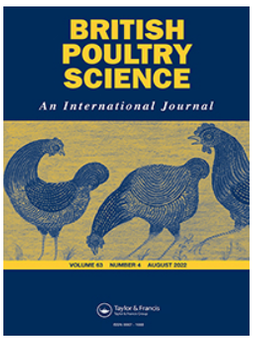
Development of an in vivo radiographic method with potential for use in improving bone quality and the welfare of laying hens through genetic selection



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Development of an *in vivo* radiographic method with potential for use in improving bone quality and the welfare of laying hens through genetic selection

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ABSTRACT

1. Genetic selection for bone quality can improve this, as it is heritable. A method was established using digital X-ray which took around 40 s in total and gave an image that allowed quantification of bone density from many appendicular bones.
2. The tibiotarsus measurement of bone density on the live hen across the different experiments had correlations with *post-mortem* whole bone radiographic density from 0.62 to 0.7, similar to that between density and material properties for example. Differences between groups of hens, where calcium and phosphorus in the diet were manipulated, were detected within 3 weeks of treatment using live hen measurement ($P < 0.001$, $n = 24$).
3. In a gage analysis, 'hen' explained more than 86% of the variance, demonstrating the ability to observe clear differences between hens. The effect of different operators' analysis on the contribution to variance was very low as was the repeated measurement of the same hen.
4. The measurement of bone density on the live hen described in this paper represented major progress to a usable method for genetic selection to improve bone strength in laying hens. The method has the potential to reduce the number of animals needed to test nutritional and management interventions to improve bone health.

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Breeding; osteoporosis; calcium; bone biology; techniques; radiographic

Introduction

Bone fractures have long been recognised as a welfare issue in laying hens (FAWC, 2010), since the physiological adaptations for egg laying increase the likelihood of bone loss during the life of the hen (Whitehead, 2004). The current drive to extend the laying period to 100 weeks might place additional demands on the skeleton (Bain et al., 2016), although recent studies suggested that the number of eggs a hen lays may not be so important as issues around the timing of the onset of lay (Dunn et al., 2021). The switch to more extensive housing systems, which allow greater movement and, consequently, increased functional loading of the skeleton, should provide opportunities for improving bone quality. However, these alternative systems provide increased opportunities for collisions and falls, and are associated with a greater incidence of bone damage (Sandilands, 2011), often featuring the keel (Rorvang et al., 2019).

Quantitative genetic selection, using a weighted index of economically important traits, has been used by commercial breeders for over 60 years to improve the performance of laying hens (Preisinger, 2018). A weighted index, or breeding value, of important traits combines information on an individual's own performance with that of all relatives of the selection candidates, with correction for known non-genetic differences between animals (for example, week of hatch or house effect). By selecting and breeding from only the best birds, favourable alleles are passed on to the next generation. It has previously been demonstrated that genetic factors underlie the variation in the susceptibility of individual hens to osteoporosis and bone fracture, and that genetic selection for improved bone strength was possible without detriment to production traits (Bishop et al., 2000; Dunn et al., 2007, 2021). However, assessment of bone quality parameters, such as breaking strength and mineral content, is made *post-mortem*, which makes implementation in a commercial breeding programme more challenging. Thus, there is a requirement for a phenotypic assessment made during the life of the hen that can be incorporated with other phenotypes in the calculation of the breeding value. To be practical, the assessment must be quick and easy to perform, inexpensive and correlated with the accepted measurements of bone quality, such as breaking strength and mineral density. Ultimately, the measurement does not need to be exactly correlated with existing *post-mortem* measurements, which are, after all, only a proxy in themselves of skeletal damage, but must be sufficiently related to the existing measurements that would be useful in selection to improve skeletal quality. Methods that mimic potential fractures that cause injuries have successfully shown line differences and a relationship with skeletal quality, but required the hen to be dead for the measurement (Candelotto et al., 2020).

A variety of non-invasive methods exist for the determination of body and carcase composition in livestock, such as dual-energy X-ray absorptiometry (DEXA), computed tomography, magnetic resonance imaging and ultrasound (Fleming et al., 2004). Live hens were measured using DEXA which successfully detected changes in diet-induced bone density after 25 weeks of treatment, but each scan took 10 min to complete (Schreiweis et al., 2003). It has been previously demonstrated that Digitised Fluoroscopy, a low-cost radiographic technique, could be used as a predictor of breaking strength in end of lay avian bone, but was considered problematic to use (Fleming et al. 2004). The use of whole hen CT scans has potential, but the equipment is immobile which requires the movement of thousands of animals

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to where the scanner is located and, although image acquisition may be relatively rapid, the analysis is time consuming (Donko et al., 2018). However, developments in digital imaging have now made the routine use of radiographs a viable option for such criteria of speed and simplicity (Korner et al., 2007). Digital radiography uses X-ray–sensitive plates to directly capture data during X-ray exposure, immediately transferring it to a computer system. Advantages include time efficiency, bypassing chemical processing, and the ability to digitally store and transfer images immediately. In addition, less radiation is required to produce an image of similar contrast to conventional radiography. This paper investigated the use of radiographic imaging to assess bone quality in the living laying hen suitable for genetic evaluation.

Materials and methods

Ethical statement

Use of animals was approved by the Roslin Institute Animal Welfare and Ethical Review Body, and experiments were carried out under the Animals (Scientific Procedures) Act 1986, project licence 70/7909.

X-ray capture and initial optimisation and assessment of images

For initial development purposes, end of lay (~65 weeks) Lohmann brown laying hens (n = 50) were obtained, in two batches of 25, from a local egg producer. X-ray exposures of the dead hens were taken over a range of kV (kilovoltage) and mAs (milliAmpere seconds) values, under the guidance and advice of an experienced radiographer, to determine the combination that gave a clear image with a low exposure time. Each hen was radiographed and each exposure included a 16-step aluminium step wedge, with 1 mm increments. Images were saved as DICOM files. Once radiographed, the relevant bones were excised for *post-mortem* measurement.

