The effect of bone choice on quantification of mineralization in broiler chickens up to six weeks of age

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Running head: Bone type and bird age affects bone mineralization

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SUMMARY

An experiment was conducted to assess the most appropriate bone type for measuring bone mineralization in male broiler chicks up to 42 days. A total of 72 male broilers were raised in $0.64m^2$ pens on a litter floor. The study design included two dietary treatments (Control and Low) containing differing levels of total phosphorus (7.8 and 4.4g/kg for Control and Low diets respectively) and calcium (22.7 and 13.1g/kg for Control and Low diets respectively) with each fed to six replicate pens of 6 birds. Each week, six birds per diet were euthanized and leg bones removed to measure ash percentage. Foot, toe, tibia and femur ash were compared using the mean of both legs from each bird, via t tests to separate Control and Low diets. At the end of week 1, diets could not be separated using any of the bone ash measures. From week 2 to week 5, both tibia and foot ash differentiated between the control and low diets, and tibia continued to show significant differences between the diets into week 6. Femur ash did not show any dietary differences until week 3, but then showed significant differences between the diets until week 6. Toe ash only differentiated between diets at week 2, and variation both within and between birds was high, particularly with younger birds. These results suggest that bird age has implications when choosing a bone for assessing possible differences in dietary phosphorus and calcium uptake. Femur ash may be more appropriate for showing differences in broilers aged 6 weeks and older. Foot ash provides a comparable alternative to tibia ash in birds aged 2 to 5 weeks of age, providing a labor and time saving alternative.

DESCRIPTION OF PROBLEM

Skeletal development in broilers is a key factor both in terms of welfare and production with strong evidence that over- or under-supply of dietary phosphorus substantially impacts on bird growth performance [1, 2]. Typically, broiler diets are supplemented with vitamin D, calcium and phosphorus in order to improve skeletal development. However, phosphorus is expensive and in limited supply globally, and oversupply leads to increased soil run off and eutrophication of water sources [3]. It is therefore essential to accurately quantify both the total and available phosphorus in poultry diets so bird requirements are precisely matched.

Dietary available phosphorus content is often measured indirectly by quantifying bone mineralization. There are several methods which are routinely used based around the comparison of ashed bone to dry bone weight. Bone ash is a critical measure as chicks with bone disorders usually have lower percentage ash content. Commonly, toe or tibia ash are used, with tibia ash the most frequently used criteria for assessment of commercial calcium and phosphorus content [4, 5, 6]. Other possible measurements are whole foot and femur ash, but there is little consensus between research groups with respect to methodology.

Tibia ash is time consuming and labor intensive, both in terms of collection of material and preparation, and differences in methodology can affect results [7]. Toe ash has been shown to have a linear relationship with tibia ash up to 21 days post-hatch and may be more sensitive to dietary changes [8]. However, toe ash allows a certain amount of subjectivity when collecting the material and the sample size can be so small that any errors are disproportionately large. Foot ash has been shown to give comparable results to toe and tibia ash up to two weeks of age [9] and has been shown to reflect dietary phosphorus levels [10], but its value as a measure in older chicks is not fully established. Shastek *et al*. [11] investigated two age periods (11-21 and 25-35 days post hatch) and found the sensitivity of foot ash was comparable to tibia ash when evaluating mineral sources of phosphorus but there is no information on weekly foot ash measures and how they compare to other bone ash measures. The advantages of foot ash as a measure of bone mineralization are speed of sample collection and reproducibility due to larger sample weight.

It is important to consider which bone ash measure will accurately reflect dietary phosphorus differences in birds of different ages. Several historical studies have suggested that the femur is more representative of total skeletal mineralization than the tibia, and suggested that it may be more sensitive than the tibia to dietary changes [12, 13].

The objective of this study was to establish whether, tibia, toe, foot or femur ash would differentiate between diets formulated with low and control phosphorus and calcium levels over a six-week period.

MATERIALS AND METHODS

A total of 72 male Ross 308 broiler chicks were fed one of two wheat/soya based mash diets from day of hatch to 42 days. The chicks were housed in groups of six, in 0.64 m^2 floor pens bedded on fresh wood shavings. Six pens of birds were fed each dietary treatment and feed and water were available *ad libitum*, and provided with age-appropriate supplemental heating and ventilation. A dark period of 6 hours was provided from 6d onwards including an unbroken 4-hour period of darkness as required by EU legislation. The study was approved by the Nottingham Trent University ethics committee and all animal care met the guidelines approved by the institutional animal care and use committee (IACUC).

