as the difference between the amount of phosphorus ingested and the total voided from the gastrointestinal and urinary tracts at a particular phosphorus intake and stage in bird development with the following equation:

$$\text{Total phosphorus retained} = \text{non-phytate phosphorus retained} + \text{phytate phosphorus retained}$$

$$\text{Phosphorus retention (\%)} = \frac{(\text{TPI} - \text{TPE})}{\text{TPI}} \times 100$$

Where:
TPI = total phosphorus ingested
TPE = total phosphorus excreted

Although the retained phosphorus assay has been used extensively to evaluate phosphorus availability in poultry (Rodehutscord and Dieckmann, 2005; Manangi and Coon, 2008), a major drawback is that it is dependent on measuring the phosphorus content in the excreta. This requires considerable experimental effort (Rodehutscord, 2009), prompting Shastak and Rodehutscord (2013) to propose the use of the prececal digestibility assay.

1.4.2.3. Prececal digestible phosphorus

The prececal digestibility assay which is an established method for evaluating protein quality in poultry (Ravindran *et al.*, 1999) has also been used to evaluate phosphorus availability in poultry (Van der Klis *et al.*, 1997). This assay is thought to have an advantage over the retained phosphorus assay due to its simplicity of not requiring a metabolic cage. Also values are not affected by post-ileal microbial activity (Rodehutscord, 2009). It involves feeding birds graded levels of phosphorus formulated below requirement and with an indigestible marker included, and collecting digesta from birds post-mortem. The section between Meckel's diverticulum and 2 cm prior to the ileo-caeco-colonic-junction is used and digesta collected at the terminal two-thirds. The collected digesta is frozen, dried, ground and analysed for nutrient according to standard official methods.

Prececal digestibility (%) is calculated according to the following equation:

$$100 - 100 \times [(TiO_{2Diet} \times P_{Digesta})/(TiO_{2Digesta} \times P_{Diet})],$$

where:

TiO_{2Diet} and $TiO_{2Digesta}$ = the analysed concentration of TiO_2 in the diet or excreta (g/kg).

P_{Diet} and $P_{Digesta}$ = the analysed concentration of phosphorus in the diet or digesta (g/kg).

Shastak and Rodehutscord (2015) recently investigated if previously published phosphorus availability data based bone criteria and relative bioavailability could be recalculated to produce a single phosphorus availability table of values. This was however not successful as published quantitative data had a wider range of phosphorus availability compared with the recalculated relative bioavailability values.

1.4.2.4. WPSA standard protocol for determining available phosphorus in broilers

The lack of consensus on a standardised methodology for determining phosphorus availability prompted the Working Group 2 on Nutrition of the European Federation of branches of the World Poultry Science Association to propose a standard assay protocol for determining available phosphorus in broilers. The prececal phosphorus digestibility assay was recommended, and a standard protocol produced (WPSA, 2013). The compilation of a feed table based on the preceal phosphorus digestibility assay was also proposed by the Working Group.

1.4.3. *In vitro* measurements

The use of *in vitro* tests was explored with the aim of obtaining results based on methods which were quicker and not as expensive as the standard bioassays (Shastak and Rodehutscord, 2013).

1.4.3.1. Solubility tests

The evaluation of phosphorus availability by testing the solubility of phosphate sources in various solvents such as hydrochloric acid, citric acid, neutral ammonium citrate has its historical background (Hill *et al.,* 1945). Day *et al.* (1973) however, found acid solubility was not well correlated with biological availability, and concluded it could not be used to predict bioavailability. A low correlation between water solubility and relative bioavailability of feed

phosphates was also reported in the study of Sullivan *et al.* (1992). Waldroup *et al.* (1999) who cited the work of other investigators (Sullivan *et al.,* 1992; Coffey *et al.,* 1994) related solubility tests with bioavailability values and concluded results were not consistent. This is in agreement with the review of Shastak and Rodehutscord (2013) who concluded that the solubility of phosphate sources *in vitro* solubility is not representative of availability *in vivo*.

1.4.3.2. Near-infrared reflectance spectroscopy

Near-infrared reflectance spectroscopy (NIRS) which works based on the principle of selective absorption of electromagnetic radiation (from 800 – 2500 nm) in accordance with the characteristic vibration frequencies of functional groups has been used to evaluate the nutrients of feed ingredients and nutrient analysis of mixed feed. Its potential for analysing agricultural commodities was first described by Bengera (1968) and has for long been used to evaluate nutritional values of animal feed (Hymowitz *et al.,* 1974; Miller *et al.,* 1978, Delwiche *et al.,* 2006). It has the advantage of providing quick estimates of the nutritional values of feed ingredients, and has been proven to be economical without reliance on chemical analysis unlike traditional time consuming wet chemistry laboratory assays. It also serves as a quality control tool in high paced feed manufacturing plants where results are needed quickly before dispatch.

The potential for using NIRS to measure total phosphorus and phytate phosphorus in wheat by-products and corn gluten feed was demonstrated in the study of DeBoever *et al.* (1994). Smith *et al.* (2001) determined the phosphorus content in poultry excreta and found strong coefficients of determination between chemical assays and NIRS for total phosphorus and phytate phosphorus (0.91 and 0.86 respectively). However, the wide range of relatedness between total phosphorus and phytate phosphorus NIRS values for different feed ingredients

(Tahir *et al.*, 2012) and poultry manure (Reeves, 2001) raises the question about the reliability of using such values for practical feed formulation.

Williams (1975) elaborated on the factors which affect the accuracy of NIRS assay in determining the chemical content of cereal and feed grains. These include mean particle size and particle size distribution (affected by processes involved in grinding and mixing), the nature of ingredient analysed, and the seasonal effect in which they are grown. Valdes *et al.* (1985) elaborated on the difficulty in determining the chemical analysis of mixed feed using NIRS due to the complex chemical and physical properties of feed which may affect reflectance. The authors emphasised the importance of accurate standards which depends on the accuracy of wet chemistry procedures and concluded NIRS could be a useful tool provided precautions are taken with sample preparation and calibration using wet chemistry analysis.

1.5. Phosphorus metabolism

A variety of factors affecting phosphorus and calcium absorption have been extensively reviewed (Shafey, 1993; Bar *et al.*, 2003). These include the physical and chemical form and concentration of calcium and phosphorus in diets, viscosity of digesta, passage rate of feed, gastrointestinal pH, chelating agents and mineral interactions. The variability in the phosphorus content of feed ingredients, and poor digestibility of plant phytate phosphorus by poultry (Bedford, 2000; Adeola and Cowieson, 2011) are other important factors affecting the metabolism of phosphorus. Sodium-dependent phosphate transporter IIb (NaPi-IIb) protein also plays an important role in intestinal phosphorus absorption in poultry, regulated by vitamin D_3 and dietary phosphorus (Murer *et al.,* 2004). Furthermore, phosphorus availability required to meet metabolic function depends on the efficiencies of other physiological processes such as intestinal absorption, glomerular filtration, renal tubular

reabsorption, the rate of transfer from blood to bone, and intestinal endogenous losses. These are modulated by several hormones, mainly the parathyroid hormone and the hormonal form of vitamin D_3 (Li *et al.,* 2016a; Figure 1.1).

The regulation of plasma phosphorus homeostasis, which is traditionally thought to involve the parathyroid hormone and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$), and more recently the fibroblast growth factor 23 (FGF23) is controlled by a counter-regulatory feedback mechanism involving the intestine, bone, kidney and parathyroid gland (Rao and Roland, 1990; Sapir-Koren and Livshits, 2011). High dietary phosphorus intake induces the expression of FGF23, which increases renal phosphorus excretion. Subsequently, increased FGF23 decreases the synthesis of 1,25-$(OH)_2D_3$ by the kidneys as well as the synthesis and secretion of parathyroid hormone which reduces intestinal absorption of phosphorus. In response to low dietary phosphorus leading to low plasma phosphorus, the thyroid gland is stimulated to produce parathyroid hormone which increases the synthesis of 1,25-$(OH)_2D_3$ by the kidneys, and triggers bone resorption and absorption of phosphorus and calcium from the intestines (Norman, 1987).

In response to low dietary calcium, the parathyroid gland is stimulated to secrete parathyroid hormone which then stimulates the production of renal 1α-hydroxylase prompting the synthesis of $1,25(OH)_2D_3$. This in turn regulates the DNA transcription of calcium-binding proteins which absorbs calcium from the intestines. $1,25(OH)_2D_3$ is also known to increase phosphorus absorption from the intestines, and calcium and phosphorus reabsorption from the kidneys and bones (McDonald *et al.,* 2011).

The described homeostatic response to calcium and phosphorus intake in the bird occurs as a result of the antagonising relationship between calcium and phosphorus concentrations in the intestine (Liu *et al.*, 2013). The homeostatic control of phosphorus and calcium in poultry mediated through the action of the parathyroid hormone on 1,25-$(OH)_2D_3$, as affected by the dietary concentration of calcium and phosphorus, vitamin D and NaPi-IIb is further elaborated in the report of Proszkowiec-Weglarz and Angel (2013) and is discussed briefly.

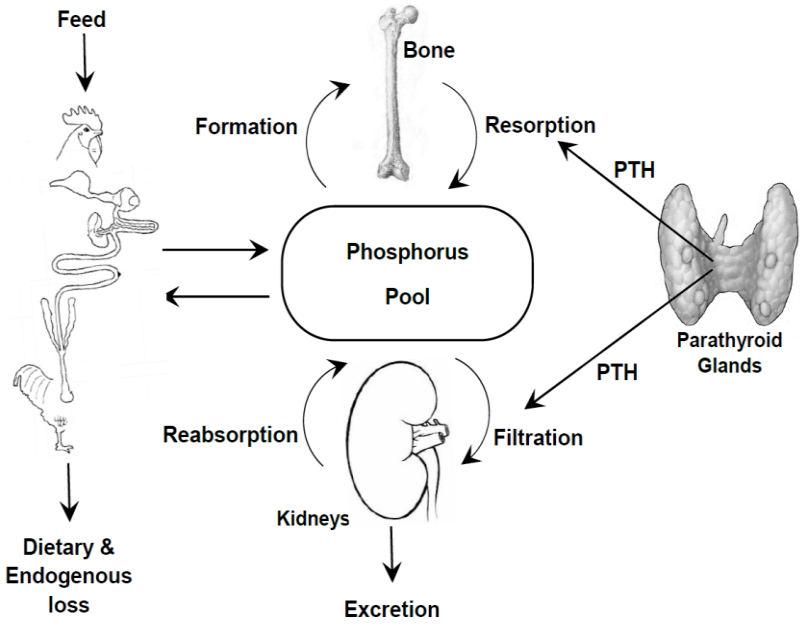

Figure 1.1. Phosphorus metabolism (Li *et al.*, 2016a: p.3)

1.5.1. Calcium and phosphorus

Calcium metabolism is affected by dietary levels of phosphorus (Edwards and Veltmann, 1983). Calcium and phosphorus are liberated from the bone, particularly when birds are fed low calcium diets, and during egg production when there is an increased demand for calcium for shell formation. This process is controlled by the parathyroid hormone secreted by the parathyroid gland, which causes resorption of bone, thereby liberating calcium in order to meet requirement. As calcium is combined with phosphorus in bone in the form of hydroxyapatite, phosphorus is also liberated and excreted.

A normal commercial chick diet has an approximate calcium:phosphorus ratio of 2:1, and it is important that this ratio is maintained as an abnormal ratio may be as harmful as a deficiency of either element in the diet (Waldenstedt, 2006). Calcium deficient diets, or high phosphorus diets have been linked to a high incidence of rickets and tibial dyschondroplasia (Long *et al.,* 1984; Riddell and Pass, 1987). The current calcium requirement for broilers (NRC, 1994) for the starter growth phase (0 - 21d), grower growth phase (21- 42d) and finisher growth phase (42 - 56d) are 1.00%, 0.90% and 0.80% respectively.

The effect of calcium inclusion on phosphorus utilisation is well documented. Calcium readily precipitates phytate, forming a calcium-phytate complex which is insoluble in the intestine (Nelson and Kirby, 1987), thereby rendering calcium unavailable for absorption (Sebastian *et al.*, 1996b). Increasing dietary calcium concentration decreases phytate phosphorus digestibility (Scheideler and Sell, 1987; Plumstead *et al.,* 2008) with consequent negative effects on feed intake, weight gain, efficiency of feed utilisation and tibia ash concentration (Letourneau-Montminy *et al.,* 2010). Similarly, feeding broilers highly soluble forms of dietary calcium have also been reported to have a negative impact on feed intake, body weight gain,

tibia ash and bone phosphorus concentration (Walk *et al.,* 2012). In contrast, decreasing dietary calcium concentration increases phytate phosphorus digestibility in chicks (Mohammed *et al.*, 1991; Selle *et al.,* 2009). Increasing dietary calcium, and widening calcium:phosphorus ratios decreases apparent phosphorus utilisation from dietary phytate in chicks (Hurwitz and Bar, 1971; Qian *et al.,* 1997).

1.5.2. Vitamin D

Vitamin D is involved in the absorption of calcium and phosphorus (Jande and Dickson, 1980) and its metabolism is a complex process involving several metabolites. Supplied through the feed, vitamins D_2 (ergocalciferol) and D_3 (cholecalciferol) are absorbed from the small intestines and are transported in the blood to the liver where they are converted into $25(OH)D_3$ by 25-hydroxylase. $25(OH)D_3$ is then transported to the kidneys where it is converted by 1α-hydroxylase (Jones *et al.*, 1998) into the most biologically active form of vitamin D_3: 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$). $1,25(OH)_2D_3$ functions to control calcium and phosphorus homoeostasis by acting on the intestines and kidneys, and regulating the secretion of parathyroid hormone produced in the parathyroid gland (Pike *et al.,* 2007).

Once ingested, phosphorus homoeostasis is heavily regulated by the rate of intestinal absorption and renal excretion. It is absorbed across the brush border membranes in the small intestine and kidney (Renfro and Clark, 1984; Quamme, 1985) through a sodium-dependent process of 3 classes of sodium-phosphate (NaPi) cotransporters: the types I, II and III (Proszkowiec-Weglarz and Angel, 2013). These represent 15, 84 and 1% of the total mRNA expressed by these transporters respectively (Miyamoto *et al.,* 1997; Tenenhouse *et al.*, 1998). The NaPi-IIb accounts for over 90% of the sodium-dependent phosphate transport in mammals (Sabbagh, 2009). Adedokun *et al.* (2012) highlighted the importance of gut health in

the expression of the NaPi-IIb gene in orally gavaged broiler chickens treated with mild coccidial vaccines. Significantly higher NaPi-IIb gene expression was observed in the challenged group of birds despite having the same adequately balanced diets with the control (unvaccinated) birds.

1.5.3. Sodium-phosphate cotransporters

The type I NaPi cotransporter was first identified in the kidney cortex of the rabbit (Werner *et al.,* 1991) and then other species including poultry (Werner and Kinne, 2001). It is not specific for sodium-dependent phosphate transport (Busch *et al.,* 1996) and its precise physiological role in phosphate homoeostasis is not well defined (Zhao and Tenenhouse, 2000). The characteristics of the type I NaPi cotransporter suggests it does not play a significant role in sodium-phosphate transport in the brush border membranes (Yan *et al.,* 2007).

The roles of type III NaPi cotransporters are also not well defined in avian species (Proszkowiec-Weglarz and Angel, 2013) but have been identified in most mammalian tissues. They are further classified into two sub-groups: PiT1 found in the intestinal brush border membrane, and the PiT2 found in the renal brush border membrane (Breusegem *et al.,* 2009; Villa-bellosta *et al.,* 2009).

The type II NaPi cotransporters (IIa, IIb and IIc) are the most abundant and their expression have been determined for all the three sub types in mammals and chicks (Custer *et al.,* 1994; Hilfiker *et al.*, 1998; Segawa *et al.,* 2002). Of particular importance is the NaPi-IIb cotransporter due to its role in intestinal phosphorus absorption and phosphorus homoeostasis. The molecular basis of the NaPi-IIb was first identified in the mouse (Hilfiker *et al.*, 1998) and

soon after in humans, rats and goats (Feild *et al.,* 1999; Hashimoto *et al.,* 2000; Huber *et al.,* 2000). Yan *et al.* (2007) cloned and characterised the chicken NaPi-IIb cotransporter homologue. In response to calcium/phosphorus restricted diets, the authors reported that the highest expression of the NaPi-IIb cotransporter mRNA was found in the duodenum followed by the jejunum and ileum.

1.6. Phosphorus and skeletal health

The majority of the phosphorus found in the body (about 80%) is stored in the bone, where it combines with calcium to form hydroxyapatite, the from which biological demands are met during bone turnover (Li *et al*., 2016a). Feeding inadequate dietary phosphorus has been directly related to poor bone mineralisation; and feeding adequate dietary phosphorus levels, as well as the use of feed additives such as of phytases and vitamin D known to improve phosphorus digestibility have been shown to improve bone mineralisation. Adeola and Walk (2013) demonstrated that increased phosphorus digestibility is directly related to improved bone mineralisation. However, the causes of leg disorders are complex and not usually attributed to a single factor (Butterworth, 1999).

Skeletal diseases account for 2 - 8% mortality in fast-growing broilers (Thorp, 1994), and have been reported to cost the US broiler industry between $80 and $120 million annually (Sullivan, 1994). They are of a serious welfare concern in broiler production (Venäläinen *et al.,* 2006). Yogaratnam (1995) investigated the causes of carcass rejection in UK processing plants and found that 19.5% of rejected carcasses were related to leg health issues. In a moderately recent large scale UK study, 27.5% of broilers close to slaughter age showed poor locomotion, and 3.3% were almost unable to walk (Knowles *et al.,* 2008).

Leg disorders, deformity, lameness, leg weakness and leg health problems are terms which have been used synonymously to describe problems associated with the walking ability of birds (Whitehead, 1998; Butterworth, 1999; Dinev, 2012); and are considered a major welfare concern in broilers (Gentle, 2011). Yalçin *et al.* (1998) reported birds with poor walking ability have difficulties in reaching food and tend to have lower body weight when compared with birds with good walking ability. The UK Farm Animal Welfare Council requires animals to be free from discomfort under the Freedom framework for assessing welfare in animal production, but the pathology of leg disorders has been associated with pain (Brickett *et al.,* 2007a). Furthermore, the reports of McGeown *et al.* (1999) and Danbury *et al.* (2000) have shown that lame birds preferentially select diets containing analgesic compared to non-lame birds.

1.7. Common causes of leg disorders in poultry

There are many possible causes of leg disorders in poultry (Table 1.4) which makes it difficult to pinpoint causative factors, particularly as leg disorders may be of multi-factorial causes (Venäläinen *et al.,* 2006). The causes may be of infectious or non-infectious origin, and have been reviewed by various authors in great detail (Leterrier and Nys, 1992; Thorp, 1994; Julian, 1998; Butterworth, 1999; Dinev, 2012).

Table 1.4. Disorders affecting locomotion in broilers (Thorp, 1996)

Clinical signs	Histopathology	Likely cause	Contributory factors	Diagnosis
Loss of motor function and upper motor neuron responses in the hind limbs	Deformity of the thoracic spine causing spinal cord compression	Spondylolisthesis Scoliosis	Genetic	Histopathology of thoracic vertebrae and spinal cord
	Infection of thoracic spine	Infectious spondylopathy	Infection Staphylococci Fungi	Histopathology Bacteriology Mycology
Loss of motor function and lower motor neuron responses in hind limbs.	Demyelination of sciatic nerve	Riboflavin deficiency Marek's disease	Dietary	Histology of nerve Serology Virus isolation
Severely lame. Difficulty in rising wing tips may be used for support	Disintegration of proximal femur and tibia, confirmation by histology	Femoral head necrosis, bacterial chondronecrosis (BCN)	Infection Staphylococci E. coli, Salmonella, Chlamydia Reovirus? Adenovirus?	Histopathology Bacteriology Virology
Severely lame. Hot, swollen joints and/ or tendons	Synovitis/ tenosynovitis Infected hocks	Inflammation of tendon/ligament and sheaths	Infection Staphylococci Reovirus Mycoplasma Adenovirus?	Histology Bacteriology Virology Serology
Bone deformity in the absence of growth plate thickening t displacement of gastrocnemius tendon	Valgus (lateral) varus (medial) and/or torsional deformity, frequently of tibiotarsus or tarsometatarsus in the absence of growth plate abnormality	Long bone deformity	Genetic Exercise Diet Growth rate Unknown	Measurement of bone torsion and angulation
Bone deformity with thickened growth plates	Thickening of growth plates in proximal tibiotarsus and/or tarsometatarsus as a result of accumulation of nonmineralized cartilage	Dyschondroplasia	Marginal hypocalcaemia Genetic	Histopathology
Long bone deformity with shortening and no thickening of growth plates	Tibiotarsus and tarsometatarsus shortened. No effect on growth plate mineralisation	Chondrodystrophy	Dietary deficiency of manganese, 6choline, niacin, vitamin E, biotin, folic acid, or pyridoxine Mycoplasma Genetic	Histopathology of growth plates and metaphyseal bone Undecalcified histology Bone ash analysis

1.7.1. Pathological factors

Highlighting the impact of lameness on welfare, Butterworth (1999) gave a review of the pathologies of bacterial and viral infectious diseases known to cause lameness and highlighted the importance of understanding their pathologies in order to reduce the incidence of infection. The aetiology of common leg disorders is discussed briefly to better understand the possible causes of leg disorders in poultry.

Bacterial chondronecrosis with osteomyelitis (previously known as femoral head necrosis and proximal femoral degeneration) has been identified as the most common cause of lameness in 25 - 45 day-old broilers in the UK (McNamee *et al.,* 1998; McNamee and Smith, 2000). It occurs when Staphylococcus (usually *S. aureus)* overcomes the immune response and circulates through the blood, forming small abscesses and local metaphyseal bone necrosis (Reece, 1992). It is characterised by lameness, lesions at the proximal end of the femur and tibiotarsus and mortality. As a result of rapid growth rate, the excessive stress exerted on the relatively immature epiphyseal and physeal cartilage of the affected bone creates clefts within the chondrocytes which are colonised by opportunistic bacteria (Wideman, 2016). An altered immune system as a result of viral infections, e.g. infectious bursal disease (Naqi *et al.,* 1984) and pox virus (Schoemaker *et al.,* 1998) have been reported as predisposing factors. Raising birds on wire flooring have been reported to give rise to incidences of bacterial chondronecrosis, particularly in males (Wideman*, et al.,* 2013). Yogaratnam (1995) attributed a 0.5 - 0.7% loss to the total UK broiler production due to bacterial chondronecrosis, and this represents an annual loss of £4.7 million to the UK economy (Butterworth, 1999).

Other infectious organisms identified as causative agents of lameness include those of bacterial origin: Escherichia (*E.coli*), Salmonella (*S. enteritidis, S. typhimurium*), Mycoplasma (*M.*

gallisepticum, M. synoviae); and those of viral origin, e.g. Reoviridae (genus Reovirus). These have been reported to cause one or more of the following conditions: bacterial chondronecrosis, arthritis, synovitis, tenosynovitis, chondordystophy and angular bone deformity (Gomis *et al.,* 1997; Morrow *et al.,* 1997; Takase *et al.,* 1987).

1.7.2. Nutritional factors

The role of nutrition in leg disorders in poultry has been widely researched over the years (Edwards, 1992; Whitehead, 1998; Edwards, 2000; Julian, 2005; Fleming, 2008) as an adequate dietary supply of nutrients is essential for proper bone formation. The amount of important minerals and vitamins required for proper bone development (Table 1.5) stipulated by the National Research Council (NRC, 1994) was derived from a review of numerous experimental trials.

Table 1.5. Important minerals and vitamins required for bone formation (NRC, 1994)

Nutrients[1]	0-3 weeks	3-6 weeks
Minerals		
Calcium (%)	1.00	0.90
Non-phytate phosphorus	0.45	0.35
Chlorine (%)	0.20	0.15
Magnesium (mg)	600	600
Potassium (%)	0.30	0.30
Sodium (%)	0.20	0.12
Zinc (mg)	40	40
Manganese (mg)	60	60
Fat soluble Vitamins[2]		
Vitamin A (IU)	1500	1500
Vitamin D_3 (IU)	200	200
Vitamin E (IU)	10	10
Vitamin K (mg)	0.50	0.50
Water soluble vitamins		
Biotin (mg)	0.15	0.15
Choline (mg)	1300	1000
Folacin (mg)	0.55	0.55
Niacin (mg)	35	30
Pyridoxine (B6) (mg)	3.5	3.5
Riboflavin (B2) (mg)	3.6	3.6

[1]Expressed as % or as unit per kg diet; 90% dry mater.
[2]IU Vitamin A = 0.3 μg retinal; IU Vitamin D_3 = 0.025 μg cholecalciferol.

1.7.2.1. The role of vitamins and minerals

The role of various minerals and vitamins in proper bone development has for long been of interest to various researchers. Phosphorus plays an important role in proper bone development and mineralisation, and its deficiency is known to increase the incidence of mobility issues in birds (Proszkowiec-Weglarz and Angel, 2013). Calcium metabolism is affected by the dietary levels of phosphorus (Edwards and Veltmann, 1983). A deficiency of either calcium or phosphorus causes rickets; and high dietary concentration of either calcium or phosphorus which induces the deficiency of the other have been reported to cause tibial

dyschondroplasia in broilers (Riddell and Pass, 1987). The metabolism of phosphorus and its association with calcium has already been discussed in Sections 1.5 and 1.5.1.

Vitamin A is involved in the continual renewal of light sensitivity of the retina, and plays a secondary role in the formation and protection of epithelial tissues and mucous membranes. Its deficiency has been reported to impair bone development and cause leg weakness in poultry (Lowe *et al.,* 1957). The high dietary inclusion of vitamin A has been reported to induce growth depression with an increase in the incidence of gait abnormalities, impaired bone development and leg disorders (Tang *et al*., 1985). But reports are inconclusive on its effect on skeletal health. High inclusion of vitamin A was reported to increase the incidence of tibial dyschondroplasia (Veltmann and Jenson, 1986; Li *et al*., 2008) while other investigators (Ballard and Edwards, 1988; Whitehead *et al.,* 2004) reported excessive dietary levels of vitamin A either reduced the incidence of tibial dyschondroplasia or had no effect.

Vitamin E functions mainly as a biological antioxidant, protecting the cells against oxidative damage caused by free radicals but its deficiency leads to increased incidence of leg abnormalities (Summers *et al.,* 1984) and muscular dystrophy resulting in poor mobility (Austic and Scott, 1991). Vitamin K is required for the post-transitional modification of osteocalcin, a protein associated with bone growth (Hauschka *et al.,* 1989). Although deficiency of vitamin K does not impair initial bone development in growing chicks according to Lavelle *et al.* (1994), Fleming *et al.* (1998) reported its beneficial effect in improving cancellous bone volume at the proximal tarsometatarsus in 25-week old hens.

According to the review of Bradshaw *et al.* (2002), the dietary deficiency of manganese, choline, vitamin E, folic acid, pyridoxine and zinc were reported to cause chondrodystrophy - a long bone deformity characterised by the shortening and thickening of growth plates. A

deficiency in vitamin B_6 has been shown to cause stunted longitudinal bone growth (Masse *et al.,* 1996); biotin deficiency has been associated with foot pad dermatitis (Harms *et al.,* 1977); choline deficiency has been associated with tibial dyschondroplasia (Summers *et al.,* 1984) and vitamin B_2 deficiency linked to curled-toes paralysis (Jortner *et al.,* 1987).

The effect of electrolyte balance on skeletal health has also been reported by various authors and is thought to be due to the effect of dietary mineral concentration on the blood buffer capacity which impacts on bone mineralisation (Oviedo-Rondon *et al*., 2001). Sauveur and Mongin (1978) reported that excessive dietary chloride or ammonium chloride increased the occurrence of tibial dyschondroplasia; while sodium and potassium had an opposite effect. Replacing NaCl with $NaHCO_3$ in the diet can reduce tibial dyschondroplasia (Julian, 1998). Halley *et al*. (1987) concluded high dietary anion relative to cation increased the incidence of tibial dyschondroplasia.

1.7.2.2. Common skeletal disorders

Rickets and tibial dyschondroplasia are two common skeletal disorders discussed as examples of nutritional disorders. Rickets is characterised by thickened and poorly mineralised growth plates and bones. Bones of affected birds are soft and break easily, leaving birds reluctant to move. Two forms of rickets have been reported. Hypocalcaemic rickets occurs due to calcium deficiency and it is characterised by the accumulation of proliferating chondrocytes (Jande and Dickson, 1980); while hypophosphataemic rickets occurs as a result of phosphorus deficiency and leads to the accumulation of hypertrophic chondrocytes with normal metaphyseal vessel invasion (Lacey and Huffer, 1982).

Young broiler chicks are predisposed to the development of subclinical incidence of rickets due to their rate of skeletal growth and inadequate vitamin D_3 metabolism (Bradshaw *et al.,*

2002) which plays a role in the absorption of calcium and phosphorus (Jande and Dickson, 1980). Vaiano *et al.* (1994) studied the age-related changes in blood concentration of $1,25(OH)_2D_3$ and reported chicks are not able to synthesise $1,25(OH)_2D_3$ between 1-2 weeks of age. Bar *et al.* (1987) however suggested nutritionally inadequate diets may not always be a cause of rickets. For example, field rickets can occur when feed is contaminated with *Fusarium spp*, or through the ingestion of excess iron and aluminium which interferes with phosphorus utilisation (Edwards, 1992).

Tibial dyschondroplasia usually occurs in the tibiotarsus and tarsometatarsus of fast growing birds. The effect of growth, electrolyte balance, dietary calcium and phosphorus ratio, $1,25,(OH)_2D_3$, ascorbic acid and *Fusarium spp* have been cited as contributory factors to the incidence of tibial dyschondroplasia in poultry (Elliot and Edwards, 1994; Praul *et al.*, 2000; Rennie *et al.*, 1993; Wu *et al.*, 1993). It is characterised by impaired endochondral ossification and the presence of a mass avascular hypertrophic cartilage in the posteromedial section of the tibial metaphysis of meat type poultry (Lowther *et al.,* 1974). The large lesions associated with tibial dyschondroplasia can lead to a fracture in the growth plate, or more commonly the development of an abnormal tibial plateau angle causing deformity (Lynch *et al.,* 1992). It is more prevalent in the first few weeks of young modern meat type broilers when the fast growth rate of bones exceeds other growth periods (Bond *et al.,* 1991) and this coincides with when the bird cannot synthesise sufficient $1,25,(OH)_2D_3$ required for calcium absorption (Abbas *et al.,* 1985). Edwards and Veltmann (1983) reported an increase in the incidence of tibial dyschondroplasia when the calcium content of diets is decreased at constant phosphorus; or when phosphorus is increased at adequate dietary calcium concentration. However, tibial dyschondroplasia cannot be prevented by merely adjusting calcium-phosphorus ratio in the diets (Riddell and Pass, 1987).

The dietary inclusion of D_3 and its metabolites (1α hydroxycholecalciferol, 1,25-$(OH)_2D_3$ and 25-$(OH)D_3$) have been reported to decrease the incidence of tibial dyschondroplasia in controlled studies (Rennie *et al.,* 1993; Whitehead, 1998; Edwards, 2000; Fritts and Waldroup, 2003). Although 1,25-$(OH)_2D_3$, is not available for commercial use, its commercially available metabolite precursor 25-$(OH)D_3$ has been shown to reduce the incidence of tibial dyschondroplasia in poultry, but it is not as effective than 1,25-$(OH)_2D_3$ according to the study of Rennie and Whitehead (1996). Roberson (1999) however reported 25-$(OH)D_3$ did not prevent tibial dyschondroplasia in broilers. Traditionally vitamin D_3 has not been associated with alleviation of tibial dyschondroplasia in broilers, however Whitehead *et al.* (2004) reported that a high inclusion (125μ/kg) reduced the incidence of tibial dyschondroplasia in 14 day broilers and suggested a review of the current NRC requirement of 5μ/kg (NRC, 1994).

1.7.3. Rapid growth rate and genetics

It has been widely suggested that fast growth rate is one of the major causes of leg health problems in poultry (Lilburn, 1994; Kestin *et al.,* 2001; González-Cerón, 2015), particularly in broilers, which are selected for rapid growth and breast muscle deposition. This has resulted in poor walking ability, increased incidence of lameness and downgrades at processing plants (Venäläinen *et al.*, 2006; Kestin *et al.,* 1999) as the rapid growth puts a demand on poultry to have sufficiently mineralised strong bones.

Lameness has been reported to be more prominent in male broilers which have a poorer walking ability compared to females (Brickett *et al.*, 2007a). In a study comparing the effect of growth rate on leg health, Shim *et al.* (2012b) reported that the incidence of tibial dyschondroplasia was significantly higher in a fast growing breed compared to a slow growing

breed of chicken. Although increase in growth rates has been associated with an increase in the incidences of leg abnormalities, the correlation between growth and leg abnormalities is inconclusive. Cook *et al.* (1984) reported that the severity of leg deformity was independent of body weight, while other authors (Kuhlers and McDaniel, 1996; Rekaya *et al.*, 2013) reported that the correlation between tibial dyschondroplasia and body weight was negligible. Although the strategy of reducing body weight is effective in reducing leg weakness, it comes at an economic cost (Venäläinen *et al.,* 2006). Feed restriction also raises welfare concerns as birds placed on feed restriction show signs of hunger (D'Eath *et al.,* 2009).

The genetic predisposition of birds to leg disorders is well documented (Riddell, 1976; Sheridan *et al.,* 1978; Hulan *et al.,* 1980; Wong-Valle *et al.,* 1993; Kapell *et al.,* 2012; Rekaya *et al.,* 2013). The potential for reducing lameness with the possible elimination of leg disorders through genetic selection in poultry breeding has been identified (Kestin *et al.,* 1999). For example, the incidence of some leg disorders like valgus-varus deformity, rotated tibia, spondylolisthesis and tibial dyschondroplasia have been reduced in the UK through genetic selection (Pattison, 1992). Fleming *et al.* (2007a,b) compared 3 modern broiler lines with their unselected older control lines (2007 vs. 1972) and found that despite the modern lines were significantly heavier and had better FCR, they also had a higher percentage of good leg health.

1.7.4. Diet and feeding regimes

It is well established that increased nutrient density leads to better efficiency in feed conversion and improved body weight gain (Saleh *et al.,* 2004). However, rapid growth is also known to impact on skeletal health. Lowering dietary energy and protein density, implementing early feed restriction, and offering various feeds forms are nutritional management strategies used in reducing growth rate (Brickett *et al.,* 2007b). Restricting feed during growth has been directly

linked to the reduction of growth and the improvement of leg health (Riddell, 1983; Lee and Leeson, 2001). Classen and Riddell (1989) affirmed feed restriction remarkably reduced the incidence of angular deformity, dyschondroplasia and spondylolisthesis which jointly account for 65 - 80% of non-infectious causes of leg deformity and lameness in broilers.

Su *et al.* (1999) investigated the effect of feeding pattern and early feed restriction on the prevalence of leg weakness in broilers. In a first experiment, broiler chicks were given access to feeders at allocated times of the day to test the effect of feeding pattern while a second experiment was conducted to examine bird response to various feed restriction programmes. They concluded meal feeding and early feed restriction were effective at reducing the prevalence of leg weakness as evidenced in better walking ability and fewer incidences of tibial dyschondroplasia, hock burn, and angulation of the hock joints.

Although chickens tend to adjust feed intake to match energy requirements (Plavnik *et al.,* 1997), birds have difficulty in adjusting feed intake to meet energy requirement at low nutrient densities (Nielsen, 2004) thereby resulting in reduced growth rate. Jones and Wiseman (1985) reported a reduction in body weight of birds fed a low-energy starter diet *ad libitum* compared with those fed a more energy dense diet. Urdaneta-Rincon and Leeson (2002) examined the effect of feed form (pellets vs. mash diets) on growth characteristics in a broiler trial. Broilers fed mash had a lower body weight at 42 and 49 days. This is corroborated by the findings of Brickett *et al.* (2007b) who recorded lower final broiler weights of birds offered mash diets.

Recent investigations into alternating high energy/low protein diets with low energy/high protein diets have also shown commercial promise. By sequentially feeding broilers 13.39MJ/kg, 15% crude protein diets and alternating with 11.72MJ/kg, 23% crude protein diets, Leterrier *et al.* (2008) demonstrated this reduced the incidence of leg problems through

reduced early growth rates, without any compromise on expected final body weight targets typical of commercial production.

1.7.5. Stocking density and level of activity

High stocking density can increase the occurrence and severity of leg disorders. It has been reported to increase the incidence of footpad dermatitis (Martrenchar *et al.,* 1997) and poor walking ability (Sørensen *et al.,* 2000) in poultry. It has a negative effect on growth and slaughter quality (Cravener *et al.,* 1992) and in the increase in the number of leg culls (Hall, 2001). Buijs *et al.* (2012) evaluated the effect of stocking density on bone quality and found increased stocking density had a negative effect on tibia curvature and shear strength.

Broilers reared in cages with limited space for activity have shown more incidence of skeletal problems compared with birds reared in group houses (Reece *et al.,* 1971). Conversely, Veltmann and Jensen (1980;1981) reported a reduction in the incidence of tibial dyschondroplasia in birds reared in cages compared to those reared on the floor and speculated that a predisposing pathological agent or toxin susceptible to the varying environmental conditions of floor-reared broilers may have been involved in the aetiology of tibial dyschondroplasia.

The incidence of leg abnormality is increased with lack of exercise (Haye and Simmons, 1978), a proposition supported by other investigators (Classen and Riddell, 1989; Renden *et al.*, 1991) who also reported that by manipulating lighting schedules, birds were more active and this resulted in a lower incidence of twisted legs in broilers. On the contrary, Su *et al.* (2000) and Tablante *et al.* (2003) reported that providing perches to stimulate activity did not reduce the incidence of leg weakness in broiler chickens; although Ventura *et al.* (2010) reported it improved footpad health.

Adjusting dietary nutrients have also been shown to improve leg conditions in broilers by stimulating activity. Feeding low protein or low amino acid diets are known to increase the level of activities in chickens (Rovee-Collier *et al.,* 1993). Bizeray *et al.* (2002) reported birds sequentially fed low lysine diets (0.85% lysine) had better leg conditions than birds fed normal lysine diets (1.19% lysine), but care must be taken to ensure minimum dietary protein requirements are met.