Positioning and immobilisation of the live hen

For the technique to have practical application for genetic evaluation, there needed to be rapid throughput of hens. Anaesthesia and physical restraint by personnel were neither practical nor desired for reasons of safety, so a method of restraint of the hen during X-ray exposure was used. The VSP Miami Vise® Restraint (Veterinary Specialty Products, Miami, USA; Figures S1, S2) was designed to restrain hens during such procedures and was tested for suitability. For each exposure, the aim, during development, was to visualise as many bones as possible, including the keel, in one single image. The laying hen cadavers (n = 50; age ~65 weeks) were used to determine the best positioning to provide images of both keel and long bones simultaneously prior to live hen radiography. Further details and illustrations can be accessed in the supplementary material and a SOP can be found at https://doi.org/10.7488/ds/3484.

X- ray equipment

The X-ray equipment consisted of a Cuattro Slate 6 DR X-ray system with a Wireless 12 × 14" AED VW Caesium Wireless Flat Panel Detector. X-rays were generated using a MeX +20BT lite Battery-Powered X-ray Generator (90 kV/20 mA), suspended from a Stat-X Vaquero Folding Mobile Stand (IVM Imaging, Bellshill, Scotland).

Of primary importance when using X-ray equipment is safety, and the use of X-ray generators is heavily regulated. All methodology was developed in conjunction with the Radiation Protection Unit of the University of Edinburgh, to ensure compliance with all regulations.

Validation experiment 1; differences in dietary calcium and phosphorus

To assess and validate the correlations between this live radiographic measurement and current *post-mortem* measures, the bone quality of 26 weeks of age Hy-line brown laying hens (Hy-line UK Ltd., Studley, UK) was manipulated by feeding diets differing in their concentration of dietary calcium (Ca) and phosphorus (P). This was done to produce a large range of bone breaking strength and density values in order to demonstrate the ability of the methods to define such differences compared to existing *post-mortem* analysis. Three groups of 24 hens (two pens per diet; 12 hens per pen) were fed one of three diets with the following measured Ca concentration, measured P concentration and ratio Ca:P of Diet Control 3.28%, 0.46%, 7.08:1; Diet LowCa, 1.89%, 0.48%, 3.96:1; Diet LowCaP 1.39%, 0.31%, 4.54:1. Diets were fed *ad libitum* for 5 weeks. Live radiographs were taken on three occasions – immediately before feeding the diets; 3 weeks after the start of the experiment and immediately before culling.

Validation experiment 2; reproducibility and repeatability

To assess the reproducibility and repeatability of the live measurement, 60 one-day-old Hy-line brown chicks (Hy-line UK Ltd., Studley, UK) were obtained from a commercial hatchery and reared according to recommended management protocols to 30 weeks of age in 4 replicate pens of 15 hens per pen. Hens were radiographed at 30 weeks of age. On each occasion, each hen was radiographed once per day for 4 consecutive days. Hens were individually weighed before the first radiograph. To investigate the effect of repeatability of the measurement from radiographs on different days, a Gage R&R analysis was undertaken (Burdick et al., 2003). Four separate tibiotarsus measurements were made for each radiograph taken on each of the 4 d (60 hens × 4 d × 4 measurements) at each of the ages by one operator.

To investigate reproducibility, the variability in measurement when a different operator measures the same part multiple times, a single measurement of tibiotarsus AUC was made for each radiograph, taken on each of the 4 d, by two different operators (60 hens × 4 d × 1 measurement × 2 operators). To further assess the repeatability and reproducibility of the radiographic process itself, the AUC of the steps of the step wedge (16 steps; 1–16 mm) of 12 random images was measured by three different operators.

Once the last radiograph images had been captured in live hens, all hens were weighed again and then culled using an overdose of Pentobarbital (200 mg/ml) and the bones of interest (tibiotarsus, tarsometatarsus, humerus, radius, ulna and keel) dissected out for *post-mortem* assessment.

Image analysis

To satisfy the requirement to develop a technique that was easy to perform, inexpensive, with the possibility of automation and that could be used in high-throughput systems with live hens, we investigated a number of alternative methods of assessing bone quality. These measurements were correlated with accepted *post-mortem* measurements, such as breaking strength and bone mineral density. Previous work used the tibiotarsus as a suitable tool for assessing bone quality, so initial assessments focused on this bone, although, subsequently, other bones were assessed. Initial investigations settled on two potential analysis approaches using the Fiji implementation of ImageJ (Schindelin et al., 2012; http://rsb.info.nih.gov/ij/):

Method 1. In Fiji, a plot profile can be created of the intensities of pixels along a selected region within the image. For rectangular selections, the x-axis represents the horizontal distance through the selection and the y-axis the vertically averaged pixel intensity. In this case, a line of length 30 mm and 10 pixels wide which was accommodated by the tibiotarsus width was placed along the distal end of the tibiotarsus and the mean pixel intensity was recorded (Figure 1(a)). This approach did not have any capacity to control for overlying tissue. The density recorded from this approach was named the Mean Gray Value.

Method 2. A 100-pixel wide line was drawn across the tibiotarsus at the point where the three-point bending tests are made (Figure 1(b)). The plot profile of the selected region displays a two-dimensional graph of the pixel intensities along the selected region within the image (Figure 1(c)), and a measurement was made of the area under the curve (Tibiotarsus AUC) to act as a proxy for density. Measurement of the selected area was restricted to the area representing the bone in the image by creating a baseline across the image at the edges of the bone peak. This allowed for the variation in the background (created by varying intensities of muscle, skin, feathers, etc., between hens) to be at least partially accounted for. A further measurement, the mean cortical density, was made from the same selected region that comprised the mean of the pixel density created by the radiograph representation of the denser cross section of the cortical bone (Figure 1(c)).