Diets were formulated to be as nutritionally similar as possible, with the exceptions of total analyzed phosphorus (P) and calcium (Ca) content. Mineral levels were chosen in order to produce a measureable difference in bone mineralization in the birds. The Low Diet was formulated at a low but nutritionally adequate level of P and Ca, and the Control Diet contained double these inclusion levels. Both diets had common ingredients, and levels of other nutrients were formulated to conform to NRC (1994) recommendations (Table 1). Diets were analyzed for crude protein content (calculated as nitrogen multiplied by 6.25) by the AOAC standard method [14] and gross energy via bomb calorimetry [15]. Phosphorus and calcium content of the diets were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) following an aqua regia digestion step [16].

One bird per pen was euthanized weekly by cervical dislocation. Feet were removed from each bird at the tibial-tarsal joint prior to dissection of tibia and femur bones from both legs. Tibias and femurs were immediately frozen at -20^{o}C. The middle toe of each foot was removed at the joint between the second and third toe bones. Feet and toes were also stored at -20^{o}C until analysis. All bones were labelled so the individual bird and leg could be identified for comparison purposes.

Toes and feet were dried at 105^{o}C until constant weight (minimum 5 days) prior to ashing individually at 650^{o}C for 13 hours. Toe weights were added back to the corresponding foot to calculate whole foot ash.

Individual legs were autoclaved at 121^{o}C for 15 minutes, and the flesh, including cartilage caps, carefully removed by hand. The stripped bones were then dried to constant weight for a minimum of 3 days at 105^{o}C, prior to ashing at 650^{o}C for 13 hours. The ash weight of each bone, foot or toe was expressed as a percentage of dry weight.

Statistical analysis was performed using independent sample t tests to compare the control and low diets for each bone type each week, using SPSS v19 (IBM statistics), with means deemed to be significantly different at $P < 0.05$. Interactions between week and diet were not included in the analysis as the profound and well established effect of age on bone mineralization over-emphasizes the effect of bird age. The relationship between the four different ash sources was investigated using Pearson product- moment correlation coefficients after preliminary analysis to ensure normality and linearity. Interpretations of the strength of the relationships between the factors were based on guidelines by Cohen (1988) [17]; weak relationship r = 0.10 to 0.29, medium relationship r = 0.30 to 0.49 and strong relationship r = 0.50 to 1.0. Statistical significance was declared at $P < 0.05$. Coefficients of variation was determined by dividing standard deviation values by their associated mean and expressed as a percentage. Root mean square error (RMSE) for each bone type was obtained using SPSS as a measure of variability between bone types spanning all bird ages.

RESULTS AND DISCUSSION

Table 1 shows the analyzed values for the test diets which closely reflect the formulated values, with some variation in both phosphorus and calcium content. Analysis of the study diets presented substantial differences in dietary calcium and phosphorus, which translated into measureable differences in bone mineralization between the Control and Low diets in some bones. Although the calcium to phosphorus ratios of the diets was higher than formulated, the ratios for both the Control and Low diets were very similar.

Table 2 shows percentage bone ash results separated into toe, foot, tibia and femur ash for each diet on a weekly basis and broadly confirms increased bone mineralization in birds fed the Control diet. The foot and toe ash have substantially lower percentage ash values, which is due to the inclusion of skin and tissue in the dry weight. The statistical comparisons between the two diets are also shown in table 2. Dietary differences for each bone type individually (toe, foot, tibia and femur) were compared for each week of the study, in order to monitor which bone type was recording a statistical difference in bone mineralization between the two diets at each age. Table 2 shows no bone produced statistically significant differences between diets in week 1 but, from week two onwards, an overall pattern emerged with smaller bones differentiating between diets early post hatch, and larger bones not differentiating between diets until later on. The apparent lack of difference in bone mineralization between treatments at week 1 sampling is in agreement with the findings of Itoh and Hatano [12], who also found no significant differences when measuring bone minerals in chicks at 7 days of age, suggesting week 1 is too early in the lifetime of the bird for dietary treatments to have had any measurable effect on bone mineralization unless nutrient intervention begins in ovo [18].