1.7.6. Light and photoperiod

Light intensity, photoperiod, light source and wave length are all aspects of light that can be manipulated to improve productivity and management practice (Manser, 1996). Birds grown in continuous light have been shown to have more leg abnormalities, increased incidence of tibial dyschondroplasia and impaired walking ability compared with those provided intermittent light (Buckland *et al.,* 1976; Sanotra *et al.,* 2002). Conversely, intermittent or stepped lighting programmes normally practised in commercial broiler production have been reported to have positive effects in the reduction of leg disorders (Classen and Riddell, 1989), as birds reared under these conditions are more active during light period (Simons and Haye, 1985).

Although it has been previously reported that increasing light intensity could increase activity which could consequently improve leg health (Hester *et al.,* 1994; Prayitno *et al.,* 1997), other investigators (Kristensen *et al.,* 2006; Blatchford *et al.,* 2009) reported that increasing light intensity did not improve leg health. The European Union Council Directive on lighting (European Commission, 2007) stipulates that at least 20lx light intensity must be provided for all ages of birds during the lighting period.

1.8. Nutritional imprinting in poultry

1.8.1. Epigenetics

The gene within the cell bears the blueprints required to synthesise proteins; and every cell thus has the same genetic information required to potentially make all body cells, tissues and organs. The central dogma of molecular biology, which states that the DNA sequence of a gene is first transcribed into RNA and then translated into a protein sequence was first stated by Crick (1958). This is the foundational basis for Genetics, the study of heritable changes in genes activity or function which occur due to the direct alteration of DNA sequence (Moore *et al.,* 2013).

The mutations to the DNA sequences which results in diseases were once accepted as reasons for observed aberrations. However they are not sufficient in explaining certain disease situations, e.g. cancer; or why despite having the same genetic makeup and raised in similar environments, some monozygotic (identical) twins have different susceptibility to different diseases (Cardno *et al*., 2002) or sometimes react differently to the same medicine. It is now evident in certain types of cancers that the genes which control the proliferation of cells can be inactivated when methylated, thereby resulting in tumours. These changes in the epigenome commonly referred to as *epi*-genetics exist due to other causes which overlay the DNA sequence (genotype) and sometimes do not reflect the expected outcomes in gene expression (cellular phenotype). Epigenetics orchestrates the means by which particular genes are either expressed or silenced without altering the underlying DNA sequence.

The concept of Epigenetics (*epi* - ἐπί, the Greek prefix meaning over, outside of, or attached to) was originally coined by Waddington (Waddington, 2012) who referred to it as the study of causal mechanisms by which the genes of the genotype bring about phenotypic effects. It has since evolved in meaning. A more commonly cited definition is that of Riggs *et al.* (1996)

who defined it as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. Epigenetics has been used to describe other processes which have not been demonstrated to be heritable such as histone modification, leading to several debatable attempts to redefine it (Ledford, 2008; Bird, 2007; Ho and Burggren, 2010). A recent more encompassing definition is that of the Roadmap Epigenomics Project (NIH, 2016), which defines Epigenetics as both heritable changes in gene activity and expression (in the progeny of cells or individuals) and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable.

1.8.2. Mode of action

The identification of DNA methylation in mammals (Avery *et al.*, 1944) and discovery of the methylation at the 5th carbon of the cytosine base within the double helix DNA structure of the calf's thymus (Hotchkiss, 1948) opened a new paradigm to the understanding of gene function and expression. Chemical reactions occur within the gene which turns on or off particular base pairs in the DNA and alters how genes are either expressed or silenced. Specifically, cytosine methylation within CpG dinucleotides islands of DNA (short dispersed regions of unmethylated DNA located in the promoter regions of genes where cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases) in conjunction with chromatin modification via the spatial arrangement of DNA around histone proteins are the main mechanisms by which a single allele of a gene is silenced (Bird, 2002; Ashwell and Angel, 2010). It has been reported that CpG methylation is well correlated with reduced transcription (Razin, 1998) and consequently gene expression (Razin and Riggs, 1980). Alterations to DNA methylation pattern involves active DNA methylation and demethylation in the neuronal genome.

In humans, 3 enzymes are involved in the methylation process (Hsieh, 1999): DNA methyltransferase 1 (DNMT1) which functions by maintaining methyl transfer after DNA replication, and two *de novo* DNA methyltransferases (DNMT3A and DNMT3B) which act on somatic cells by establishing a new methylation pattern to unmodified DNA; although this distinction is not clear cut (Robertson and Wolffe, 2000). It is thought that all three enzymes are involved in both *de novo* and maintenance functions (Robertson *et al.,* 2000; Rountree *et al.,* 2000). In general, DNA methyl transferases are directly involved in the transfer of a methyl group from S-adenyl methionine (SAM) to the 5th carbon of a cytosine residue to form 5-methylcytosine (5mC), suppressing transcription and ultimately the expression of genes (Figure 1.2). No enzyme is directly involved in demethylation of 5mC, but 5-hydroxymethyl-cytosine (5hmC) has been reported to serve as an intermediate in the DNA methylation pathway (Moore *et al.,* 2013). Supplementation with dietary folate, vitamin B_{12}, methionine, choline, and betaine is required for SAM synthesis (Cooney, 2002; Van den Veyver, 2002).

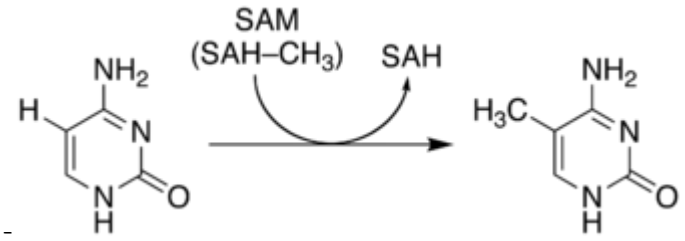

Figure 1.2. Methylation of cytosine to 5-methylcytosine by DNA methyltransferase

The mechanisms of DNA methylation have been widely reviewed in plants (Steimer *et al.*, 2004; Henderson and Jacobsen, 2007) and animals (Bird and Wolffe, 1999; Jones and Takai, 2001). In response to various stimulation, the methylation of DNA and histone methylation have also been identified in poultry, e.g. acclimation to heat (Kisliouk and Meiri, 2009; Yossifoff *et al.,* 2008), dietary deficiency e.g. phosphorus, calcium and protein (Yan *et al.,* 2005b; Rao *et al.,* 2009), and immune response to diseases such as salmonella (Gou *et al.,* 2012).

1.8.3. Nutritional imprinting and epigenetics

Nutritional imprinting, the early life experience of nutrition which has an effect on later physiological outcomes is an established phenomenon. Diet plays one of the most important roles in mediating the mechanisms of epigenetics. The regulation of gene expression through nutritional imprinting, where nutrition is manipulated (pre or postnatal) with effects on health, cognitive abilities, and lifespan is well documented (Lucas, 1998). These manipulations occur at specific developmental points leading to long term or permanent effects later in life (Lucas, 1991). Nutritional manipulation at specific stages of development is known to have different outcomes (Symonds *et al.*, 2007) thus identifying the stage of development is crucial for comparisons. This concept was further elaborated by Hanley *et al.* (2010) who also highlighted the importance of stage in developmental plasticity.

Maternal nutrition is reported to have an effect on epigenetic mechanisms and has been linked to chronic degenerative diseases in offspring (Lumey *et al.,* 2007; Heijmans *et al.,* 2008). The classic study of Waterland and Jirtle (2003) showed feeding methylated diets to pregnant agouti mice downregulated the agouti gene expression in their offspring which were darker, leaner and healthier unlike the offspring of pregnant mice fed normal diets which retained their yellow colour and were more susceptible to hyperinsulinemia and tumour formation.

1.8.4. Transgenerational effect epigenetics

The transgenerational effect of ancestral nutrition on progenies is well documented (Leon *et al.,* 1998) and has been linked to epigenetics (Bygren *et al.,* 2001; Whitelaw, 2006). For example, Martyn *et al.* (1996) showed early maternal nutrition influenced their offspring's susceptibility to cardiovascular diseases, diabetes and hypertension; similar to the findings of Kaati *et al.* (2002) who identified a significant association in increased risk of cardiovascular diseases and diabetes between the nutrition of selected Swedish male parents and grandparents and their offspring. Examining a subset of the same Swedish cohort, Pembrey *et al.* (2006) reported sex-specific transgenerational responses linking grandfather's food supply only to mortality risk ratios of their grandsons, and grandmother's food supply only to mortality risk ratios of their granddaughters. Results from the study of the Hertfordshire and Dutch Hunger Winter (Hales *et al.*, 1991; Ravelli *et al.,* 1998) have also confirmed the correlation between low birth weight and obesity in adulthood.

Transgenerational transfer of parental behaviour has been reported in animals. In nursing rats, pup licking and grooming behaviour have been shown to have a remarkable but reversible effect on the ability of offspring to better cope with stress (Weaver *et al.*, 2004; Cameron *et al.,* 2008). In humans, maternal care has been linked with offspring's ability to cope with stress (Meaney, 2001); and the epigenetic mechanisms involved explained (Champagne, 2008; Champagne and Curley, 2009).

1.8.5. Epigenetics in poultry

The potential for improved performance in poultry though the application of epigenetics was recently reviewed by Frésard *et al.* (2013). The prenatal and neonatal period have been identified as the most important stages of development in poultry during which exposure to various stimuli are capable of shaping phenotypic traits (Dixon *et al.*, 2016).

Early studies in poultry focused on the thermal conditioning of broilers early post-hatch (Yahav and Hurwitz, 1996; Yahav and McMurtry, 2001) by manipulating chicks to better tolerate heat stress later in life. This has been reported be one of the best strategies for adapting to heat stress (Lin *et al.,* 2006). Renaudeau *et al.* (2011) reported that exposure to varying heat conditions at the end of incubation could help adapt birds to heat challenges later post-hatch, a proposition supported by other investigators (Yalçın *et al.*, 2008; Piestun *et al.,* 2008; Druyan *et al.*, 2012).

The molecular basis for how this works via the expression of the brain-derived neurotrophic factor (BDNF) gene in the hypothalamus during thermotolerance acquisition has been explained (Yossifoff *et al.,* 2008; Kisliouk and Meiri, 2009). During the period of early thermal conditioning and later reexposure to heat stress, transient changes in the expression of BDNF occurs and have been related to changes in CpG methylation pattern in the BDNF promoter region of the brain in poultry. The precise timing of thermal conditioning is important otherwise such the technique could potentially lead to poor bone development and the down regulation of the gene important for adequate ossification (Oveido-Rondon and Wineland, 2012). Yalçın *et al.* (2007) demonstrated that incubating broiler eggs below (36.9°C) or above (39°C) optimal incubating temperatures (37.8°C) during early embryo development (0 – 8 days) increased the incidence of tibial dyschondroplasia at 49 days post-hatch.

More recent work in poultry nutrition have been directed towards exploring how the transgenerational effect of epigenetics could be used as a tool for improved productivity including skeletal quality. For example, it has been established that small egg size at hatch leads to small bird size at market age (Proudfoot and Hulan, 1981; Whiting and Pesti, 1984). However, some investigators (Lopez and Leeson, 1995; Rao *et al.*, 2009; Van Emous *et al.*, 2015) demonstrated that when broiler breeders were fed low protein diets, although their

offspring were hatched from smaller eggs, they were more efficient at utilising nutrients, and were heavier and had more muscle mass compared to control chicks fed normal diet. Reduced mortality and improved growth rates in offspring have also been reported when broiler breeders were fed low-density diets (Enting *et al.,* 2007). And by fortifying maternal diets with high dietary concentration of vitamin D, Atencio *et al.* (2005) and Driver *et al.* (2006b) demonstrated the positive effects it had on their progeny by reducing the incidence of rickets, tibial dyschondroplasia and increased bone ash.

The potentials for epigenetics to address nutrient pollution due to the accumulation of undigested nutrients contained in poultry manure typical of concentrated animal feeding operations have been identified. Angel and Ashwell (2008) nutritionally imprinted birds with a low phosphorus diet for 90h (0.59%Ca : 0.25%avP) and reported better growth performance characteristics, tibia ash and phosphorus retention when the birds were later challenged with a low phosphorus diet (0.4%Ca : 0.11%avP) at the finisher stage (22 - 38d), compared to control birds by 38 days. It has also been proposed that nutritional imprinting may serve as a useful tool in addressing ammonia pollution in poultry production if birds are conditioned to be more efficient at utilising nitrogen (Angel, 2010).

1.9. Current strategies for efficient phosphorus use in poultry

Nahm (2007) reviewed the detrimental environmental effects of excreted phosphorus and concerns about the cost and rapid depletion of inorganic phosphate reserves have stimulated research into ways of improving phosphorus utilisation in poultry. This includes the simple avoidance of oversupplying feed with dietary phosphates (Waldroup, 1999) as poultry can utilise only a certain amount of nutrients for maintenance and production and excrete nutrients when supplied in excess of requirement.

Commonly used poultry feed ingredients have variable available phosphorus content and therefore varying phosphorus digestibility. The use of highly digestible feed ingredients and management strategies which optimises bird health are also known to improve the efficiency of feed utilisation. This in turn leads to less feed consumed and consequently less phosphorus excreted per kg meat produced.

The nutrient requirement of birds changes with age and older birds are better able to utilise phytate (Peeler, 1972), and thus a more targeted approach of phase feeding (Angel *et al.*, 2000) where diets are formulated to meet changing nutrient requirements due to age has been shown to be effective in minimising wastage. Split-sex feeding where birds are reared and fed according to sex-specific nutritional requirement has been proposed to further improve precise feeding (Spiehs, 2005), but this has commercial limitation due to the additional costs of separating birds by sex at the hatchery.

The inclusion of feed additives known to improve phosphorus utilisation which allows for a reduction of supplemental phosphorus routinely added to poultry diets have also been investigated by various researchers. Phytase is the most important feed additive known to improve phosphorus digestibility. According to the International Union of Biochemistry (IUB, 1979), it is the only enzyme capable of initiating the release of phosphorus from phytin and has been widely investigated (Perney *et al.,* 1993; Ravindran *et al.,* 2006; Plumstead *et al.,* 2007). Phytase supplementation in poultry has been effective in improving phosphorus retention (Kornegay *et al.*, 1996; Qian *et al.,* 1997) and phytate phosphorus digestibility by 20 - 45% (Ravindran *et al.,* 1995) by the release of unavailable phosphorus stored in plant grains. The use of dietary phytase has been reported to reduce phosphorus excretion by 15 - 30% in poultry (Applegate *et al.,* 2008).

Organic acids such as citric acid have been shown to improve phosphorus utilisation in broilers (Boling-Frankenbach *et al.,* 2001; Snow *et al.,* 2004) by reducing the pH of digesta and thereby creating the right environment for the dissociation of phytic acid and minerals (Maenz *et al.,* 1999). The synergistic effect of citric acid with phytase was demonstrated in the study of Woyengo *et al.* (2010) who reported that the combination of the two additives further improved phosphorus digestibility. This was corroborated by Demirel *et al.* (2012) who reported citric acid significantly improved phosphorus retention, and in combination with phytase significantly increased tibia ash percentage. However, the mechanism of action of citric acid on phosphorus availability is not well defined. Pileggi *et al.* (1956) suggested that by binding to calcium, citric acid had anti-rachitogenic effect in rats by reducing the inhibitory effect of calcium on intestinal phytic acid hydrolysis. Citric acid is a strong chelator of calcium and renders phytate more susceptible to endogenous phytase by complexing with calcium bound to phytate (Boling *et al.,* 2000).

The dietary inclusion of the probiotic lactobacillus (Nahashon *et al.*, 1994; Angel *et al.,* 2005); and Vitamin D_3 and its metabolites: 25-hydroxycholecalciferol, 1,25-dehydroxycholecalciferol (Edwards, 1993; Angel *et al.,* 2006) have been shown to reduce phosphorus requirement by improving phosphorus utilisation.

New plant genotypes that contain lower levels of phytate and more amounts of available phosphorus have been developed. For example, the use of high available phosphorus (HAP) maize (Waldroup *et al.,* 2000) and soybeans (Dilger and Adeola, 2006) reduces the need for supplementary inorganic phosphorus. More recently the use of transgenic corn varieties with innate ability to express phytase within the endosperm (Nyannor and Adeola, 2008; Nyannor *et al.,* 2009) have been shown to improve phytate phosphorus utilisation in poultry.

Despite these strategies, phosphorus is not completely digested in poultry, and the safe disposal of phosphorus contained in manure is important from the environmental stewardship viewpoint. Treating poultry litter with manure amendments such as aluminium sulphate has been shown to reduce the water soluble phosphorus content (Moore *et al.,* 1995; Miles *et al.,* 2003; Bolan *et al.,* 2010). Suitable land-based agricultural practices such as terracing and contour tillage which reduces surface runoff by increasing soil resistance to erosion and thereby reducing the impact of rainfall on soils are other well-practised measures which have been proven effective in reducing excessive phosphorus pollution in water.

Transporting poultry manure to areas where it is needed for land application has been identified as the most direct way to resolve surpluses (Sims *et al.,* 2005). However, the cost of haulage remains an economic bottleneck (Keplinger and Hauck, 2006). Greaves *et al.* (1999) identified the need for processing manure with a view of reducing volume whilst creating a valuable product. Sharpley (1999) also noted the potentials of using manure as a source of energy via combustion and anaerobic digestion. By composting manure, bulk size is reduced, and this could potentially reduce the cost of haulage. This is of advantage as the composted material is more uniform in physical and chemical properties and therefore its application on land more accurate.

1.10. Conclusion

Adequate supply of dietary phosphorus is important from the bird welfare and commercial standpoints, as it plays an important role in bone development, growth and efficient feed conversion. However, it is a finite resource, and careful consideration must be given to its judicial use in order to avoid oversupply whilst ensuring the bird's welfare is not compromised.

Presently, nutrition, health, and improved management practices are the main areas being explored in the quest to improve the efficiency of phosphorus utilisation in poultry.

The NRC recommendation for phosphorus requirements in broilers (NRC, 1994) is widely used as a reference publication for diet formulation in research and commercial feed manufacture. However, it is based on studies dated between 1952 – 1983 and derived from methodologies different from the recently updated phosphorus availability assays (Shastak and Rodehutscord, 2013; WPSA, 2013). It has also been criticised for not being reflective of the present day broiler strains which are characterised by fast growth rate and larger muscle mass (Havenstein *et al.*, 2003a; Schmidt *et al.,* 2009) but poorly mineralised skeleton (Williams *et al.*, 2000b; Leeson, 2012). The lack of a centralised, up-to-date publication on poultry phosphorus requirements, and confusion over the potential phosphorus contribution of different feed materials with or without supplemental phytase (Applegate and Angel, 2014) has led to feeding dietary phosphorus with excesses of between a fifth and twice over published requirements (Applegate and Angel, 2008).

The first part of this research project examines 4 common divergences in the bone ash methodology for evaluating bone mineralisation in broilers, and the established findings from the bone ash methodology study will be used in other studies in this project. The next part of this project focuses on characterising the normal range of tibia phosphorus content and whole body phosphorus content of broilers raised in commercial settings. This is followed by examininig strategies for maximising the efficiency of phosphorus utilisation: nutritional imprinting for improved phosphorus utilisation, and the use of a high phytase novel wheat cultivar to provide a more bioavailable form of phosphorus in broilers.

The key novel contribution of this project is providing the range of tibia ash and phosphorus content dataset applicable to normal healthy commercial broilers which are not currently available. Another novel contribution is examining how nutritional imprinting improves phosphorus utilisation and its impact on bone quality; and the potential use of a high phytase wheat cultivar not reported in the literature.

Findings from this project addresses the contemporary issue of the dwindling phosphorus reserves through better utilisation whilst ensuring bird welfare is not compromised. It provides the poultry industry with an up-to-date reference dataset of bone and whole body phosphorus content which could be used to evaluate bone mineralisation and phosphorus requirement in broilers.

1.11. Aims and objectives

The overarching aim of this project was to characterise the bone and whole body phosphorus content in commercial broilers, and to examine 2 nutritional techniques for improving phosphorus utilisation in broilers. The specific objectives were to:

- Examine the tibia bone ash methodology in evaluating bone mineralisation.
- Characterise tibia morphometry and mineralisation in commercial broilers.
- Characterise bone and whole body phosphorus content in commercial broilers.
- Examine nutritional imprinting as a tool for improving phosphorus utilisation.
- Evaluate the use of a high phytase novel wheat cultivar.

CHAPTER 2: MATERIALS AND METHODS

2.1. Introduction

The general materials and methods used in this thesis are described in this chapter. A total of 8 studies involving 6 experimental trials were conducted as summarised below in Table 2.1. Four common divergences to the bone ash methodology were examined in Studies 1-4. The normal range of phosphorus and calcium content in the tibia of healthy commercial broilers was characterised in Study 5, while the relatedness between the phosphorus content of various bones (tibia, femur, and feet) with the whole body phosphorus content was examined in Study 6. The use of nutritional imprinting for improving phosphorus utilisation was evaluated in Study 7, and the potential use of a high phytase wheat cultivar on phosphorus digestibility and bone mineralisation was investigated in Study 8.

Table 2.1. Outline of studies conducted

Study	Investigation	Chapter
1	The effect of fat extraction on tibia ash content	3
2	The effect of including cartilage caps on fat extracted tibia ash content	3
3	The effect of increasing fat extraction time on fat extraction efficiency	3
4	The effect of autoclaving prior to fat extraction on tibia ash content	3
5	Characterisation of tibia bone mineralisation in commercial broilers	4
6	An investigation into the relationship between bone phosphorus content and whole body phosphorus content in commercial broilers	5
7	Nutritional imprinting as a tool for improving phosphorus utilisation in broilers	6
8	The effect of a high phytase wheat cultivar on phosphorus digestibility and bone mineralisation in broilers	7

All experimental trials were conducted at the Poultry Research Unit located at the Nottingham Trent University, UK; with the exception of Studies 4 & 5 which were conducted at 6 commercial broiler farms located in the UK.

2.2. Birds and management

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny *et al.*, 2010) were followed, and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee.

For Studies 1 - 4, 7 & 8, Ross 308 day-old male broiler chicks were sourced from PD Hook Hatcheries Ltd, Oxfordshire. On arrival, birds were allocated to pens (0.64m^2). Chicks were raised in-house in a thermostatically controlled room and provided with an initial room temperature of 32°C which was gradually reduced to 21°C by day 21 and maintained until the end of the experiment. Lighting was provided with 1h darkness from day 1 which was increased by 1h a day to 6h, then maintained until the end of the experiment. Wood shavings were spread on the pen floors (approximately 3cm thick) and was topped up as required during each trial. Birds were allocated in such a way that they were evenly distributed by weight across treatments. Experimental diets and water were provided *ad libitum*, and birds were checked twice daily to monitor birds and environmental conditions. Mortalities were recorded along with the date, and weight of the bird and reason if culled.

For Studies 5 & 6 Ross 308 day-old chicks from a mixed flock were raised at one of six participating commercial farms which complied with the Ross 308 management guidelines (Aviagen, 2014). Birds were raised on wood shavings in open sheds which were thermostatically controlled to provide an initial room temperature of 30°C which was gradually

reduced to 20°C by day 27 and maintained until the end of the experiment. Lighting was provided with 1h darkness from days 1 - 3, and then increased by 1h a day up until day 6. Lighting was then maintained on a daily 4 and 2 hourly split darkness period between days 7 up until day 32. From days 33 - 35, total darkness was reduced daily to 3, 2 and 1h respectively. 1h darkness was then maintained until the end of production cycle when birds were transported to processing plants. Standard commercial pelleted diets and water were provided *ad libitum*. Birds were regularly checked to monitor environmental conditions and mortalities recorded. When birds were culled, the reason for culling was also recorded.

2.3. Diet formulation and feed preparation

For Studies 1 - 4, 7 & 8, experimental diets of the mash type were manufactured on site. A production sheet specifying the quantity of ingredients based on nutrient requirement was produced. The dry feed ingredients were weighed on a top pan balance (Mettler, Toledo, Leicester, UK) as specified in the production sheet and mixed for 5 minutes in a ribbon mixer (Rigal Bennett, Goole, UK) in a maximum of 100kg batches. Oil was then added and feed mixed for an additional 5 minutes. During the mixing process, care was taken to brush down clumps of oil to ensure homogeneity. Details of the feed specification and calculated nutritional composition are given in the relevant chapters. 1kg samples of diets were taken for analysis. Nutrient composition was confirmed by laboratory analysis of diets later described in this chapter.

For Studies 5 & 6, pelleted feed manufactured in a commercial feed mill was supplied to commercial farms. The feed was manufactured to meet the Ross 308 nutrient requirements (Aviagen, 2014). Strict compliance with national regulatory guidelines on the safe manufacture

of feed such as the Universal Feed Assurance Scheme and Foods Standard Agency were adhered to. These regulatory bodies operate within a wider EU legislative directive.

2.4. Sampling and preparation methods

2.4.1. Bird performance, lameness, and pododermatitis scores

In Studies 1 - 4, 7 & 8, the experimental feed was preweighed into bags designated to particular pens and recorded. During the trials, additional feed was weighed into the feed bags and recorded. Feed intake was determined on each sampling day by calculating the difference between the total amount of feed added to the feed bags at the start of the sampling period and what was left at the end of the sampling period.

Birds were individually weighed at the start of the trial and were evenly distributed to pens ensuring each treatment had a similar average pen weight. Birds were weighed by pen on each sampling day, and the average bird weight was determined by dividing the total pen weight by the number of birds left in pen.

On each sampling day prior to post-morterm tissue collection, birds were culled by cervical dislocation and weighed individually. Body weight gain by pen was determined by calculating the difference between pen weight at the start and end of the sampling period. Individual body weight gain was determined by dividing the weight gain by the number of birds in a pen. Daily weight gain (by pen or individual bird) was calculated by dividing the average weight gain (by pen or individual bird) by the number of days between sampling. Feed conversion ratio (FCR) was determined by dividing the total feed intake by total weight gain. For Studies 5 & 6, daily feed intake and body weight gain was measured using computerised scales.

Each bird was individually assessed for lameness (Studies 4 & 5) prior to selection using a 5 point qualitative gait score system after the method of Leterrier *et al.* (2008). Briefly, this

involved classifying birds based on walking ability from 0 (no detectable gait abnormality) to 4 (severe gait defect), and only birds which scored 0 were selected. Birds in Study 4 were also scored for the incidence of pododermatitis (Appendix 1) based on the welfare quality assessment protocol for poultry (Welfare Quality, 2009). Briefly, the feet of each bird were inspected and given a score based on the severity of pododermatitis ranged from 0 (no evidence of pododermatitis), 1 & 2 (minimal pododermatitis) and 3 & to 4 (severe pododermatitis).

2.4.2. Duodenal tissue, digesta, and bone samples

Birds were culled by cervical dislocation on each sampling day by a trained person before duodenal tissues, digesta or bones were collected.

2.4.2.1 Duodenal tissues

In Study 7, duodenal tissues were collected from 2 birds per replicate pen at days 4, 18 and 28 for mRNA isolation and quantification. Dissecting utensils were baked at 240°C overnight before use and work surface cleaned with RnaseZap (Life Technologies, UK). 100mg duodenal tissue sample was cut from the middle of the duodenal loop and stored at -20°C in RNAlater (Life Technologies, UK) until further processing.

2.4.2.2. Digesta

Before digesta was collected, care was taken to ensure birds had access to feed for a minimum of 1h to ensure gut fill. Digesta was collected post-mortem by gently squeezing along the distal end of the small intestine identified as the portion between the Meckel's diverticulum and the ileal-ceco-colonic junction. Digesta was pooled per pen into labelled pots and stored at -20°C until further processing. At processing, they were freeze-dried (LTE Scientific, Oldham, UK) for 5 days. Dried samples were finely ground in a mortar to ensure homogeneity.

2.4.2.3. Bone samples

Tibia bones were separated from the feet at the tibiotarsal junction, and from the femur at the tibiofemoral junction. The femur was separated from the hip by carefully dislocating it with the aid of a scalpel where necessary. The bones were individually stored in sealed plastic zip-lock bags at -20°C until further processing. At processing, bones were completely thawed at room temperature before all adhering tissues including fibula were removed following one of 2 processing methods. Bones were either manually removed using laboratory scalpels whilst ensuring cartilage caps were kept intact (Studies 1, 3 - 8), or were autoclaved (Study 2) at 121°C and 15bars for 15 minutes (Boxer Laboratory Equipment, UK) and then allowed to cool to room temperature before manually removing adhering flesh from bones whilst preserving cartilage caps.

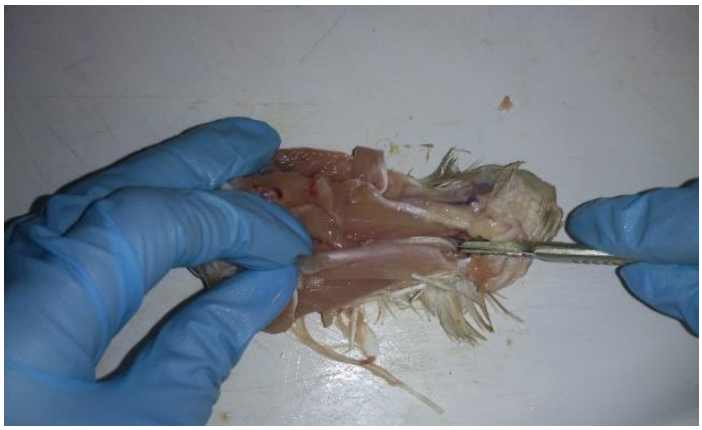

Figure 2.1. Removal of adhering flesh from the tibia bone carefully done to ensure no cut is made through the cartilage caps at both ends

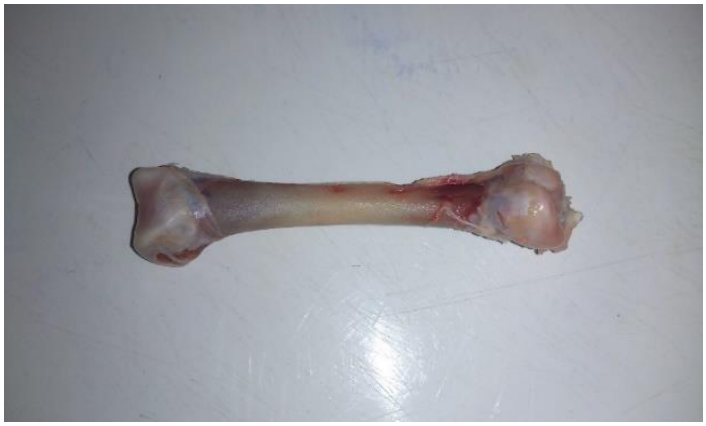

Figure 2.2. Tibia bone completely defleshed with cartilage caps intact

2.4.2.4. Whole bird sample preparation

In Study 6, birds from a mixed flock raised in commercial farms were euthanised by cervical dislocation at one of 3 sampling points: days 14, 28 and 36. Six birds were collected per bone type studied (tibia, femur, and feet) on each sampling day making a total of 18 birds per sampling day. At processing, birds were initially weighed to determine live bird weight. The digestive tract was opened to empty the digestive content in the crop, proventriculus, gizzard, intestines, and caeca after which the organs were rinsed with ultrapure water. Right and left bones (for each bone type studied) were removed from the related bird for onward processing as described in Section 2.4.2.3. The carcass of each bird (whole bird excluding bones) was then cut into small pieces and stored at -20°C until further processing.

At processing, the carcass of each bird was thoroughly minced from slightly frozen using an electric mincer (Andrew James, Durham, UK) 4 times until a homogeneous mix was obtained. The mincer was thoroughly cleaned after each bird was minced to prevent carry over between bird samples. Minced birds were stored at -20°C until further processing.

2.5. Analytical procedures

2.5.1. Bone morphometry and strength

After defleshing, each tibia and femur bone was weighed to determine fresh bone weight accurate to 4d.p. using an analytical scale (Sartorius, UK). Feet was measured for fresh. The length and width of the tibia and femur were measured using a set of Vernier callipers. Tibia and femur strength was measured using a 3 point-bend TA-XT Plus Texture Analyser (Stable Micro Systems, UK; Figure 2.3), configured with a 50kg load cell and a test speed of 1.0mm/s, after the method of Shaw *et al.* (2010). The bones were supported on a fixture which was adjusted to match bone length which varied with age. Computer generated data for peak force in Newtons was then recorded.

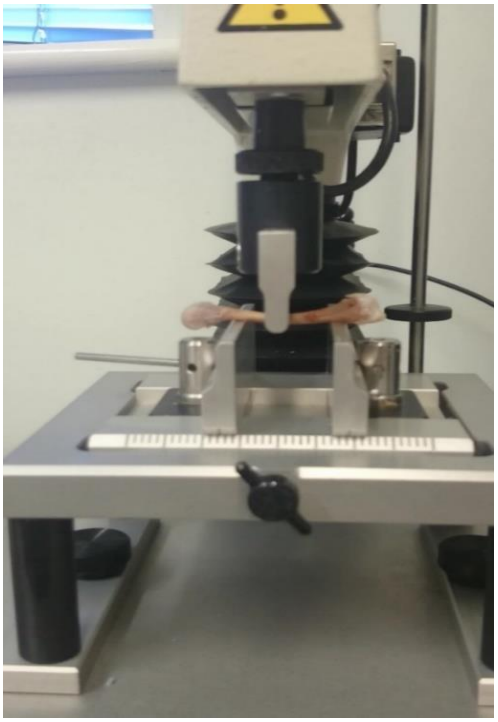

Figure 2.3. TA-XT Plus Texture Analyser

2.5.2. Dry matter

Whole bones (tibia, femur, and feet), 5 - 10g of diets, or 70 - 100g of the whole minced body were carefully weighed into pre-weighed crucibles to determine fresh weight. They were then dried at 105°C in an oven until constant weight was achieved. Dried samples were re-weighed after they had cooled down in a desiccator. Moisture content was determined by the following formula:

$$\text{Moisture content (\%)} = \frac{\text{fresh sample weight (g)} - \text{dry sample weight (g)}}{\text{fresh sample weight (g)}} \times 100$$

Dry matter (%) = 100 - moisture content (%)

2.5.3. Crude protein

Diets were analysed for nitrogen content using the Kjeldahl method (AOAC official method 2001.11). Approximately 1g of a sample was accurately weighed into distillation tubes, into which copper and selenium catalyst tablets (Fisher Scientific, UK) were also added. This was done in duplicate and starch was used as a blank. 12.5ml concentrated nitrogen-free sulphuric acid was then added to the distillation tubes and heated in a digestion unit (1007 Digester, Foss Tecato, UK) at 450°C for 45 minutes. Upon complete digestion of the samples, the distillation tubes were left to cool for 30 minutes after which 75ml distilled water was added to each tube. 50ml of 10M NaOH was then added to the samples by a distillation unit (2100 Kjeltec, Foss Tecato, UK) and distilled for 3 minutes. During the process, liberated ammonia was captured into 25ml of 4% boric acid pH indicator solution which was previously measured into conical flasks. This caused a colour change from orange to blue. The resultant alkali solution was then titrated with 0.1M HCl using a burette and care was taken to record the volume of HCl required to neutralise the solution (indicated by a colour change from blue to rose pink).

The following calculations were used to determine % crude protein:

% nitrogen = 1.4 x (V_1 – V_2) x M/W

% crude protein = 6.25 x % nitrogen

Where:
W = original sample weight
V_1 = volume of acid used to titrate the sample
V_2 = volume of acid used to titrate the blank
M = molarity of acid

2.5.4. Extractable fat

The extractable fat in diets, bones and the whole body of birds was done using either a conventional Soxhlet apparatus (Studies 1- 4, 7) or a rapid Soxhlet apparatus (Studies 5, 6 & 8).

2.5.4.1. Soxhlet fat extraction

5g of dried diet, dried whole tibia or femur, or dried whole bird sample were inserted in thimbles which were placed in a fat extraction apparatus (Figure 2.4). A clean dry 250ml round bottom flask which also contained some anti-bumping granules was carefully weighed. 150ml petroleum ether (CAS 64742-49-0, Fisher Scientific, UK) was then carefully poured into the round bottom flask and connected to the fat extraction apparatus. The solvent was refluxed for 6h (or otherwise stated in the methodology study, Chapter 3) by heating the solvent on an electric heating mantle (Electrothermal, Staffordshire, UK) above its boiling point. The round bottom flasks with the remaining petroleum ether in the flasks were then placed on a hot plate to evaporate off the solvent. Flasks were then dried in an oven for 2h set at 105°C until constant weight was achieved. The flasks including content (fat and anti-bumping granules) were weighed after they had cooled down in a desiccator.

Fat was determined using the following formula: % extractable fat = $[(M_2 – M_1) \div M_O] \times 100$

Where:
M_O = weight of sample (g)
M_1 = weight of flask + anti-bumping granules (g)
M_2 = weight of flask + fat + anti-bumping granules (g)

Figure 2.4. Soxhlet fat extraction apparatus

2.5.4.2. Soxtherm fat extraction

The Soxtherm fat extraction system (Gerhardt, UK; Figure 2.5) is based on the conventional principles of the traditional Soxhlet fat extraction method. Clean dry extraction flasks which had boiling stones were accurately weighed at the start of the fat extraction process. 5g of dried diet, dried bone, or 10g of dried minced whole bird was weighed and inserted into extraction thimbles which were then placed in fat extraction beakers.

The fat extraction process took a total of 2 hours and constituted of the following programmable steps:

a. Hot extraction phase: 170ml petroleum ether (CAS 64742-49-0, Fisher Scientific, UK) was poured into the extraction flask containing dried samples and brought to boil at 150°C for 30 minutes. Fat was liberated from the sample during this process.
b. Evaporating phase A: the level of the solvent was lowered below the extraction thimble. Excess solvent was collected in the rear solvent recovery tank.
c. Extraction phase: petroleum ether was then refluxed for 1h to further extract fat from the samples.
d. Evaporating phase B: the remaining solvent was distilled and collected in the rear solvent recovery tank.
e. Evaporating phase C: a further recovery of the remaining solvent which was distilled and collected at the rear solvent recovery tank.