Post-mortem assessment

Excised bones were radiographed in a Faxitron 43855D soft X-ray apparatus fitted with an NTB EZ240 digital X-ray scanner (NTB GmbH, Germany). Exposure was at a voltage suitable for the bone type and age of hen. Each exposure included a 16-step aluminium step wedge, with 0.25-mm increments, for calibration purposes. Image acquisition was made using the IX-Pect acquisition and imaging software supplied with the scanner. For the measurement of *post-mortem* bone density, each bone was delineated from the background and the mean radiographic density (pre-calibrated in mm of aluminium equivalent) of the whole bone was measured. Measurements on *post-mortem* images were made using the Fiji implementation (Schindelin et al. 2012) of the software package ImageJ (http://rsb.info.nih.gov/ij/).

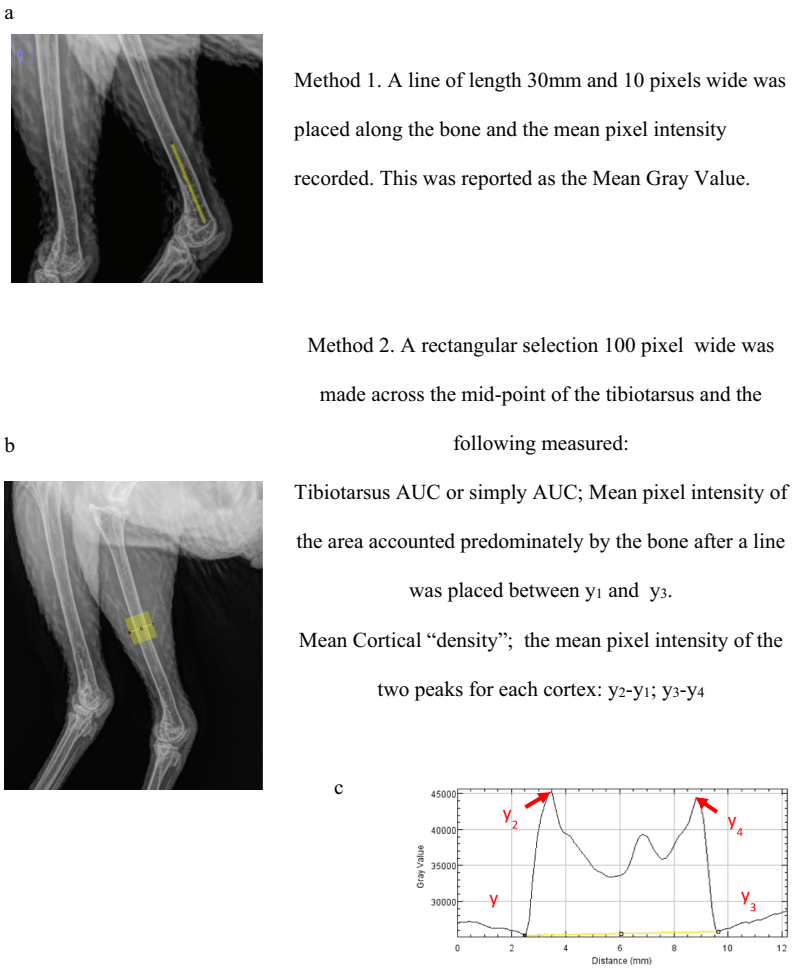

Figure 1. Diagrammatic representation of the measurement options used to calculate radiographic density from tibiotarsus of living laying hens.

Bone breaking strengths were determined on all bones, except the keel, by three-point destructive bending tests, using a JJ Lloyd LS5 5kN advanced materials testing system (Ametek (GB) Ltd., West Sussex, UK) running the software package NEXYGEN*Plus* and fitted with a 2500 N load cell for the tibiotarsus, tarsometatarsus and humerus, and a 500 N load cell for the radius and ulna. The bending jig consisted of two 10 mm diameter steel bar supports, 30 mm apart at centre, and a 10 mm diameter cross head which approached at 30 mm/min. Breaking strength was determined as the maximum load achieved before failure, and the failure point was set at a load that was 30% of the maximum. Stiffness was calculated from the load/displacement curve and was a measure of the bone's resistance to bending.

Statistical analysis

Data were analysed using Genstat v18 (VSN International Ltd., www.vsni.co.uk) and Minitab (www.minitab.com) statistical packages. Data were checked for normal distribution using the Shapiro–Wilk test for Normality, and log transformed where necessary.

Summary statistics were produced from Genstat, and Pearson correlations were calculated in Minitab. Treatment effects in validation experiment 1 were produced using the repeated measures ANOVA option in Genstat including pen in the model. Differences between means were attributed using least significance differences. In validation experiment 2, repeatability of the measurement from radiographs was analysed using Gage Repeatability & Reproducibility analysis in Minitab to calculate variance components from the variables of hen, day and operator and their contribution to overall variance. A similar analysis was carried out on the Aluminium density standards to determine whether the differences between radiographs were a significant variable or could effectively be ignored. Repeatability was denoted from the variance due to the same person making repeated measurements whilst reproducibility was the variance from different people making the measurements.

The data from the validation experiments were examined for agreement between the AUC measurement and the *post-mortem* measurement of radiographic density (Bland and Altman, 1986). Data were log transformed for analysis. Data from the experiments in the paper can be accessed using https://doi.org/10.7488/ds/3484.

Results

X-ray capture and initial optimisation and assessment of images

The mean grey value for all steps of the aluminium step wedge decreased significantly ($P < 0.001$) with increasing exposure (Table S1). Mean Gray Value reduced by just over 4% as exposure changed from 60 kV/2mAs to 65 kV/5mAs and by 50% when increased from 60 kV/2mAs to 75 kV/5mAs. The variance on the steps was lowest for the 65 kV/5mAs. Lastly, examination of the radiographs of the hen suggested that the setting for optimal contrast and visualisation of the bones was 65 kV/5mAs. No evidence of saturation of step wedge 'steps' was evident at this setting and densities of bones in the hen image were within a similar range. These settings were used for all subsequent images. The best positioning of the hen for good image generation is shown in Figure S3.

The hens tolerated the positioning well, and the whole procedure, from getting and positioning the hen to removal and return of the hen post radiography, took around 45 s.