The RMSE values and inter bird coefficients of variation (week 1 - 28.5%; weeks 2 through 6 - 10%) show that the toe ash measurements were highly variable; particularly in young chicks where the low weight of the toe makes small variations in sampling technique result in a relatively large standard deviation. This is confirmed by the intra bird coefficients of variation, which were over 10% in week 1 for toe ash and although reduced to 4.7% in weeks 2 through 6, were notably higher than for the other bone sources. Toe ash showed a significant difference between diets only when the birds were 2 weeks old, suggesting that toe ash may be an inappropriate measure of bone mineralization, particularly in older chicks (from 21 days old). Ravindran [8] however found that toe ash was a sensitive measure of phosphorus availability in chicks of 21 days old, and found a strong correlation between toe and tibia ash, which was not reflected in this study, where no relationship was observed (see table 3), although a relationship was seen between toe and foot ash. In some studies, all the toes on each foot have been pooled in order to increase the sample weight and thereby reduce variation [9]. However, this method would increase the time required for the collection of the toes, when compared with foot ash collection.

Pearson correlations found between all bone types are shown in table 3, with correlations between all bone types with the exception of toe ash, which was only correlated with foot ash. The correlation plot for foot and tibia ash is shown in Figure 1.

Overall, foot ash appears to be comparable with tibia and femur ash for reproducibility across the study: little difference was found within bird, with variation of 2.7, 2.8% and 1.3% for tibia, femur and foot ash respectively. Foot and tibia ash were significantly ($p<0.05$) higher in birds fed the Control diet than the low diet for weeks 2 through 5 but in week 6, foot ash did not differentiate between the diets, whereas tibia ash still showed a significant difference. This suggests that the foot bones were approaching full mineralization by 6 weeks of age, and were therefore less effective at showing differences between dietary treatments. These results suggest that foot ash is comparable with tibia ash at showing dietary mineral differences up to 5 weeks of age. Yan *et al*. [9] extended variation in dietary phosphorus levels beyond those seen in commercial practice and found a strong correlation between tibias and foot ash (R^2=0.92) which was not shown as strongly in this trial, although it is clear from Figure 1 that there is some relationship between the two methods (R^2 =0.455). This may be due to the small number of replicates in this study (6 birds per diet per week), restricted range of dietary phosphorus levels and increased variation caused by analysis at a range of ages, in contrast to the single age used by Yan *et al.* [9]. However, even with the small number of replicates, differences in sensitivity were still observed between bone types.

Tibia ash, with an ether pre extraction, is recommended by the AOAC [19] for the measurement of vitamin D activity and as a method for bone mineralization analysis. Although the bones in this study were not ether extracted, the average inter-bird coefficients of variation for tibia ash were lower than for any other leg bone type, which evidences the precision of the method. Ether extraction can reduce variation via fat removal and therefore sensitivity could be expected to improve further if bones were pre extracted. Previous authors have assessed percentage tibia ash over the same time period as this study and presented the same trend to increasing tibia ash content as the birds age [4].

Femur ash has been reported to be the most similar bone in terms of mineralization to whole skeletal values. It has been reported to be more responsive to dietary changes than the tibia [12], and more sensitive to differing ratios of Ca: P in older birds [13]. In this study, the femur ash showed no differences between the control and low diet in birds up to 3 weeks of age, but was significantly greater for the control diet compared with the low diet in weeks 4 to 6. At week 6, the differences between the diets were highly significant ($P > 0.001$) for femur ash, contrasting with the results for foot and tibia ash, which resulted in less significant differences between treatments with increasing bird age. This suggests that the femur bone is still mineralizing at 6 weeks of age and may be a more appropriate bone to use when making dietary comparisons in older chicks (over 6 weeks of age). Percentage femur ash was found to correlate with tibia ash and, to a lesser extent, with foot ash (Table 3). The regression coefficients are comparatively modest, which is likely to be due to the small replicate size and narrow range of ash values achieved with only two phosphorus levels.

Variability in organic matter and lipid content may have reduced the sensitivity of foot ash and tibias as a measure in older birds. A potential solution to this problem is to extract the fat from larger feet prior to ashing [10]. Hall *et al*. [7] found that fat extraction improved the power of the tibia ash method. However, the un-extracted tibia ash coefficients in this study averaged less than 5%, compared to that study, where un-extracted, autoclaved tibias had high coefficients of variation (21.53%) [7]. This suggests that it may not be necessary to extract fat from bones prior to mineralization analysis, which would be advantageous because fat extraction is labor intensive and uses harmful chemicals, but this requires further investigation.