The extraction flasks with remaining petroleum ether and boiling stones were placed on a hot plate to evaporate off the solvent. Flasks were then placed in an oven for 2h set at 105°C until constant weight was achieved. Flasks including content (fat and boiling stones) were weighed after they had cooled down in a desiccator. Fat was determined using the following formula:

$$\% \text{ extractable fat} = [(M_2 - M_1) \div M_O] \times 100$$

Where:
M_O = weight of sample (g)
M_1 = weight of flask + anti-bumping granules (g)
M_2 = weight of flask + fat + anti-bumping granules (g)

Figure 2.5. Soxtherm fat extractor (Gerhardt, UK)

2.5.5. Ash content

2.5.5.1. Diets and digesta

Ash content of diets and digesta were determined by weighing 5 - 10g of dried samples into pre-weighed crucibles. These were then placed into a muffle furnace (Nabertherm, Germany) for 24h at 650°C. Ashed samples were cooled in a desiccator and re-weighed. Ash weight was determined by weighing the ash residue accurate to 4 d.p using an analytical balance (Sartorius, UK).

2.5.5.2. Bones and whole body

Ash content of bones was determined after the tibia and femur bones were defleshed and dried at 105°C for 24h. Fat was extracted from the dried bones in Studies 1- 4, & 7 by refluxing petroleum ether for 1 - 6h, using a conventional Soxhlet apparatus as described in the particular studies. The Soxtherm fat extraction apparatus was used in Studies 5, 6 & 8 to extract fat from bones and whole dried bird due to its rapid processing time and solvent

recovery features. Feet were dried at 105°C for 24h without any prior fat extraction. Following fat extraction, the bones were dried at 105°C for 24h, cooled in a desiccator, weighed and placed in into pre-weighed crucibles. These were then ashed in a muffle furnace (Nabertherm, Germany) for 24h at 650°C.

Ash content of whole bird was determined using a similar procedure. Representative aliquots of thoroughly minced whole bird (70 - 100g per bird) were first dried for 4 days at 105°C until constant weight was achieved. They were then extracted of fat using the Soxtherm fat extraction apparatus as described in Section 2.5.4.2. Samples were then dried at 105°C for 2 days prior to ash determination. 10g samples were ashed in a muffle furnace (Nabertherm, Germany) for 9h at 450°C, and then for a further 15h at 650°C.

Ashed samples were cooled in a desiccator and reweighed to determine the ash weight. Ash % was determined using the following formula:

$$\text{Ash \%} = \frac{\text{Dry sample weight} - \text{Ash sample weight}}{\text{Fresh sample weight}} \times 100$$

2.5.6. Titanium dioxide

In Studies 7 & 8, 0.5% titanium dioxide (TiO_2) was included in diets as an inert marker for digestibility studies. It was analysed in diets and digesta using a UV spectrophotometer after the method of Short *et al.* (1996). Standard titanium solution (0.5mg ml^{-1}) was prepared by dissolving 250 mg TiO_2 in 100ml concentrated H_2SO_4 (Fisher Scientific, UK) which was brought to 500ml by adding distilled water. 1-10ml of the TiO_2 solution was poured into 100ml volumetric flasks labelled 1-10. Concentrated H_2SO_4 was then added to each flask to reach a total volume of 10ml, to which 10ml 30% hydrogen peroxide (Fisher Scientific) was

added. The volumetric flasks were then brought to volume with distilled water and stored in darkness in glass vials.

0.3 - 0.5g of feed or freeze dried digesta were weighed in duplicates into ceramic crucibles and ashed in a muffle furnace (Nabertherm, Germany) for 14h at 650°C. After cooling in a desiccator, 10ml 7.4M H_2SO_4 was pipetted into each crucible and heated on a hot plate for 2h when the samples were completely dissolved. Additional extra 5ml H_2SO_4 was added to the samples during this process if required.

Cooled down samples were filtered through Whatman 541 hardened ashless filter papers into 100ml volumetric flasks. 10ml of 30% hydrogen peroxide was added to each volumetric flask which was then brought to volume by adding distilled water. The absorbance of the samples and standards was read at 410nm using a UV spectrophotometer (Unicam Helios, USA). A regression analysis of the standard curve was performed to obtain the coefficient used in determining TiO_2 concentration. The amount of TiO/mg in the solution was calculated using the following equation:

$$TiO_2/mg = \frac{\text{Absorbance x 100}}{\text{Coefficient x sample weight (mg)}}$$

2.5.7. Calcium and phosphorus

The calcium and phosphorus content in the diets, digesta, tibia, femur and feet ash were analysed for calcium and phosphorus using an inductively coupled plasma optical emission spectrometry (ICP-OES) assay (Optima 2100 DV, PerkinElmer, USA). All glassware used were soaked in 1% nitic acid overnight to remove all mineral contamination. 0.5g of each sample was incubated in duplicate with 10ml aqua regia (made up by mixing 1 part HNO_3 with 3 parts HCl) in 50ml flasks conical and incubated for 16h. Samples were then boiled for

90 minutes and then cooled down for 30 minutes. 5ml aqua regia was then added to the samples and boiled for another 60 minutes. Cooled down samples were filtered through Whatman 541 hardened ashless filter papers into 50ml volumetric flasks. Ultrapure water was used to rinse the conical flasks 3 times after which the volumetric flasks were brought to volume (50ml). These were thoroughly mixed and transferred into 15ml tubes.

Ca standards (10 - 1000ppm) and P standards (0 - 350ppm) were prepared by diluting 1000ppm ICP-OES standards (Fisher Scientific, UK) with ultra-pure water and used for ICP-OES analysis. The Ca and P content of the samples were analysed by ICP-OES set at a wavelength of 317.933nm for Ca and 213.617nm for P. Analysed Ca and P emission intensities of standards and samples were recorded. Standard curves were determined by regressing Ca and P emission intensities against the range of standards prepared. Mineral concentration (ppm) was calculated by the following formula:

$$\text{Mineral conc. (ppm)} = \frac{\text{Dilution volume (ml)}}{\text{Sample weight (g)}} \times \frac{\text{MEI} - \text{Intercept}}{\text{Gradient}}$$

$$\text{Mineral conc. (g/kg)} = \text{Mineral conc. (ppm)} \div 1000$$

Where:
Mineral conc. = mineral concentration of Ca or P
Dilution volume = final volume in which samples were dissolved, i.e. 50ml
Sample weight = weight of sample analysed
MEI = Ca or P mineral emission intensity analysed by ICP-OES
Intercept = intercept of the standard curve of Ca or P
Gradient = gradient of the standard curve of Ca or P

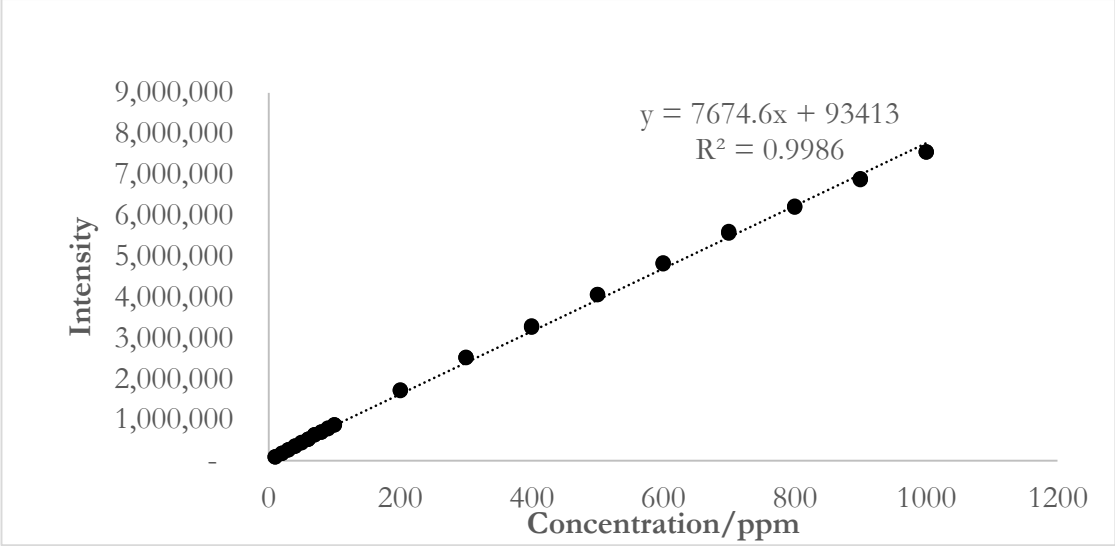

Figure 2.6. Example of a standard curve for Ca emission intensity

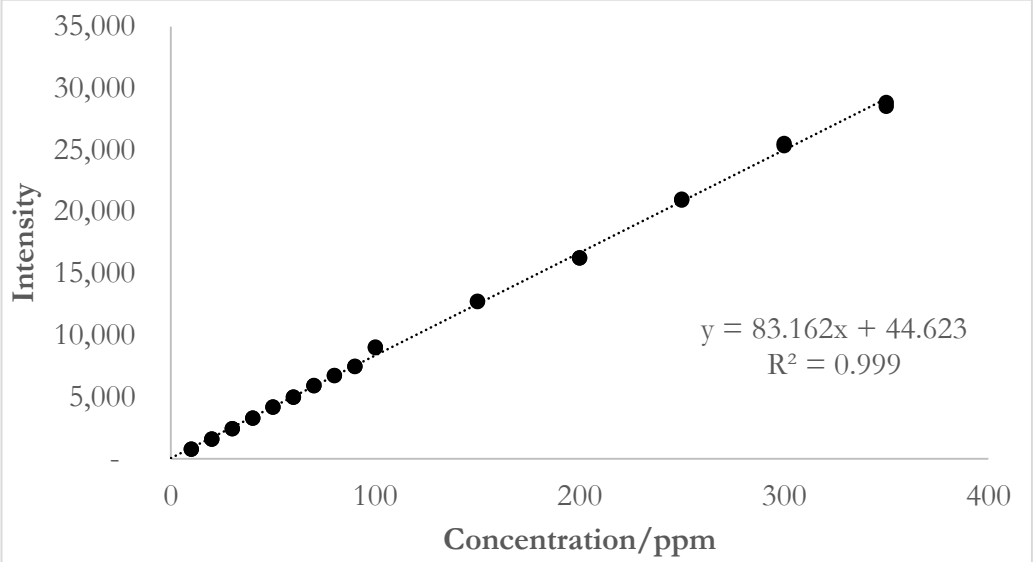

Figure 2.7. Example of a standard curve for P emission intensity

2.5.8. Total phytate

The total phytic acid of ileal digesta collected from birds in Studies 7 & 8; and trial diets including feed ingredients used in Study 8 were analysed for total phytic acid using a Megazyme™ K-PHYT assay (Megazyme International, Ireland).

Sample extraction was done by carefully adding 20mL 0.66M HCl to 1g of accurately weighed feed, diet or ileal digesta. These were stirred with a magnetic stirrer for a minimum of 3h. 1mL of the resulting solution was pipetted into a 1.5mL microfuge tube and centrifuged for 10 minutes at 13,000rpm. 0.5mL of the resulting extract supernatant was then transferred into a clean unused microfuge tube to which 0.5mL 0.75M NaOH was added to neutralise the sample.

An enzymatic dephosphorylation reaction was carried out for free and total phosphorus as follows. For free phosphorus, 0.62mL ultra-pure water, 0.20mL acidic buffer solution (pH of 5.5 containing sodium azide 0.02% w/v as preservative) and 0.05mL neutralised sample were pipetted into fresh 1.5mL microfuge tube. These were then mixed by vortex and incubated at 40°C for 10 minutes in a water bath. 0.02mL ultra-pure water and 0.20mL of an alkaline buffer (pH of 10.4 containing $MgCl_2$, $ZnSO_4$ and sodium azide 0.02% w/v as a preservative) were then added, mixed by vortex and incubated at 40°C for 15 minutes in a water bath. 0.3mL trichloroacetic acid (50% w/v) was added to terminate the reaction. For total phosphorus, 0.6mL ultra-pure water, 0.20mL acidic buffer solution (of pH 5.5 containing sodium azide 0.02% w/v as preservative), 0.05mL neutralised sample and 0.02mL phytase suspension were pipetted into fresh 1.5mL microfuge tube. These were then mixed by vortex and incubated at 40°C for 10 minutes in a water bath. 0.20mL of an alkaline buffer (of a pH of 10.4 containing $MgCl_2$, $ZnSO_4$ and sodium azide 0.02% w/v as a preservative) and 0.02mL alkaline phosphatase suspension were then added, mixed by vortex and incubated at 40°C for 15

minutes in a water bath. 0.3mL trichloroacetic acid (50% w/v) was added to terminate the reaction.

The terminated reactions for free and total phosphorus were centrifuged for 10 minutes at 13,000rpm. 1mL of supernatants were carefully pipetted into a 1.5mL microfuge tube to which 0.50mL colour reagent (made by mixing 5 parts of ascorbic acid 10% w/v / 1M H_2SO_4 with 1 part ammonium molybdate 5% w/v) was added.

Standards were prepared by diluting phosphorus standard solution (50µg/mL with 0.02% w/v sodium azide preservative) with ultrapure water into 15mL tubes as follows:

Sample	**Standard 0 (0 µg)**	**Standard 1 (0.5 µg)**	**Standard 2 (2.5 µg)**	**Standard 3 (5 µg)**	**Standard 4 (7.5 µg)**
Distilled water (mL)	5.00	4.95	4.75	4.50	4.25
Phosphorus standard (mL)	-	0.05	0.25	0.5	0.75

1mL of made up standards and 0.5mL colour reagent were pipetted in duplicates into 1.5 microfuge tubes. All samples including standards were incubated for 1h at 40°C, mixed by vortex, transferred into cuvettes and read at 655nm on a UV-VIS spectrophotometer (Unicam Helios, USA).

The absorbance of each phosphorus standard was determined by subtracting absorbance of standard 0 from the other standards (1 - 4).

Factor M ($\mu g/\Delta A_{phosphorus}$) was determined using the formula:

$$M = \frac{P\ (\mu g)}{\Delta A_{phosphorus}}$$

Mean value for M ($\mu g/\Delta A_{phosphorus}$) was calculated using the formula:

$(M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4}) \div 4 \quad [\mu g/\Delta A_{phosphorus}]$

Where STD = standard.

Concentration of phosphorus (c) was calculated using the following formula:

$$c = \frac{\text{mean M} \times 20 \times F}{10{,}000 \times 1.0 \times v} \times \Delta A_{phosphorus} \quad [g/100g]$$

Where:
Mean M = mean value of phosphorus standards [$\mu g/\Delta A_{phosphorus}$]
20 = original sample extract volume (mL)
F = dilution factor
ΔA = absorbance change of sample
10,000 = conversion from μg/g to g/100g
1.0 = weight of original sample matter (g)
v = sample volume (used in the colorimetric determination step)

It follows for phosphorus:

$$c = \frac{\text{mean M} \times 20 \times 55.6}{10{,}000 \times 1.0 \times 1.0} \times \Delta A_{phosphorus} \quad [g/100g]$$

$$= \text{mean M} \times 0.1112 \times \Delta A_{phosphorus} \quad [g/100g]$$

$$\text{Phytic acid content} = \frac{\text{phosphorus [g/100g]}}{0.282}$$

The calculation of phytic acid content assumes that the amount of phosphorus measured is exclusively released from phytic acid and that this comprises 28.2% phytic acid (Kumari *et al.,* 2014). These calculations were done using the Mega-Calc™ software tool available for free from the Megazyme website (www.megazyme.com).

2.5.9. Nutrient digestibility

Titanium dioxide concentration was determined by UV spectrophotometer described in Section 2.5.6, after the method of Short *et al.* (1996). Ca and P content of diets, feed, ileal digesta and bones were determined by ICP-OES described in Section 2.5.7. The following

calculations were used in determining ileal digestibility coefficient and amount of nutrient digested, after the method of Mutucumarana *et al.* (2014).

$$\text{The apparent ileal digestibility coefficient} = 1 - \frac{(\text{Ca or P in digesta} \times \text{TiO}_2 \text{ in diet})}{(\text{TiO}_2 \text{ digesta} \times \text{Ca or P diet})}$$

Ca or P digested = apparent ileal digestibility coefficient x Ca or P in diet

2.5.10. Energy determination

Gross energy (GE) content of diets, digesta and excreta was determined using a bomb calorimeter (Instrument 1261, Parr Instruments, Illinois, USA). Approximately 1g sample was mixed with small amount of water and made into pellets with a pellet press (Parr Instruments, USA). The pellets were dried overnight at 105°C in a drying oven before being weighed into tin crucibles and placed in the bomb calorimeter (Parr Instruments, USA). 10cm of fuse wire was threaded through the holes in the bomb with the electrodes attached, ensuring the wire was in contact with the pellet. The bomb was then assembled, ensuring the top was tightly screwed on, and then filled with oxygen. The bomb was then carefully placed into the calorimeter bucket of water filled with 2L of water, and the lid of the bomb jacket was shut, and the process was started. The pellets were exploded in the calorimeter and the energy produced was measure in MJ/kg.

Apparent metabolisable energy (AME) was calculated by:

GE diet - (GE digesta x (TiO_2 in the diet/TiO_2 in digesta))

The nitrogen content of the digesta was analysed by Dumas method, and metabolisable nitrogen was calculated using the following equation:

Diet N – Digesta N x (Diet Ti/Digesta Ti)

The apparent ileal metabolisable energy was also corrected to zero N balance (AMEn) using the figure of 34.4 kg/g N retained as detailed by Hill and Anderson (1958).

2.5.11. Gene expression of sodium-phosphate cotransporter IIb (NaPi-IIb)

2.5.11.1. Isolation and quantification of NaPi-IIb total mRNA

100g tissue was collected from duodenal tissue samples and stored in RNAlater at -80ºC until further processing. At processing, tissue samples were homogenised in 1ml Tri-Reagent (Life Technologies) with 5mm stainless steel bead (Qiagen) in the Qiagen Tissue Lyser II. Phase separation was performed using molecular grade 1-bromo-3-chloropropane (Sigma). RNA was then purified from the aqueous phase using the MagMAX-96 for Microarray Isolation kit (Life Technologies) and the RNA subsequently stored at -20°C. The concentration of RNA was determined spectrophotometrically using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific). RNA (5µl) was reverse-transcribed into cDNA using 20µl RT premix 2 (Primerdesign). The reaction was performed at 42°C for 20 minutes and 72°C for 10 minutes. cDNA was stored at -20°C until used in the PCR reaction. The NaPi-IIb gene-specific primers were designed by Primerdesign Ltd, UK. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene.

The primers for NaPi-IIb were as follows:

Forward, 5'- AATAGCGTTGATAGATGAGACAAGG-3'
Reverse, 5'- AATCCCATAGAGTACACGAATGATTT-3'

2.5.11.2 Quantitative Real-Time PCR

Quantitative Real-Time PCR was performed using Stratagene Mx3005p (Agilent Technologies). PCR was carried out in duplicates in Stratagene PCR plates (Agilent Technologies) under the following conditions: 95°C for 2 minutes, 40 cycles of 95°C for 10s

and 60°C for 1 minute. 10µl cDNA was diluted in 90µl PCR water (Solis BioDyne) and 5µl diluted cDNA used in each PCR reaction. 1µl of each primer/probe mix was combined with 10µl PrecisionPLUS Mastermix and 4µl PCR water (all from Primerdesign). Relative gene expression was determined using the comparative cyclic threshold (C_T) method of Livak and Schmittgen (2001).

2.6. Data analysis

All data were analysed using IBM SPSS statistical software, version 23 (IBM Statistics, 2016). Normality of data was confirmed -by performing the Kolmogorov-Smirnov test. Levene's test to assess the equality of variances between treatment groups. Details of the statistical methods used are described in the relevant sections. All data were considered statistically significant when $P < 0.05$.

CHAPTER 3: EVALUATING THE BONE ASH METHODOLOGY USED IN ASSESSING PHOSPHORUS MINERALISATION IN BROILERS

3.1. Introduction

There have been significant improvements in the growth rate of commercial broilers (Havenstein *et al.,* 2003b; Gous, 2010) as breeding companies continue to intensively select for rapid growth and high meat yield (Druyan *et al.,* 2007; Talaty *et al.,* 2010). However, the improved growth rate has had a negative impact on metabolic disorders with an increase in the incidences of skeletal deformities (Lilburn, 1994; Angel, 2007; Buzala *et al.,* 2014). Consequently, the economic cost incurred from increased mortality during rearing and transportation, and rejects or downgrades at processing plants remains a major challenge in commercial poultry production (Rath *et al.,* 2000; Driver *et al.,* 2006a; Talaty *et al.*, 2009; Shim *et al.,* 2008).

Phosphorus is required for adequate skeletal development, and it is widely researched in broiler nutrition (Waldroup *et al.,* 2000). The low availability and variability of phytate phosphorus from plant sources necessitates the inclusion of additional inorganic phosphorus in the feed (Perney *et al.,* 1993; Watson *et al.,* 2006), often at levels which exceed requirement in order to provide a margin of safety (Dhandu and Angel, 2003). However there are concerns regarding the rapid depletion of the world phosphate reserves (Cordell *et al.,* 2009), and the detrimental effects of undigested dietary phosphorus to the environment (Sharpley, 1999; Dilger *et al.,* 2004). This underscores the importance of optimising the utilisation of dietary phosphorus in poultry whilst avoiding any compromise on skeletal integrity. To this end, an accurate evaluation of the effect of dietary phosphorus on the skeleton is required.

Bone mineralisation is routinely used to assess phosphorus availability in poultry. It has been demonstrated that broilers respond to increase in dietary phosphorus with an increase in tibia

ash weight (Venäläinen *et al*., 2006); and a corresponding reduction in tibia ash percentage when dietary phosphorus is reduced (Watson *et al*., 2006). However, there is a lack of up-to-date bone mineralisation data derived from commercial broiler production in the literature. The various bone measurements that have been used to evaluate bone mineralisation in broilers include: bone mineral content (Shang *et al.,* 2015), bone ash concentration (Shastak *et al.,* 2012a), bone densitometry (Barreiro *et al.,* 2009), bone breaking strength (Shaw *et al.,* 2010), and bone ash (Atteh and Leeson, 1983). However, according to the report of Sullivan and Douglas (1990) cited in the work of Qian *et al.* (1996), bone ash is the preferred criterion by researchers due to its simplicity. Different bone types have been examined by various investigators for ash content. These include the femur (Dickey *et al*., 2012), toe (Karimi *et al*., 2013), foot (Garcia and Dale, 2006; Shastak *et al*., 2012a) and tibia (Olukosi and Fru-Nji, 2014). Tibia ash is the most common bone used in evaluating bone mineralisation in poultry research (Hall *et al*., 2003).

Only very few studies have directly characterised bone morphology and mineralisation in poultry (Bond *et al.,* 1991; Skinner and Waldroup, 1995; Williams *et al.,* 2000b; Applegate and Lilburn, 2002), but the publication dates and experimental rearing conditions (small pens or cages) suggest the bone data reported from these studies may not be applicable to current commercial practices. It was therefore of interest to characterise the range of tibia bone mineralisation found in a current strain of healthy, commercially reared broilers to create a database of benchmarks applicable to current commercial practice. This was examined in Chapter 4, but before embarking on that study, it was important to evaluate the methodology for evaluating bone ash in broilers.

As already discussed in Chapter 1, Section 1.4.1.1.1; a review of the available literature (Table 1.3) shows common divergences in the various processing methods employed in the tibia

bone ash methodology. For example, it is necessary to remove all adhering flesh from the bones, and this is usually done by manual excision. However, this a laborious and time-consuming process and therefore different processing methods have been employed to make the process easier. These include enzymatic maceration techniques (Shastak *et al.,* 2012a), autoclaving (Boling-Frankenbach *et al.,* 2001; Kim *et al.,* 2008) and boiling (Ruiz-Feria *et al.,* 2014). Another divergence in the bone processing method prior to ash determination is the inclusion of the cartilage caps of the defleshed bones (Lumpkins and Batal, 2005), or removing them (Snow *et al.,* 2004; Angel *et al.,* 2006). Extracting fat from the bones prior to ash determination is a commonly cited methodology, although this step has been omitted in some previous research (Qian *et al.,* 1996; Baird *et al.,* 2008). It is also common practice not precisely state what procedures were followed when extracting fat from the bones.

3.1.2. Aims and objectives

The overall aim of this study was to investigate four common divergences in the tibia bone processing methods employed prior to ash determination in poultry, and to examine the sensitivity of these procedures in identifying bone ash responses.

The specific objectives were:

- To examine the effect of fat extraction on tibia ash content.
- To investigate the effect of including cartilage caps on fat extracted tibia ash.
- To investigate the effect of increasing fat extraction time on fat extraction efficiency.
- To evaluate the effect of autoclaving prior to fat extraction on tibia ash content.

The hypothesis of these series of studies was that different bone processing method would result in different bone ash values.

3.2. Materials and methods

3.2.1. Bird husbandry and trial design

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny *et al.*, 2010) were followed, and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee. Two trials were conducted to evaluate the effect of 4 divergences in the tibia bone processing methods employed prior to ash determination.

Ross 308 day-old male broiler chicks were sourced from PD Hook Hatcheries Ltd, Oxfordshire. On arrival, the birds were allocated to pens (0.64m^2). The birds were allocated in such a way that they were evenly distributed by weight across treatments. The birds were raised in-house in a thermostatically controlled room and provided with an initial room temperature of 32°C which was gradually reduced to 21°C by day 21 and maintained till the end of the experiment. Lighting was provided with 1h darkness from day 1 and was increased by 1h a day to 6h, then maintained until the end of the experiment. Wood shavings were spread on the pen floors (approximately 3cm thick) and was topped up as required during the course of each trial. Experimental diets and water were provided *ad libitum*, and birds were checked twice daily to monitor birds and environmental conditions. Mortalities were recorded along with the date and weight of the bird and reason if culled.

In Trial 1, 288 day-old male broiler chicks were fed one of six mash type diets which varied in phosphorus and phytase content (Tables 3.1 and 3.2), formulated to produce a range of bone ash content. Each diet was allocated to 8 replicate pens, with 6 birds allocated per replicate pen. On day 35, 2 birds per replicate pen were euthanised by cervical dislocation, and right and left tibia bones removed and individually stored at -20°C until further processing. In Study 1, the effect of fat extraction prior to bone ash determination was examined by

comparing fat extracted right tibia bones including cartilage caps (n = 98) with un-extracted left tibia bones including cartilage caps (n = 98). The right fat extracted tibia bones examined in Study 1 were used to evaluate the effect of including cartilage caps in Study 2.

In Trial 2, 264 day-old male broiler chicks were fed a standard commercial mash diet (Tables 3.3 and 3.4). The diet was formulated to meet or exceed the Ross 308 nutrient requirements (Aviagen, 2014) and 8 birds per pen were allocated to 33 replicate pens. On day 42, 1 bird per pen was euthanised by cervical dislocation and right and left tibia bones removed and individually stored at -20°C until further processing. Right tibia bones including cartilage caps (n = 33) were used in Study 3 to examine the effect of fat extraction time on fat extraction efficiency using the Soxhlet fat extraction method. In Study 4, the effect of autoclaving bones prior to fat extraction on bone ash content was examined. Left tibia bones including cartilage caps (n = 33) were autoclaved before fat extraction and bone ash determination, and were compared with the ash content of fat extracted right tibia bones obtained in Study 3.

Table 3.1. Feed composition of the experimental diets (g/kg as fed basis), Trial 1

Ingredient	Starter (1 - 10d)	Grower 1 (10 - 20d)	Grower 2 (20 - 26d)	Finisher (26 - 35d)
Corn	400	270	260	250
Wheat	215.79	331.86	363.38	379.83
Wheat meal		20		17
Soya - HiPro	131	240	238	149
Soya pellets	186			56
Rapeseed meal		60	30	20
Rapeseed expeller			30	50
Vegetable oil	20	46	49	50
Limestone, fine	12.1	11.4	9.2	10.3
Ca-Na-P fine	3	5	7.8	3.6
Monocap.	11.5	2.8		
Salt	2.4	2.0	1.5	1.8
Sodium bicarbonate	1	0.9	0.9	1.3
[1]Additives/Premix	17.21	10.04	10.22	11.17

[1]Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 µg; folic acid, 1.5 mg; and biotin 125 mg

Table 3.2. Calculated feed composition of the experimental diets (g/kg as fed basis), Trial 1

Treatment group	Phytase inclusion/kg	Diet composition	Starter	Grower 1	Grower 2	Finisher
		Ca	10	8	7.5	6.5
		[1]Crude protein	220.2	219	218	216
		[1]Ether extract	79	88	92	94
		[1]Ash	5.6	49	50	47
Control	0 FTU	Total digestible P	4.5	3.4	3.1	2.5
		Digestible P from phytase	0	0	0	0
		Digestible P from feed	1.3	1.4	1.4	1.4
		Digestible P from mineral additive	3.2	2	1.7	1.1
Group 1	500 FTU	Total digestible P	4.5	3.4	3.1	2.5
		Digestible P from phytase	1.1	1.1	1.1	1.1
		Digestible P from feed	1.3	1.4	1.4	1.4
		Digestible P from mineral additive	2.1	0.9	0.6	0
Group 2	500 FTU	Total digestible P	4.5	2.8	2.5	2.5
		Digestible P from phytase	1.1	1.1	1.1	1.1
		Digestible P from feed	1.3	1.4	1.4	1.4
		Digestible P from mineral additive	2.1	0.3	0	0
Group 3	500 FTU	Total digestible P	4.5	2.5	2.5	2.5
		Digestible P from phytase	1.1	1.1	1.1	1.1
		Digestible P from feed	1.3	1.4	1.4	1.4
		Digestible P from mineral additive	2.1	0	0	0
Group 4	750 FTU	Total digestible P	4.7	2.7	2.7	2.7
		Digestible P from phytase	1.3	1.3	1.3	1.3
		Digestible P from feed	1.3	1.4	1.4	1.4
		Digestible P from mineral additive	2.1	0	0	0
Group 5	1000 FTU	Total digestible P	4.8	2.8	2.8	2.8
		Digestible P from phytase	1.4	1.4	1.4	1.4
		Digestible P from feed	1.3	1.4	1.4	1.4
		Digestible P from mineral additive	2.1	0	0	0

[1]Analysed.

Table 3.3. Feed composition of experimental diets (g/kg as fed basis), Trial 2

Ingredient	Starter (1 - 14d)	Grower (15 - 28d)	Finisher (29 - 42d)
Wheat - Feed	585.2	612.4	694.8
Soybean meal 48	351.5	317.8	240.1
Soy oil	15.9	31.7	28.7
Salt	4.3	4.0	4.2
DL-Methionine	3.3	2.0	1.3
Lysine HCl	2.5	0.5	0.3
Threonine	0.9		
Limestone	13.3	11.2	10.3
Dicalcium phosphate	18.0	15.3	15.3
[1]Vitamin premix	4.0	4.0	4.0

[1]Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 μg; folic acid, 1.5 mg; and biotin 125 mg

Table 3.4. Analysed feed composition of the experimental diets (as fed basis), Trial 2

Diet	[1]DM (%)	Ash (%)	[2]GE (MJ/kg)	Protein (%)	Fat (%)	Ca (g/kg)	P (g/kg)
Starter	88.1	5.11	18.61	24.99	6.14	12.85	6.70
Grower	88.1	4.43	19.02	23.18	7.34	11.69	5.59
Finisher	88.1	4.10	20.30	20.37	6.09	8.75	4.32

[1]Dry matter
[2]Gross energy

3.2.2. Bone processing

All tibia bones were processed as previously described in Chapter 2, Sections 2.4.2.3, 2.5.2, 2.5.4.1 and 2.5.5.2. Briefly, right and left tibia bones were completely thawed at room temperature before all adhering tissues including fibula were either manually excised using laboratory scalpels, whilst ensuring cartilage caps were kept intact (Studies 1 - 3) or autoclaved at 121°C and 15bars for 15 minutes (Study 4) before removing all adhering tissues including fibula but ensuring cartilage caps were kept intact. After further processing dependent on the study as described below, bones were then ashed at 650°C for 24h in a muffle furnace to determine ash weight and ash percentage.

3.2.2.1. Study 1: the effect of fat extraction on tibia ash content

Right tibia bones including cartilage caps (n = 98) were dried, fat extracted using the Soxhlet extraction method and then dried until constant weight was achieved. The corresponding left tibia bones including cartilage caps (n = 98) were not fat extracted but dried until constant weight was achieved. Ash weight and ash percentage of the right and left bones were then compared to examine the effect of the 2 bone processing methods on ash content, and sensitivity in elucidating treatment means.

3.2.2.2. Study 2: the effect of including cartilage caps on fat extracted tibia ash content

The effect of including cartilage caps on bone ash was evaluated using the right tibia bones analysed in Study 1, by comparing ash values when cartilage caps were included, with ash values when cartilage caps were not included prior to ash determination.

3.2.2.3. Study 3: the effect of increasing fat extraction time on fat extraction efficiency

Right tibia bones including cartilage caps of birds sampled in Trial 2 (n = 33) were dried at 105°C for 24h prior to 1h fat extraction in petroleum ether using the Soxhlet extraction method to determine the amount of fat extracted. This process was cumulatively repeated 8 times in total. Additional hourly fat extracted was determined to evaluate fat extraction efficiency.

3.2.2.4. Study 4: the effect of autoclaving prior to fat extraction on tibia ash content

Autoclaved left tibia bones, including cartilage caps from birds sampled in Trial 2 (n = 33) were extracted of fat for 8h using the Soxhlet extraction method, then dried until constant weight was achieved. Bones were then ashed, and compared with ashed right tibia bones examined in Study 3.

3.2.3. Statistical analysis

Results were analysed using using IBM SPSS statistical software, version 23 (IBM Statistics, 2016). The General Linear Model for one-way between-groups ANOVA was used to analyse data obtained in Studies 1 and 2. Data were analysed in Study 3 using the General Linear Model for one-way repeated-measures ANOVA, while the independent *t*-test procedure was used to compare data obtained in Study 4. Significant differences between treatment means were elucidated using the Bonferroni procedure for Studies 1-3. Bone ash data of the different bone processing methods determined within Studies 1, 2 & 4 were compared using the independent *t*-test procedure.

The strength of relationships between bone ash data for the different bone processing methodologies was examined using the bivariate model of the Pearson correlation procedure. The interpretation of the strength between relationships was based on those of Cohen (1988): small when r = 0.1 - 0.29, medium when r = 0.30 - 0.49 and large when r = 0.50 - 1.0. Data were considered statistically significant when $P < 0.05$.

3.3. Results

From the results obtained in Study 1 (Table 3.5), although the mean un-extracted dry bone weights were significantly heavier than the extracted dry bone weights (7.17g vs. 5.66g; $P < 0.001$), mean ash weight was similar for both processing methods (2.07g vs. 2.05g; $P = 0.727$). The un-extracted bones were more sensitive in elucidating differences in treatment means for ash weight compared with extracted bones ($P = < 0.001$ and 0.009 respectively). Conversely, mean ash percentage of the fat extracted bones was significantly greater (36.50% vs. 28.74%; $P < 0.001$) than the un-extracted bones by 22%; and was also more sensitive in elucidating differences in treatment means than the un-extracted bones ($P = 0.001$ and 0.005 respectively). When the fat extracted bones were compared with un-extracted bones, a large correlation was

found for ash weight (r = 0.592), while a medium strength correlation found for ash percentage (r = 0.367).

Table 3.5. The effect of fat extraction on tibia ash content

	Mean ash weight (g)		Mean ash percentage (%)	
Treatment[1]	**+ Fat extraction**	**- Fat extraction**	**+ Fat extraction**	**- Fat extraction**
1	2.21[ab]	2.31[a]	38.70[a]	30.19[a]
2	2.29[a]	2.27[a]	38.52[ab]	29.68[ab]
3	2.05[ab]	1.90[b]	35.98[abc]	27.21[ab]
4	1.83[b]	1.77[b]	34.04[c]	26.84[b]
5	1.93[ab]	2.02[ab]	35.11[bc]	29.12[ab]
6	2.12[ab]	2.04[ab]	36.61[abc]	29.40[ab]
Mean	2.07	2.05	36.50	28.74
Pooled SEM	0.042	0.035	0.378	0.319
P value	0.009	<0.001	0.001	0.005

[1]Experimental diets.
[a-c]Means within the same column with no common subscript differ significantly ($P < 0.05$).

In Study 2, although mean ash weight was significantly greater when cartilage caps were included compared to when they were not (2.07g vs. 1.88g; P = 0.001), both bone processing methods (cartilage inclusion vs. non-inclusion) elucidated identical differences in treatment means for ash weight, Table 3.6. Ash percentage was significantly greater (44.54% vs. 36.50%; $P < 0.001$) when cartilage caps were not included compared to when they were included prior to ash determination. Interestingly when ash percentage was determined, method sensitivity was improved when cartilage caps were removed compared to when they were left on.

Table 3.6. The effect of including cartilage caps on fat extracted tibia ash content

Treatment[1]	Mean ash weight (g) + Cartilage cap	- Cartilage cap	Mean ash percentage (%) + Cartilage cap	- Cartilage cap
1	2.21 [ab]	2.00[ab]	38.70[a]	47.48[a]
2	2.29[a]	2.08[a]	38.52[ab]	46.94[a]
3	2.05[ab]	1.88[ab]	35.98[abc]	44.23[ab]
4	1.83[b]	1.68[b]	34.04[c]	41.71[b]
5	1.93[ab]	1.75[ab]	35.11[bc]	42.43[b]
6	2.12[ab]	1.92[ab]	36.61[abc]	44.43[ab]
Mean	2.07	1.88	36.50	44.54
Pooled SEM	0.042	0.039	0.378	0.409
P value	0.009	0.025	0.001	<0.001

[1]Experimental diets.
[a-c] Means within the same column with no common subscript differ significantly ($P < 0.05$).

In Study 3, the cumulative mean fat extracted from tibia bones was significantly different ($P < 0.001$) when fat was determined on an hourly basis up to 8 hours, Table 3.7. Additional hourly mean fat extracted was also significantly different up to 6 hours ($P < 0.001$), beyond which no significant differences were observed.