Image analysis

The mean grey values (Mean ± SD, Coefficient of Variation) obtained for method 1 (line) was: 27481 ± 2219, 8.1%; for method 2–1 (AUC): 80986 ± 9257, 11.5% and for method 2–2 (mean cortical 'density'): 18509 ± 2127, 11.5%. *Post-mortem* measurements of the bones were made from tibiotarsus breaking strength (in Newtons (N); 259.6 ± 105.2, 40.5%), tibiotarsus stiffness (in N/m; 377841 ± 114978, 30.4%) and whole bone density from radiography of dissected bones (in mm Al equiv.; 2.18 ± 0.25, 11.4%).

The live hen bone 'density', which was termed tibiotarsus AUC (or just AUC) measurement, correlated best with the *post-mortem* measurements of bone quality. For example, 0.77 between the live hen tibiotarsus AUC value and *post-mortem* tibiotarsus whole bone density (Table 1). The mean cortical density was surprisingly poor, with, at best, an 'r' value of around 0.35 with stiffness or breaking strength, and the mean grey value (method 1) was intermediate between those values (Table 1). For that reason, it was decided to concentrate on the AUC which measured the mean pixel intensity with effectively a degree of background subtraction for surrounding tissue. To ascertain whether a different cross-sectional area might improve the AUC measurement, the radiographs were re-analysed. The rectangular selection was placed at the mid-point of the bone (as in Figure 1(b)) and three sizes of the box were examined – 50 pixels, 100 pixels and 200 pixels in depth.

Table 1. Correlation coefficient between traditional measures of bone quality and live hen radiographic based area under the curve (AUC) density measurement. The significance of the correlation is in brackets.

	Tibiotarsus Breaking Strength (N)	Tibiotarsus Stiffness (N/m)	Tibiotarsus Whole Bone Density (mm Al Equiv.)	Mean Gray Value (method 1)	Tibiotarsus AUC
Tibiotarsus Stiffness (N/m)	0.863*(P = 0.000)*				
Tibiotarsus Whole Bone Density (mm Al Equiv.)	0.724*(P = 0.000)*	0.762*(P = 0.000)*			
Mean Gray Value (method 1)	0.493*(P = 0.000)*	0.498*(P = 0.000)*	0.527*(P = 0.000)*		
Tibiotarsus AUC	0.590*(P = 0.000)*	0.617*(P = 0.000)*	0.770*(P = 0.000)*	0.487*(P = 0.000)*	
Mean Cortical Density	0.344*(P = 0.017)*	0.355*(P = 0.015)*	0.135*(P = 0.355)*	0.219*(P = 0.130)*	0.270*(P = 0.061)*

Table 2. Correlation coefficients between traditional measures of bone quality and live hen radiographic based area under the curve (AUC) density measurement at three different widths. The significance of the correlation is in brackets. Significant correlations are in bold.

Trait	Maximum Load (N)	Stiffness (N/m)	Tibiotarsus Whole Bone Density (mm AlEquiv.)	Bone 'Density ' Tibiotarsus AUC (50 pixel width)	Bone 'Density' Tibiotarsus AUC (100 pixel width)
Stiffness (N/m)	**0.863***(P = 0.000)*				
Whole Bone Density (mm Al Equiv.)	**0.724***(P = 0.000)*	**0.762***(P = 0.000)*			
Bone 'Density' (Tibiotarsus AUC (50 pixel width)	**0.482***(P = 0.001)*	**0.467***(P = 0.001)*	**0.761***(P = 0.000)*		
Bone 'Density' (Tibiotarsus AUC (100 pixel width)	**0.519***(P = 0.000)*	**0.582***(P = 0.000)*	**0.748***(P = 0.000)*	**0.756***(P = 0.000)*	
Bone 'Density' (Tibiotarsus AUC (200 pixel width)	**0.433***(P = 0.002)*	**0.455***(P = 0.002)*	**0.598***(P = 0.000)*	**0.707***(P = 0.000)*	**0.819***(P = 0.000)*

The mean grey values obtained for line of 50 pixels: 80645 ± 10911, 13.5%; for a line of 100 pixels: 87311 ± 11619, 13.5% and for line of 200 pixels: 85029 ± 10607, 12.5%.

The 50-pixel depth AUC 'Density' and 100-pixel depth AUC 'Density' measurements had similar correlations with *post-mortem* whole bone density (Table 2). The 200-pixel depth AUC density measurement did not correlate as well as either of the other depth measurements. The best correlations with *post-mortem* measures of breaking strength and stiffness were achieved with the AUC density measurement made with a line of 100-pixel depth (Table 2), and so this size continued to be used for subsequent analyses.

Although the tibiotarsus has always been favoured for making an assessment of skeletal quality, a range of other bones were examined using the AUC technique, the tarsometatarsus, radius, ulna, humerus and keel, to see whether these were superior to the measurements made with the tibiotarsus (Table 3).

Although the Pearson correlation on these bones between the live hen AUC density and the *post-mortem* values was not poor (Table 4), none were as high as observed for the tibiotarsus (Table 2).

The humerus is often pneumatised and has a highly variable content of medullary bone as illustrated (Figure 2(a,b)) which made measurement difficult.

The difficulty of getting reliable keel measurements was due to its relatively low density and the pectoral muscle overlaying the keel meant suitable measurements were not considered at this stage. Taking into account all of the above optimisations, the study concentrated on the measurement of the tibiotarsus AUC measurement from the live hen using a 100-pixel width region to deliver a method that best reflected the skeletal quality.

Table 3. Descriptive statistics of traditional measures of bone quality and live hen radiographic based AUC density measurement for radius, tarsometatarsus and ulna.