The current global drive to reduce lameness in poultry requires robust, efficient measures. Whilst bone mineralization is not the only factor to be considered, this study demonstrates that the age of the bird needs to be taken into account when monitoring this aspect of lameness. Foot ash provides a rapid and simple measure which can be considered as an alternative to tibia ash in chicks 5 weeks of age and younger. In birds 6 weeks of age and above, this study shows that femur ash may be the most appropriate measure for assessing bone mineralization. Further investigations based on corn-soy diets that included examination of the impact of bird weight alongside age on optimum bone sampling for ash determination would provide further insight in this area.

CONCLUSIONS AND APPLICATIONS

1. Percentage foot ash is an efficient and comparable alternative to percentage tibia ash for assessing bone mineralization in birds between 2 and 5 weeks of age
2. Percentage femur ash may be a more appropriate measure for use in birds aged 6 weeks and older than tibia ash.
3. Operator care needs to be taken when using toe ash measures to ensure consistency of sampling.

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Table 1 Composition and analysis of low and control diets

Ingredient / Analyses	Control Diet (g/kg)	Low Diet (g/kg)
Wheat[1]	663.5	677.5
High protein soya bean meal[2]	250.0	250.0
Lysine[3]	3.0	3.0
Methionine[3]	2.5	2.5
Soya Oil	40.0	40.0
Limestone[4]	20.0	10.0
Monocalcium Phosphate[5]	8.0	4.0
Sodium Chloride	2.5	2.5
Sodium Bicarbonate	1.5	1.5
Vitamin E/Biotin 75/125 Premix	1.0	1.0
Vitamin Mineral Premix[6]	2.5	2.5
Choline Chloride	0.5	0.5
Titanium Dioxide	5.0	5.0
Calculated Composition		
Phosphorus g/kg	5.14	4.30
Calcium g/kg	10.81	6.19
Non phytate phosphorus g/kg	2.94	2.04
Calculated Poultry ME kcal/kg	3066.3	3109.8
Analyzed Composition		
Phosphorus g/kg	7.8	4.4
Calcium g/kg	22.7	13.1
Protein g/kg	208	204

Gross energy kcal/kg	4,610	4,750

[1] Wheat: Ca 0.04%; P 0.24%; non phytate P 0.06%

[2] Soya bean meal: protein 48.7%; Ca 0.31%; P 0.72%; non phytate P 0.32%

[3] Lysine and Methionine sourced from Evonik

[4] Limestone: Ca 38%; P 0%

[5] Monocalcium phosphate: Ca 16%; P 25.3%

[6] Premix content (volume/kg diet): Mn 100mg, Zn 80mg, Fe 20mg, Cu 10mg, I 1mg, Mb 0.48mg, Se 0.2mg, Retinol 13.5mg, Cholecalciferol, 3mg, Tocopherol 25mg, Menadione 5.0mg, Thiamine 3mg, Riboflavin 10.0mg, Pantothenic acid 15mg, Pyroxidine 3.0mg, Niacin 60mg, Cobalamin 30µg, Folic acid 1.5mg, Biotin 125mg

Table 2 Mean values for percentage ash for each bone type for weeks 1 through 6 for low and control dietary groups with statistical differences between diets.

	Toe ash (%)		Foot ash (%)		Tibia ash (%)		Femur ash (%)	
Week	Low	Control	Low	Control	Low	Control	Low	Control
1	10.04	10.08	12.93	12.99	35.97	37.26	36.32	37.12
2	9.93*	10.82*	12.30**	13.50**	38.89*	41.63*	39.03	40.68
3	10.01	11.10	13.22**	14.97**	42.95**	45.42**	42.93	45.34
4	11.46	12.22	13.76*	15.40*	41.07**	44.22**	41.70*	44.56*
5	11.59	12.09	13.84*	15.09*	40.58*	43.62*	40.33*	44.43*
6	10.59	10.88	14.47	15.48	43.57*	47.83*	41.50**	45.06**
RMSE[1]	1.525		0.823		2.052		2.767	

[1]RMSE Root mean square error for each bone type obtained using SPSS

Statistical comparisons of dietary treatments by t test. Significant differences denoted by * $P<0.05$; ** $P<0.01$;

Table 3 Pearson product moment correlations between different bone types

	Toe ash	Foot ash	Tibia ash	Femur ash
Foot ash	0.623[1]	0.632	0.132*	0.315
Tibia ash	-	-	0.675	0.644
Femur ash	-	-	-	0.687

[1]n=141; all other data sets, n=144

*correlation not significant at $P<0.05$