Table 3.7. The effect of increasing fat extraction time on fat extraction efficiency

Fat extraction time (h)	Cumulative mean fat extracted[1] (%)	Additional mean fat extracted[1] (%)
1	5.72[a]	5.72[a]
2	9.39[b]	3.89[b]
3	11.57[c]	2.41[c]
4	12.90[d]	1.50[d]
5	13.53[e]	0.72[e]
6	13.91[f]	0.45[ef]
7	14.21[g]	0.35[f]
8	14.51[h]	0.35[f]
Pooled SEM	0.243	0.131
P value	< 0.001	< 0.001

[1]Fat extracted from a total of 33 right tibia bones.
[a-h] Means within the same column with no common subscript differ significantly ($P < 0.05$).

In Study 4, autoclaving bones prior to fat extraction did not have any significant effect on ash weight in 42d old broilers. Although ash percentage was also not significantly different between both processing methods, autoclaved bones tended to have greater ash percentage compared to the bones which were not autoclaved ($P = 0.084$).

Table 3.8. The effect of autoclaving tibia bone before fat extraction on ash content

Bone processing method	[1]Dry bone weight (g)	[1]Bone ash weight (g)	[1]Bone ash %
Fat extracted bones	5.49	2.33	42.15
Autoclaved bones	5.35	2.31	42.95
P value	0.603	0.858	0.084
Pooled SEM	0.141	0.065	0.231

[1]Mean values determined from a total of 33 tibia bones per processing method.

3.4. Discussion

The need for standardising the methodology for determining the ash content of bones has for long been recognised. Previous investigators (St. John *et al.*,1933; Bethke and Record, 1934) observed variations in the processing methods employed in determining bone ash content in poultry e.g. use of different fat extraction solvents, different drying temperature, inclusion or removal of cartilage caps, and determining bone ash percentage either on air dry or moisture-free basis. These variations in methodology may affect the result obtained (Orban *et al.,* 1993). It was therefore of interest to identify which bone processing method and ash criterion (weight or %) best reflect sensitivity to changes in bone mineralisation in order to improve accuracy when comparing bone data, especially from unrelated studies which may have been determined from different processing methods

3.4.1. Study 1: the effect of fat extraction on tibia ash content

The fat extraction procedure is a well-cited methodology routinely employed prior to ash determination (Waldroup *et al.,* 2000; Yan *et al.,* 2005a; Driver *et al.,* 2006a); and is known to reduce variation between samples (Garcia and Dale, 2006). However, this process requires substantial use of organic solvents and laboratory processing time, which raises the question of whether fat extraction is an essential step in the methodology used to determine bone ash content.

Garcia and Dale (2006) analysed bones from 2-week old broilers and reported sensitivity in tibia ash percentage was not improved when the fat extraction method was compared with the non-fat extraction method. They however acknowledged that measurements in older birds might be more variable due to higher bone lipid content. Age-related increases in the fat content of eviscerated male broiler carcasses have been reported (Perreault and Leeson, 1992). Although the bone fat content was not determined in this study, it is expected that the fat content in the bones of the 35d birds used would be greater than the 2 week-old birds used in the study of Garcia and Dale (2006), which may partly explain why differences in assay sensitivity were observed in this study.

A medium strength correlation ($r = 0.367$) was found for ash percentage when fat extracted bones were compared with un-extracted bones. This is in contrast with the findings of Yan *et al.* (2005a) who reported a much higher coefficient of determination for ash percentage ($R^2 = 0.95$) when fat extracted tibia bones were compared with un-extracted tibia bones in 21d old broilers. This may be due to different reasons. The broilers in the study of Yan *et al.* (2005a) were fed a wide range of dietary phosphorus (0.39 – 0.73% total P) which resulted in a wide range of tibia ash values (29.57% - 41.57%). This would have extended the correlation plot resulting in a higher coefficient of determination. The observed differences may also be due

to the physiological differences at the age of sampling. It is expected that the older birds in this study (35d) would have retained more dietary fat in the bones compared to 21d broilers. This may have led to increased variation between bone samples and subsequently weaker correlation in bone ash percentage between the two processing methods.

Considering the significant differences in actual bone ash percentage values and improvement in method sensitivity when ash percentage was determined, this study suggests accuracy is improved when fat is extracted prior to ash determination when evaluating bone mineralisation, especially when comparing bone samples from unrelated studies. Extracting fat from the bones prior to ash determination will remove variations in the fat content of the bones, particularly in older birds fed varied dietary fat content.

3.4.2. Study 2: the effect of including cartilage caps on fat extracted tibia ash content

Ash percentage was significantly greater ($P < 0.001$) when cartilage caps were not included compared to when they were included prior to ash determination. This stands to reason as the cartilage caps had lower mineral content and resulted in lower ash weight and % when included in ash determination. Cartilage caps are sometimes removed prior to ash determination (Baird *et al.,* 2008; Chowdhury *et al.,* 2009); and comparison of ash measurements derived from bones where this is the case, with ash values of bones when cartilage caps are included may be misleading, particularly in disease situations. For example Hamilton and Garlich (1971) and Huff *et al.* (1980) reported aflatoxin decreases bone ash content by inhibiting fat metabolism. Bacterial chondronecrosis with osteomyelitis, one of the main causes of lameness in broilers (Wideman *et al.*, 2013), affects the cartilaginous epiphysis of the proximal tibiotarsus in broilers and has been shown to correlate with high bone ash

values. Thorp and Waddington (1997) determined higher ash values in birds diagnosed with chondronecrosis compared with normal healthy birds (52.5% vs. 47.7%).

In this study, removing the cartilage caps was more sensitive in elucidating differences in treatment means. In controlled research trials where the health status of a flock is known, removing the cartilage caps in order to improve the accuracy of comparisons may be adopted. However, it may be beneficial to include the cartilage caps when comparing bone ash values from unrelated studies, particularly when the health status is unknown. Although strong correlations were observed between both methods for both ash weight and ash percentage (r = 0.995 and 0.893 respectively); the result from this study suggests accuracy in comparing ash data between the two methods is improved when ash weight data is used. This demonstrates how different processing method can result in various ash values (weight and %) and highlights the importance of precisely stating what methods were followed in order to accurately compare results.

3.4.3. Study 3: the effect of increasing fat extraction time on fat extraction efficiency

Organic solvents are routinely used to extract fat from the bone. However, there is a lack of common procedure adopted by researchers in extracting fat from the bone. Variations of this method reported in the literature include soaking in diethyl alcohol for 48h (Perney *et al.*, 1993), a 2-phase fat extraction process with ethyl alcohol for 36h followed by diethyl ether for either 36h (Sun *et al.,* 2013); or 48h (Watson *et al.,* 2006; Hamdi *et al.,* 2015); or a 2-phase extraction process with ethanol for 48h followed by ether for 48h (Payne *et al.,* 2005). Fat extraction with petroleum ether using the Soxhlet method is very well cited in the literature but has been carried out for varied times, e.g. 6h (Olukosi and Fru-Nji, 2014) and 16h (Li *et al.,* 2016b). Researchers are also sometimes not specific in stating the fat extraction time

followed. Although it has for long been reported by Bethke and Record (1934) that the use of different organic solvent did not affect bone ash percentage, an understanding of the minimum time required to extract fat from bones is important, considering laboratory cost, bone processing time required and the potential detrimental effects on the environment when organic solvents are used.

No significant differences were observed in additional percentage fat extracted from bones beyond 6h using the Soxhlet fat extraction method. This suggests 6h is adequate to extract fat from bones using petroleum ether. This has obvious benefits for reduced processing time and costs compared with other methods that take longer or use multiple solvents.

3.4.4. Study 4: the effect of autoclaving prior to fat extraction on tibia ash content

The manual removal of flesh from the bones is a laborious and time-consuming process. Autoclaving tibia bones prior to ash determination is a technique sometimes employed to make the process of flesh removal easier by softening the adhering tissues (Boling-Frankenbach *et al.,* 2001; Kim *et al.,* 2008). However, the effect of autoclaving prior to fat extraction on bone ash has not been investigated.

The autoclaved bones tended to have a greater ash percentage ($P = 0.084$) compared to the ones which were not autoclaved. The autoclaved bones had numerically lower dry bone weight compared to the bones which were not autoclaved (5.53g vs. 49g, $P = 0.603$). Autoclaving the bones may have liberated some fat from the bones and improved subsequent fat extraction efficiency compared to when the bones were not autoclaved. This however needs to be verified considering the small sample size used in this study. Nonetheless, there is the need for subsequent fat extraction after autoclaving to adequately remove fat from the bones and also reduce variability in bone fat content as already discussed.

Comparing the strength of the relationship between the two bone processing methods, ash weight was more strongly correlated than ash percentage (r = 0.620 and 0.346 respectively; Figures 3.1 & 3.2). This suggests that accuracy in comparing bone mineralisation data from unrelated studies where these two processing methods are employed is improved when ash weight is used.

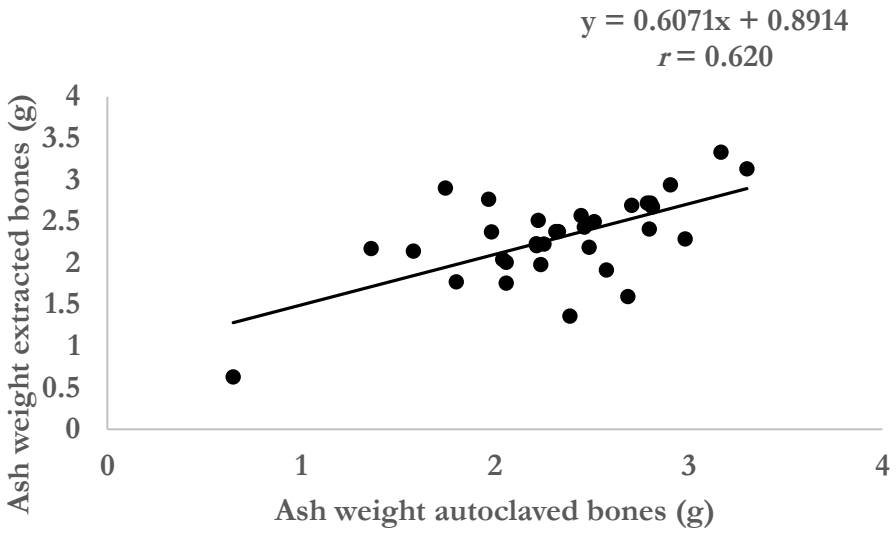

Figure 3.1. Correlation between autoclaved and unautoclaved fat extracted tibia ash weight

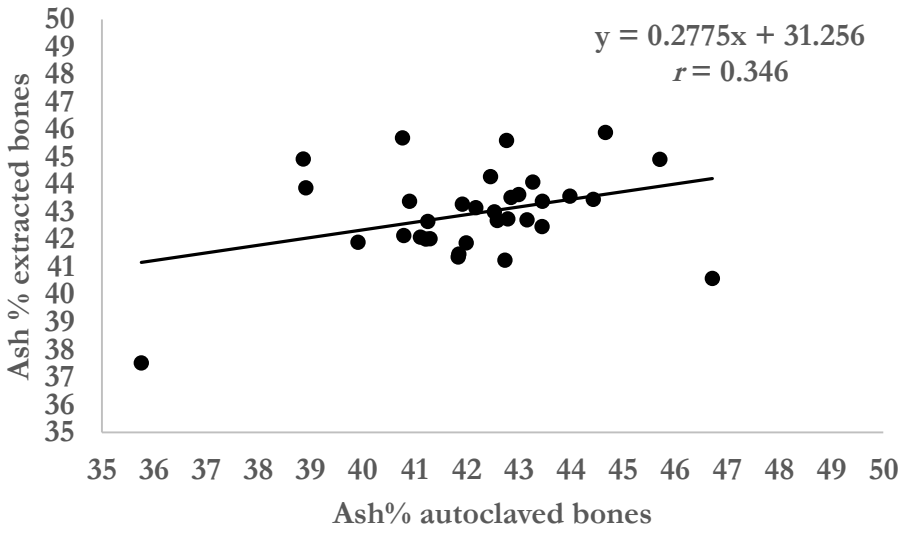

Figure 3.2. Correlation between autoclaved and unautoclaved fat extracted tibia ash %

Autoclaving bones before fat extraction helps to soften the adhering flesh, and enables the process of defleshing bones easier. However, considering the time required to label and autoclave the bones, it is doubtful if any significant time would be gained by autoclaving. Once the technique for manually removing flesh from bones is learnt (manual excision without prior autoclaving), defleshing bones can be quickly done. In agreement, Orban *et al.* (1993) reported it took about 25% less time to manually excise flesh from bones compared to boiling bones in water for 10 minutes due to the time required to process the bones (labelling, boiling, cooling and de-fleshing). Not autoclaving the bones also provides an opportunity to capture other bone measurements, particularly bone strength and histology which cannot be accurately obtained from soft autoclaved bones. These will help in further understanding bone mineralisation data.

3.5. Implication

The logistics involved in rapidly determining bone ash from a large dataset can be onerous. There is therefore the need to develop a rapid way of obtaining results without compromising reliability. The recommended 6h fat extraction time determined in this study may significantly reduce the bone processing time and increase sample throughput compared to other bone processing methods that take longer. This is advantageous when analysing a large dataset. More work needs to be done to develop a consensus on what method gives the best reliability with least input.

3.6. Conclusion

The lack of a standardised procedure for evaluating bone mineralisation, which leads to differences in results as previously highlighted in the report of Orban et al. (1993), remains a pertinent issue in contemporary poultry research. The findings from these studies support the hypothesis that different bone processing methods result in different bone ash values. This could potentially hamper meaningful comparison of bone ash measurements particularly from unrelated studies where different processing methods are employed.

There are significant differences in ash percentage when cartilage caps are included or not included in the bone processing methodology, and it would be misleading to directly compare values obtained from different studies where these two processing methods are employed. From the results obtained in these studies, it is has been demonstrated that sensitivity in elucidating treatment means is improved when cartilage caps are removed from tibia bones prior to ash determination. This could be adapted for use in controlled research trials where the health status of the flock is known. However, some previous reports have shown that ash percentage is increased in disease conditions affecting the cartilage caps. It may therefore be beneficial to include the cartilage caps when comparing bone ash values from unrelated

studies, particularly when the health status is unknown. It is also recommended that tibia bones are extracted of fat for a minimum of 6h with the cartilage caps included when using the Soxhlet fat extraction method. Fat extraction will help remove any variation in bone lipid content which may arise due to the effect of diet, disease or age-related differences. These established methods will be used in further studies in this PhD project.

The results obtained from these studies highlight the effect of different bone processing methods on bone ash (weight and %), and therefore the importance of a making careful consideration on what criterion to use when evaluating bone mineralisation. Researchers must therefore adopt a common method in the bone processing which will enable accurate comparisons of results from unrelated studies.

CHAPTER 4: CHARACTERISATION OF TIBIA BONE MINERALISATION IN COMMERCIAL BROILERS

4.1. Introduction

Tremendous increases in growth rate have occurred in commercial broiler strains (Zuidhof *et al.,* 2014) but bone development has not kept pace with overall growth, and the skeleton remains a weak link in physically supporting heavy carcasses in young birds (Applegate and Lilburn, 2002). The current NRC recommendations for phosphorus requirement in broilers (NRC, 1994) are based on research conducted from 1952 – 1983 and may not be applicable to the modern broiler strain. The need for an update of nutrient requirements has therefore been suggested (Applegate and Angel, 2014) which will better reflect the current physiological needs of modern broilers.

Phosphorus retention in the bones is widely studied to evaluate phosphorus availability of raw materials and efficacy of phytase enzymes, but studies are often conducted in research settings that do not represent commercial rearing conditions for modern broilers. Only very few studies have directly characterised bone morphology and mineralisation (Bond *et al.,* 1991; Skinner and Waldroup, 1995; Williams *et al.,* 2000b; Applegate and Lilburn, 2002), but the publication dates and experimental rearing conditions (small pens or cages) suggest the bone data reported from these studies may not be applicable to current commercial practices.

In order to appropriately update the broiler nutrient requirements, an understanding of the current range of normal bone morphology and mineralisation values of birds is required. The lack of such benchmarks was particularly noted by practicing Poultry Veterinarians and Feed Nutritionists, who commented on the difficulty in determining the cause of flock lameness in broilers without readily available reference values of normal bone mineralisation data.

4.1.2. Aims and objectives

The overall aim of this study was to characterise the range of tibia bone mineralisation found in healthy, commercially reared broilers to create a database of benchmarks applicable to current commercial practice.

The specific objectives were:

- To characterise the normal range of age and sex-related tibia bone parameters in Ross 308 broilers.
- To examine the relatedness between various bone measurements in Ross 308 broilers.

4.2. Materials and methods

4.2.1. Trial procedure

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny *et al.*, 2010) were followed, and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee. Age and sex-related tibia bone morphology and mineralisation values of healthy commercial broilers were sequentially sampled across 6 commercial farms from 24 flocks of birds. The sampling process described in detail below was repeated 4 times for each participating farm, i.e. 4 different flock cycles of birds were studied at each participating farm. The trial was conducted between February 2015 and September 2015.

On each farm, a mixed flock of Ross 308 day-old broilers was raised on wood shavings in open plan houses which were thermostatically controlled to provide an initial room temperature of 30°C. This was gradually reduced to 20°C by day 27°C and maintained till the end of the production cycle. Lighting was provided with 1h darkness from days 1-3 and then increased by 1h a day until day 6. Lighting was then maintained afterward on a daily 4 and 2

hourly split darkness period between days 7 and 32. On days 33 - 35, total daily darkness was reduced to 3, 2 and 1h respectively. 1h darkness was then maintained until the end of production cycle when birds were transported to processing plants. Standard commercial pelleted diets were formulated for 4 growth phases to meet or exceed the Ross 308 broiler nutrient specification (Aviagen, 2014). Diets and water were provided *ad libitum*. Birds were regularly checked to monitor environmental conditions and mortality recorded.

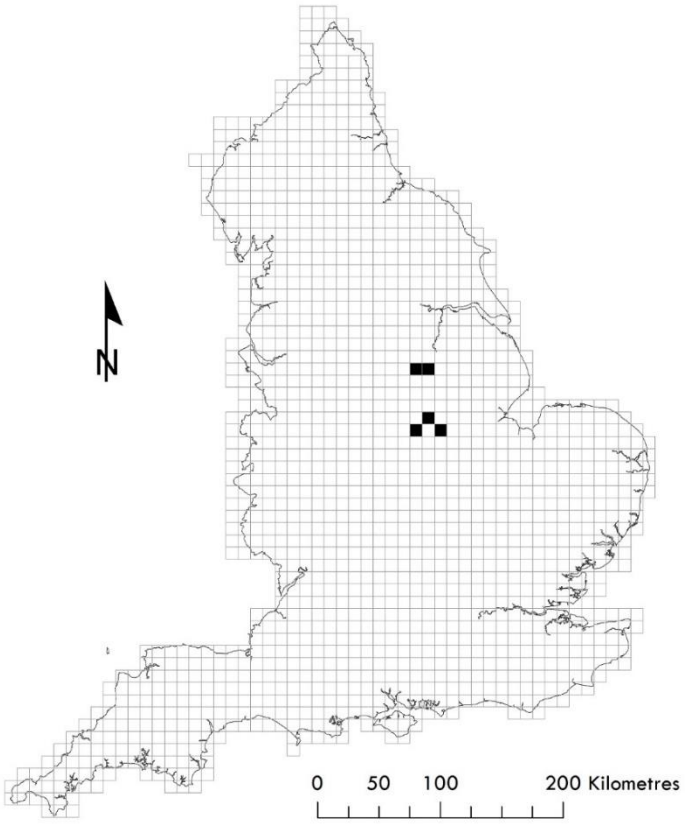

Figure 4.1. UK broiler production sites for the six participating farms
N.B. 2 farms shared the same grid reference on the map

4.2.2. Sample collection

At the start of the trial, farm performance data for all flock of birds on each participating farm were evaluated in order to identify a rearing house which produced healthy birds with no diagnosed incidence of lameness or other health-related problems. This was a crucial screening criterion as this study was intended to characterise the normal range of bone mineralisation in healthy birds. This process of examining flock records for health and performance was repeated each time a flock of birds was studied. From the selected house, 6 birds were collected at 3 different sampling points: six unsexed birds at day 14; and three cockerels and three pullets at day 28 and at the end of crop (varied between 34 – 40 days) just before the birds were sent for processing, hence 18 birds per flock. Each bird was individually assessed for lameness prior to selection using a 5 point qualitative gait score system after the method of Leterrier *et al.* (2008). Briefly, this involved classifying birds based on walking ability from 0 (no detectable gait abnormality) to 4 (severe gait defect), and only birds which scored 0 were selected. Birds were also scored for the incidence of pododermatitis (Appendix 1) based on the welfare quality assessment protocol for poultry (Welfare Quality, 2009). Briefly, the feet of each bird were inspected and given a score based on the severity of pododermatitis which ranged from 0 (no evidence of pododermatitis), 1 & 2 (minimal pododermatitis) and 3 & to 4 (severe pododermatitis). A questionnaire (Appendix 2) was administered to the Farm Managers to capture the relevant flock performance data. Iindividual bird data was also recorded (Appendix 3).

At sampling, the selected birds were euthanised by cervical dislocation and individually weighed, after which left tibia bones were collected and individually stored at -20^{o}C until further processing. These were collected as described in Chapter 2, Sections 2.4.2.3.

4.2.3. Determined parameters

The parameters measured were body weight, pododermatitis score, bone length, bone width, fresh bone weight, bone strength, dry fat extracted weight, bone ash weight, bone ash percentage, bone calcium content and bone phosphorus content, as previously described in Chapter 2, Sections 2.5.1, 2.5.2, 2.5.4.2 and 2.5.5.2. Briefly, right and left tibia bones were separated at the tibiotarsal junction where the feet were removed, and the tibio-femoral junction where the femurs were removed, and then individually stored at -20°C until further processing. At processing, the bones were manually cleaned and weighed but ensuring the cartilage caps were intact. Bone length and width were measured using a set of Vernier callipers. Bone strength was measured using a TA-XT Plus Texture Analyser (Stable Micro Systems, Surrey, UK). The average measurement of both right and left bones were used to determine bone length, width, and strength. Left tibia bones including cartilage caps were dried to constant weight, defatted and bone ash weight determined. The bones were then dried at 105°C for 24h prior to fat extraction using the Soxtherm hot fat extraction method for 2h. The left tibia bones including cartilage caps were dried at 105°C for 24h until constant weight was achieved and then ashed for 24h at 650°C in a furnace to determine ash weight. Bone ash was analysed for total calcium and total phosphorus content using an ICP-OES assay as previously described in Chapter 2, Sections 2.5.7.

4.2.4. Data analysis

Data were analysed using IBM SPSS version 23 for Windows (IBM Statistics, 2016). Descriptive statistics were used to characterise the bone parameters measured at each sampling point (day 14, day 28 and the end of crop). Differences due to sex for day 28 and end of crop were separated using the independent *t*-test procedure. The General Linear two-way ANOVA model was used to evaluate the main effects of bird sex (cockerel or pullets)

and age (days), and their interactions on all bone parameters measured for the combined dataset. At each sampling point, the strength of relationship between bone measurements was examined using the Pearson correlation procedure. Interpretations of the strength of relationships were based on those of Cohen (1988): small r = 0.1 - 0.29, medium r = 0.30 - 0.49 and large r = 0.50 - 1.0. Data were considered statistically significant when $P < 0.05$.

4.3. Results

As expected, there was a significant increase in bone parameters with age, and greater measurements in cockerels compared to pullets (Tables 4.1 – 4.4). At day 28, no significant differences were found in podo score between both sexes ($P = 0.922$) and bone Ca:P ($P = 0.818$). Ash weight tended to be higher in cockerels compared to pullets ($P = 0.091$); while all other parameters measured were significantly greater in cockerels compared to pullets ($P < 0.0001$). At the end of crop, no significant differences were found in podo score between cockerels and pullets ($P = 0.144$) and bone Ca:P ($P = 0.652$). Ash weight was significantly higher in pullets compared to cockerels ($P = 0.036$); while all other parameters measured were significantly greater in cockerels compared to pullets ($P < 0.0001$). The strength of relationships between bone parameters was analysed (Tables 4.5 – 4.9), and further ranked according to sex and sampling age (Appendix 4 – 8). At all sampling age, calcium was more positively correlated with phosphorus ($r \geq 0.92$) compared to all other parameters, as was the correlation between bone ash weight and dry bone weight ($r \geq 0.94$), and fresh bone weight and dry fat extracted bone weight ($r \geq 0.86$). Bone ash weight consistently ranked second in strength of correlation with bone calcium content or bone phosphorus content at day 28 and end of crop compared to all parameters measured. In general, medium to weak correlations were found between bone parameters and bone ash percentage or bone strength respectively across sampling age or sex.

Table 4.1. Tibia bone morphology, strength, and mineralisation in 14 day-old unsexed broilers

	N	Mean	Std. Deviation	Std. Error of Mean	Median	Range	Minimum	Maximum
Bird weight (g)	144	496.56	89.17	7.431	504.6	498.7	269.5	768.2
Podo score	144	0.15	0.51	0.042	0	3	0	3
Bone length (mm)	144	61.17	3.45	0.287	61.03	17.8	52.52	70.32
Bone width (mm)	144	4.22	0.44	0.036	4.18	2.53	3.21	5.74
Fresh bone weight (g)	144	3.76	0.75	0.062	3.76	4.36	2.04	6.4
Bone strength (N)	144	103.9	40.92	3.41	97.46	245.55	37.32	282.87
Dry fat extracted bone weight (g)	144	1.21	0.24	0.02	1.22	1.43	0.7	2.13
Bone ash weight (g)	144	0.48	0.1	0.008	0.48	0.65	0.27	0.92
Ash %	144	39.59	1.86	0.155	39.73	11.92	32.01	43.94
Ca content/dry bone weight (mg)	144	160.37	44.24	3.687	153.59	322.11	15.8	337.91
P content/dry bone weight (mg)	144	62.26	18.41	1.534	58.68	135.23	5.95	141.18
Bone Ca:P	144	2.59	0.2	0.017	2.6	2.14	1.9	4.03

Table 4.2. Tibia bone morphology, strength, and mineralisation in 28 day-old cockerels and pullets

	Sex	N	Mean	Std. Dev.	Std. Error of Mean	Median	Range	Minimum	Maximum	*P*
Bird weight (g)	Cockerels	72	1605.61	182.61	21.521	1642.00	809.20	1137.50	1946.70	<0.0001
	Pullets	72	1324.85	193.70	22.828	1326.15	979.40	881.20	1860.60	
Podo score	Cockerels	72	0.74	0.80	0.095	1.00	3.00	0.00	3.00	0.922
	Pullets	72	0.75	0.88	0.104	0.50	3.00	0.00	3.00	
Bone length (mm)	Cockerels	72	91.58	3.03	0.357	91.89	17.59	81.56	99.15	<0.0001
	Pullets	72	88.10	3.73	0.440	88.08	18.21	78.84	97.05	
Bone width (mm)	Cockerels	72	7.05	0.52	0.061	6.97	2.34	5.85	8.19	<0.0001
	Pullets	72	6.25	0.51	0.060	6.26	2.45	4.88	7.33	
Fresh bone weight (g)	Cockerels	72	12.79	1.50	0.176	12.96	7.33	9.37	16.70	<0.0001
	Pullets	72	10.12	1.52	0.179	10.06	6.40	7.14	13.54	
Bone strength (N)	Cockerels	72	248.35	67.43	7.947	234.84	304.67	156.22	460.89	<0.0001
	Pullets	72	201.17	51.09	6.021	194.58	216.32	123.87	340.19	
Dry fat extracted bone weight (g)	Cockerels	72	4.56	0.51	0.060	4.61	2.74	3.26	6.00	<0.0001
	Pullets	72	3.67	0.53	0.062	3.71	2.30	2.51	4.81	
Bone ash weight (g)	Cockerels	72	1.82	0.23	0.027	1.84	1.24	1.28	2.52	<0.0001
	Pullets	72	1.48	0.23	0.027	1.49	1.05	0.99	2.04	
Ash %	Cockerels	72	39.93	1.69	0.200	39.69	7.66	36.93	44.59	0.091
	Pullets	72	40.42	1.80	0.212	40.32	8.33	35.97	44.30	
Ca content/dry bone (mg)	Cockerels	72	596.47	124.63	14.687	588.29	729.71	220.42	950.13	<0.0001
	Pullets	72	481.47	87.28	10.287	471.74	498.51	208.47	706.98	
P content/dry bone (mg)	Cockerels	72	226.48	44.19	5.208	225.14	264.46	88.87	353.33	<0.0001
	Pullets	72	183.49	37.43	4.411	183.37	212.09	53.75	265.84	
Bone Ca:P	Cockerels	72	2.63	0.19	0.022	2.63	1.80	2.08	3.88	
	Pullets	72	2.65	0.24	0.209	2.63	2.00	1.97	3.97	0.818

Table 4.3. Tibia bone morphology, strength, and mineralisation by the end of crop in cockerels and pullets

	Sex	N	Mean	Std. Dev.	Std. Error of Mean	Median	Range	Minimum	Maximum	*P*
Bird weight (g)	Cockerels	69	2392.36	370.81	44.640	2393.30	1798.40	1542.60	3341.00	<0.0001
	Pullets	69	1947.04	248.89	29.963	1915.00	1019.40	1412.60	2432.00	
Podo score	Cockerels	69	0.62	0.94	0.113	0.00	3.00	0.00	3.00	0.144
	Pullets	69	0.87	1.03	0.124	0.00	3.00	0.00	3.00	
Bone length (mm)	Cockerels	69	105.40	5.54	0.667	105.28	28.09	92.79	120.88	<0.0001
	Pullets	69	101.01	4.10	0.493	101.14	19.95	90.24	110.19	
Bone width (mm)	Cockerels	69	8.25	0.77	0.093	8.29	3.09	6.70	9.79	<0.0001
	Pullets	69	7.20	0.46	0.055	7.17	2.17	6.15	8.32	
Fresh bone weight (g)	Cockerels	69	18.73	2.76	0.332	18.87	13.07	12.17	25.24	<0.0001
	Pullets	69	14.31	1.63	0.196	14.23	7.21	10.61	17.82	
Bone strength (N)	Cockerels	69	303.83	66.51	8.007	296.38	312.42	177.04	489.46	<0.0001
	Pullets	69	258.46	56.09	6.753	241.98	298.79	133.72	432.51	
Dry fat extracted bone weight (g)	Cockerels	69	7.14	1.12	0.135	7.14	5.35	4.37	9.72	<0.0001
	Pullets	69	5.64	0.77	0.093	5.65	3.79	3.73	7.52	
Bone ash weight (g)	Cockerels	69	2.78	0.47	0.056	2.80	2.08	1.76	3.84	<0.0001
	Pullets	69	2.23	0.30	0.037	2.19	1.67	1.39	3.06	
Ash %	Cockerels	69	38.89	1.79	0.216	38.89	8.68	35.02	43.70	0.036
	Pullets	69	39.53	1.78	0.214	39.46	8.20	35.88	44.08	
Ca content/dry bone (mg)	Cockerels	69	910.81	208.08	25.050	923.60	1019.36	248.27	1267.63	<0.0001
	Pullets	69	719.27	144.38	17.382	732.33	924.83	166.84	1091.67	
P content/dry bone (mg)	Cockerels	69	343.91	74.61	8.982	347.67	376.32	103.17	479.49	<0.0001
	Pullets	69	270.53	48.45	5.833	282.53	306.01	69.29	375.30	
Bone Ca:P	Cockerels	69	2.64	0.10	0.012	2.65	0.59	2.41	3.00	
	Pullets	69	2.65	0.20	0.025	2.63	1.53	2.37	3.89	0.652

Table 4.4. Effect of sex and sampling age on bone morphology, strength, and mineralisation in Ross 308broilers

	Day 14 unsexed	Day 28 pullets	Day 28 cockerels	End of crop pullets	End of crop cockerels	Pooled SEM	*P* (ANOVA) Sex	Age	Sex x age
Bird weight (g)	496.55[a]	1324.85[b]	1605.61[c]	1947.03[d]	2392.36[e]	35.551	< 0.001	< 0.001	0.005
Podo score	0.15[a]	0.62[b]	0.73[b]	0.75[b]	0.87[b]	0.041	0.035	< 0.001	0.322
Bone length (mm)	61.17[a]	88.09[b]	91.58[c]	101.01[d]	105.40[e]	0.875	< 0.001	< 0.001	0.064
Bone width (mm)	4.22[a]	6.25[b]	7.05[c]	7.20[c]	8.25[d]	0.078	< 0.001	< 0.001	0.074
Fresh bone weight (g)	3.76[a]	10.12[b]	12.79[c]	14.31[d]	18.73[e]	0.275	< 0.001	< 0.001	0.002
Bone strength (N)	103.90[a]	201.17[b]	248.35[c]	258.46[c]	303.83[d]	4.544	< 0.001	< 0.001	0.545
Dry fat extracted bone weight (g)	1.21[a]	3.67[b]	4.56[c]	5.64[d]	7.14[e]	0.11	< 0.001	< 0.001	0.006
Bone ash weight (g)	0.48[a]	1.48[b]	1.82[c]	2.22[d]	2.78[e]	0.043	< 0.001	< 0.001	0.003
Ash %	39.59[bc]	40.42[a]	39.93[ab]	39.53[bc]	38.89[c]	0.09	0.075	0.077	0.462
Ca content/dry bone weight (mg)	160.37[a]	481.47[b]	596.47[c]	719.27[d]	910.81[e]	14.592	< 0001	< 0.001	< 0.001
P content/dry bone weight (mg)	62.26a	183.79[b]	226.48[c]	270.53[d]	343.91[e]	5.439	< 0.001	< 0.001	0.001
Bone Ca:P	2.59	2.65	2.63	2.64	2.64	0.009	0.843	0.117	0.955

[a-e]Means within the same row with no common subscript differ significantly ($P < 0.05$).