Variable	n	Mean	SD
Radius			
Breaking strength (N)	48	74.8	12.1
Stiffness (N/m)	41	65271	10826
Whole Bone Density (mm Al Equiv.)	48	0.90	0.12
AUC (pixel)	49	40971	3813
Tarsometatarsus			
Breaking strength (N)	49	267.6	45.6
Stiffness (N/m)	48	299263	69371
Whole Bone Density (mm Al Equiv.)	49	1.45	0.16
AUC (pixel)	47	61978	9166
Ulna			
Breaking strength (N)	49	146.5	40.5
Stiffness (N/m)	48	194710	39977
Whole Bone Density (mm Al Equiv.)	49	1.16	0.19
AUC (pixel)	49	76458	9384

Table 4. Correlation coefficients of traditional measures of bone quality and live hen radiographic based AUC density measurement for radius, tarsometatarsus and ulna. Significant correlations are in bold.

Radius	Breaking Strength (N)	Stiffness (N/m)	Whole Bone Density (mm Al Equiv.)
Stiffness (N/m)	**0.857***(P = 0.000)*		
Whole Bone Density (mm Al Equiv.)	**0.598***(P = 0.000)*	**0.611***(P = 0.000)*	
AUC (100 pixel depth)	**0.487***(P = 0.000)*	**0.411***(P = 0.008)*	**0.545***(P = 0.000)*
Tarsometatarsus			
Stiffness (N/m)	**0.518***(P = 0.000)*		
Whole Bone Density (mm Al Equiv.)	**0.372***(P = 0.008)*	0.091*(P = 0.537)*	
AUC (100 pixel depth)	**0.347***(P = 0.017)*	0.133*(P = 0.378)*	**0.386***(P = 0.007)*
Ulna			
Stiffness (N/m)	**0.874***(P = 0.000)*		
Whole Bone Density (mm Al Equiv.)	**0.614***(P = 0.000)*	**0.722***(P = 0.000)*	
AUC (100 pixel depth)	0.197*(P = 0.176)*	**0.459***(P = 0.001)*	**0.591***(P = 0.000)*

Validation experiment 1; differences in dietary calcium and phosphorus

Data from this experiment are summarised in Table 5. The feeding of diets ranging in Ca and P concentrations resulted in hens with significantly different bone quality parameters. There were strong correlations between the tibiotarsus AUC measurement at both 3 weeks and at cull with tibiotarsus breaking strength, stiffness and radiographic density (Table 6).

Importantly, significant differences in tibiotarsus AUC were calculated after just 3 weeks of feeding the diets, which were very similar to that seen in the *post-mortem* measurements (after 5 weeks) of bone quality such as breaking strength (Table 5, Figure 3).

These methods are, of course, not similar, and in a Bland Altman plot, as expected, the agreement was not zero, but within the 95% confidence intervals, there were 34 out of 36 values above the line of agreement and 35 out of 36 below, which indicated there was no obvious bias between the different measurements.

There were significant Pearson correlations between the tibiotarsus AUC measurements at both 3 weeks and at 5 weeks after treatment commenced, with the *post-mortem* measurements of tibiotarsus breaking strength, stiffness and radiographic density (r = 0.619–0.700). AUC measurements were significantly correlated with body weight at cull (r = 0.485), similar to that of body weight at cull correlation with tibiotarsus breaking strength (r = 0.411), stiffness

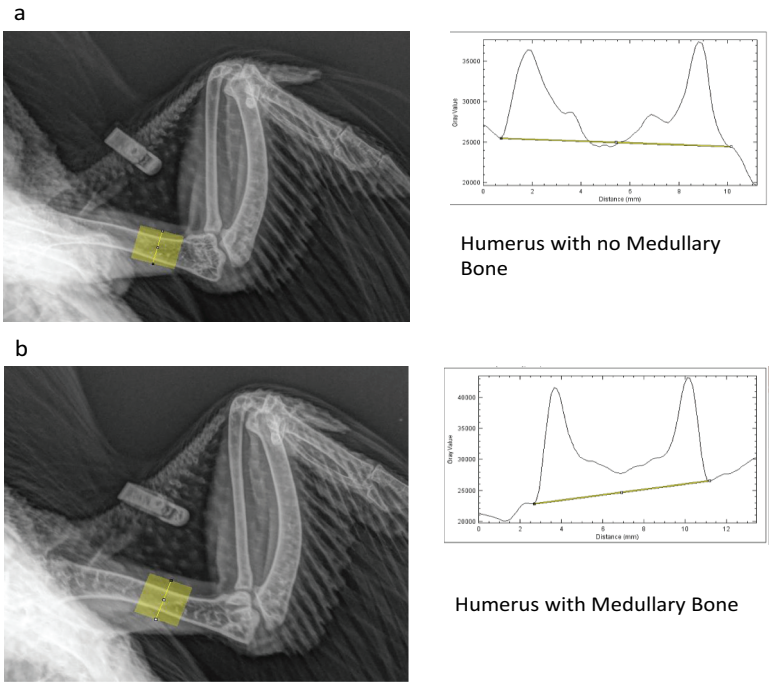

Figure 2. Diagrammatic representation of the measurement of radiographic density from the humerus of living laying hens with a) little or no medullary bone or b) with medullary bone.

Table 5. Descriptive statistics of body weight, traditional measures of bone quality and live hen radiographic based AUC density measurement of the tibiotarsus of laying hens fed ad libitum diets of differing Ca and P concentrations for 5 weeks. The measured Ca and P concentration and ratio Ca:P of Diet Control 3.28%, 0.46%, 7.08:1; Diet LowCa, 1.89%, 0.48%, 3.96:1; Diet LowCaP 1.39%, 0.31%, 4.54:1. Except where stated, measurements were made after cull at 5 weeks of treatment.