Table 4.5. Pearson correlation values between bone parameters in 14 day-old unsexed broilers

	Bird weight (g)	Podo score	Bone length (mm)	Bone width (mm)	Fresh bone weight (g)	Bone strength (N)	Dry fat extracted bone weight (g)	Bone ash weight (g)	Ash %	Ca content/dry bone weight (mg)	P content/dry bone weight (mg)
Bird weight (g)		0.156	0.593	0.559	0.628	0.287	0.627	0.598	0.052	0.465	0.449
Podo score	0.156		0.102	0.132	0.187	-0.106	0.142	0.120	-0.058	0.137	0.147
Bone length (mm)	0.593	0.102		0.768	0.903	0.522	0.889	0.847	0.047	0.585	0.560
Bone width (mm)	0.559	0.132	0.768		0.880	0.669	0.851	0.830	0.131	0.563	0.549
Fresh bone weight (g)	0.628	0.187	0.903	0.880		0.560	0.946	0.895	0.020	0.649	0.642
Bone strength (N)	0.287	-0.106	0.522	0.669	0.560		0.617	0.682	0.414	0.418	0.388
Dry fat extracted bone weight (g)	0.627	0.142	0.889	0.851	0.946	0.617		0.975	0.129	0.719	0.704
Bone ash weight (g)	0.598	0.120	0.847	0.830	0.895	0.682	0.975		0.344	0.714	0.693
Ash %	0.052	-0.058	0.047	0.131	0.020	0.414	0.129	0.344		0.164	0.131
Ca content/dry bone weight (mg)	0.465	0.137	0.585	0.563	0.649	0.418	0.719	0.714	0.164		0.963
P content/dry bone weight (mg)	0.449	0.147	0.560	0.549	0.642	0.388	0.704	0.693	0.131	0.963	

Table 4.6. Pearson correlation values between bone parameters in 28 day-old cockerels

	Bird weight (g)	Podo score	Bone length (mm)	Bone width (mm)	Fresh bone weight (g)	Bone strength (N)	Dry fat extracted bone weight (g)	Bone ash weight (g)	Ash %	Ca content/dry bone weight (mg)	P content/dry bone weight (mg)
Bird weight (g)		0.009	0.414	0.416	0.525	0.136	0.541	0.541	0.190	0.369	0.348
Podo score	0.009		-0.031	-0.146	-0.012	-0.136	-0.103	-0.138	-0.138	-0.109	-0.048
Bone length (mm)	0.414	-0.031		0.402	0.757	0.233	0.741	0.677	0.031	0.356	0.416
Bone width (mm)	0.416	-0.146	0.402		0.730	0.421	0.706	0.741	0.303	0.491	0.537
Fresh bone weight (g)	0.525	-0.012	0.757	0.730		0.444	0.947	0.900	0.137	0.523	0.561
Bone strength (N)	0.136	-0.136	0.233	0.421	0.444		0.489	0.605	0.465	0.579	0.582
Dry fat extracted bone weight (g)	0.541	-0.103	0.741	0.706	0.947	0.489		0.939	0.112	0.561	0.581
Bone ash weight (g)	0.541	-0.138	0.677	0.741	0.900	0.605	0.939		0.445	0.638	0.642
Ash %	0.190	-0.138	0.031	0.303	0.137	0.465	0.112	0.445		0.378	0.345
Ca content/dry bone weight (mg)	0.369	-0.109	0.356	0.491	0.523	0.579	0.561	0.638	0.378		0.938
P content/dry bone weight (mg)	0.348	-0.048	0.416	0.537	0.561	0.582	0.581	0.642	0.345	0.938	

Table 4.7. Pearson correlation values between bone parameters in 28 day-old pullets

	Bird weight (g)	Podo score	Bone length (mm)	Bone width (mm)	Fresh bone weight (g)	Bone strength (N)	Dry fat extracted bone weight (g)	Bone ash weight (g)	Ash %	Ca content/dry bone weight (mg)	P content/dry bone weight (mg)
Bird weight (g)		0.177	0.623	0.474	0.736	0.350	0.679	0.703	0.241	0.532	0.462
Podo score	0.177		0.045	-0.225	-0.037	0.289	-0.027	-0.008	0.064	0.071	0.089
Bone length (mm)	0.623	0.045		0.549	0.855	0.071	0.740	0.676	-0.051	0.471	0.427
Bone width (mm)	0.474	-0.225	0.549		0.705	0.185	0.677	0.645	0.035	0.486	0.449
Fresh bone weight (g)	0.736	-0.037	0.855	0.705		0.165	0.859	0.813	0.047	0.531	0.486
Bone strength (N)	0.350	0.289	0.071	0.185	0.165		0.119	0.235	0.423	0.456	0.390
Dry fat extracted bone weight (g)	0.679	-0.027	0.740	0.677	0.859	0.119		0.957	0.087	0.620	0.569
Bone ash weight (g)	0.703	-0.008	0.676	0.645	0.813	0.235	0.957		0.371	0.695	0.629
Ash %	0.241	0.064	-0.051	0.035	0.047	0.423	0.087	0.371		0.397	0.335
Ca content/dry bone weight (mg)	0.532	0.071	0.471	0.486	0.531	0.456	0.620	0.695	0.397		0.950
P content/dry bone weight (mg)	0.462	0.089	0.427	0.449	0.486	0.390	0.569	0.629	0.335	0.950	

Table 4.8. Pearson correlation values between bone parameters by the end of crop in cockerels

	Bird weight (g)	Podo score	Bone length (mm)	Bone width (mm)	Fresh bone weight (g)	Bone strength (N)	Dry fat extracted bone weight (g)	Bone ash weight (g)	Ash %	Ca content/dry bone weight (mg)	P content/dry bone weight (mg)
Bird weight (g)		0.040	0.852	0.755	0.902	0.300	0.914	0.918	0.241	0.607	0.604
Podo score	0.040		0.044	0.240	0.092	0.221	0.113	0.100	-0.028	0.146	0.118
Bone length (mm)	0.852	0.044		0.676	0.901	0.174	0.900	0.837	-0.012	0.542	0.527
Bone width (mm)	0.755	0.240	0.676		0.837	0.274	0.823	0.847	0.271	0.528	0.509
Fresh bone weight (g)	0.902	0.092	0.901	0.837		0.213	0.981	0.950	0.121	0.583	0.574
Bone strength (N)	0.300	0.221	0.174	0.274	0.213		0.235	0.373	0.569	0.399	0.390
Dry fat extracted bone weight (g)	0.914	0.113	0.900	0.823	0.981	0.235		0.964	0.105	0.607	0.596
Bone ash weight (g)	0.918	0.100	0.837	0.847	0.950	0.373	0.964		0.363	0.644	0.637
Ash %	0.241	-0.028	-0.012	0.271	0.121	0.569	0.105	0.363		0.303	0.314
Ca content/dry bone weight (mg)	0.607	0.146	0.542	0.528	0.583	0.399	0.607	0.644	0.303		0.988
P content/dry bone weight (mg)	0.604	0.118	0.527	0.509	0.574	0.390	0.596	0.637	0.314	0.988	

Table 4.9. Pearson correlation values between bone parameters by the end of crop in pullets

	Bird weight (g)	Podo score	Bone length (mm)	Bone width (mm)	Fresh bone weight (g)	Bone strength (N)	Dry fat extracted bone weight (g)	Bone ash weight (g)	Ash %	Ca content/dry bone weight (mg)	P content/dry bone weight (mg)
Bird weight (g)		0.134	0.706	0.593	0.868	0.171	0.847	0.853	0.035	0.400	0.389
Podo score	0.134		0.232	0.115	0.237	0.278	0.217	0.172	-0.120	0.279	0.191
Bone length (mm)	0.706	0.232		0.520	0.821	-0.054	0.840	0.754	-0.245	0.330	0.267
Bone width (mm)	0.593	0.115	0.520		0.727	0.181	0.719	0.737	0.058	0.287	0.297
Fresh bone weight (g)	0.868	0.237	0.821	0.727		0.095	0.957	0.925	-0.087	0.363	0.314
Bone strength (N)	0.171	0.278	-0.054	0.181	0.095		0.078	0.243	0.505	0.372	0.385
Dry fat extracted bone weight (g)	0.847	0.217	0.840	0.719	0.957	0.078		0.940	-0.162	0.395	0.354
Bone ash weight (g)	0.853	0.172	0.754	0.737	0.925	0.243	0.940		0.179	0.491	0.467
Ash %	0.035	-0.120	-0.245	0.058	-0.087	0.505	-0.162	0.179		0.292	0.342
Ca content/dry bone weight (mg)	0.400	0.279	0.330	0.287	0.363	0.372	0.395	0.491	0.292		0.920
P content/dry bone weight (mg)	0.389	0.191	0.267	0.297	0.314	0.385	0.354	0.467	0.342	0.920	

4.4. Discussion

Tremendous changes have occurred in the productivity of the broiler chicken over the past six decades. Notably among which includes a significant increase in growth rate, better feed efficiency and carcass yield as reported in the studies of Havenstein *et al.* (1994a,b; 2003a,b) who compared a 1957 broiler strain with a 1991 and 2001 improved broiler strain. These findings have also been recently confirmed by other investigators (Schmidt *et al.*, 2009; Collins *et al.,* 2014; Zuidhof *et al.,* 2014). Although the development of new and improved feedstuff, advances in animal health, better husbandry techniques and more precise feed formulation techniques have contributed to the observed changes; the application of quantitative genetics through selective breeding programmes (Hunton, 2006) is the single most important contributory factor accounting for 85 – 90% of the observed changes (Havenstein, 2006).

Broiler breeding programmes are usually aimed at improved growth rate and feed efficiency (Petracci *et al.,* 2013), but poor bone mineralisation relative to body weight has been a direct consequence (Leeson, 2012). An understanding of the age-related pattern of bone mineralisation is therefore important for the precise evaluation of nutritional needs (Skinner and Waldroup, 1995), and to benchmark baseline limits which will help in properly assessing field problems relating to poor bone mineralisation in broilers.

Bone mineralisation values are normally derived from research recommendations. However, it has been questioned how applicable these values are to current commercial practice. Although it has been noted that not all bones contribute equally to structural support, and that a single bone may not be a true representation of skeletal integrity (Van Wyhe *et al.,* 2012), long bones have been widely evaluated in bone mineralisation studies in broilers. The tibia bone was evaluated in this study as it is most common bone used in evaluating bone mineralisation in poultry research (Shaw *et al.,* 2010).

4.4.1. Growth performance

Body weight at each sampling age was higher than the NRC (1994) typical broiler body weight for male and female broilers, and also that of previous studies that characterised bone growth in broilers (Bond *et al.,* 1991; Skinner and Waldroup, 1995; Applegate and Lilburn, 2002). However it is in good agreement with the Ross 308 performance objectives (Aviagen, 2014), and reflect the changes in improved growth rate that have occurred due to selective breeding between previous reports and when this trial was conducted.

4.4.2. Bone morphometry, breaking strength and mineralisation

The bone morphometric data determined in this study was greater than those from previous age and sex-related tibia data (Bond *et al.,* 1991; Skinner and Waldroup, 1995; Applegate and Lilburn, 2002). This was expected as increased body size results in increased bone morphometry (Williams *et al.,* 2000b). The mean bird weight at each sampling age in this study was greater in comparison to these previous studies.

There is a paucity of recent age and sex-related characterisation of broiler bone mineralisation in the literature. Talaty *et al.* (2010) examined the relationship between bone mineralisation and gait score in 4 commercial male broiler crosses but captured only bone morphometric and densitometric data, with no information on bone strength or phosphorus content. Shim *et al.* (2012a) examined the effect of growth on bone mineralisation by comparing a slow and fast growing strain of unsexed broilers. However the birds were partly raised in wired floor cages from 4 - 6 weeks and significantly smaller and weaker bones have been reported in cage reared birds compared to birds raised on the floor (Bond *et al.*, 1991; Fleming *et al.,* 1994). Neither authors (Talaty *et al.*, 2010; Shim *et al.*, 2012a) characterised the age-related differences in bone measurements but collected bone data at the end of the experiment (42d), past the period of rapid bone development. Barreiro *et al.* (2009) evaluated age-related bone mineralisation and

densitometric values in broilers, but the study was based on a small dataset of 29 male broilers. Charuta *et al.* (2013) characterised the age and sex-related densitometric and geometric differences in broilers using quantitative computed tomography but did not include other bone measurements such as length, width, strength, ash and mineral content.

Comparisons of the mean bone measurements determined in this study were made with published data of control birds given standard diets from different studies. Kleczek *et al.* (2012) investigated the effect of dietary supplementation of propolis (a natural alternative to antibiotic growth promoter) on bone physiochemical properties and strength in 35d broilers (mixed flock). The tibia length in 35d control broilers was shorter when compared to the end of crop data observed in this study (93.0 mm vs. 103.2mm), although bone weight and maximum shear force were greater (22.71g/445N vs. 16.5g/281N respectively). In another study, Swiatkiewicz *et al.* (2012) investigated the effect of organic acids (short and medium chain fatty acids) on tibia bone characteristics. The authors reported remarkably greater bone strength in a 42d mixed flock of Ross 308 control broilers compared to that determined in this study (416N vs. 281N respectively), despite reporting a comparable mean fresh bone weight and bone length (15g and 102mm vs. 16.5g and 103.2mm, respectively). Age-related increases in bone parameters have been reported (Rath *et al.*, 2000) and this may partly explain the observed differences, as the end of crop data in this study was sampled from a wider age range (34 - 40d).

Bone ash weight and bone ash percentage are two common criteria used in evaluating bone mineralisation. De Groote and Huyghebaert (1997) reported 80% of the total body phosphorus is retained in the bones, and the amount of bone ash weight is indicative of how well a bone is mineralised (Shim *et al.*, 2012a). A range of bone ash percentage data have been cited in the literature, probably as a result of the effect of methodology differences already

discussed in Chapter 3, which makes the comparison of data between studies difficult but patterns and changes within a study may still be usefully discussed.

Kleczek *et al.* (2012) reported high bone ash percentage (54% at day 35), while Mirakzehi *et al.* (2013) reported 48 – 54% at days 21 and 42 respectively. However, much lower values have been reported in broilers. For example, Khodambashi *et al.* (2013) investigated the effect of phytase and organic acid supplementation and reported 41.5% bone ash percentage in 28d Ross 308 male broilers fed control diets. This is slightly higher than the 39.93% determined in 28d cockerels in this study. Skinner and Waldroup (1995) reported a numerical increase in bone ash percentage with age in floor-reared male and female broilers (39.3% and 40.4% at day 14; and 42.8% and 43.8% at day 42 respectively). In contrast, Barreiro *et al.* (2009) reported bone ash percentage was 43%, 47.7% and 43.7% on days 8, 22 and 43 respectively; and concluded that the higher bone ash percentage determined on day 22 was due to a greater demand for mineral in response to rapid growth at that age. This pattern was not observed in this study. The mean bone ash percentage at each sampling age and sex determined in this study was between 39 - 40%. The overall mean bone ash percentage (39.67%) was in close comparison to that determined by Shim *et al.* (2012a) who reported 39.76% bone ash percentage at 42d for fast-growing unsexed broilers.

It was remarkable to find the wide range of bone ash percentage (minimum of 32% and a maximum of 44.6%) in this study. This however can be explained. Because of the variability between batches of feed ingredients and the difficulty in precisely evaluating nutrients at the point of manufacture, there may be a difference between the nutrient content of the feed formulated to what is delivered in commercial feed production (Bedford *et al.*, 2016). Also, an individual bird within a large flock may respond differently to environmental or husbandry challenges which may affect nutrient metabolism and bone growth pattern. The mean

population size of the birds used in this study was 33,000 per shed, from which only 6 birds were selected at random from a particular shed at each sampling day. It is possible that healthy birds with extreme bone mineralisation values were selected, which resulted in the wide range of values determined.

4.4.3. Sexual dimorphism in bone parameters

Sexual dimorphism in bone parameters has been reported in poultry (Bond *et al.*, 1991; Yalçin *et al.*, 2001). In general, cockerels had greater bone measurements than pullets, and it was hypothesised this might be due to the sex-related differences in body weight. Data was further analysed to examine if the observed differences were influenced by body weight or other factors. Day 28 and end of crop combined data were analysed separately for each sex to examine the strength of correlation between body weight and bone weight or strength; and strength of correlation between bone weight and 3 other bone measurements: bone strength, calcium content, and phosphorus content (Table 4.10).

Body weight was more strongly correlated with bone weight in cockerels than in pullets (0.95 and 0.93 respectively), and variation in body weight accounted for 90% and 87% of the variability in bone weight for cockerels and pullets respectively. This is in agreement with the findings of Applegate and Lilburn (2002) who characterised the relationship between growth and skeletal development in commercial broilers and reported variation in body weight accounted for 99 % variability of fat extracted tibia weight. The higher variability reported by the authors might be due to the wider age range of birds sampled in that study (1 - 43d) which would have extended the correlation plot, compared to 28 - 40d examined in this study. Similarly, the strength of correlation between bone weight and bone mineral content (calcium and phosphorus) was higher in cockerels than in pullets. In contrast, medium strength correlations were found between bone weight and bone strength in both cockerels and pullets

(0.472 and 0.440 respectively), and variation in bone weight accounted for only 22% and 19% variability in bone strength in cockerels and pullets respectively. Similarly, variation in body weight accounted for only 19% and 26% variability in bone strength in cockerels and pullets respectively. Data from this study suggests that the sex-related differences in bone parameters can be partly explained by the differences in bone weight as influenced by the differences in body weight. However, growth is controlled by a complex interaction of genetic, hormonal, and nutritional factors (Tesseraud *et al.*, 2003) which may directly or indirectly affect growth.

Table 4.10. Sex-related correlation of some bone parameters in broilers[1]

Measurement	Equation	R^2	*r*	*P*
Pullets				
Bone weight vs. strength	$22.496x^2 + 124.9$	0.1939	0.440	<0.001
Bone weight vs. bone calcium content	$109.2x^2 + 91.513$	0.5955	0.772	<0.001
Bone weight vs. bone phosphorus content	$39.273x^2 + 43.994$	0.5785	0.761	<0.001
Body weight vs. bone weight	$0.0029x^3 - 0.0663$	0.8663	0.931	<0.001
Body weight vs. bone strength	$0.0811x^3 + 97.049$	0.2622	0.512	<0.001
Cockerels				
Bone weight vs. strength	$21.938x^2 + 147.82$	0.2229	0.472	<0.001
Bone weight vs. bone calcium content	$120.26x^2 + 50.376$	0.6513	0.807	<0.001
Bone weight vs. bone phosphorus content	$44.268x^2 + 26.299$	0.6624	0.814	<0.001
Body weight vs. bone weight	$0.003x^3 - 0.1785$	0.8980	0.948	<0.001
Body weight vs. bone strength	$0.0644x^3 + 147.24$	0.1901	0.436	<0.001

[1]Days 28; 34 - 40
r = correlation
R^2 = coefficient of determination

Sex-related differences in skeletal mass and body weight have been associated with differences in metabolic processes and endocrine function (Tatara *et al.*, 2012). For example, functional amino acids are essential in building protein and polypeptide structures, and their metabolism is also known to play a vital role in metabolic processes involved in body growth, reproduction and health (Wu *et al.*, 2009). The skeletal tissues are the main body reservoir for functional amino acids, and they contribute to the overall body growth by improving protein accretion and feed utilisation.

Increase in body weight has been linked to better skeletal metabolism of functional amino acids such as glutamine and tryptophan (Wagenmakers, 1998). For example, significantly higher glutamine and tryptophan concentrations were found in the breast muscle of male turkeys which were significantly heavier than females turkeys fed the same diet (Tatara *et al.*, 2012). Nonetheless, other sex-related factors may also explain the differences found in bone parameters between cockerels and pullets. For example, gonadal steroids have long been recognised to play a major role in the maintenance of skeletal homoeostasis by regulating proliferation and resorptive processes involved in bone mineralisation (Rath *et al.,* 1996). Oestrogen is known to induce medullary osteogenesis (Turner and Schraer, 1977), but the effect of androgens on bone development in poultry is inconclusive, and the mechanism of action is not clear. Fennell and Scanes (1992) reported androgens suppressed skeletal growth in white leghorn chickens but in contrast, Deyhim *et al.* (1992) reported testosterone did not affect bone growth in 28d and 48d old male and female broilers. Rath *et al.* (1996) compared the effect of different gonadal steroids on the bones of male broiler chickens and reported that although testosterone did not affect bone weight, bone length, and percentage tibia and femur ash; significantly stronger bones were observed in 6 week-old male broilers. Although neither functional amino acids nor gonadal hormones were measured in this study, they may

have played a role, resulting in the sex-related differences of bone parameters reported in this study. Further work is warranted to examine the effect of functional amino acids and gonadal hormones on sexual dimorphism in commercial broilers.

4.4.3. Bone Ca:P content

As previously noted by Skinner and Waldroup (1995), there is a scarcity of the typical broiler bone Ca and P content in the literature. The overall mean Ca:P molar ratio determined in this study was 2.64, and was not significantly different across the various sampling age or sex (Table 4.4). Also remarkable was the range (1.90 - 3.89) found across the overall dataset which is substantially higher than the 1.67:1 Ca:P molar ratio found in calcium phosphate hydroxyapatite (Pellegrino and Biltz, 1968). Similar to the findings in this study, Williams *et al.* (2000a) observed wide Ca:P molar ratios (1.82 – 3.89) in birds given a range of dietary Ca and available phosphorus content. The authors reported some of the diets contained almost double the recommended 0.45% avP (NRC, 1994) and concluded that high bone Ca:P molar ratio could not be attributed to only mineral deficiency. In contrast, when the dataset published by Skinner and Waldroup (1995) is recalculated, the overall mean Ca:P molar ratio were 2.01 (1.82 – 2.40) and 2.02 (1.82 – 2.30) for male and female broilers respectively (aged 1 – 56d). Similarly, Barreiro *et al.* (2009) reported mean tibia Ca:P molar ratios of 2.0 - 2.01. It is unclear if the wide range of Ca:P molar ratios observed in this study is due to the effect of selective breeding on bone quality, or the difficulty in precisely delivering nutrients to all birds in large-scale bird husbandry which may have affected bone mineralisation and is worthy of further investigation. However, it is important to note that the mineral status of a bird is not fixed and that bone resorption occurs regularly in response to requirements.

4.5. Implication

This dataset provides a detailed examination of bone parameters within a fixed range of conditions. The use of 6 farms owned by a single UK integrator which provided uniform feed to just one bird strain concomitantly increases the rigour of the data but limits the breadth of application. In order to increase the value of the database to the commercial sector, other strains of broilers, reared by other integrators and independent producers across a range of geographical regions should be examined similarly. Also, as already discussed, the evolution of bird strains through selective breeding and changes to industry practice would necessitate regularly updating the database to ensure it reflects the current industry standards.

4.6. Conclusion

There is a growing consensus for the need to update the NRC recommendation of nutrient requirements in broilers due to the continually increasing growth rate and a concomitant reduction in slaughter age. The data collected and presented in this chapter makes a significant contribution to the knowledge of the current range of normal bone morphology, and calcium and phosphorus mineralisation values of commercial broilers. Standard mean bone mineralisation values are typically used for evaluation purposes; however in this study, a range of bone morphology and mineralisation values was determined in healthy birds and should be considered for evaluation purposes. Although only one bird strain is represented, it may serve as a benchmark of values applicable to commercial practice rather than solely relying on data derived from controlled research trials.

The next study (Chapter 5) was to verify if the bone is a reliable model for assessing phosphorus requirement in broilers. This fundamental question will be examined by evaluating bone calcium and phosphorus content, and whole body phosphorus content in commercial broilers.

CHAPTER 5: AN INVESTIGATION INTO THE RELATIONSHIP BETWEEN BONE PHOSPHORUS CONTENT AND WHOLE BODY PHOSPHORUS CONTENT IN COMMERCIAL BROILERS

5.1. Introduction

Recommendations for nutrient requirement are usually derived using the factorial approach based on a theoretical framework, or growth balance trials that give realistic estimates (McDonald *et al.,* 2011). These, however, require a knowledge of nutrient retention or comparative whole body analysis, which are not only laborious but expensive to determine. Consequently, the use of other simpler assays such as bone, growth, and blood criteria have been used to rank phosphorus availability (Rodehutscord, 2009). Results from these assays however are known to depend on the test material used, and criterion evaluated (Shastak *et al.,* 2012a), and do not provide biological retention values (Coon *et al.,* 2002) useful for comparing phosphorus availability from different sources. Nonetheless, bone measurements are still widely used as a simpler alternative to the whole body phosphorus retention assay to evaluate phosphorus availability. The question remains as to whether an individual bone is a reliable estimate of whole body phosphorus retention (Shastak *et al.,* 2012c).

Considerably more information is available on the relatedness between bone phosphorus content and phosphorus availability, but only very few studies have examined the relationship between bone mineral content and whole body phosphorus (WBP) content. Previous studies examining WBP content were conducted in small controlled trials, and have only examined its relatedness with tibia phosphorus content (Huyghebaert *et al*., 1980; Shastak *et al*., 2012c). Considering the differences between experimental trials and commercial husbandry practices, it is not known if previous results accurately model phosphorus retention in commercially raised chickens. Also, the femur and feet bones are commonly used in evaluating bone

mineralisation but, there is a current lack of understanding of the relatedness between WBP content and the phosphorus content of these bones.

5.1.2. Aims and objectives

The overall aim of this study was to characterise the normal range of WBP content of commercial broilers, and to examine its relatedness with the phosphorus content of different bones, in order to provide values that are applicable to commercial practice when evaluating phosphorus requirements.

The specific objectives were:

- To characterise the WBP content in commercial broilers.
- To examine the relatedness between WBP content and the phosphorus content of the tibia, femur, and feet in commercial broilers.

The hypothesis of this trial was that for each age assessed, any of the three leg bone types sampled would reflect one WBP content of the bird.

5.2. Materials and methods

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny *et al.*, 2010) were followed, and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee. 37 209 Ross 308 day-old mixed broiler flock were raised on wood shavings in an open plan house (1840m^2) which was thermostatically controlled to provide an initial room temperature of 30^{o}C. This was gradually reduced to 20°C by day 27 and maintained until the end of the experiment. Lighting was provided with 1h darkness from days 1-3 and then increased by 1h a day until day 6. Lighting was then maintained on a daily 4 and 2 hourly split darkness period between 7 – 32 days. On days 33 - 35, total daily darkness was reduced to 3,

2 and 1h respectively. 1h darkness was then maintained until the end of production cycle when birds were transported to processing plants. Standard commercial pelleted diets were formulated for 4 growth phases to meet or exceed the Ross 308 broiler nutrient specification (Aviagen, 2014). Diets and water were provided *ad libitum*. Birds were regularly checked to monitor environmental conditions and mortality recorded.

5.2.1. Sample collection

At the start of the trial, farm performance data for all flock of birds were evaluated in order to identify a rearing house which produced healthy birds with no diagnosed incidence of lameness or other health-related problems. This was a crucial screening criterion as this study was intended to characterise the normal range of WBP content and bone measurements in healthy birds.

In order to characterise age-related developmental changes, birds were collected at 3 different sampling points: days 14, 28 and 36 post-hatch. Each bird was individually assessed for lameness prior to selection using a 5 point qualitative gait score system after the method of Leterrier *et al.* (2008). Briefly, this involved classifying birds based on walking ability from 0 (no detectable gait abnormality) to 4 (severe gait defect), and only birds which scored 0 were selected. Birds were collected and grouped according to the 3 different bones studied, i.e. tibia, femur and feet as follows: 6 unsexed bird for each bone type studied at day 14; and 3 cockerels and 3 pullets each for each bone type studied at days 28 and 36 respectively, hence 54 birds in total. A questionnaire (Appendix 2) was then administered to the Farm Manager to capture the relevant flock performance data.

5.2.2. Bird preparation

Upon selection, each bird was individually euthanised by cervical dislocation and weighed. The birds were then dissected, and content in the crop, proventriculus, intestine, and caeca, emptied and flushed with ultra-pure water. The birds including the emptied organs were re-weighed to determine whole body weight after which both right and left bones (tibia, femur, and feet, according to the bone type studied) were removed as described in Chapter 2, Section 2.4.2.3. Whole body (WB) as used in this study is defined as the whole bird including feather, cleaned viscera and bones but emptied of digestive tract content. Briefly, flesh from the tibia and femur was carefully removed with a scalpel whilst ensuring cartilage caps were kept intact. Care was taken to ensure the flesh removed from the bones was added back to the remaining carcasses. The feet were not defleshed. The whole bird including feathers and viscera but excluding the tibia, femur or feet were then individually cut into small pieces and stored at -20°C until further processing. Their associated bones (tibia, femur or feet) were also individually stored at -20°C until further processing.

At processing, whole body (excluding sampled bones) were individually ground from slightly frozen in an electric mincer as described in Chapter 2, Section 2.4.2.4. Briefly, the cut pieces of the whole body were fed through the mincer and kneaded by hand before re-mincing. This process was repeated 4 times until the sample was thoroughly ground and homogenised. Between each bird minced, the electric mincer was fully cleaned and rinsed with ultra-pure water in order to prevent carryover of tissue. A 200g representative sample of each ground bird was then collected and stored at -20°C until further analysis.

5.2.3. Determined parameters

Bones were defleshed, and bone weight, length, width, and strength measured, and ash weight determined as previously described in Chapter 2, Sections 2.4.2.3, 2.5.1, 2.5.4.2 and 2.5.5.2.

Briefly right and left tibia and femur bones were individually weighed, and measured for length and width using a set of Vernier callipers. Bone strength was measured using a TA-XT Plus Texture Analyser (Stable Micro Systems, Surrey, UK) and dried at 105°C for 24h prior to fat extraction using the Soxtherm hot fat extraction method for 2h. Tibia and femur bones including cartilage caps were dried at 105°C for 24h until constant weight was achieved. Right and left feet bones were not fat extracted but dried at 105°C until constant weight was achieved. All bones were then ashed for 24h at 650°C in a furnace to determine ash weight.

Whole body samples were processed as described in Chapter 2, Section 2.4.2.4. Briefly, ground whole body samples (excluding bones) oven dried at 105°C for approximately 5 days until constant weight was achieved to determine moisture content and then extracted of fat using the Soxtherm hot fat extraction method for 2h as previously described in Chapter 2, Section 2.5.4.2. Whole body samples were then dried at 105°C for approximately 5 days until constant weight was achieved to obtain dried fat extracted samples. The, dry fat extracted samples were then ground, and 10g subsamples weighed to determine ash content. The 10g subsamples were gradually ashed in a furnace for 9h at 450°C, and then for a further 15h at 650°C. Both bone and whole body ash were analysed for total calcium and total phosphorus content using the ICP-OES assay as previously described in Chapter 2, Section 2.5.7.

5.2.4. Data analysis

Mean values of all right and left bone measurements were used for statistical analysis. WBP and WBCa content for each bird were calculated as the sum of calcium and phosphorus content determined in the whole bird plus that determined in their respective bones. Data were analysed using IBM SPSS version 23 for Windows (IBM Statistics, 2016). The General Linear one-way ANOVA model was used to evaluate the main effects of either bird sex (cockerels or pullets) or age (days) on bone parameters and WBP content for the whole

dataset. Significant differences in bone measurements, and whole body phosphorus and calcium content between cockerels and pullets at days 28 and 36 were separated using the Tukey's HSD procedure. The strength of the relationship between the calcium and phosphorus content in tibia, femur, feet and whole body; and with all other bone measurements were examined using the Pearson correlation procedure. Regression analysis was performed to examine the relatedness between the phosphorus content of the tibia, femur and feet bones and their respective WBP content. Statistically significant difference was declared at $P < 0.05$.

5.3. Results

None of the birds sampled in this study showed any signs of ill health, nutrient deficiency or lameness, nor revealed any signs of skeletal disease post-mortem. The body weight of the birds sampled ranged between 348g – 2747g, accounting for age and sex-related differences at the time of sampling. The overall growth rate (analysed from farm data) was in very close comparison with the Ross 308 performance objectives (Aviagen, 2014; Figure 5.1). The apparent deviation from the Ross 308 performance objectives is an artefact of the thinning process, where the largest birds are removed for slaughter and the rest left to grow a bit longer.

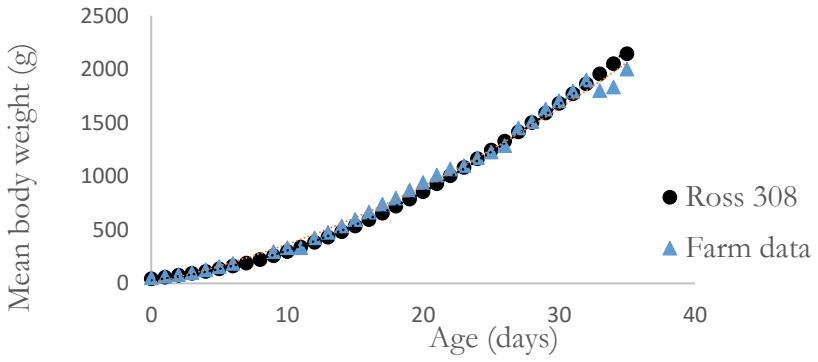

Figure 5.1. Growth performance of trial birds in comparison with Ross 308 growth performance objectives (Aviagen, 2014)

Mean body weight loss due to the emptying of the intestinal tract was 7.72 ± 2.44% of all birds sampled. An overall mean of 5.06g/kg WBP and 6.95 g/kg WBCa were determined in this study, and were not significantly different when analysed by age or sex (Tables 5.1). With an increase in age, significant differences were found for all bone parameters with the exception of ash percentage, WBP and WBCa (Tables 5.2 - 5.4). In general, greater bone measurements were found in cockerels compared with pullets and in older birds compared with younger birds.

Table 5.1. WBP and WBCa content in broilers

Bird	[1]N	WBCa (g/kg)	WBP (g/kg)	WBCa:WBP
Sex				
Day 14 unsexed bird	18	6.73	5.01	1.34[a]
Day 28 pullets	9	7.05	5.11	1.38 [ab]
Day 28 cockerels	9	7.38	5.20	1.42[b]
Day 36 pullets	9	6.98	5.07	1.37 [ab]
Day 36 cockerels	9	6.81	4.98	1.37[ab]
P value		0.359	0.764	0.020
Age (days)				
14	18	6.73	5.01	1.34[a]
28	18	7.21	5.17	1.40[b]
36	18	6.89	5.03	1.37[ab]
P value		0.179	0.498	0.010
Overall mean		6.95	5.06	1.37
Pooled SEM		0.107	0.054	0.008

[1]Number of birds.
[a, b]Means within the same column with no common subscript differ significantly ($P < 0.05$).

Table 5.2. Characterisation of tibia morphology, strength, mineralisation, WBP and WBCa content in broilers

	D14 unsexed	D28 pullets	D28 cockerels	D36 pullets	D36 cockerels	[1]SEM	*P* value
Whole bird weight (g)	509.57[a]	1341.13[b]	1505.4[bc]	1903.5[cd]	2192.46[d]	162.080	<0.001
Fresh tibia weight (g)	4.61[a]	11.25[b]	13.51[bc]	16.08[cd]	18.90[d]	1.362	<0.001
Tibia length (mm)	63.14[a]	90.32[b]	93.53[b]	104.42[c]	105.09[c]	4.318	<0.001
Tibia width (mm)	4.46[a]	6.14[b]	7.05[bc]	7.75[c]	8.20[c]	0.377	<0.001
Tibia strength (N)	88.43[a]	170.91[b]	197.08[b]	206.63[b]	226.88[b]	14.873	<0.001
[2]Dry tibia weight (g)	1.43[a]	4.08[b]	4.73[bc]	6.34[cd]	7.11[d]	0.549	<0.001
Tibia ash (g)	0.54[a]	1.63[b]	1.90[b]	2.41[bc]	2.71[c]	0.214	<0.001
Tibia ash %	37.77	40.10	40.11	37.97	37.89	0.415	0.145
Tibia P content (mg)	75.89[a]	238.03[b]	265.12[b]	344.07[bc]	387.92[c]	30.888	<0.001
Tibia Ca content (mg)	182.86[a]	580.88[b]	651.26[b]	841.92[bc]	951.89[c]	76.153	<0.001
WBP content (g/kg)	4.65	4.94	5.18	4.91	4.98	0.073	0.143
WBCa content (g/kg)	6.18	6.80	7.38	6.78	6.75	0.144	0.061

[1]Pooled standard error of mean.
[2]Fat extraxted.
[a, b, c, d] Means within the same row with no common subscript differ significantly ($P < 0.05$).

Table 5.3. Characterisation of femur morphology, strength, mineralisation, WBP and WBCa content in broilers

	D14 unsexed	D28 pullets	D28 cockerels	D36 pullets	D36 cockerels	[1]SEM	*P* value
Whole bird weight (g)	499.13[a]	1241.73[b]	1429.30[bc]	1714.10[c]	2506.40[d]	171.712	<0.001
Fresh femur weight (g)	3.16[a]	7.70[b]	10.12[c]	10.51[c]	14.58[d]	1.007	<0.001
Femur length (mm)	46.52[a]	63.46[b]	67.74[c]	73.26[d]	75.27[d]	2.846	<0.001
Femur width (mm)	5.10[a]	7.35[b]	8.38[bc]	8.58[c]	10.11[d]	0.455	<0.001
Femur strength (N)	116.51[a]	206.99[b]	227.90[b]	219.24[b]	315.24[c]	17.117	<0.001
[2]Dry femur weight (g)	1.01[a]	2.79[b]	3.56[c]	3.76[c]	5.43[d]	0.384	<0.001
Femur ash (g)	0.39[a]	1.05[b]	1.32[c]	1.41[c]	2.02[d]	0.141	<0.001
Femur ash %	38.54	37.50	37.07	37.62	37.18	0.209	0.066
Femur P content (mg)	55.15[a]	156.02[b]	197.53[c]	221.23[c]	315.76[d]	22.558	<0.001
Femur Ca content (mg)	131.06[a]	376.15[b]	487.18[c]	534.07[c]	763.50[d]	54.927	<0.001
WBP content (g/kg)	5.35	5.64	5.62	5.09	4.95	0.093	0.088
WBCa content (g/kg)	7.42	7.97	8.20	7.03	6.70	0.190	0.101

[1]Pooled standard error of mean.
[2]Fat extraxted.
[a, b, c, d]Means within the same row with no common subscript differ significantly ($P < 0.05$).

Table 5.4. Characterisation of feet mineralisation, WBP and WBCa content in broilers

Bird	D14 unsexed	D28 pullets	D28 cockerels	D36 pullets	D36 cockerels	[1]SEM	*P* value
Whole bird weight (g)	497.70[a]	1200.83[b]	1359.80[bc]	1763.30[c]	2260.80[d]	159.153	<0.001
Fresh feet weight (g)	9.25[a]	21.03[b]	26.16[bc]	32.91[c]	43.91[d]	3.084	<0.001
Dry feet weight (g)	3.64[a]	8.22[b]	10.33[bc]	12.38[c]	16.24[d]	1.129	<0.001
Feet ash (g)	0.51[a]	1.19[b]	1.51[bc]	1.88[c]	2.40[d]	0.170	<0.001
Feet ash %	14.14	14.48	14.69	15.19	14.81	0.165	0.295
Feet P content (mg)	77.33[a]	150.24[b]	219.47[c]	254.16[c]	316.61[d]	22.157	<0.001
Feet Ca content (mg)	166.25[a]	351.76[b]	515.20[c]	612.31[c]	765.63[d]	55.365	<0.001
WBP content (g/kg)	5.04	4.76	4.80	5.22	5.00	0.082	0.503
WBCa content (g/kg)	6.61	6.38	6.56	7.13	6.98	0.165	0.704

[1]Pooled standard error of mean.
[a, b, c, d]Means within the same row that do not have the same subscript differ significantly ($P < 0.05$).

5.4. Discussion

Increase in growth rate has been reported to affect growth allometry (Schmidt *et al.,* 2009) and skeletal integrity (Williams *et al.,* 2000b; Angel, 2007). Consequently, there is the need to investigate the effect of changing nutrient requirements in relation to rapidly changing carcass characteristics (Applegate and Angel, 2014).

In order to accurately meet phosphorus requirement while avoiding over supply, precise knowledge of the available phosphorus contribution of an ingredient is required (Liu *et al.,* 2012). This is usually determined by feeding an animal a test phosphorus source in a dose-response trial, taking into account the amount of phosphorus ingested and excreted. Phosphorus availability could also be determined by the direct measurement of WBP content, although there is difficulty in obtaining homogeneous samples (Haag, 1939). In general, both assays require substantial laboratory effort hence easier alternatives such as the use of growth, blood or bone characteristics to rank phosphorus availability are often employed (Rodehutscord, 2009).

The use of various bone criteria (morphology, strength, and ash) which dates back its historic use in relative phosphorus bioavailability studies (Hurwitz, 1964; Ravindran *et al.,* 1995) is still widely used in evaluating phosphorus availability (Karimi *et al.,* 2013; Faridi *et al.,* 2015). This study builds on findings from previous studies by evaluating the phosphorus content of various bone types (tibia, femur and feet) and their relatedness with WBP content in commercial broilers.

5.4.1. WBP and WBCa content

There is a paucity of WBP and WBCa data in the literature. In this study, there were no significant differences when WBP or WBCa content was analysed by age or sex (Table 5.1). The overall mean WBP and WBCa retention (5.06 and 6.95 g/kg respectively) were in very close agreement with the WPSA report (WPSA, 1985) and that of Nieß *et al.* (2005), but in contrast higher than that reported in other studies (Table 5.5) used for comparison. The mean values from those studies (Table 5.5) were however derived from phosphorus/calcium dose-response trials in which WBP and WBCa content were determined in birds given increasing concentration of dietary phosphorus and calcium.

Table 5.5. WBP (g/kg) and WBCa (g/kg) from previous reports

Age (days)	[1]Cockerels	Pullets	[1]Unsexed	Source
0			3.4 (4.3)	
21			4.9 (6.8)	
42			4.8 (6.7)	WPSA, 1985
21 – 42	5.1 (6.9)			Nieß *et al.*, 2005
21			2.2 – 3.9 (2.4 – 5.5)	
35			3.0 – 4.5 (3.4 – 6.8)	Shastak *et al.*, 2012b
35 and 43	4.7	4.5		Van Krimpen *et al.*, 2013
10	4.5 (5.3 –6.1)			
21	4.75 (5.7 – 6.8)			
30	4.48 (6.0)			
38	4.19 (5.6)			Van Krimpen *et al.*, 2016

[1]Corresponding WBCa content in parenthesis

Shastak *et al.* (2012c) reported a range of WBP content: 2.2 – 3.9 g/kg at day 21, and 3.0 – 4.5 g/kg at day 35 respectively. In that study, a linear and significant increase in WBP was observed in birds given increasing levels of dietary phosphorus concentration (3.5 - 5.9 g/kg total phosphorus) intended to be below requirement across 7 treatments. Interestingly, a relatively constant 5g/kg WBP was determined in this study despite the increase in body weight (Figure 5.2.). This is similar to the findings of Narcy (2014) cited by Bedford *et al.* (2016) who reported 5g/kg WBP content in 500g and 2500g broilers. This is also in agreement with the report of Nieß *et al.* (2005) who concluded body weight had no effect on the concentration of major minerals and trace elements.

WBCa:P data determined in this study were significantly different according to age or sex (Table 5.1) but these were in close comparison to that of the WPSA report (WPSA, 1985) for 0, 3 and 6 weeks broilers.

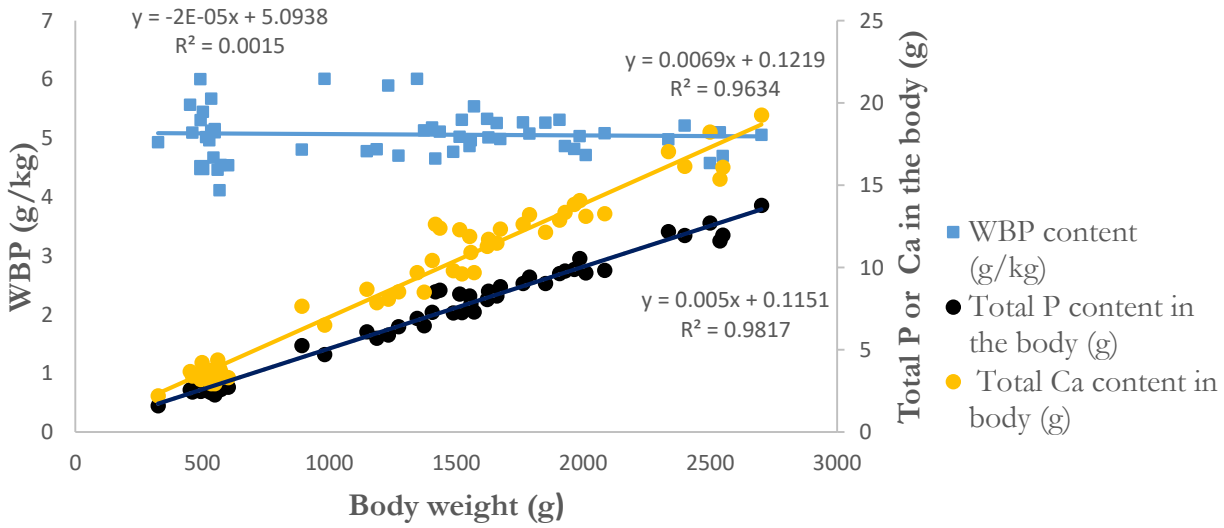

Figure 5.2. Whole body phosphorus and whole body calcium retention as a function of body weight

The lower WBCa and WBP values reported by other researchers listed in Table 5.5 compared to this study can be explained. In those trials, the diets were formulated to be lower than phosphorus requirement which resulted in the lower amount of phosphorus retained in the birds. On the contrary, the WBCa and WBP data determined in some other reports (Nieß *et al.*, 2005; Narcy, 2014) was similar to the findings of this study where broilers were fed nutritionally adequate calcium and phosphorus diets. This suggests there is a limit to the amount of calcium and phosphorus retained in the whole body, orchestrated by the process of homoeostasis which controls their assimilation and metabolism.

Phosphorus utilisation is improved when dietary calcium concentration is low, and Ca:P ratios are narrow (Selle *et al.*, 2009). However, improved phosphorus absorption could lead to increased phosphorus excretion when dietary calcium is low. Plumstead *et al.* (2008) evaluated the effect of calcium on phosphorus utilisation by feeding broilers varying levels of Ca:P diets. The authors reported that although feeding low dietary calcium concentration increased phosphorus absorption in the intestine, there was a corresponding increase in phosphorus excretion due to lack of sufficient calcium needed to combine with the circulating phosphorus to form hydroxyapatite in the bone. The authors concluded that the kidneys play an important role in regulating plasma calcium and phosphorus concentration and therefore any excess phosphorus is excreted in the urine. It can thus be concluded that the nutritional state of the birds determines the amount of phosphorus retained; and that when fed adequate amount of dietary phosphorus and calcium, there is a limit to the amount retained in the whole body. This conclusion highlights the relevance of the WBP data determined in this study, which was conducted using diets formulated to meet or exceed the estimated phosphorus requirements for each growth phase, in contrast to WBP data determined in previous studies that focussed on phosphorus bioavailability, where marginally deficient phosphorus diets were used.

Table 5.6. Bone Ca:P and the relatedness between bone P content and WBP content

Age (days)	Tibia Ca:P	Tibia WBP:Bone P	Femur Ca:P	Femur WBP:Bone P	Feet Ca:P	Feet WBP:Bone P
14	2.41[a]	31.41	2.37	48.50[a]	2.11[a]	32.38
28	2.45[b]	28.65	2.44	42.57[b]	2.34[b]	33.77
36	2.45[b]	27.90	2.42	40.49[b]	2.41[b]	34.01
P value	0.005	0.092	0.158	0.001	<0.001	0.217
Mean	2.43	29.33	2.41	43.85	2.29	34.05
Pooled SEM	0.007	0.711	0.014	1.054	0.039	0.844

[a, b]Means within the same column with no common subscript differ significantly ($P < 0.05$).

WBCa:P ratio in this study at days 14, 28 and 36 were 1.34, 1.40 and 1.37 respectively (Table 5.1). These were in close comparison to that of the WPSA report for 0, 3 and 6 weeks broilers fed adequate dietary calcium and phosphorus diets: 1.3, 1.4 and 1.4 respectively (WPSA, 1985). Given that all the birds received nutritionally adequate diets and that relatively more calcium was retained in the bones at days 28 and 36 compared to day 14, it is therefore not surprising that with an increase in body weight, relatively more calcium was retained in the whole body compared with phosphorus as clearly shown in Figure 5.2.

The overall bone Ca:P ratio determined in this study was 2.38, indicating a higher proportion of calcium relative to phosphorus was retained in the bone. Also notable is the lower bone Ca:P ratio determined at day 14 in the 3 bone types compared days 28 and 36 (Table 5.6). Williams *et al.* (2000b) observed bone mineral is not a pure substance. Bone acts as a reservoir, and it is involved in the removal and replacement of calcium and phosphorus in the bone crystal lattice. This may disrupt the Ca:P molar ratio especially during the critical period of bone development. In young broilers, the period of rapid bone formation (4 - 18d) and mineralisation (4 - 11d) occurs during early development (Williams *et al.*, 2000b). The young immature bones are likely to have a greater amount of the mineral precursors of hydroxyapatite present (McLean and Urist, 1968); and the numerous exchange of

calcium/phosphate groups in the crystal lattice of the bone as it functions as a mineral reservoir may have resulted in the lower molar Ca:P ratios observed at day 14 compared to days 28 and 36. This suggests that with an increase in age, more calcium is retained, and explains the divergence in the greater amount of WBCa relative to WBP in larger birds (Figure 5.2).

5.4.2. Relatedness between bone ash or phosphorus content with WBP content

Strong correlations ($r \geq 0.985$) were found between the ash weight of the various bones and their respective WBP content (Tables 5.7 – 5.9). Strong correlations ($r \geq 0.978$) were also found between the phosphorus content of the various bones and their respective WBP content. This indicates bone phosphorus content or ash weight are reliable indicators of WBP retention. In contrast, bone ash percentage was weakly correlated with tibia phosphorus content and WBP content (Table 5.7); and negatively correlated with femur phosphorus content and WBP content (Tables 5.8). Medium strength correlations were found between feet ash percentage and feet phosphorus content and WBP content (Tables 5.9).

Although bone ash percentage is routinely used to evaluate bone mineralisation in poultry, the negative to medium strength correlations observed indicates it is not a sensitive measure of WBP content. The poor reliability of ash percentage in indicating phosphorus availability or adequately assessing bone mineralisation has been questioned by other investigators (Hall *et al.*, 2003; Li *et al.*, 2015). Coon *et al.* (2007) observed that the slope of percentage bone ash measurements was not as sensitive as bone ash weight or bone breaking force in evaluating the relative biological availability of a phosphorus source. From this study, it can be concluded that bone ash weight and retained phosphorus in the bone are reliable indicators of WBP content, the contrary being the case for bone ash percentage.

Acknowledging the difficulty in obtaining WBP content, Hurwitz (1964) proposed tibia phosphorus content might be a reliable estimate of WBP content after observing a constant WBP:tibia phosphorus ratio of 19.6 in broilers irrespective of dietary treatment. Huyghebaert *et al.* (1980) reported a WBP:tibia phosphorus ratio of 17.95 when broilers were fed dietary phosphorus content of less than 0.6%; and 16.8 at higher dietary phosphorus levels indicating more phosphorus was retained in the tibia at higher dietary phosphorus concentration. Recently, Shastak *et al.* (2012c) fed broilers incremental levels of phosphorus and reported a WBP:tibia phosphorus ratio of 21.3 and 19.8 at days 21 and 35 respectively. The mean WBP:tibia phosphorus ratio determined in this study (29.33, Table 5.6) is much higher than the values previously reported by other investigators (Hurwitz, 1964; Huyghebaert *et al.,* 1980; Shastak *et al.*, 2012c). Although not significant, there was a numerical decrease in WBP:tibia phosphorus ratio with age. This indicates that as the birds aged, relatively more phosphorus was retained in the bones compared to what was retained in the whole body.

Data from this study suggests WBP:tibia phosphorus ratio may not be as constant as previously assumed. As noted by Shastak *et al.* (2012c), this might also be due to differences in the length of experimental period, varying dietary phosphorus content, age of bird at sampling, and the changes in bird composition due to selection for improved growth rate, each of which might have altered the WBP:bone phosphorus composition of broilers. It might also be due to environmental or husbandry effects as previous data were derived from birds reared in controlled experimental conditions where measurements are more precise compared to the birds in this study which were raised in a commercial setting. Lighting, litter quality, health management and humidity are factors that could affect bone quality (Hester, 1994).

Table 5.7. Pearson correlation values between tibia and whole body P and Ca content

	Whole bird weight (g)	**Tibia weight (g)**	**Tibia length (mm)**	**Tibia width (mm)**	**Tibia Strength (N)**	**Dry fat extracted tibia weight (g)**	**Tibia ash weight (g)**	**Tibia ash %**	**Tibia P content (mg)**	**Tibia Ca content (mg)**	**WBP content (mg)**	**WBCa content (mg)**
Tibia P content (mg)	0.994	0.987	0.967	0.983	0.963	0.991	0.998	0.284		1.000	0.986	0.981
Tibia Ca content (mg)	0.994	0.987	0.966	0.984	0.964	0.991	0.998	0.287	1.000		0.986	0.981
Whole body P content (mg)	0.995	0.992	0.980	0.979	0.944	0.993	0.990	0.207	0.986	0.986		0.999
Whole body Ca content (mg)	0.990	0.989	0.983	0.978	0.945	0.989	0.986	0.213	0.981	0.981	0.999	

Table 5.8. Pearson correlation values between femur and whole body P and Ca content

	Whole bird weight (g)	**Femur weight (g)**	**Femur length (mm)**	**Femur width (mm)**	**Femur Strength (N)**	**Dry fat extracted femur weight (g)**	**Femur ash weight (g)**	**Femur ash %**	**Femur P content (mg)**	**Femur Ca content (mg)**	**WBP content (mg)**	**WBCa content (mg)**
Femur P content (mg)	0.990	0.994	0.958	0.984	0.967	0.997	0.997	-0.590		1.000	0.984	0.975
Femur Ca content (mg)	0.988	0.995	0.959	0.986	0.968	0.998	0.998	-0.593	1.000		0.984	0.975
Whole body P content (mg)	0.989	0.985	0.948	0.966	0.948	0.984	0.985	-0.585	0.984	0.984		0.997
Whole body Ca content (mg)	0.975	0.977	0.950	0.962	0.939	0.976	0.977	-0.593	0.975	0.975	0.997	

Table 5.9. Pearson correlation values between feet and whole body P and Ca content

	Whole bird weight (g)	**Feet weight (g)**	**Dry feet weight (g)**	**Feet ash weight (g)**	**Feet ash %**	**Feet P content (mg)**	**Feet Ca content (mg)**	**WBP content (g/kg)**	**WBCa content (g/kg)**
Feet P content (mg)	0.981	0.981	0.988	0.992	0.493		0.999	0.978	0.977
Feet Ca content (mg)	0.981	0.984	0.990	0.994	0.480	0.999		0.978	0.977
Whole body P content (mg)	0.996	0.989	0.988	0.990	0.480	0.978	0.978		0.999
Whole body Ca content (mg)	0.993	0.988	0.986	0.988	0.471	0.977	0.977	0.999	

5.4.3. Relatedness between femur and feet bone phosphorus content and WBP content

Bone measurements are known to vary in relatedness and level of sensitivity to mineralisation (Ravindran *et al.,* 1995; Cheng and Coon, 1990). The relatedness between tibia phosphorus content and WBP content have been examined and discussed. Although the ash content of the femur and feet ash have also been used to evaluate bone mineralisation in broilers (Hemme *et al.,* 2005; Garcia and Dale, 2006), the relationship between the phosphorus content of these bones (femur and feet) and WBP content have not been reported in the literature. Strong correlations linear were found between the phosphorus content of all three bones and WBP content (Figure 5.3).

Similar to the tibia bone, there was a numerical decrease in WBP:femur phosphorus content with age (Table 5.6). The age-related decrease in WBP:bone phosphorus ratio of the tibia and femur can be explained by the increase in size and mineralisation of these bones with age. The amount of minerals deposited in the bone collagen matrix is known to increase with age (Rath *et al.*, 2000). But in contrast, an increase was observed in WBP: feet phosphorus content with age. This indicates that relatively more phosphorus was retained in the whole body compared with that retained in the feet. Feet ash percentage was considerably less ($P = 0.001$) than the ash percentage determined in the other bones suggesting it contained less mineral content per unit weight.

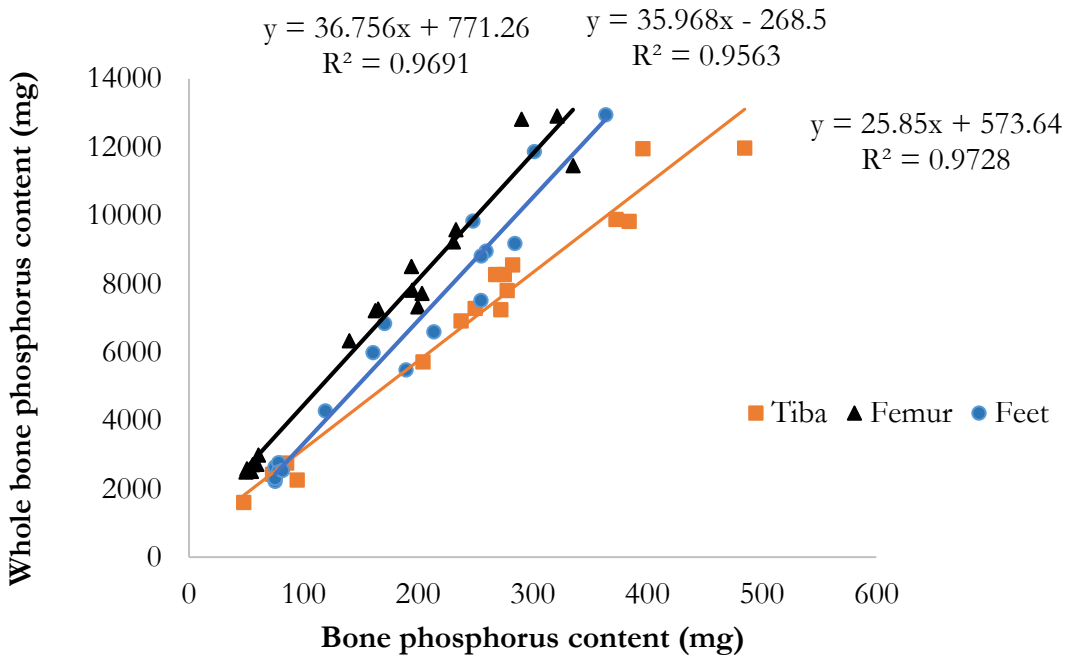

Figure 5.3. Regression of bone phosphorus content on WBP

Shastak *et al.* (2012c) determined a constant slope of 17.7 in day 21 and 35 birds when the phosphorus content of tibia bones was regressed against WBP content, indicating that for each mg of phosphorus retained in the tibia, 17.7mg was retained in the whole body. The authors suggested the slope may be a suitable criterion for determining WBP retention in broilers. Data from this study was further analysed to evaluate the correlation between phosphorus retained in the 3 bones and phosphorus retained in the whole body.

Linear increases in WBP content relative to increase in bone phosphorus were observed. From the slopes of regression (Figure 5.3), for each mg retained in the tibia, femur, and feet, 25.85mg, 36.76mg and 35.97mg phosphorus were retained in the whole body respectively. The slope of regression for the femur and feet bone is not available in the literature to make any comparisons. The slope of WBP retention relative to retained phosphorus in the tibia determined in this study was higher than that reported by Shastak *et al.* (2012c). It is possible

that the differences in diet composition between experimental trials (adequate dietary phosphorus in this study vs. a range of dietary phosphorus concentration formulated below requirement) may have contributed to the observed differences and warrants further investigation. The variability in phosphorus availability is known to affect bone mineral status in poultry (Adeola, 2010); and this directly determines the amount of phosphorus retained in the bone or whole body. The possible difference in husbandry methods (commercial husbandry practice in this study vs. precise experimental trial) might also be a possible explanation.

It was interesting to note that the slopes of the femur and feet phosphorus content regressed against WBP content were parallel and greater than the slope of tibia phosphorus content. The proximal region of the tibia is the fastest growing growth plate in broilers (Julian, 1998); and as a result of rapid growth retained more phosphorus. The mean ash weight was consistently greater in the tibia compared to the femur or feet ash weight at all sampling age (Table 5.10), and consequently contained more phosphorus than the femur and feet. Thus while the ratio between WBP and the phosphorus retained in the femur and feet followed a similar linear pattern, with an increase in age, disproportionately more phosphorus was retained in the tibia relative to that retained in the whole body. The findings from this study do not support the hypothesis that for each age assessed, any of the 3 leg bone type sampled reflects one WBP content of the bird.

Table 5.10. Ash weight and phosphorus content of the tibia, femur and feet

Bone parameter	D14	D28	D36
Bone ash (g)			
Tibia bone ash	0.54[a]	1.76[a]	2.56[a]
Femur bone ash	0.39[b]	1.18[b]	1.72[b]
Feet bone ash	0.51[a]	1.35[b]	2.14[ab]
P value	0.004	0.001	0.010
Pooled SEM	0.022	0.076	0.124
Bone phosphorus content (mg)			
Tibia phosphorus content	76[a]	252[a]	366[a]
Femur phosphorus content	55[b]	177[b]	268[b]
Feet phosphorus content	77[a]	185[b]	285[ab]
P value	0.002	0.003	0.031
Pooled SEM	3.242	11.134	16.937

[a, b]Means within the same column with no common subscript differ significantly ($P < 0.05$)

5.4.4. Implication

As already discussed, it is important to continually re-evaluate the optimal phosphorus requirement in poultry. Given that the amount of phosphorus retained in the body is fairly constant as demonstrated in this study, digestible phosphorus requirement was re-calculated (Figure 5.4) using the method of Bedford *et al.* (2016) by dividing the weight gain increment in phosphorus (derived using the mean WBP) by feed intake (using the Ross 308 performance objectives, Aviagen, 2014).

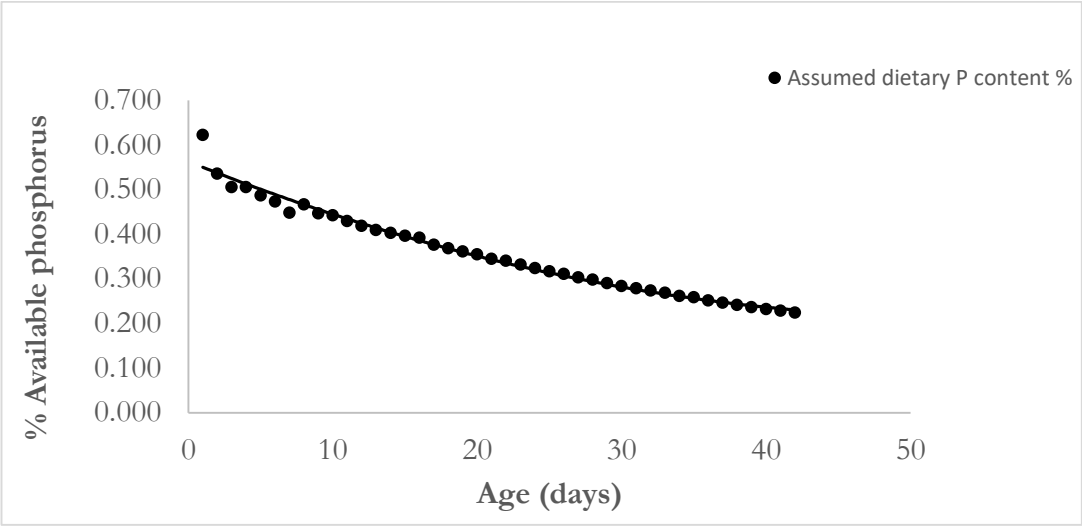

Figure 5.4. Estimated digestible phosphorus requirement as a function of age

The current NRC requirement for 3 - 6 week old broilers is 0.35% nPP, however by day 22, 0.34% avP was determined in this study, and this decreased with age. Although the absolute requirement will depend on environmental, husbandry and nutritional status of the bird as noted by Bedford *et al.* (2016), this study shows that beyond 21 days, the calculated available requirement is less than 0.35%. This implies that the current NRC recommendation for 3 - 6 week old broilers may exceed requirement, as previously suggested by Dhandu and Angel (2003). This study also suggests a mean value of 2.6g/kg available phosphorus is required to meet the Ross 308 performance objective between 31 - 38 days, and this is in exact agreement with the findings of Van Krimpen *et al.* (2016).

5.5. Conclusion

Phosphorus plays an important role in growth and bone mineralisation. As a finite resource, preserving the global phosphate reserves in a sustainable way is of major concern. To this end, the precise use of dietary phosphorus in order to minimise wastage has long been recognised (Summers, 1997).

WBP determined in this study was in close comparison to findings of other reports (WPSA, 1985; Nieß *et al.*, 2005; Narcy, 2014) where broilers were fed adequate calcium and phosphorus diets. This study demonstrates that despite the improvements in growth rate and muscle mass observed in modern broiler strains, WBP and WBCa content in broilers, including the partitioning of calcium in relation to phosphorus in the whole body of broilers has remained the same irrespective of age and sex. The strong linear relationship between WBP content and various bone measurements (including retained phosphorus content) suggests the bone is a reliable indicator of WBP retention in broilers. However, the lack of agreement between the data determined in this study and that reported in previous studies further highlights the difficulty in defining the relationship between bone phosphorus retention and WBP phosphorus content by a mathematical constant and needs to be examined further.

More studies are required to validate the data obtained in this study, and to establish if the results are as a result of environmental and husbandry effects common to commercial husbandry, or are due to the effect of selective breeding on the current broiler strains. Further work is also required to ascertain what dietary phosphorus requirements are, particularly at the finishing stage of production when feed intake in increased. This should be done in light of the vast array of nutritional strategies currently utilised to aid phosphorus utilisation and improve bone mineralisation in poultry, giving careful consideration to bird welfare and economic objectives

CHAPTER 6: NUTRITIONAL IMPRINTING AS A TOOL FOR IMPROVING PHOSPHORUS UTILISATION IN BROILERS

6.1. Introduction

Earlier studies in this thesis examined appropriate tools and methodologies for assessing phosphorus requirement in broilers. The next two studies examine strategies for maximising the efficiency of phosphorus utilisation in broilers.

Reducing excessive dietary levels of phosphorus is one of the nutritional strategies adopted to minimise wastage (Waldroup, 1999). Beyond efforts to precisely meet the bird's needs, restricting phosphorus supply in broiler diets has been investigated in older birds (21 days or more) without adverse effect on performance (Skinner *et al.*, 1992; Dhandu and Angel, 2003). However, feeding phosphorus deficient diet continuously from hatch through to finishing (Moran and Todd, 1994), or omitting dietary phosphorus at the finishing stage (Chen and Moran, 1995) have resulted in poor bone mineralisation and a high incidence of broken bones at processing. Such nutritional strategies are therefore not ideal for minimising phosphorus usage from both the commercial and welfare perspectives.

The nutritional imprinting of birds for better nutrient absorption is a new and emerging approach reported to improve flock performance and reduce environmental pollution through better nutrient absorption (Angel, 2010). Angel and Ashwell (2008) demonstrated the benefits of nutritionally imprinting broilers with low phosphorus diets early post-hatch. In that study, birds in the test group were fed a moderately deficient Ca/P diet (0.59% Ca / 0.25% avP) for 90 hours immediately post-hatch; followed by a standard Ca/P diet (1.11% Ca / 0.5% avP) until day 22 (starter phase). They were then compared with birds fed the same standard diet (1.11% Ca / 0.5% avP) throughout the starter phase (1 - 22d). Marginally deficient diets (0.4% Ca / 0.11% avP) were then fed to both bird groups afterward until 38

days (finishing phase). The authors reported that the nutritionally imprinted birds were significantly heavier, had a better feed to gain ratio, higher phosphorus absorption, and greater tibia ash weight compared to the control group birds by 38 days. However, despite the promising results of nutritional imprinting of broilers for better phosphorus utilisation conducted in experimental battery cages, it has not been adopted for use in commercial poultry farming.

6.1.2. Aims and objectives

The overall aim of this trial was to verify the impact of nutritional imprinting on phosphorus utilisation.

The specific objectives were:

- To evaluate the effect of nutritional imprinting on growth performance.
- To evaluate the effect of nutritional imprinting on bone morphometry and bone mineralisation.
- To examine the effect of nutritional imprinting on ileal phosphorus and calcium digestibility.
- To evaluate the effect of nutritional imprinting on the gene expression of sodium-phosphate cotransporter type IIb used as a marker for phosphate transport.

The hypothesis of this trial was that phosphorus utilisation may be manipulated by nutritional imprinting. Broilers were raised in floor pens to simulate current commercial rearing practice.

6.2. Materials and methods

6.2.1. Trial procedure

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny *et al*., 2010) were followed, and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee. 144 one day-old male Ross 308 broiler chicks were used in a 28-day experiment. Chicks from a parent flock aged 45 weeks were sourced from PD Hook Hatcheries Ltd, Oxfordshire, and housed in an environmentally controlled room as previously described in Chapter 2, Section 2.2. The experimental design consisted of two groups of birds: a control group and test group of birds. Each group consisted of 6 replicate pens, each containing 12 birds per pen at placement. On arrival, the birds were weighed to determine individual bird weight, and were then allocated to pens ensuring there were no significant differences in mean pen weight across treatments. Maize/soya mash diets (Table 6.1) were formulated for two growth phases (starter: 1 - 18 days and grower: 19 - 28 days).

Table 6.1. Feed composition of experimental diets (g/kg as fed basis)

Ingredient	Starter diet	Low P starter diet	Low P grower diet
Maize	546.9	579.1	603.5
Soya 48	367.9	362.4	335.1
Soya oil	35	25	33.9
Calcium carbonate	16.5	8.7	5.5
Monocalcium phosphate	16.7	7.8	3.6
DL-Methionine	1.6	1.6	1.7
L-lysine HCL	2.2	2.2	3.2
Vitamin premix*	4	4	4
Titanium dioxide	5	5	5
Salt	3	3	3
Threonine	1.2	1.2	1.5

Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg; retinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 μg; folic acid, 1.5 mg; and biotin 125 mg

During the starter phase (day 1 – day 18), birds in the control group were fed a standard mash starter diet formulated to meet the Ross 308 (Aviagen, 2014) nutrient specification for all nutrients including Ca and P (1.05% Ca and 0.5% nPP). The test group of birds were fed a low Ca/P mash diet (0.6% Ca and 0.3% nPP) for the first four days from hatch, and were then fed the same standard starter diet as the control group of birds (1.05% Ca and 0.5% nPP) from day 5 to day 18. A marginally low phosphorus diet (0.4% Ca and 0.2% nPP) was then fed to both groups of birds from 19 - 28 days. Diets and water were provided *ad libitum* to all birds during the experimental procedure. Birds were observed twice daily to monitor environmental conditions and mortality recorded. Nutrient analysis of experimental diets are presented in Table 6.2.

Table 6.2. Nutrient analysis of experimental diets (% as fed basis)

	Starter diet		**Low P starter diet**		**Low P grower diet**	
Composition	**Calculated**	**Analysed**	**Calculated**	**Analysed**	**Calculated**	**Analysed**
Dry matter	90.15	84.54	89.89	85.25	88.90	85.54
ME (MJ/kg)	12.9		12.9		13.4	
Crude protein	22.5	22.2	22.5	22.7	21.5	21.4
Crude fat	5.98	6.01	5.11	5.13	6.07	6.09
Lysine	1.44		1.43		1.43	
Threonine	0.96		0.96		0.94	
Methionine	0.51		0.51		0.51	
Total calcium	1.05	1.05	0.60	0.62	0.40	0.40
Total phosphorus	0.76	0.77	0.56	0.55	0.46	0.42
nPP	0.50	0.49	0.30	0.30	0.20	0.19
Titanium dioxide	0.50	0.52	0.50	0.52	0.50	0.49

6.3. Sample collection

Sampling was carried out on days 4 and 18, planned to coincide with diet change, and on day 28 when the experiment was concluded. Six birds per replicate pen were euthanised on day 4 by cervical dislocation to ensure sufficient digesta was obtained for analysis as described in

Chapter 2, Section 2.4.2.2. Briefly, digesta was collected from the distal end of the small intestine, identified as the portion between Meckel's diverticulum and the ileal-ceco-colonic junction, for ileal digestibility studies. Digesta was pooled per pen and frozen at -20°C until analysis. 100g duodenal tissue was collected from 1 bird per replicate pen for total NaPi-IIb mRNA quantification as described in Chapter 2, Section 2.5.11, and stored in RNAlater at -80°C until further processing. Left tibia bones were collected from two birds per pen as described in Chapter 2, Section 2.4.2.3 for bone measurements. Briefly, the bones were separated at the tibiotarsal junction where the feet were removed, and the tibio-femoral junction where the femurs were removed, and then individually stored at -20°C until further processing. Digesta, duodenal tissue, and left tibia bones were also collected on days 18 and 28 from two birds per replicate pen after euthanasia by cervical dislocation as described for sampling day 4.

6.4. Determined parameters

6.4.1. Growth performance

Body weight, body weight gain, feed intake, and feed conversion ratio were evaluated per pen on sampling days (days 4,18 and 28); and on a weekly basis as described in Chapter 2, Section 2.4.1. FCR of birds was calculated by dividing feed intake by body weight gain, taking into account any mortalities.

6.4.2. Tibia bone morphometry, strength, and ash

Tibia weight, length, width, strength, and ash content of the left tibia bones were measured as previously described in Chapter 2, Sections 2.5.1, 2.5.4.1 and 2.5.5.2. Briefly, flesh from all bones was manually removed and weighed whilst leaving the cartilage caps intact. Bone length and width were measured using a set of Vernier callipers. Bone strength was measured using a TA-XT Plus Texture Analyser (Stable Micro Systems, Surrey, UK) before fat extraction

using the Soxhlet fat extraction method for 6h. The tibia bones including cartilage caps were then dried at 105°C for 24h until constant weight was achieved and then ashed for 24h at 650°C in a furnace to determine ash weight.

6.4.3. Diet nutrient analysis

Diets were analysed for dry matter, protein content and fat content as previously described in Chapter 2, Sections 2.5.2, 2.5.3 and 2.5.4.1.

6.4.4. Titanium dioxide, calcium and phosphorus analysis

Diets and digesta were analysed for titanium dioxide content using the ICP-OES assay previously described in Chapter 2, Section 2.5.6. Diets, digesta, and bone ash were also analysed for total calcium and total phosphorus content using the ICP-OES assay previously described in Chapter 2, Section 2.5.7.

6.4.5. Non-phytate phosphorus

Digesta samples were freeze-dried and finely ground using a pestle and mortar. The total phytic acid content of ileal digesta samples was analysed using a Megazyme™ K-PHYT assay (Megazyme International, Ireland) previously described in Chapter 2, Section 2.5.8. The non-phytate phosphorus (nPP) content of diets and digesta samples were calculated as the difference between total phosphorus and phytate phosphorus.

6.4.6. Sodium phosphate cotransporter IIb mRNA

Sodium phosphate cotransporter (NaPi-IIb) mRNA used as a marker for phosphate transport was isolated and quantified according to the method outlined in Chapter 2, Section 2.5.11. Briefly, 100g duodenal tissue was collected from one bird per pen, placed in RNAlater, and subsequently stored at −80°C. At processing, tissue samples were homogenised in 1ml Tri-Reagent (Life Technologies) with 5mm stainless steel beads (Qiagen) in the Qiagen Tissue

Lyser II. Phase separation was performed using molecular grade 1-bromo-3-chloropropane (Sigma). RNA was purified from the aqueous phase using the MagMAX-94 for Microarray Isolation kit (Life Technologies) and the RNA subsequently stored at -20°C.

RNA (5µl) was reverse-transcribed into cDNA using 20µl RT premix 2 (Primerdesign). The reaction was performed at 42°C for 20 minutes and 72°C for 10 minutes cDNA was stored at -20°C until used in the PCR reaction. The NaPi-IIb gene-specific primers were designed by Primerdesign Ltd, UK. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping. Quantitative Real-Time PCR was performed using Stratagene Mx3005p (Agilent Technologies). Relative gene expression was determined by the comparative cyclic threshold (C_T) method of Livak and Schmittgen (2001).

6.5. Data analysis

Results were analysed using IBM SPSS statistical software, version 23 for Windows (IBM Statistics, 2016). Statistically significant differences between groups were declared at $P < 0.05$ using the independent *t*-test procedure.

6.6. Results

6.6.1. Bird performance

No significant differences were found between the control and the test group of birds when bird performance data were analysed for body weight, body weight gain, feed intake, or feed conversion ratio at all 3 sampling points (Tables 6.3 – 6.5).

Table 6.3. Mean bird performance data (0 – 4d)

Treatment group	BW (g)	BWG (g)	FI (g)	FCR
Control	80	37	67	1.80
Treatment	82	39	74	1.90
Pooled SEM	0.878	0.964	1.852	0.063
P value	0.274	0.322	0.071	0.455

Table 6.4. Mean bird performance data (5 – 18d)

Treatment group	BW (g)	BWG (g)	FI (g)	FCR
Control	492	411	424	1.54
Treatment	459	377	780	1.81
Pooled SEM	11.753	11.203	46.126	0.128
P value	0.346	0.311	0.266	0.141

Table 6.5. Mean bird performance data (19 – 28d)

Treatment group	BW (g)	BWG (g)	FI (g)	FCR
Control	1149	438	1102	1.74
Treatment	1085	413	1094	1.82
Pooled SEM	26.276	17.505	32.904	0.067
P value	0.423	0.664	0.953	0.694

6.6.2. Bone measurements

Bone measurement data for days 4, 18 and 28 are presented in Tables 6.6 - 6.8.

No significant differences were found between treatment groups at all sampling points.

Table 6.6. Effect of nutritional imprinting on broilers: d4 mean tibia measurements

Treatment group	[1]Dry weight (g)	Length (mm)	Width (mm)
Control	0.17	36.14	2.08
Treatment	0.17	36.70	2.17
SEM	0.004	0.235	0.031
P value	0.857	0.251	0.193

[1]Fat extracted

Table 6.7. Effect of nutritional imprinting on broilers: d18 mean tibia measurements

Treatment group	[1]Dry weight (g)	Length (mm)	Width (mm)
Control	1.41	63.28	4.56
Treatment	1.30	61.38	4.46
Pooled SEM	0.073	0.934	0.117
P value	0.460	0.319	0.682

[1]Fat extracted

Table 6.8. Effect of nutritional imprinting on broilers: d28 mean tibia measurements

Treatment group	[1]Dry weight (g)	Length (mm)	Width (mm)
Control	3.68	84.99	7.22
Treatment	3.68	84.16	7.46
Pooled SEM	0.138	0.878	0.151
P value	0.994	0.647	0.453

[1]Fat extracted

6.6.3. Bone mineralisation

Data for bone strength, bone ash weight, ash percentage, calcium and phosphorus content for days 4, 18 and 28 are presented in Tables 6.9 – 6.11. Apart from bone strength which was significantly greater in the test group of birds compared to the control group of birds at day 28 ($P = 0.012$), no other significant differences were found.

Table 6.9. Effect of nutritional imprinting on broilers: d4 mean tibia mineralisation

Treatment group	Strength (N)	Ash weight (g)	Ash %	Ca content/dry bone (g/kg)	P content/dry bone (g/kg)
Control	15.67	0.06	32.79	19.97	7.82
Treatment	16.17	0.05	32.81	19.66	8.05
Pooled SEM	0.749	0.001	0.482	0.574	0.286
P value	0.751	0.985	0.793	0.694	0.694

Table 6.10. Effect of nutritional imprinting on broilers: d18 mean tibia mineralisation

Treatment group	Strength (N)	Ash weight (g)	Ash %	Ca content/dry bone (g/kg)	P content/dry bone (g/kg)
Control	122.56	0.55	38.63	198.93	75.98
Treatment	123.37	0.51	38.65	191.51	74.51
Pooled SEM	9.500	0.324	0.665	12.427	4.678
P value	0.967	0.577	0.990	0.772	0.880

Table 6.11. Effect of nutritional imprinting on broilers: d28 mean tibia mineralisation

Treatment group	Strength (N)	Ash weight (g)	Ash %	Ca content/dry bone (g/kg)	P content/dry bone (g/kg)
Control	217.71	1.38	37.20	481.93	183.71
Treatment	286.36	1.41	38.21	515.90	193.33
Pooled SEM	14.158	0.057	0.450	20.298	7.702
P value	0.012	0.802	0.275	0.415	0.544

6.6.4. Phosphorus and Calcium ileal digestibility

By day 4, the apparent P ileal digestibility coefficient was significantly greater in the control group of birds compared with the treatment group of birds ($P = 0.016$) which also digested significantly more P and Ca ($P < 0.001$ respectively; Table 6.12). No significant differences were found in the apparent calcium digestibility between the two treatment groups.