Trait	Diet control	Diet LowCa	Diet LowCaP	SED	P
Body weight at start of diet (g)	1833	1840	1834	39.3	0.983
Body weight after 3 weeks of diet (g)	1837	1733	1695	37.2	<0.001
Body weight at after 5 weeks of diet (cull) (g)	1882	1742	1677	43.0	<0.001
Tibiotarsus Breaking strength (N)	273.8	225.6	192.4	22.7	0.003
Tibiotarsus Stiffness (N/m)	403736	333059	266321	30722	<0.001
Tibiotarsus Whole Bone Density (mm Al Equiv.)	2.343	2.120	1.981	0.722	<0.001
Tibiotarsus AUC at start of diet	79151	78524	81403	2047	0.341
Tibiotarsus AUC after 3 weeks of diet	84504	77950	76063	2206.8	<0.001
Tibiotarsus AUC after 5 weeks of diet (pre-cull)	85794	77656	77409	2424.7	<0.001

Table 6. Correlation coefficients of traditional measures of bone quality and live hen radiographic based AUC density measurement for tibiotarsus of laying hens fed ad libitum diets of differing Ca and P concentrations for 5 weeks. The measured Ca and P concentration and ratio Ca:P of Diet Control, 3.28%, 0.46%, 7.08:1; Diet LowCa, 1.89%, 0.48%, 3.96:1; Diet LowCaP, 1.39%, 0.31%, 4.54:1. Except where stated, measurements were made after cull at 5 weeks of treatment. Significant correlations are in bold.

	Tibiotarsus Breaking Strength (N)	Tibiotarsus Stiffness (N/m)	Tibiotarsus Whole Bone Density (mm Al Equiv.)	Weight at cull (g)	Tibiotarsus AUC at start	Tibiotarsus AUC at 3 weeks
Tibiotarsus Stiffness (N/m)	**0.904***(P = 0.000)*					
Tibiotarsus Whole Bone Density (mm Al Equiv.)	**0.866***(P = 0.000)*	**0.870***(P = 0.000)*				
Weight at cull (g)	**0.411***(P = 0.000)*	**0.486***(P = 0.000)*	**0.479***(P = 0.000)*			
Tibiotarsus AUC at start	0.131*(P = 0.279)*	0.114*(P = 0.350)*	0.213*(P = 0.076)*	0.175*(P = 0.146)*		
Tibiotarsus AUC at 3 weeks	**0.619***(P = 0.000)*	**0.624***(P = 0.000)*	**0.700***(P = 0.000)*	**0.501***(P = 0.000)*	**0.534***(P = 0.000)*	
Tibiotarsus AUC at 5 weeks	**0.667***(P = 0.000)*	**0.633***(P = 0.000)*	**0.689***(P = 0.000)*	**0.485***(P = 0.000)*	**0.576***(P = 0.000)*	**0.869***(P = 0.000)*

(r = 0.486) and radiographic density (r = 0.479); however, these were relatively weak.

Validation experiment 2; reproducibility and repeatability

The data for body weight, traditional measurements of bone quality and live hen radiographic based area under the curve (AUC) density measurement are shown in Table 7.

Tibiotarsus AUC measurements at 30 weeks showed significant correlation with all *post-mortem* measures of bone quality, tibiotarsus breaking strength, tibiotarsus stiffness and tibiotarsus whole bone density (Table 8).

The Gage R&R analysis using variance components indicated that 92% of the variance was attributable to hen, but only 8% from the repeated measurement of the AUC on each day's radiography (Table 9). Of that, the least was attributed to the repeated radiographs on different days and the majority to the operator's analysis of the AUC on each radiograph (0.31%).

When all the measurements by day and operator for each hen were displayed, the relatively large individual differences in the AUC between hens could be seen quite clearly (Figure 4).

A similar analysis showed that the variation came almost entirely from the step wedge values and very little from operator or the 12 repeated measurements (Table 10). The

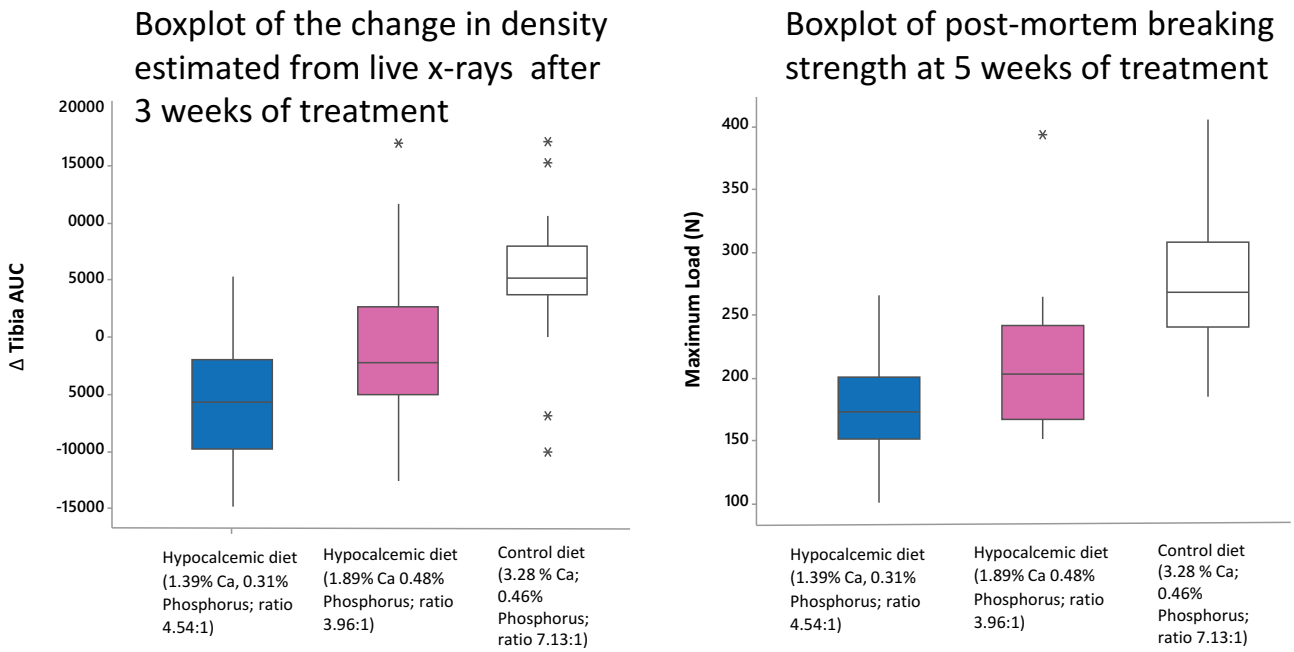

Figure 3. Boxplot of the change in density estimated from live radiographs after 3 weeks of treatment and *post-mortem* measurement of breaking strength at 5 weeks after feeding diets differing in Ca and P content.