Table 6.12. Effect of nutritional imprinting on apparent ileal digestible coefficient and amount of mineral digested, d4

	Apparent ileal digestibility coefficient		Amount of mineral digested (g/kg diet)	
Treatment group	**Ca**	**P**	**Ca**	**P**
Control	0.61	0.73	6.54	5.61
Treatment	0.66	0.69	4.06	3.76
Pooled SEM	0.016	0.008	0.397	0.282
P value	0.165	0.016	<0.001	<0.001

No significant differences were found in the Ca and P digestibility coefficient, or the amount of Ca and P digested between the two group of birds by day 18 (Table 6.13).

Table 6.13. Effect of nutritional imprinting on apparent ileal digestible coefficient and amount of mineral digested, d18

	Apparent ileal digestibility coefficient		Amount of mineral digested (g/kg diet)	
Treatment group	**Ca**	**P**	**Ca**	**P**
Control	0.67	0.72	7.14	5.54
Treatment	0.65	0.71	6.96	5.52
Pooled SEM	0.018	0.008	0.193	0.059
P value	0.701	0.687	0.676	0.885

By day 28, the apparent ileal P digestibility coefficient and the amount of P digested were significantly greater in the nutritionally imprinted group of birds ($P < 0.001$ respectively) compared to the control group of birds (Table 6.14). The apparent ileal Ca digestibility

coefficient, and the amount of Ca digested were however similar in both treatment groups of birds.

Table 6.14. Effect of nutritional imprinting on apparent ileal digestible coefficient and amount of mineral digested, day 28

Treatment group	Apparent ileal digestibility coefficient		Amount of mineral digested (g/kg diet)	
	Ca	P	Ca	P
Control	0.51	0.58	2.06	2.44
Treatment	0.51	0.62	2.06	2.60
Pooled SEM	0.020	0.007	0.079	0.029
P value	0.984	<0.001	0.984	<0.001

6.6.5. Isolation and quantification of sodium-phosphate cotransporter iib total mRNA

Nutritional imprinting had no significant effect on the gene expression of NaPi-IIb total mRNA on days 4, 18 and 28 (Table 6.15).

Table 6.15. Effect of nutritional imprinting on NaPi-IIb gene expression

Treatment group	Gene expression		
	GAPDH[1]	NaPi-IIb mRNA	n-Fold[2]
Day 4			
Control	19.06	21.75	1.0
Treatment	19.09	22.09	1.23
Pooled SEM	0.175	0.179	
P value	0.926	0.377	
Day 18			
Control	19.87	22.65	1.0
Treatment	19.92	23.04	1.36
Pooled SEM	0.209	0.286	
P value	0.910	0.517	
Day 28			
Control	20.41	23.93	1.0
Treatment	21.38	24.73	1.13
Pooled SEM	0.480	0.388	
P value	0.334	0.324	

[1]GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as the reference gene
[2]Mean fold change of NaPi-IIb mRNA expression was calculated using the 2–ΔΔCt relative quantification method (Livak and Schmittgen, 2001).

6.7. Discussion

Nutritional imprinting, defined as the early conditioning of life events where specific conditions give rise to later physiological outcomes is a well-established phenomenon (Hanley *et al.*, 2010). In poultry, it provides a means by which the bird responds and adapts to challenges during early development such as heat stress or nutritional deficiency. This is achieved by regulating the genes that are expressed or suppressed in the adult phenotype (Feil and Fraga, 2012). The effect of nutrition on health, behaviour, and cognition in poultry is further elaborated in the review of Dixon *et al.* (2016). Nutritional imprinting of phosphorus was examined in this study to evaluate its effect on bone mineralisation and phosphorus utilisation in broilers.

6.7.1. Growth performance

No significant differences in growth performance were found between the control and test group of birds at all sampling ages in this study. This implies that transiently feeding broilers 0.6%Ca / 0.3% nPP diets for 4 days immediately post-hatch does not have a deleterious effect on bird performance. It has been shown that growth performance and bone mineralisation are not negatively affected when dietary phosphorus is reduced concurrently with dietary calcium (Driver *et al.*, 2005). Rama Rao *et al.* (2006) reported similar body weight gain and feed intake in broilers given 6g Ca and 3g nPP /kg diet compared to broilers given 9g Ca and 4.5g nPP/kg diet between 2 and 14 days. This is corroborated by the report of Letourneau-Montminy *et al.* (2007) who conducted a meta-analysis study to evaluate phosphorus utilisation in broilers. The authors arrived at the same conclusion that a dietary concentration of 6g Ca and 3g nPP/kg had a similar effect on growth performance and bone mineralisation as the NRC recommended levels.

Compensatory growth as an adaptive response to low dietary phosphorus has been previously reported in poultry (Yan *et al.*, 2005b; Letourneau-Montminy *et al.*, 2008). However in this study, giving the treatment group of birds adequate phosphorus diets for 2 weeks after the initial 4 day challenge period of transiently feeding them low phosphorus diets was not sufficient enough to elicit compensatory growth by day 18. Although no significant differences were found in weight gain and feed conversion ratio by day 18, the control group of birds had numerically greater growth performance measurements. Similar to the findings of this study, Angel and Ashwell (2008) reported no significant differences in weight gain and feed to gain ratio between a control group and nutritionally imprinted group of birds. These authors however reported that the control group of birds were significantly heavier than the nutritionally imprinted birds by days 8 and 22.

When birds were re-challenged with a marginally low phosphorus diet for 10 days in this study (between 19 - 28 days), no significant changes were observed in growth performance, and the control group of birds still had numerically greater growth measurements. This is in contrast with the finding of Angel and Ashwell (2008) who reported that the nutritionally imprinted broilers had a significantly higher body weight, body weight gain and feed to gain ratio compared with the control birds fed the same diet by day 38. They however did not provide growth performance data for the birds at 28 days, the age at which growth performance was evaluated in this study, hampering direct comparison. The difference in length of dietary challenge (10 days in this study vs. 16 days in the study of Angel and Ashwell, 2008) may have resulted in the observed differences.

6.7.2. Bone morphometry, strength, and mineralisation

No significant differences were found in bone measurements (length, width, weight) at any of the sampling points, and there was no available data in the literature to compare results.

However, this was expected considering that no differences were found in body weight between the control group of birds and the test group of birds in this study.

With the exception of the significantly greater bone strength ($P = 0.012$) determined in the nutritionally imprinted birds at day 28 (Table 6.11), no other significant differences were found in the bone mineralisation data measured at all sampling points (Tables 6.9 – 6.11). Interestingly by the end of the experiment (day 28), ash weight, ash percentage and bone calcium and phosphorus content were numerically greater (but not significantly different) in the nutritionally imprinted group of birds compared with the control group. Surprisingly, although the tibia bones in the test group of birds were numerically shorter, they had numerically greater bone width was measured at day 28 compared with the control group of birds. This may have contributed to increased bone strength. Williams *et al.* (2004) compared bone growth in a fast and slow-growing strain of broilers and reported bone width plays an important functional role in bone strength by increasing the periosteal apposition and production of new osteons at the periosteal surface in response to increased load during growth in fast-growing chickens. Data from this study suggests that an increase in bone width resulting from increased bone mineralisation could also be initiated by other factors, e.g. response to the dietary challenge as result of nutritional imprinting; and not only growth as alluded to by Williams *et al.* (2004). More work is needed to examine the effect of nutritional imprinting with low dietary phosphorus on broiler bone mineralisation.

Tibia ash (Waldroup *et al.*, 2000; Driver *et al.*, 2006a) and bone strength (Cheng and Coon, 1990; Sohail and Roland, 1999) are two measuremets traditionally used as indicators of bone quality in poultry. Tibia ash weight was not sensitive in elucidating bone mineralisation differences between the two treatment groups in this study, although significant differences

in bone strength were found at day 28 ($P = 0.012$). It was therefore of interest to examine the effect of nutritional imprinting on the relationship between bone ash weight and strength.

By day 4, with an increase in bone ash weight, relatively less bone strength was observed in the nutritionally imprinted group of birds compared to the control group of birds (Figure 6.1). Phosphorus contributes to the compressional strength in the bone, but it is also required for growth and a variety of other physiological functions in the body. It is possible that in response to increase in size, more phosphorus was liberated from the bones of the test group of birds to meet growth requirement which led to relatively less bone strength.

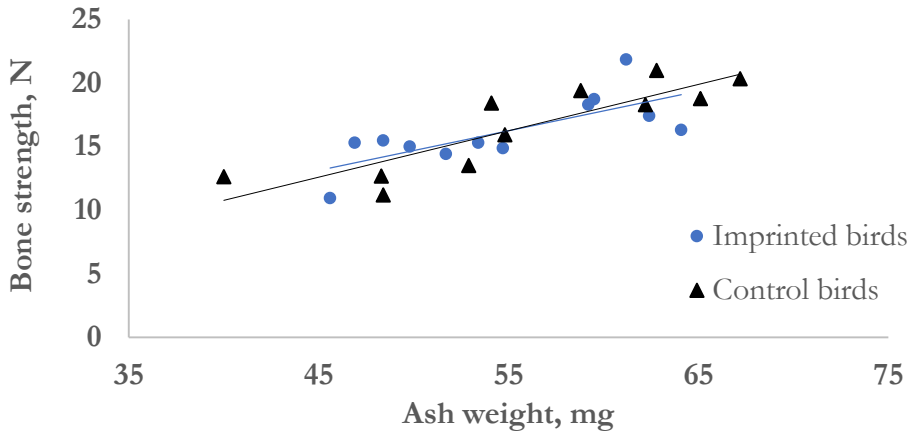

Figure 6.1. Regression of tibia bone strength as a function of ash weight, day 4
Control group of birds: $y = 0.3452x - 3.8337$, $R^2 = 0.7515$. $P < 0.001$
Imprinted group of birds $y = 0.311x - 0.84$, $R^2 = 0.5452$. $P = 0.004$

Similar large linear correlations were found between bone strength and bone ash for both groups of birds at day 18 (Figure 6.2). This suggests that nutritionally imprinting birds with transiently low phosphorus diets had no lasting effect on bone ash and bone strength after two weeks of feeding adequate phosphorus diets.

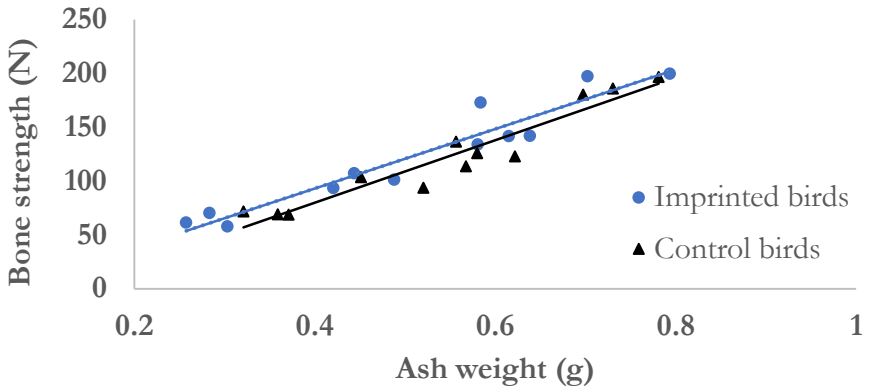

Figure 6.2. Regression of tibia bone strength as a function of ash weight, day 18
Control group of birds: $y = 290.43x - 34.142$, $R^2 = 0.9145$. $P < 0.001$
Imprinted group of birds $y = 274.27x - 17.304$, $R^2 = 0.9158$. $P < 0.001$

By day 28 however, re-challenging the test group of birds with low phosphorus diets triggered a more than an ordinary response in bone strength. While a large correlation was found between bone strength and bone ash weight in the control group of birds, the test group of birds exhibited disproportionately high bone strength relative to their bone ash weight as evidenced by the low correlation values (Figure 6.3). This may be due to the significantly greater amount of phosphorus digested by the test group of birds compared to the control group at day 28 (Table 6.14) which led to the numerically greater amount of phosphorus retained in the tibia ($P = 0.544$) and significantly greater bone strength ($P < 0.012$) as presented in Table 6.11.

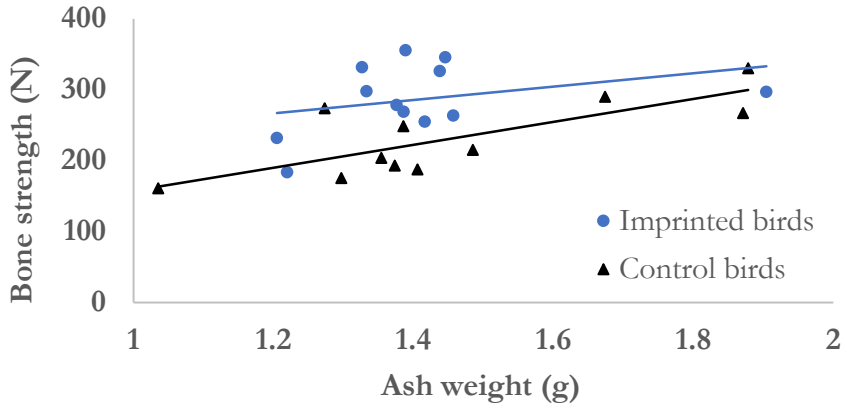

Figure 6.3. Regression of tibia bone strength as a function of ash weight, day 28
Control group of birds: $y = 169.79x - 16.315$, $R^2 = 0.7821$. $P < 0.001$
Imprinted group of birds $y = 94.632x + 153.09$, $R^2 = 0.1112$. $P = 0.289$

The level of bone mineralisation is related to bone strength (Boivin and Meunier, 2002). Calcium and phosphorus (hydroxyapatite) are the major minerals found in the bone which gives it compressional strength (Rath *et al.*, 2000). However, hydroxyapatite combines with the bone organic matrix (mainly made of collagen) which contributes to the tensile strength of bones (Einhorn, 1996). Collagen and other bone biochemical constituents have been reported to have an effect on increased bone strength and integrity by further strengthening bones through the interfibrillar interactions of its molecules to form hydroxyllysylpyridinoline and lysylpyridinoline, which are later oxidised to form pyridinium links (Knott and Bailey, 1998). Rath *et al.* (1999) reported substantially greater bone strength was strongly correlated with increases in pyridinium crosslinks in 72-week old broilers compared to 7-week old broilers. The contributory effect of collagen on bone strength was not examined in this study, and it is unknown if it contributed to the increased bone strength observed in the nutritionally imprinted birds.

6.7.3. Sodium phosphate cotransporter IIb gene expression

It is well established that sodium phosphate cotransporter (NaPi-IIb) is involved in phosphate uptake in poultry (Segawa *et al.*, 2002; Huber *et al.*, 2006). Yan *et al.* (2007) characterised the NaPi-IIb in broiler chickens and reported a higher mRNA expression in the duodenum compared to the jejunum and ileum.

In this study, nutritional imprinting had no significant effect on the expression of the NaPi-IIb mRNA, although the expression of NaPi-IIb mRNA in the test group of birds increased by 23, 36 and 13% at days 4, 18 and 28 respectively (Table 6.15). This is much lower than the findings of Ashwell and Angel (2008) who reported a 3.1 fold increase in duodenal NaPi-IIb cotransporter expression on day 4 when broilers were transiently fed a low phosphorus diet (0.59% Ca / 0.25% avP). They also reported a 2.5 fold increase in duodenal NaPi-IIb cotransporter expression by day 38 when the broilers were later challenged with low phosphorus diets. The observed differences may be due to several reasons, and comparisons will be made with the study of Ashwell and Angel (2008).

Firstly, these authors fed a much lower dietary phosphorus (0.4% Ca / 0.11% avP) during the period of phosphorus challenge compared to this study (0.4% Ca / 0.20% nPP), and it is possible that the dietary phosphorus in this study was not low enough to trigger the same effect the other authors observed. Secondly, the period of the challenge was shorter in this study (10 days) compared to 16 days (day 22 to d 38), and it is possible that greater expression of the NaPi-IIb cotransporter mRNA may have been observed after a longer period of low dietary phosphorus challenge (16 days or more). Thirdly, unlike the study of Ashwell and Angel (2008) which was conducted in battery cages where dietary feed intake would have been precise, birds in this study were raised in floor pens to simulate commercial farm practice. The birds had access to litter and may have consumed additional dietary phosphorus from the litter

in addition to that supplied in the diet to meet requirement. Thus, the relatively higher dietary phosphorus used to challenge the birds in this study, shorter period of dietary phosphorus challenge, and possible access to supplementary phosphorus in the litter may have contributed to why significant differences in NaPi-IIb mRNA expression were not observed between the 2 treatment groups in this study.

6.7.4. Apparent calcium and phosphorus ileal digestibility coefficient

The significantly higher amount of calcium and phosphorus digested by the control group of birds compared with the test group of birds at day 4 ($P < 0.001$ respectively) is consistent with the findings of Angel and Ashwell (2008). This is attributable to the different dietary concentrations of calcium and phosphorus; the control group of birds had higher concentrations in their diets and thus consumed and digested more nutrients. When both groups of birds were fed the same dietary calcium and phosphorus concentration between day 5 to day 18, as expected, no significant difference was found in the calcium and phosphorus digested.

At day 28 when both birds were challenged with low phosphorus diets, the amount of phosphorus digested by the test group of birds was significantly higher ($P < 0.001$) compared to the control group of birds (Table 6.14). This shows that the test group of birds were more efficient in phosphorus absorption, consistent with the report of Angel and Ashwell (2008). However, no significant differences were found in the amount of calcium digested by day 28. It is possible that the control group of birds, similar to the nutritionally imprinted group of birds responded to the low dietary calcium diet by increased calcium absorption to maintain calcium homoeostasis in the short term.

Improved efficiency in intestinal calcium absorption in response to calcium deficiency has previously been reported (Blahos *et al.*, 1987), and is thought to be coordinated by the up-regulation of 1, 25 dihydroxycholecalciferol (Shafey, 1993) when calcium is deficient (Hurwitz, 1996). This is supported by the study of Rousseau *et al.* (2012) who examined the effect of dietary calcium on phosphorus utilisation in finishing broilers (22 - 38d) and reported increased calcium retention (51.52% vs. 42.52%) in broilers fed a low dietary Ca/nPP concentration (0.37% and 0.18% respectively) compared to those fed a higher dietary Ca/nPP concentration (0.77% and 0.35% respectively).

6.8. Implication

Maximising bone mineralisation is known to improve bone strength (Cheng and Coon, 1990) and improves the ability to withstand mechanical stress (Orban *et al.*, 1993); while poor bone mineralisation can increase the incidence of fractures resulting in downgrades at processing (Driver *et al.,* 2006a). Transiently feeding poultry low phosphorus diets early post-hatch has been shown to improve bone mineralisation and strength, and may help improve processing yields by reducing downgrades due to fracture at processing. Nutritional imprinting could thus be adopted as a tool for improved bone strength whilst conserving the rapidly depleting global phosphate reserves.

6.9. Conclusion

Nutritional imprinting did not have any adverse impact on growth performance and bone mineralisation in broilers transiently feed low phosphorus diets for four days early post-hatch. Interestingly when later challenged with a low phosphorus diet during the growing phase, the nutritionally imprinted birds were more efficient at utilising dietary phosphorus and developed significantly stronger bones compared to unconditioned birds. The findings from this study

support the hypothesis that phosphorus is more efficiently utilised when birds are nutritionally imprinted with low phosphorus diets immediately post hatch.

Future work aimed at understudying the changes that may have occurred by examining bone histology and organic matrix is needed to elucidate the remarkable effect nutritional imprinting had on bone strength. There is also the need to establish the optimal dietary phosphorus concentration broilers can be imprinted with at the early stage of development; and the minimum dietary concentration they can be challenged with at the finishing phase without compromising on growth, bird welfare or economic returns. This should be evaluated in combination with other feed additives (e.g. phytase and vitamin D) routinely used to improve phosphorus utilisation.

The next study examines another nutritional intervention used to improve phosphorus utilisation by adapting feed to provide a more bioavailable form of dietary phosphorus.

CHAPTER 7: THE EFFECT OF A HIGH PHYTASE WHEAT CULTIVAR ON PHOSPHORUS DIGESTIBILITY AND BONE MINERALISATION IN BROILERS

7.1. Introduction

The previous study focused on aiding the bird in becoming more efficient at utilising dietary phosphorus. This current study considers the complementary approach of adapting feed to provide a more bioavailable form of phosphorus.

The development of feed ingredients with increased available phosphorus as an alternative nutritional strategy to supply phosphorus in the diets has attracted interest over recent decades (Yan *et al.,* 2000). For example, high available phosphorus (HAP) hybrids of plants with low phytate phosphorus content have been developed by using the low phytic acid 1-1 (*lpal-1*) allele of such plant species. Improved varieties of such plant hybrids include maize (Ceylan *et al.,* 2003; Snow *et al.,* 2004), barley (Jang *et al.,* 2003; Linares *et al.,* 2007) and soybean (Sands *et al.,* 2003; Dilger and Adeola, 2006). When fed to broilers, they have been shown to contain more available phosphorus leading to reduced inorganic phosphorus requirements (Huff *et al.,* 1998). When compared with standard plant varieties, they have also resulted in a significant reduction in faecal phosphorus content without any compromise in bird performance (Waldroup *et al.,* 2000; Yan *et al.,* 2000). However, these seeds are not commercially available for several reasons: lower yields compared to normal breeds (Raboy *et al.,* 2001), higher handling costs i.e. seed segregation, storage and testing costs (Makki *et al.,* 2001), and consumers reluctance in accepting genetically modified grains.

Another strategy identified is the use of transgenic phytase expressing seeds (Dionisio *et al.,* 2011). Improved knowledge in recombinant DNA technology has been used to develop transgenic corn varieties with innate ability to express phytase within the endosperm of canola (Zhang *et al.,* 2000) and maize (Gao *et al.,* 2013). By using the phytase-encoding gene from a

microbe (e.g. *Aspergillus niger*), these transgenic plant varieties contain phytase which is expressed in the endosperm of the seed kernel. Transgenic maize varieties have been reported to improve broiler growth performance and phosphorus utilisation in phosphorous deficient diets (Nyannor and Adeola, 2008); and are as effective as microbial phytases in maize-soybean diets (Gao *et al.,* 2012).

7.1.2. Aims and objectives

The overall aim of this study was to evaluate the impact of substituting standard wheat with a novel cultivar of wheat containing high intrinsic phytase activity (HIGHPHY) in broiler diets on phosphorus release from phytate in diets containing marginally low levels of available phosphorus.

The specific objectives were:

- To evaluate the effect of feeding broilers HIGHPHY on growth performance.
- To evaluate the effect of feeding broilers HIGHPHY on bone morphometry and bone mineralisation.
- To examine the effect of feeding broilers HIGHPHY on ileal phosphorus and calcium digestibility.

The hypothesis of this trial was that phosphorus utilisation may be improved by feeding broilers a high phytase wheat cultivar.

7.2. Materials and methods

7.2.1. Trial procedure

Institutional and UK national NC3R ARRIVE guidelines for the care, use and reporting of animals in research (Kilkenny *et al*., 2010) were followed, and all experimental procedures involving animals were approved by the University's College of Arts and Science ethical review committee. Wheat grains used in the feeding trial were standard field grown wheat *Triticum aestivum* L. cv Skagen with a phytase activity on 1060 FTU/kg, and a high phytase wheat variety HIGHPHY *Triticum aestivum* L. with a phytase activity on 6196 FTU/kg provided by Plant Bioscience Ltd, UK.

180 male Ross 308 day-old broiler chicks from a flock aged 43 weeks were sourced from PD Hook Hatcheries Ltd, Oxfordshire and housed in an environmentally controlled room as previously described in Chapter 2, Section 2.2. On arrival, the birds were weighed to determine individual bird weight and then allocated to pens ensuring there were no significant differences in mean pen weight across treatments. Birds were observed twice daily to monitor environmental conditions and mortality recorded. Diets and water were provided *ad libitum* to all birds during the experimental procedure. Birds were observed twice daily to monitor environmental conditions and mortality recorded.

The experimental design consisted of 5 dietary treatments (maize/soya mash diets) which were mixed in-house using a ribbon mixer (Table 7.1). The five dietary treatments were based on a control diet of standard wheat containing a putative marginally low phosphorus supply and no phytase or HIGHPHY wheat added. A phytase containing positive control which provided adequate phosphorus supply through use of standard wheat with 500FTU/kg Quantum Blue phytase (AB Vista, Marlborough, UK), but with no HIGHPHY wheat added was used to allow comparison with commercial standards,. Three further diets were as per

control but with replacement of standard wheat with HIGHPHY wheat at 33%, 66% or 100% respectively. Titanium dioxide was added to all diets at 5g/kg inclusion as an inert marker for digestibility measures. The birds were allocated to 9 replicate pens per diet, each containing 4 birds per pen.

7.2.2. Sample collection

At the end of the experiment (day 21), three birds per replicate pen were euthanised by cervical dislocation. Digesta was collected post-mortem as described in Chapter 2, Section 2.4.2.2. Briefly, digesta was collected from the from the distal end of the small intestine identified as the portion between the Meckel's diverticulum and the ileal-caeco-colonic junction. Digesta was pooled per pen and frozen at -20°C until further processing. Left tibia bones were collected from three birds per pen as described in Chapter 2, Section 2.4.2.3. Briefly, the bones were separated at the tibiotarsal junction where the feet were removed, and the tibio-femoral junction where the femurs were removed, and then individually stored at -20°C until further processing.

7.2.3. Determined parameters

7.2.3.1. Growth performance

Body weight, body weight gain, feed intake, and feed conversion ratio were evaluated per pen on a weekly basis on sampling days 7, 14 and 21 as described in Chapter 2, Section 2.4.1. Mortality was monitored and dead birds removed, weighed, and recorded. FCR of birds was calculated by dividing feed intake by body weight gain, taking into account any mortalities.

7.2.3.2. Tibia bone morphometry, strength, and ash

Tibia weight, length, width, strength, and ash content of the left tibia bones were measured as previously described in Chapter 2, Sections 2.5.1, 2.5.4.2 and 2.5.5.2. Briefly, flesh from all left tibia bones was manually removed and weighed whilst leaving the cartilage caps intact. Bone

length and width were measured using a set of Vernier callipers. Bone strength was measured using a TA-XT Plus Texture Analyser (Stable Micro Systems, Surrey, UK) before fat extraction extraction using the Soxtherm hot fat extraction method for 2h. The tibia bones including cartilage caps were then dried at 105°C for 24h until constant weight was achieved and then ashed for 24h at 650°C in a furnace to determine ash weight. Bone ash was analysed for total calcium and total phosphorus content using the ICP-OES assay previously described in Chapter 2, Section 2.5.7.

Table 7.1. Dietary formulations HIGHPHY wheat Trial (% as fed)

Diet	Control	Control + 500 FTU phytase	Control with 33% HIGHPHY[1]	Control with 67% HIGHPHY[1]	Control with 100% HIGHPHY[1]
Standard Wheat	56.71	56.70	37.61	18.61	0
Extruded Soya, 48% protein	35.00	35.00	35.00	35.00	35.00
Soya oil	3.78	3.78	3.78	3.78	3.78
Limestone	1.28	1.28	1.28	1.28	1.28
Salt	0.17	0.17	0.17	0.17	0.17
Sodium bicarbonate	0.26	0.26	0.26	0.26	0.26
Monocal phosphate, HCL	1.23	1.23	1.23	1.23	1.23
Lysine HCl	0.21	0.21	0.21	0.21	0.21
Methionine	0.32	0.32	0.32	0.32	0.32
Threonine	0.13	0.13	0.13	0.13	0.13
Econase XT	0.01	0.01	0.01	0.01	0.01
Quantum Blue Phytase	0	0.01	0	0	0
Vitamin Mineral Premix[2]	0.40	0.40	0.40	0.40	0.40
High Phytase Wheat	0	0	19.10	38.10	56.71
Titinium dioxide	0.5	0.5	0.5	0.5	0.5

[1]Percentage standard wheat replaced with HIGHPHY wheat (based on 570 g/kg wheat in total diet).
[2]Supplied per kilogram of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1 mg; molybdenum, 0.48 mg; selenium, 0.2 mg;r etinol, 13.5 mg; cholecalciferol, 3 mg; tocopherol, 25 mg; menadione, 5.0 mg; thiamine, 3 mg; riboflavin, 10 mg; pantothenic acid, 15 mg; pyroxidine, 3.0 mg; niacin, 60 mg; cobalamin, 30 µg; folic acid, 1.5 mg; and biotin 125 mg.

Table 7.2. Analysed content of diets and grain - HIGHPHY wheat Trial

	Control	Control + 500 FTU phytase	Control with 33% HIGHPHY[1]	Control with 67% HIGHPHY[1]	Control with 100% HIGHPHY[1]	Control Wheat	HIGHPHY Wheat
DM (g/kg)	879.70	880.43	888.38	878.85	908.67	890.22	889.04
Ash (g/kg)	61.86	59.83	58.11	58.39	62.47	17.20	16.94
Protein (g/kg DM)	267.28	269.23	272.84	274.46	276.93	127.73	163.20
GE (MJ/kg DM)	19.61	19.62	20.27	20.53	20.45	18.73	18.94
Ca (g/kg DM)	7.83	7.96	7.73	7.82	7.82	0.93	0.80
P (g/kg DM)	5.84	5.70	5.24	5.55	5.58	3.86	2.37
Phytate (g/kg DM)	10.14	10.15	10.22	12.07	11.92	3.18	3.40
Phytate-P (g/kg DM)[2]	2.84	2.86	2.88	3.40	3.36	2.59	0.96
Non-phytate-P (g/kg DM)[3]	2.98	2.84	2.36	2.15	2.07	1.27	1.41
Total Phytase Activity (FTU/kg)[4]	605	1150	1804	3954	5925	1060	6196
Analysed amino acid content (g/kg)							
CYS	6.031	5.398	5.437	6.448	6.963	5.544	4.444
ASP	17.610	17.147	15.642	12.492	21.286	6.497	6.454
THR	7.561	7.484	6.684	8.061	9.034	3.577	3.056
SER	8.431	8.334	7.765	9.909	10.169	5.678	4.494
GLU	42.077	38.556	38.324	44.916	50.346	39.656	30.748
GLY	7.945	7.897	7.624	8.307	9.563	5.169	4.302
ALA	7.830	7.815	7.485	8.185	9.435	4.445	3.797
VAL	9.229	9.037	8.533	9.151	11.016	5.836	4.533
MET	8.352	9.158	7.722	9.033	14.687	3.961	3.516
ILE	8.125	8.471	7.211	8.195	9.473	4.810	3.715
LEU	13.243	13.618	12.296	14.205	15.794	8.815	6.706
TYR	3.538	4.799	4.986	4.872	5.094	2.184	1.466
PHE	8.908	9.409	8.567	9.705	10.797	5.997	4.446
LYS	10.534	10.822	9.757	10.870	12.508	3.531	3.255
HIS	5.159	4.612	4.328	4.436	6.157	2.402	2.872
ARG	12.087	11.590	10.841	11.704	13.916	4.679	5.641

[1]Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet).
[2]Phytate-P was calculated as 28.2% of phytate (Tran and Sauvant, 2004).
[3]Non-phytate P was calculated as the difference between total P and phytate-P.
[4]Total phytase activity was analysed by a colorimetric enzymatic method and calculated as (net optical density at 415nm*dilution volume)/(slope of standard curve x mass x incubation time) (Engelen *et al.* 2001).

7.2.3.3. Diet nutrient analysis

All diets were analysed for dry matter, protein content and gross energy as described in Chapter 2, Sections 2.5.2, 2.5.3, 2.5.10. Gross energy of the digesta was measured as described previously for diets, and apparent ileal metabolisable energy (AME) was calculated by the following equation:

GE diet - (GE digesta x (TiO_2 in the diet/TiO_2 in digesta)).

The nitrogen content of the digesta was analysed by Dumas method, and metabolisable nitrogen was calculated using the following equation:

Diet N – Digesta N x (Diet Ti/Digesta Ti)

The apparent ileal metabolisable energy was also corrected to zero N balance (AMEn) using the figure of 34.4 kg/g N retained as detailed by Hill and Anderson (1958).

Amino acid content of diets and protein sources was determined using a Biochrom 30 amino acid analyser (Biochrom, Cambridge, UK) based on ion exchange chromatography. Briefly, samples were oxidised with performic acid before acid hydrolysis with nor-leucine added as an internal standard and then analysed against prepared standards.

7.2.3.4. Titanium dioxide, calcium and phosphorus analysis

Digesta samples were freeze-dried and finely ground using a pestle and mortar. Diets and digesta were analysed for titanium dioxide content using ICP-OES assay as previously described in Chapter 2, Section 2.5.6. Diets, digesta and bone ash were also analysed for total calcium and total phosphorus content using an ICP-OES assay as previously described in Chapter 2, Section 2.5.7.

7.2.3.5. Total phytic acid, non-phytate phosphorus and phytase activity

Total phytic acid (PP) content of ileal digesta samples was analysed using a Megazyme™ K-PHYT assay, described in Chapter 2, Section 2.5.8. Non-phytate phosphorus (nPP) of diet and digesta samples was determined as the difference between total phosphorus and phytate phosphorus. Phytase activity was determined according to the method of Engelen *et al.* (2001), where samples were incubated with sodium phytate to liberate inorganic phosphate.

7.2.4. Data analysis

All data were analysed using IBM SPSS statistical software, version 23 (IBM Statistics, 2016). Data were analysed using General Linear one-way ANOVA model to evaluate the effect of dietary treatment on growth performance, tibia mineralisation and strength, ileal Ca and P digestibility, and phytate hydrolysis. Statistical significance was declared at $P < 0.05$. Duncan *post hoc* testing was used to elucidate differences between diets.

7.3. Results

7.3.1. Bird performance

Feeding broilers the HIGHPHY wheat up to 21 days had no detrimental effect on growth performance or health. Low mortality was observed across treatments (2 birds; 1.1%) and no losses incurred from the birds fed HIGHPHY wheat (Table 7.3).

Table 7.3. Bird mortality per week and by treatment - HIGHPHY wheat Trial

Treatment	0 – 7d	7 – 14d	14 – 21d	Total per diet
Control	0	0	1	1
Control plus 500 FTU phytase	1	0	0	1
Control with 33% HIGHPHY[1]	0	0	0	0
Control with 67% HIGHPHY[1]	0	0	0	0
Control with 100% HIGHPHY[1]	0	0	0	0
Total per week	1	0	1	2

[1]Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet)

Results of feed intake, body weight gain and feed conversion ratio are presented in Table 7.4. Cumulative data analysed for the whole experimental period (21 days) showed improvement in feed intake and feed conversion ratio with increased supplementation of HIGHPHY wheat compared with the standard wheat variety (Table 7.4). Birds fed 100% HIGHPHY had the greatest body weight gain, and incremental improvement in FCR was recorded with an increase in the dietary inclusion of HIGHPHY (Table 7.4).

Table 7.4. Growth performance of birds fed varying replacement levels of HIGHPHY wheat from 0 to 21d

Treatment	[2]FI/bird (g)	[3]BWG/bird (g)	[4]FCR
Control	1238.0	764.5	1.63
Control plus 500 FTU phytase	1162.3	722.2	1.57
Control with 33% HIGHPHY[1]	1139.0	737.8	1.54
Control with 67% HIGHPHY[1]	1124.2	726.9	1.49
Control with 100% HIGHPHY[1]	1073.9	790.4	1.46
Pooled SEM	24.03	11.47	0.030
P value	0.070	0.268	0.317

[1] Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet).
[2]Feed intake
[3]Bod weight gain
[4]Feed conversion ratio

No significant differences were observed between the diets when AME and AMEn were determined in this study (Table 7.5).

Table 7.5. Apparent ileal metabolisable energy (AME) and AME corrected to zero N balance from birds fed varying replacement levels of HIGHPHY wheat from 0 to 21d

Treatment	[2]AME (MJ/kg)	[3]AMEn
Control	12.8	11.6
Control plus 500 FTU phytase	12.9	11.8
Control with 33% HIGHPHY[1]	13.0	11.9
Control with 67% HIGHPHY[1]	13.2	12.0
Control with 100% HIGHPHY[1]	13.1	11.9
Pooled SEM	0.17	0.17
P value	0.492	0.543

[1]Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet).
[2]Apparent ileal metabolisable energy
[3]Apparent ileal metabolisable energy corrected to zero N balance

7.3.2. Bone measurements

No significant differences were found in bone measurements: length, width, weight, strength, and calcium and phosphorus content (Tables 7.6 and 7.7).

Table 7.6. Tibia bone measurements of birds fed varying replacement levels of HIGHPHY wheat at d21

Treatment	Tibia length (mm)	Tibia width (mm)	Tibia weight (g)	Tibia Ash (%)	Tibia Strength (N)
Control	72.74	5.24	4.43	39.83	135.07
Control plus 500 FTU phytase	71.45	5.11	4.20	39.92	119.90
Control with 33% HIGHPHY[1]	72.14	5.18	4.53	39.71	128.67
Control with 67% HIGHPHY[1]	71.49	5.02	4.12	39.93	127.18
Control with 100% HIGHPHY[1]	73.41	5.23	4.48	40.13	131.80
Pooled SEM	0.280	0.051	0.060	0.462	5.144
P value	0.241	0.290	0.222	0.987	0.416

[1]Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet)

Table 7.7. Calcium and phosphorus content of tibia bones at d21 of birds fed varying replacement levels of HIGHPHY wheat at d21

Treatment	Ca content (mg/dry tibia)	P content (mg/dry tibia)
Control	307.3	118.4
Control plus 500 FTU phytase	298.2	116.9
Control with 33% HIGHPHY[1]	307.4	121.5
Control with 67% HIGHPHY[1]	287.4	113.3
Control with 100% HIGHPHY[1]	314.3	122.9
Pooled SEM	12.68	4.81
P value	0.612	0.643

[1]Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet)

7.3.3. Calcium and phosphorus ileal digestibility HIGHPHY wheat Trial

Significant improvements were found in the coefficient of digestibility for Calcium and phosphorus in all diets containing HIGHPHY compared to the control diet. Broilers fed 100% HIGHPHY showed the highest calcium and phosphorus digestibility coefficient across all treatment groups by day 21 (Table 7.8).