Table 7. Descriptive statistics of traditional measures of bone quality and live hen radiographic based AUC density measurement for tibiotarsus of laying hens at 30 weeks of age used for reproducibility and repeatability estimation.

Variable	n	Mean	SD
Tibiotarsus Breaking strength (N)	60	300.91	54.25
Tibiotarsus Stiffness (N/m)	60	418487	61459
Tibiotarsus Whole Bone Density (mm Al Equiv.)	60	2.56	0.18
Body weight (g)	60	1912	145
Tibiotarsus AUC	60	86540	6301

coefficient of variance calculated across the step range varied between 0.86% and 1.2% (Table 10).

Discussion

These studies aimed to establish a method for the genetic selection of laying hens to improve their skeletal health and reduce the risk of fractures. The method of immobilisation, radiography and data capture proved to be extremely quick, making this a practical approach to measure large numbers of hens. The hens did not show any signs of discomfort when restrained using the Miami vise and remained calm throughout. They might have been showing tonic immobility, which is a well-recognised response to gentle head and leg restraint in a lateral recumbent position (Gilman et al., 1950). Importantly, the radiation dosage received by operators was very small, allowing for over 60,000 radiographs per year to be performed for a person to reach current regulatory maximum yearly dosage. A simple lead PVC screen could improve this even further.

The method did not need to correlate perfectly with existing *post-mortem* methods that have been regarded as the gold standard. Ultimately, such measurements are themselves only proxies for the desired trait of reduced skeletal damage and fracture. However, given that *post-mortem* measurements have been the only reliable quantitative measurement available, it remains important that the measurements showed correlation. The method chosen to make measurements from the live hen X-rays was an area under the curve from a cross section of the tibiotarsus. The tibiotarsus AUC measurement had correlations across the different experiments with *post-mortem* whole bone radiographic density measurements that ranged from 0.62 to 0.77. For comparison, the correlation between radiographic density measurements of the whole bone *post-mortem* and measurement of breaking strength was not so dissimilar (0.57–0.86). Although there was no guarantee of success, in previous genetic experiments, the phenotypic correlation between tibiotarsus *post-mortem* radiographic density and breaking strength was 0.56 and 0.69 and the genetic correlation was 0.84 and 0.83 for White Leghorn and Rhode Island Red lines, respectively (Dunn et al. 2021). In this study the correlation between the *post-mortem* whole bone density and live AUC measurement, which were probably the most comparable live hen and *post-mortem* measurements ranged from 0.62 to 0.7, which was reasonable. Ultimately, the measure of success will be an actual improvement in bone quality if the measurement is used in selection. The clear differentiation of bone quality between individual hens was graphic evidence that the method should be useful for genetic selection.

After manipulating dietary Ca and phosphate levels, there was a significant effect after 3 weeks of treatment on the AUC of the tibiotarsus. The measurement of the reduction in bone density was obtained without the need to kill the hens. This rapid detection of the effect of a change in diet was in part because the AUC bone density was available before the trial commenced to allow the change in bone properties to be observed rather than relying on a single *post-mortem* measurement. This may mean that the method offers a sensitive way to appraise nutritional interventions to improve bone quality in laying hens and would require less animals, because of the reduction in variance afforded by measuring changes in individual hens rather than absolute values at cull.

Table 8. Correlation coefficients of traditional measures of bone quality and live hen radiographic AUC density measurement for tibiotarsus of laying hens at 30 weeks of age used for reproducibility and repeatability. Significant correlations are in bold.

	Tibiotarsus Breaking Strength (N)	Tibiotarsus Stiffness (N/m)	Tibiotarsus Whole Bone Density (mm Al Equiv.)	Tibiotarsus AUC
Tibiotarsus Stiffness (N/m)	**0.617** *(P = 0.000)*			
Tibiotarsus Whole Bone Density (mm Al Equiv.)	**0.577** *(P = 0.000)*	**0.651** *(P = 0.000)*		
Tibiotarsus AUC	**0.444** *(P = 0.000)*	**0.560** *(P = 0.000)*	**0.619** *(P = 0.000)*	
Body weight (g)	−0.023 *(P = 0.860)*	0.057 *(P = 0.667)*	0.254 *(P = 0.050)*	0.214 *(P = 0.101)*

Table 9. Gage R&R analysis of variance components of live hen radiographic based AUC density measurement for tibiotarsus of laying hens at 30 weeks of age with repeated images taken on 4 d and estimates made by two different operators.

Source	Variance component	% Contribution of variance component
Total Gage R&R	2892044	7.9
Repeatability	2775649	7.58
Reproducibility	116395	0.32
Radiograph Day	2587	0.01
Operator	113809	0.31
Hen	33706780	92.1
Total Variation	36598824	100