Table 7.8. Apparent ileal calcium and phosphorus digestibility coefficients at d21 of birds fed varying replacement levels of HIGHPHY wheat

	Apparent ileal digestibility coefficient	
Treatment	**Ca**	**P**
Control	0.567[d]	0.561[d]
Control plus 500 FTU phytase	0.608[c]	0.615[c]
Control with 33% HIGHPHY[1]	0.645[b]	0.703[b]
Control with 47% HIGHPHY[1]	0.618[bc]	0.644[c]
Control with 100% HIGHPHY[1]	0.697[a]	0.755[a]
Pooled SEM	0.0106	0.0168
P value	<0.001	<0.001

[1] Percentage standard wheat replaced with HIGHPHY wheat (based on 570g/kg wheat in total diet).
[a-d]Means within the same column with no common subscript differ significantly ($P < 0.05$).

7.4. Discussion

Intrinsic mature grain phytase activity (MGPA) of cereal seeds provides a means for phytate digestion by the seeds and play an important role during seed germination by liberating phosphates from phytate. MGPA varies considerably between species (Brinch-Pedersen *et al.*, 2014), and most plants varieties in use make negligible mature grain phytase activity contribution. Eeckhout and de Paepe (1994) found high intrinsic mature grain phytase activity in rye, triticale, wheat and barley (5130, 1688, 1193 and 582 FTU/kg respectively); although the authors also reported low intrinsic phytase activity (less than 100 FTU/kg) in other seeds such as maize, oats, sorghum, peanut, and soya.

Due to limited endogenous phytase activity in poultry, birds are unable to efficiently utilise plant phosphorus which is present in seeds as phytate. In addition, the denaturation of these phytases during steam pelleting (Jongbloed and Kemme, 1990) and in the stomach (Zeller *et al.*, 2015b) renders them inefficient in releasing phytate-bound phosphorus, necessitating the addition of inorganic phosphorus in the feed. However, this practice is not sustainable (Dionisio *et al.*, 2011) due to concerns about the depletion of the world's phosphate reserves (Steen, 1998). Dietary supplementation with exogenous phytase is a common approach that is effective in improving phosphorus utilisation from phytate (Angel *et al.,* 2005; Plumstead *et al.,* 2007). However, the additional cost of using feed enzymes including feed preparation constraints (Li *et al.*, 1997; Gontia *et al.*, 2011) remains a major challenge in feed manufacture.

Another approach is the use of transgenic plants with innate ability to express phytases. Based on their catalytic mechanisms, phytases can be grouped into four families: histidine acid phosphatase, purple acid phosphatases (PAPhy), β-propeller phytases and cysteine phosphatases (Mullaney and Ullah, 2003; Puhl *et al.*, 2007). The majority of phytases, however, belong to the histidine acid phosphatase group (Mitchell *et al.*, 1997), to which the bacterial phytases from *E. coli* also belongs (Lim *et al.*, 2000).

Wheat is an extensively used feed ingredient in poultry feed, and its enzymes containing phytase activity consists of at least two types phytases: purple acid phosphatases (PAPhy), and multiple inositol phosphate phosphatases. PAPhy is the major contributor to MGPA in wheat (Nakano *et al.*, 1999) and is further classified into the PAPhy_a isoform primarily expressed during grain filling and PAPhy_b expressed during germination. Its potential either in the purified (Morgan *et al.*, 2015) or in recombinant purple acid phosphatase form (Brejnholt *et al.,* 2011) to degrade phytate has been investigated but has received very little evaluation in animal feed trials (Brejnholt *et al.,* 2011).

Brinch-Pedersen *et al.* (2000) reported a 4-fold increase in phytase activity in the *Aspergillus niger* phytase-encoding gene (*PhyA*) in transgenic wheat seeds suggesting its potential in phytate phosphorus digestibility but literature is sparse on its efficacy in broilers. Dionisio *et al.* (2011) cloned and characterised the biochemical parameters for wheat grain PAPhy and reported a pH optimum of 5.5 ± 0.14, broad temperature curve with optimum at 55°C ± 1.8°C, and Km of 35 μM with phytate as substrate. These are comparable in range to two readily available commercial phytase products (Menezes-Blackburn *et al.,* 2015) previously evaluated in broiler feed simulation studies: *Aspergillus niger* based phytase – Natuphos (BASF, Germany) which belong to the 3-phytase group; and *Peniphora lycii* based phytase - Ronozyme P and Ronozyme NP (DSM, Switzerland) which belong to the 6-phytase group. Both products are commonly used in broiler diets, although new generation commercial phytase additives based on *E. Coli* – Quantum (AB Enzymes, Germany) or *Citrobacter braakii* - Ronozyme HiPhos (DSM, Switzerland) are now commercially available (Ariza *et al*., 2013).

The effect of increasing levels of plant-derived PAPhy phytase on phosphorus release from phytate was evaluated in this study by substituting a standard wheat variety with graded levels of HIGHPHY wheat in broiler diets. The efficacy of HIGHPHY was examined by comparing it with a commercial histidine acid phosphatase phytase (Quantum Blue; pH optimum 4.5) supplied via a thermostabilized *E. coli* in releasing phosphorus in the standard wheat variety.

Mortality across the trial was 1.1% (2 birds), with no losses from any diet containing the HIGHPHY wheat, indicating that feeding broilers the novel wheat cultivar had no adverse effect on bird health. No significant differences were found when feed intake, body weight gain and feed conversion rate (FCR) were evaluated cumulatively after 21 days of feeding (Table 7.4). Although data was not significantly different, body weight gain was highest for broilers fed diets where 100% of the standard wheat was replaced by HIGHPHY wheat, and

FCR was incrementally improved with increasing inclusion of HIGHPHY. This provides further evidence that the HIGHPHY wheat has no detrimental effect on health. Interestingly, diet did not affect tibia bone length, width, weight, strength or tibia mineral content (Tables 7.6 and 7.7). This suggests that the level of marginally adequate phosphorus concentration in the diet was not low enough to impact on bone parameters (morphometry, strength, ash, or mineral content) and that the study did not reveal the full potential of the experimental diets.

The data presented in Table 7.8 shows that the HIGHPHY MGPA had a significant, positive impact on the amount of calcium and phosphorus digested in the ileum at d21. Significantly more calcium and phosphorus were digested in birds fed diets containing 100% HIGHPHY wheat compared to those fed any other diet, indicating that the wheat PAPhy is functional in the broiler digestive tract. The ileal calcium and phosphorus digestibility coefficients for the 100% and 33% inclusion HIGHPHY wheat diets were significantly higher than the control diet, and the diet supplemented with exogenous phytase (Table 7.8). This is not surprising given the linear increase in total phytase activity levels when HIGHPHY wheat was substituted with standard wheat (Table 7.2). Further improvement in phosphorus digestibility when phytase concentration is increased beyond the recommended 500 FTU used commercially (super-dosing) is well established in poultry (Walk *et al.,* 2013, 2014). A 100% substitution of standard wheat with HIGHPHY resulted in higher phosphorus and calcium ileal digestibility coefficients than the conventional wheat diet (34.6% and 22.9% respectively). Also, 22.8% and 14.6% higher ileal digestibility coefficients for phosphorus and calcium respectively was determined when 100 % HIGHPHY diets were compared with the diet supplemented with exogenous histidine acid phosphatase phytase (HAP). Strangely, although the intermediate replacement level (67% HIGHPHY) significantly improved calcium and phosphorus digestibility over the control diet, it was not significantly better in improving calcium and

phosphorus digestibility compared to either the 33% HIGHPHY diet (lowest replacement level), or the diet supplemented with exogenous HAP Phytase. This response was not expected and requires further investigation.

7.5. Limitation of the study

Phytate is known to react with dietary proteins to form aggregates which are less accessible to proteases (Cheryan, 1980); and protein digestion can be adversely affected by the presence of phytate (Vaintraub and Bulmaga, 1991). Greater concentrations of protein and phosphorus were determined in HIGHPHY wheat compared with the conventional wheat variety (Table 7.2). The high protein content of the HIGHPHY wheat is worthy of further investigation via amino digestibility assessment, considering that apart from reducing the anti-nutritional effects of phytate in feed, it may provide a useful contribution to digestible amino acids. This may have greater significance when fed over longer feeding periods.

Phytases are thought to act in the upper gastrointestinal tract of broilers (Brejnholt *et al.*, 2011), and a range of pH have been reported in broilers: 4.4 - 5.6 in the crop, 3.6 – 4.8 in the proventriculus and 2.6 – 4.1 in the gizzard (Taylor and Jones, 2004; Jiménez-Moreno *et al.*, 2009). However, 60% of phytate remains after the gizzard, and may be hydrolyzed further along the gastrointestinal tract (Morgan *et al.*, 2015). The efficacy of phytases is optimised at specific pH, and high pH optimum may thus facilitate phytate breakdown in the small intestine where the pH tends to be higher. The pH optimum of 5.5 for wheat grain PAPhy is higher than the optimum pH of the exogenous phytase used in this study (Quantum Blue; pH optimum 4.5). There may therefore be a synergistic effect on phytate degradation when PAPhy phytase is fed in conjunction with a traditional histidine acid phosphatase phytase thereby creating broader pH optimum. This is also worthy of further investigation.

Although this study did not evaluate the effect of heat treatment during feed production on HIGHPHY PAPhy phytase activity, a broad optimum temperature curve has been previously reported. Brinch-Pedersen *et al.* (2012) estimated the optimum temperature curve of HIGHPHY PAPhy phytase by incubating it in flour at 80°C and 100% relative humidity for 10, 20 and 40 minutes and reported residual activity after were 70, 42 and 22%, respectively. This indicates the potential of HIGHPHY PAPhy in resisting high temperature and moist treatments. This however needs to be determined in a commercial feed manufacture setting before full incorporation into pelleted poultry diets.

7.6. Conclusion

In this study, the potential of intrinsic PAPhy phytase expressed in wheat on phosphorus release from phytate was evaluated in a broiler diet. It was found that just 33% replacement of standard wheat with HIGHPHY is required to significantly improve calcium and phosphorus digestibility coefficients compared to conventional supplementation of broiler diets with exogenous phytase. Replacement of standard wheat by 100% HIGHPHY further improved both calcium and phosphorus digestibility. Findings from this study support the hypothesis that wheat PAPhys improves phosphorus digestibility in broilers and indicates its promising potential, particularly where there are barriers to the use of genetically modified plants or supplements.

7.7. Acknowledgements

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CHAPTER 8: OVERALL CONCLUSIONS AND RECOMMENDATIONS

8.1. Overall conclusions

One of the challenges faced in commercial poultry production is how to avoid phosphorus deficiencies which negatively impacts bird welfare and productivity, or oversupply which leads to wastage and environmental pollution. It has also been suggested that the NRC guidelines for phosphorus requirements in poultry (NRC, 1994) may not adequately reflect phosphorus requirements in the current strain of broilers which are selected for fast growth (Applegate and Angel, 2014). Consequently, there is the need for a review of the current phosphorus requirements in broilers, which will better reflect current physiological needs.

Bone ash and phosphorus content are widely studied to evaluate the phosphorus availability of raw materials. However, there is a lack of available dataset in the literature, of the normal range of bone ash values in commercial broilers. Therefore, in order to appropriately update phosphorus requirements in broilers, an understanding of the current range of normal bone ash and phosphorus content of broilers is required.

The first part of this thesis was to characterise the range of tibia bone measurements (including bone ash and phosphorus content) found in healthy commercially reared broilers in order to create a database of benchmarks applicable to current commercial practice. The lack of such benchmarks was particularly noted by practicing Poultry Veterinarians and Feed Nutritionists, who commented on the difficulty in determining the cause of flock lameness without readily available reference values of normal bone mineralisation data for commercially raised broilers. However, it soon became clear that unless Scientists established a consensus over clear methods, the industry could not be served by science in this project.

The first two years were spent examining 4 common variations in the bone processing methodology to establish their relative impact on results. The additional cost incurred in

laboratory processing time when bones are extracted of fat prior to bone ash determination, and concerns over the use of organic solvents have often raised the question if fat extraction is an essential step in the bone ash methodology. As a result, many research groups continue to omit this step.

Results presented in Chapter 3 (Section 3.3.1) suggests that sensitivity in elucidating differences in treatment means is improved for ash percentage when fat is extracted from the bones prior to ash determination. Although defatting bones is laborious and increases analysis cost, the fat extraction assay has been shown to reduce variation between bone samples (Garcia and Dale, 2006) and is therefore recommended to be adopted for more accurate comparison of results. Sensitivity in elucidating differences in treatment means was improved for ash percentage when cartilage caps were removed from the bones prior to ash determination (Section 3.3.2). However, increased ash percentages have been reported when cartilage caps are affected by disease (Thorp and Waddington, 1997) and it may be advantageous to include the cartilage caps for better comparison of bone ash data, particularly when the health status of a bird flock is unknown. A minimum fat extraction time of 6h using the Soxhlet procedure was adequate in extracting fat from bones as shown in Section 3.3.3; while autoclaving the bones prior to fat extraction did not have any significant effect on bone ash or ash percentage (Section 3.3.4). These established methods, i.e., fat extraction of bones with cartilage caps included were then used in the other studies reported in this PhD project.

The next 2 studies in this project focused on the characterisation of bone and whole body phosphorus content of a common commercial broiler strain, the Ross 308. Rather than solely providing a mean value for each parameter measured as commonly reported in the literature, a range of bone morphometric and mineralisation data (which overlapped previously reported data) was sequentially determined from hatch to slaughter in healthy male and female broiler

chicks and data presented (Chapter 4). This dataset provides a detailed examination of bone parameters within a fixed range of conditions and provides a significant contribution to the gap in knowledge of the current range of age and sex-related normal bone morphology and mineralisation values of healthy commercial broilers. It is now being used by a major UK integrated broiler production company.

The use of 6 farms owned by a single UK integrator which provided uniform feed to just one bird strain concomitantly increases the rigour of the data but limits the breadth of application. In order to increase the value of the database to the commercial sector, other strains of broilers, reared by other integrators and independent producers across a range of geographical regions should be examined in a similar way to with a view of increasing the overall sample size and to ascertain the findings of this study. It will require regular updating to ensure it reflects the current industry standards due to the evolution of bird strains through selective breeding, and changes to industry practice. The major limitations however are researchers agreeing on a common consensus of the bone processing methodology, the appropriate sample size, the strain of birds to be evaluated, and frequency of sampling. It is hoped that published findings of this study will generate new and enhanced collaborations on future work between the academia and industry. It is also hoped that the poultry industry will lend more support in ensuring the onerous task of maintaining this database is achieved by funding poultry research groups.

It has also been suggested that the tibia phosphorus content might be a reliable estimate of WBP content, but this relationship has been examined in only very few studies (Hurwitz, 1964; Huyghebaert *et al.,* 1980; Shastak *et al.*, 2012c). Extensive literature searches indicate WBP content has not been determined in commercially raised broilers, and no data has been published on the relationship between the femur or feet phosphorus content and WBP

content. Surprisingly, despite the changes in bird composition due to selection for improved growth rate (Collins *et al.,* 2014; Zuidhof *et al.,* 2014). WBP content determined in Chapter 5 of this project (5g/kg) suggests it has remained the same. Strong correlations ($r \geq 0.97$) were found between the phosphorus content of the tibia, femur and feet and WBP content (Chapter 5, Tables 5.7 – 5.9), indicating the phosphorus content of these bones are reliable indicators of WBP retention. However, the steeper slope of regression when tibia phosphorus content was regressed against WBP content in this study compared to the work of Shastak *et al.* (2012c) require further investigation. It was interesting to note the slope of the femur and feet phosphorus content were parallel (36.76 and 35.97 respectively) and steeper than the slope of tibia phosphorus content (25.85), suggesting that although all three bones strongly indicate WBP content, there are differences in the phosphorus retained in the 3 bone types relative to WBP content. However since no other published data is available, further research is warranted to determine the biological veritability of the present finding.

The first 3 studies in this project (Chapters 3 – 5) established appropriate tools and methods for assessing phosphorus requirement in broilers. The next two studies examined strategies for maximising the efficiency of phosphorus utilisation in broilers. The first (Chapter 6) focused on aiding the bird in becoming more efficient at utilising dietary phosphorus, while the complimentary approach of adapting feed to provide a more bioavailable form of phosphorus was examined in Chapter 7.

The nutritional imprinting of birds for better phosphorus absorption in later life is a new and emerging technique that could help conserve the dwindling global phosphate reserve through reduced diet phosphorus inclusions. Angel and Ashwell (2008) demonstrated clear benefits of nutritionally imprinting broilers with low phosphorus diets early post-hatch, but interestingly the practice has not been adopted commercially, suggesting further investigation into the level

of treatment response was required. The present study (reported in Chapter 6) confirms the findings of Angel and Ashwell (2008) that significantly more phosphorus ($P < 0.001$) is digested when birds are nutritionally imprinted early post-hatch. Most interestingly, the imprinted birds in this study had significantly stronger bones ($P < 0.012$) and disproportionately exhibited significantly higher bone strength relative to their bone ash. Findings from this study strongly suggest that the practice of nutritional imprinting for improved phosphorus absorption should be commercially re-considered by the poultry industry on the basis that leg health may be improved alongside the potential reduction in rock phosphate usage.

Scientific initiatives in recent years have led to a substantially increased knowledge base on the complementary effect of supplementary phytases in cereals that can form the basis for integrating nutrition, breeding, molecular biology and genetics, but the use of intrinsic mature grain phytase activity determined in seed grains have not been widely examined. In Chapter 7, the efficacy of naturally expressed purple acid phosphatases (PAPhy) found in a novel wheat cultivar (HIGHPHY) was compared with a new generation commercially available phytase. For the first time, the promising potential for improving phosphorus digestibility in animal feed using wheat purple acid phosphatases was demonstrated. Further characterisation of the HIGHPHY wheat is required to determine the optimum inclusion in a commercial feed formulation matrix.

8.2. Future research and development

8.2.1. Characterisation of bone mineralisation in poultry

The characterisation of bone mineralisation in lame commercial broilers is warranted as it is expected that bone data determined in lame birds might be different to the data reported in this project, especially in cases directly related to poor bone mineralisation. Such dataset will be beneficial in understanding what values might be found in lame birds, and in combination with the data reported in this project, will serve as a useful tool in drawing conclusions on the bone status of broiler flocks.

Poor bone mineralisation is known to increase the incidence of fractures resulting in downgrades at processing plants (Driver *et al.,* 2006a). Therefore a comprehensive study examining the correlation between bone mineralisation in commercial poultry flocks and factory processing data will help fine-tune the optimisation of dietary phosphorus for bird welfare and best economic returns.

Applegate and Lilburn (2002) reported less mineralisation in the diaphyseal region of the femur compared to the tibia, and suggested that the femur may be important in evaluating long bone skeletal abnormalities during the latter period of broiler growth. Similar work characterising the femur bone is recommended for comparative analysis. Similar work characterising bone mineralisation in commercial layers is recommended.

8.2.2. Nutritional imprinting

Nutritional imprinting improved bone mineralisation and significantly improved bone strength. Future work aimed at understudying the changes that may have occurred by examining bone histology and organic matrix is needed to elucidate the remarkable effect nutritional imprinting had on bone strength.

The effect of breeder nutrition on subsequent progeny performance such as growth performance, immune status, and leg health is well documented (Hocking, 2007; Oveido-Rondon and Wineland, 2012; Chang *et al.*, 2016). However, published data on the effects of nutritional imprinting of broiler breeders for improved phosphorus utilisation in progeny are not available in the literature and presents a valuable area of further research.

More work is also required to establish the optimal level of dietary phosphorus concentration and length of dietary challenge required to give best results considering bird welfare and economic returns. This should be examined early post-hatch when the chick consumes its first meal, and at a subsequent period of challenge during the growing/finishing stage.

8.2.3. Additive effect of nutritional techniques

In this project, nutritional imprinting and the use of a high phytase expressing wheat cultivar to improve phosphorus utilisation were not examined in combination with other feed additives (phytase and vitamin D) routinely used to improve phosphorus utilisation. The synergistic effect of these feed additives in combination with nutritional imprinting and the use of a high phytase expressing wheat cultivar presents a valuable area of further research.

8.3. Recommendations

- There is the need to agree on a common consensus for the bone ash methodology used by researchers for assessing bone mineralisation in broilers. A minimum Soxhlet fat extraction time of 6h is recommended. In controlled research trials where the health status of a flock is known, removing the cartilage caps in order to improve the accuracy of comparisons may be adopted. However, disease conditions have been reported to increase bone ash percentage, and it may be beneficial to include the cartilage caps when comparing bone ash values from unrelated studies, particularly when the health status is unknown, in order to improve accuracy when comparing bone ash data from unrelated studies.
- The current database of bone mineralisation of commercial broilers will serve as a useful tool in understanding values applicable to commercial practice but will need to be regularly updated in line with changes due to selective breeding.
- Retainable whole body phosphorus content is constant (5g/kg) irrespective of bird size. This has remained constant despite improvement in bird growth rate and may be useful in better understanding phosphorus requirement in broilers.
- Nutritional imprinting for improved phosphorus utilisation (0.6% Ca and 0.3% nPP for 4 days immediately post-hatch) and the use of the high phytase wheat variety (at 100% replacement for standard wheat in a wheat/soya broiler diet) are useful techniques that can be employed to improve phosphorus utilisation in broilers. This could be further improved through the use of other feed additives such as vitamin D and phytases.

In conclusion, phosphorus plays an important role in poultry nutrition, and its judicial use is important from the environmental stewardship viewpoint. Improving phosphorus utilisation in poultry will not only help minimise wastage and reduce the negative effect undigested phosphorus has on the environment, but will also help in preserving the world phosphorus reserves.

Science should serve society, and this thesis was undertaken to meet the request of the commercial broiler sector. Continued collaboration between the poultry industry and academia is required to examine and improve on the promising results presented in this project.

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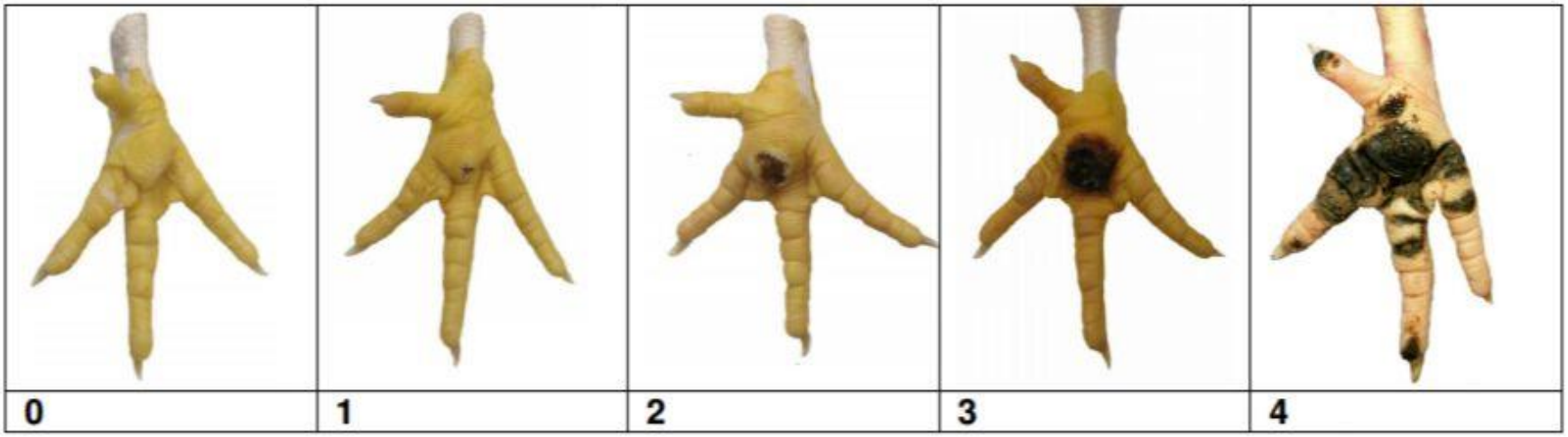
Appendix 1: Scoring scale for pododermatitis



Classification of the severity of pododermatitis) based on the welfare quality assessment protocol for poultry (Welfare Quality, 2009)

Score	Severity of pododermatitis
0	No evidence of foot pad dermatitis
1 & 2	Minimal evidence of foot pad dermatitis
3 & 4	Severe evidence of foot pad dermatitis

Appendix 2: Poultry data form

Date of sampling _____

Name of farm and address _____

Poultry house code_____

Parent flock code(s) and age (weeks) _____

Age of birds at sampling (days) _____

Sex of birds: Males _____ Females _____ Mixed _____

Number of birds housed _____

Floor area of poultry house _____

-Litter condition: wet _____fair ___ dry ___

Current body weight _____ (if not available age and weight at last weigh_____)

Target market weight: Male _____ Female _____ Mixed _____

Target market age Male _____ Female _____ Mixed _____

Feed conversion ratio _____

Gait score _____

Current cumulative number of culls related to lameness _____

Current cumulative number of culls due to other factors including disease _____

Mortality (including all culls) _____

Do you use additives in water (in addition to that supplied)?

None _____

Vitamin D_3 _____

Antibiotics _____

Others _____ (please specify)

Lighting programme _____ (please indicate pattern)

Please attach feed ticket

Appendix 3: Individual bird data form

Date **Farm** **House** **Sampling age**

Bird 1	
Storage bag code	
Sex	
Bird weight (g)	
Fresh bone weight (g)	
Bone length (mm)	
Bone width (mm)	
Bone strength (N)	

Bird 2	
Storage bag code	
Sex	
Bird weight (g)	
Fresh bone weight (g)	
Bone length (mm)	
Bone width (mm)	
Bone strength (N)	

Bird 3	
Storage bag code	
Sex	
Bird weight (g)	
Fresh bone weight (g)	
Bone length (mm)	
Bone width (mm)	
Bone strength (N)	

Bird 4	
Storage bag code	
Sex	
Bird weight (g)	
Fresh bone weight (g)	
Bone length (mm)	
Bone width (mm)	
Bone strength (N)	

Bird 5	
Storage bag code	
Sex	
Bird weight (g)	
Fresh bone weight (g)	
Bone length (mm)	
Bone width (mm)	
Bone strength (N)	

Bird 6	
Storage bag code	
Sex	
Bird weight (g)	
Fresh bone weight (g)	
Bone length (mm)	
Bone width (mm)	
Bone strength (N)	

Appendix 4: Ranking of Pearson correlation values according to bone measurements in 14 day-old unsexed broilers

Ca content/dry bone weight (mg)

P content/dry bone weight (mg)	0.963
Dry fat extracted bone weight (g)	0.719
Bone ash weight (g)	0.714
Fresh bone weight (g)	0.649
Bone length (mm)	0.585
Bone width (mm)	0.563
Bird weight (g)	0.465
Bone strength (N)	0.418
Ash percentage	0.164
Podo score	0.137

P content/dry bone weight (mg)

Ca content/dry bone weight (mg)	0.963
Dry fat extracted bone weight (g)	0.704
Bone ash weight (g)	0.693
Fresh bone weight (g)	0.642
Bone length (mm)	0.560
Bone width (mm)	0.549
Bird weight (g)	0.449
Bone strength (N)	0.388
Podo score	0.147
Ash percentage	0.131

Bone ash weight (g)

Dry fat extracted bone weight (g)	0.975
Fresh bone weight (g)	0.895
Bone length (mm)	0.847
Bone width (mm)	0.830
Ca content/dry bone weight (mg)	0.714
P content/dry bone weight (mg)	0.693
Bone strength (N)	0.682
Bird weight (g)	0.598
Ash percentage	0.344
Podo score	0.120

Ash percentage

Bone strength (N)	0.414
Bone ash weight (g)	0.344
Ca content/dry bone weight (mg)	0.164
Bone width (mm)	0.131
P content/dry bone weight (mg)	0.131
Dry fat extracted bone weight (g)	0.129
Bird weight (g)	0.052
Bone length (mm)	0.047
Fresh bone weight (g)	0.020
Podo score	-0.058

Bone strength (N)

Bone ash weight (g)	0.682
Bone width (mm)	0.669
Dry fat extracted bone weight (g)	0.617
Fresh bone weight (g)	0.560
Bone length (mm)	0.522
Ca content/dry bone weight (mg)	0.418
Ash percentage	0.414
P content/dry bone weight (mg)	0.388
Bird weight (g)	0.287
Podo score	-0.106

Dry fat extracted bone weight (g)

Bone ash weight (g)	0.975
Fresh bone weight (g)	0.946
Bone length (mm)	0.889
Bone width (mm)	0.851
Ca content/dry bone weight (mg)	0.719
P content/dry bone weight (mg)	0.704
Bird weight (g)	0.627
Bone strength (N)	0.617
Podo score	0.142
Ash percentage	0.129

Appendix 5: Ranking of Pearson correlation values according to bone measurements in day 28 cockerels

Ca content/dry bone weight (mg)

P content/dry bone weight (mg)	0.938
Bone ash weight (g)	0.638
Bone strength (N)	0.579
Dry fat extracted bone weight (g)	0.561
Fresh bone weight (g)	0.523
Bone width (mm)	0.491
Ash percentage	0.378
Bird weight (g)	0.369
Bone length (mm)	0.356
Podo score	-0.109

P content/dry bone weight (mg)

Ca content/dry bone weight (mg)	0.938
Bone ash weight (g)	0.642
Bone strength (N)	0.582
Dry fat extracted bone weight (g)	0.581
Fresh bone weight (g)	0.561
Bone width (mm)	0.537
Bone length (mm)	0.416
Bird weight (g)	0.348
Ash percentage	0.345
Podo score	-0.048

Bone ash weight (g)

Dry fat extracted bone weight (g)	0.939
Fresh bone weight (g)	0.900
Bone width (mm)	0.741
Bone length (mm)	0.677
P content/dry bone weight (mg)	0.642
Ca content/dry bone weight (mg)	0.638
Bone strength (N)	0.605
Bird weight (g)	0.541
Ash percentage	0.445
Podo score	-0.138

Ash percentage

Bone strength (N)	0.465
Bone ash weight (g)	0.445
Ca content/dry bone weight (mg)	0.378
P content/dry bone weight (mg)	0.345
Bone width (mm)	0.303
Bird weight (g)	0.190
Fresh bone weight (g)	0.137
Dry fat extracted bone weight (g)	0.112
Bone length (mm)	0.031
Podo score	-0.138

Bone strength (N)

Bone ash weight (g)	0.605
P content/dry bone weight (mg)	0.582
Ca content/dry bone weight (mg)	0.579
Dry fat extracted bone weight (g)	0.489
Ash percentage	0.465
Fresh bone weight (g)	0.444
Bone width (mm)	0.421
Bone length (mm)	0.233
Bird weight (g)	0.136
Podo score	-0.136

Dry fat extracted bone weight (g)

Fresh bone weight (g)	0.947
Bone ash weight (g)	0.939
Bone length (mm)	0.741
Bone width (mm)	0.706
P content/dry bone weight (mg)	0.581
Ca content/dry bone weight (mg)	0.561
Bird weight (g)	0.541
Bone strength (N)	0.489
Ash percentage	0.112
Podo score	-0.103

Appendix 6: Ranking of Pearson correlation values according to bone measurements in day 28 pullets

Ca content/dry bone weight (mg)

P content/dry bone weight (mg)	0.950
Bone ash weight (g)	0.695
Dry fat extracted bone weight (g)	0.620
Bird weight (g)	0.532
Fresh bone weight (g)	0.531
Bone width (mm)	0.486
Bone length (mm)	0.471
Bone strength (N)	0.456
Ash percentage	0.397
Podo score	0.071

P content/dry bone weight (mg)

Ca content/dry bone weight (mg)	0.950
Bone ash weight (g)	0.629
Dry fat extracted bone weight (g)	0.569
Fresh bone weight (g)	0.486
Bird weight (g)	0.462
Bone width (mm)	0.449
Bone length (mm)	0.427
Bone strength (N)	0.390
Ash percentage	0.335
Podo score	0.089

Bone ash weight (g)

Dry fat extracted bone weight (g)	0.957
Fresh bone weight (g)	0.813
Bird weight (g)	0.703
Ca content/dry bone weight (mg)	0.695
Bone length (mm)	0.676
Bone width (mm)	0.645
P content/dry bone weight (mg)	0.629
Ash percentage	0.371
Bone strength (N)	0.235
Podo score	-0.008

Ash percentage

Bone strength (N)	0.423
Ca content/dry bone weight (mg)	0.397
Bone ash weight (g)	0.371
P content/dry bone weight (mg)	0.335
Bird weight (g)	0.241
Dry fat extracted bone weight (g)	0.087
Podo score	0.064
Fresh bone weight (g)	0.047
Bone width (mm)	0.035
Bone length (mm)	-0.051

Bone strength (N)

Ca content/dry bone weight (mg)	0.456
Ash percentage	0.423
P content/dry bone weight (mg)	0.390
Bird weight (g)	0.350
Podo score	0.289
Bone ash weight (g)	0.235
Bone width (mm)	0.185
Fresh bone weight (g)	0.165
Dry fat extracted bone weight (g)	0.119
Bone length (mm)	0.071

Dry fat extracted bone weight (g)

Bone ash weight (g)	0.957
Fresh bone weight (g)	0.859
Bone length (mm)	0.740
Bird weight (g)	0.679
Bone width (mm)	0.677
Ca content/dry bone weight (mg)	0.620
P content/dry bone weight (mg)	0.569
Bone strength (N)	0.119
Ash percentage	0.087
Podo score	-0.027

Appendix 7: Ranking of Pearson correlation values according to bone measurements in end of crop cockerels

Ca content/dry bone weight (mg)

P content/dry bone weight (mg)	0.988
Bone ash weight (g)	0.644
Bird weight (g)	0.607
Dry fat extracted bone weight (g)	0.607
Fresh bone weight (g)	0.583
Bone length (mm)	0.542
Bone width (mm)	0.528
Bone strength (N)	0.399
Ash percentage	0.303
Podo score	0.146

P content/dry bone weight (mg)

Ca content/dry bone weight (mg)	0.988
Bone ash weight (g)	0.637
Bird weight (g)	0.604
Dry fat extracted bone weight (g)	0.596
Fresh bone weight (g)	0.574
Bone length (mm)	0.527
Bone width (mm)	0.509
Bone strength (N)	0.390
Ash percentage	0.314
Podo score	0.118

Bone ash weight (g)

Dry fat extracted bone weight (g)	0.964
Fresh bone weight (g)	0.950
Bird weight (g)	0.918
Bone width (mm)	0.847
Bone length (mm)	0.837
Ca content/dry bone weight (mg)	0.644
P content/dry bone weight (mg)	0.637
Bone strength (N)	0.373
Ash percentage	0.363
Podo score	0.100

Ash percentage

Bone strength (N)	0.569
Bone ash weight (g)	0.363
P content/dry bone weight (mg)	0.314
Ca content/dry bone weight (mg)	0.303
Bone width (mm)	0.271
Bird weight (g)	0.241
Fresh bone weight (g)	0.121
Dry fat extracted bone weight (g)	0.105
Bone length (mm)	-0.012
Podo score	-0.028

Bone strength (N)

Ash percentage	0.569
Ca content/dry bone weight (mg)	0.399
P content/dry bone weight (mg)	0.390
Bone ash weight (g)	0.373
Bird weight (g)	0.300
Bone width (mm)	0.274
Dry fat extracted bone weight (g)	0.235
Podo score	0.221
Fresh bone weight (g)	0.213
Bone length (mm)	0.174

Dry fat extracted bone weight (g)

Fresh bone weight (g)	0.981
Bone ash weight (g)	0.964
Bird weight (g)	0.914
Bone length (mm)	0.900
Bone width (mm)	0.823
Ca content/dry bone weight (mg)	0.607
P content/dry bone weight (mg)	0.596
Bone strength (N)	0.235
Podo score	0.113
Ash percentage	0.105

Appendix 8: Ranking of Pearson correlation values according to bone measurements in end of crop pullets

Ca content/dry bone weight (mg)

P content/dry bone weight (mg)	0.920
Bone ash weight (g)	0.491
Bird weight (g)	0.400
Dry fat extracted bone weight (g)	0.395
Bone strength (N)	0.372
Fresh bone weight (g)	0.363
Bone length (mm)	0.330
Ash percentage	0.292
Bone width (mm)	0.287
Podo score	0.279

P content/dry bone weight (mg)

Ca content/dry bone weight (mg)	0.920
Bone ash weight (g)	0.467
Bird weight (g)	0.389
Bone strength (N)	0.385
Dry fat extracted bone weight (g)	0.354
Ash percentage	0.342
Fresh bone weight (g)	0.314
Bone width (mm)	0.297
Bone length (mm)	0.267
Podo score	0.191

Bone ash weight (g)

Dry fat extracted bone weight (g)	0.940
Fresh bone weight (g)	0.925
Bird weight (g)	0.853
Bone length (mm)	0.754
Bone width (mm)	0.737
Ca content/dry bone weight (mg)	0.491
P content/dry bone weight (mg)	0.467
Bone strength (N)	0.243
Ash percentage	0.179
Podo score	0.172

Ash percentage

Bone strength (N)	0.505
P content/dry bone weight (mg)	0.342
Ca content/dry bone weight (mg)	0.292
Bone ash weight (g)	0.179
Bone width (mm)	0.058
Bird weight (g)	0.035
Fresh bone weight (g)	-0.087
Podo score	-0.120
Dry fat extracted bone weight (g)	-0.162
Bone length (mm)	-0.245

Bone strength (N)

Ash percentage	0.505
P content/dry bone weight (mg)	0.385
Ca content/dry bone weight (mg)	0.372
Podo score	0.278
Bone ash weight (g)	0.243
Bone width (mm)	0.181
Bird weight (g)	0.171
Fresh bone weight (g)	0.095
Dry fat extracted bone weight (g)	0.078
Bone length (mm)	-0.054

Dry fat extracted bone weight (g)

Fresh bone weight (g)	0.957
Bone ash weight (g)	0.940
Bird weight (g)	0.847
Bone length (mm)	0.840
Bone width (mm)	0.719
Ca content/dry bone weight (mg)	0.395
P content/dry bone weight (mg)	0.354
Podo score	0.217
Bone strength (N)	0.078
Ash percentage	-0.162