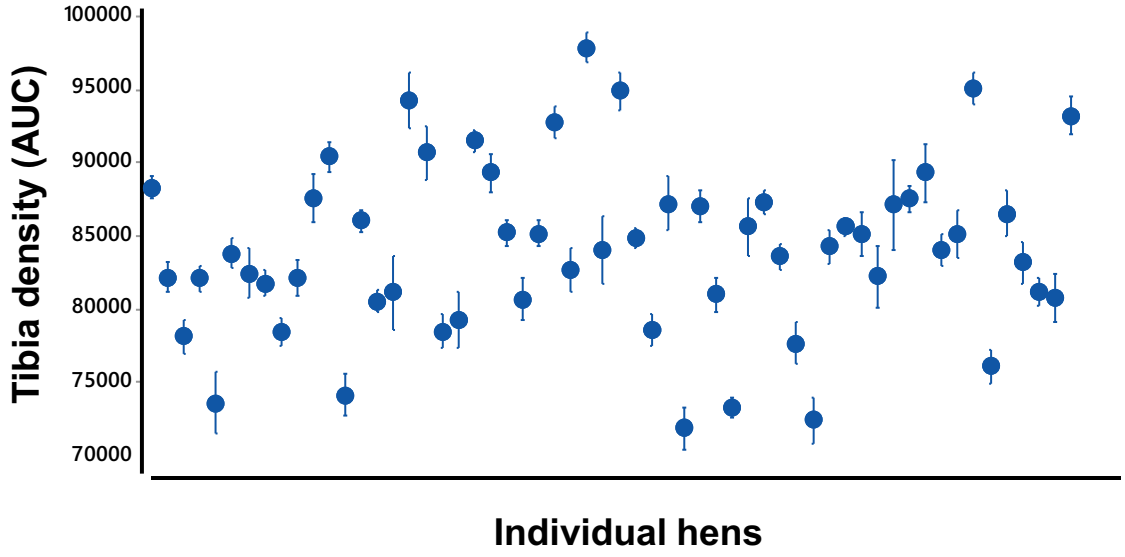

Figure 4. Interval plot with 95% confidence intervals of the mean for tibiotarsus AUC measurements made by two operators from 4 radiographs of 60 hens at 30 weeks of age from validation experiment 2. Clear differences between individual hens can be seen which underlies the data on measurement variance coming primarily from the hen rather than the method.

Table 10. Gage R&R analysis of variance components radiographic based AUC density measurement for the step wedge from repeated images taken on 4 d and estimates made by two different operators.

Source	Variance component	% Contribution of variance component
Total Gage R&R	5.45E+07	0.21
Repeatability	1.44E+07	0.06
Reproducibility	4.01E+07	0.15
Operator	1.04E+07	0.04
Radiograph Day	2.96E+07	0.11
Step	2.60E+10	99.79
Total Variation	2.60E+10	100.00

Experiments to measure reproducibility and repeatability are important because, if the contribution of the measurement or the operator was too large in comparison to the variation present between individual animals, it would potentially make the measurement useless for selection. This is because the hen-to-hen variation sets the upper boundary for any estimate of heritability, the genetic contribution to variation. Therefore, the relatively low contribution of variance from the measurement and the consequent large observed value of 92% for variation between hens was extremely encouraging for use of the measurement for genetic selection. The majority of, albeit, the small variation in the measurement method came from repeated radiography of the same hen rather than the operator or the same operator measuring the same images repeatedly, which was represented by reproducibility. Although relatively unimportant, the variability from measuring the same hen on consecutive days probably came from the small differences in positioning the hen on each occasion rather than the operator analysis of the image.

Measurement alternatives to the AUC of the tibiotarsus were examined. Density measurements (mean grey area) from the tibiotarsus, although showing correlation with *post-mortem* measurements, were not as good as the cross-section

of the tibiotarsus. The cross-sectional area approach to measuring density included contributions from the cortex and any medullary bone that was present in the tibiotarsus as well as from muscle, skin and feathers. The cross-sectional AUC density measurement included a simple attempt to control for non-bone tissue that may be a reason why it was superior to the mean grey area bone density estimate. Although it may be that the settings for X-ray power and duration could be further investigated, the setting used gave good contrast in a relatively short period with a minimal variance of the standards. In the longer term, this setting would allow the exposure for hens of different ages radiographed at different times to be accommodated.

Although AUC measurements from other bones did give respectable correlations with *post-mortem* measurements, the correlation between the AUC measurement from a live hen and the *post-mortem* measurements of stiffness, breaking strength and whole bone radiographic density of the tibiotarsus was always greatest. The keel bone is currently receiving a lot of attention, as it seems to suffer damage or deformation, especially in alternative systems (Toscano et al., 2020). It proved difficult to make reliable measurements from the keel because of the overlying pectoral muscle. It is possible that, in recent years, it has been difficult to make useful measurements to calculate genetic parameters for keel density heritability (Dunn et al. 2021), perhaps because of the confounding effects of callus formation from healed fractures on radiographic density measurements from hens kept in furnished cages. However, in older studies when hens were housed in single cages, moderate heritability was calculated from keel radiographic density and, most importantly, there was good genetic correlation between the bone quality traits measured on the tibiotarsus and keel bone radiographic density (Bishop et al. 2000). Because the humerus was often pneumatised, it created a very different plot profile compared to the other bones and a highly variable content of medullary bone between hens ranging from none to full, meant it was potentially not suitable. However, future studies might be able to use the occurrence of medullary bone in the humerus as a potential selection tool, as shown with medullary bone mineralisation as a potential factor in bone quality overall (Dunn et al. 2021).

When steps of the step wedge were measured to assess variability, they contributed almost all the variation and there was very little variation attributed from radiographs over time or operator. The fact that virtually no variation was attributable to the day of measurement suggests that calibration for every radiograph is not necessary to give reliable results. The component for the operator was lower compared to its contribution to the tibiotarsus AUC measurement variance. However, in all cases, the repeated measurement in terms of measurement from different radiographs remained at or less than about 0.1% of the variance. The coefficient of variances for step measurements was very low at around 1%. This ability to avoid calibration is important for developing a simple and quick method to estimate bone quality. However, this would need to be checked for any new radiograph or detection system.

For the reasons outlined, the AUC measurement of the tibiotarsus from live hen radiographs represents a major step forward in getting a usable method for genetic selection to improve bone strength in laying hens. The method has the potential to reduce the number of animals needed to test nutritional and management interventions to improve bone health. Of course, further improvements will be possible and researchers may consider many different improvements to the method. However, this offers significant improvements over other methods, although, to some extent, this could be seen as an extension of previous radiographic methods (Fleming et al. 2004), the improved technology has given a step change in what is practically possible. The use of a continuous variable rather than a score, such keel bone damage, avoids complex analysis and should aid measurement automation.

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