

## Assessment of avian osteoporosis by a quantitative radiographic method

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### Summary

The aim of this study was to develop a quantitative radiographic method for assessment of experimental osteoporosis in Leghorn breed laying hens. Three groups of 24 Leghorn pullets were reared in cage and fed a ration containing different calcium levels, including 3.55, 2.075 and 0.6 percent (for groups 1-3, respectively). The hens were fed this diet from 17 weeks of age to the end of the experiment. At 20, 28 and 36 weeks of age, 8 hens from each group were selected randomly. Radiographs were obtained from the tibiotarsus and the humerus of each hen. Radiographs were digitized using a camera and assessed by "Image J" software. Bone radiopacities and bone cortex/diameter (C/D) ratios were measured. The hens were sacrificed and the bone ash and calcium contents were measured. Bone densities of the birds in different groups were significantly different at just 36 weeks of age; they were greater in the tibiotarsus bone of the control group than in the tibiotarsus bone of median (P=0.02) and with the low calcium (P=0.007) groups. Humerus densities were also greater in control group compared with that of median (P=0.04) and with low calcium (P=0.0004) diet group. Cortex/diameter index of the tibiotarsus bone was different in all three stages between control and the two other groups, while there were no significant differences between the humeri C/D indices and the three groups in the first stage. Humeri C/D indices of the second and third stages had significant differences between control and the two other groups (P≤0.05). This study showed that radiographic evaluation of bone density is valuable just in progressed osteoporosis, while C/D index can be used for diagnosis of osteoprotic bones in earlier stages.

**Key words:** Laying hens, Osteoporosis, Radiography

### Introduction

Bones have an essential role in poultry production including provision of some of the calcium (Ca) required for eggshell formation and Ca homeostasis (Korver *et al.*, 2004). High demand for calcium during eggshell formation makes the birds susceptible to Ca deficiency (Elaroussi *et al.*, 1994) that takes place mainly during the night when the majority of eggshell formation performs (Etches, 1987) or in aged hens in which decreased efficiency of Ca metabolism occurs (Al-Batshan *et al.*, 1994).

Osteoporosis is defined as a local or systemic deficiency in the quantity of fully

mineralized structural bones and is a common problem in caged egg-laying strains of hens (Mansoori *et al.*, 2008). This decrease in structural bones leads to fracture susceptibility and has been shown to be the main reason for bone loss and subsequent fractures in laying hens (Randall and Duff, 1988; Whitehead and Fleming, 2000). It is estimated that 15 to 30% of hen mortality is due to osteoporosis, a problem which leads to economic losses (McCoy *et al.*, 1996).

Changes in bone integrity have historically been measured using invasive techniques such as bone breaking force, ashing, histomorphometry, and mineral analysis of the bones. These invasive techniques require a greater number of

animals euthanized for measurements over time than what the current radiographic method needs. Another superiority of the presenting method is its facility to be performed. Radiographic instruments are much more accessible in comparison with the other non invasive diagnostic tools such as computed tomography scan, digitized fluoroscopy, ultrasound and densitometry. This method can even be easily performed using portable radiographs in the field (Schreiweis *et al.*, 2003; Fleming *et al.*, 2004; Korver *et al.*, 2004; Martinez-Cummer *et al.*, 2006). The aims of this study were to investigate the possibility of using radiographic indices including quantitative radiographic densities and bone cortex/diameter ratio for diagnosis of osteoporosis in laying hens in comparison with bone mineral contents in different stages.

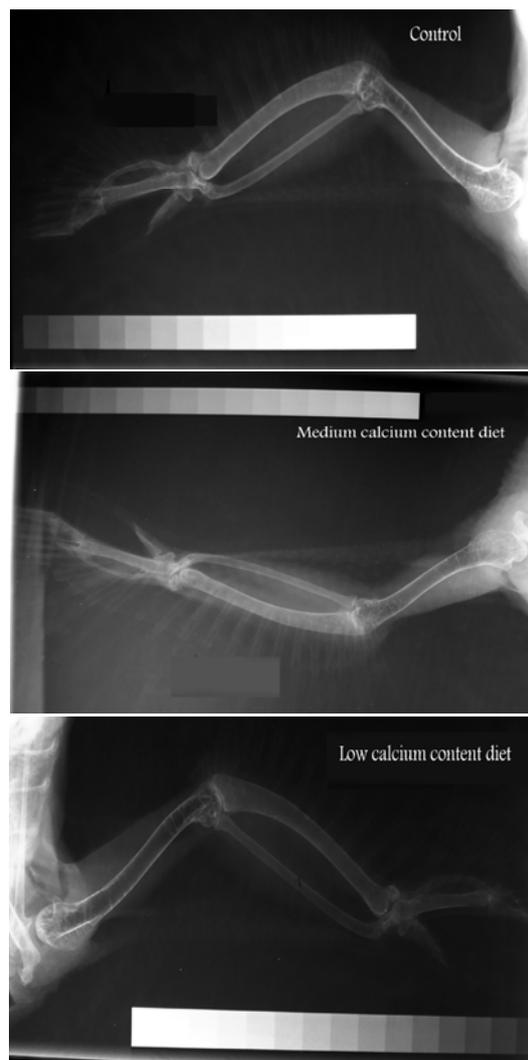
## Material and Methods

### Housing and feeding

One flock of 72 Hy line W36 Leghorn pullets of 15 weeks age were caged randomly in three groups and fed similarly for 2 weeks. The experiment was conducted in an environmentally controlled room with temperature of 27°C and a photoperiod of 13 to 16 h of light per day. This photoperiod started from 13 h/d and increased 20 min per week to reach a constant light of 16 h/d for the remained period. Twenty four birds were assigned to one of three different diets designed with different levels of calcium content; 3.55, 2.075 and 0.6% for the first (control), second (medium calcium supplement) and third (low calcium supplement) groups, respectively. A total of eight birds in each group were examined radiologically and sacrificed for Ca and ash content measurements at 20, 28 and 36 weeks of age.

### Radiography

Lateral radiographs of humerus and tibiotarsus were obtained using an X-ray apparatus (Ralco s.r.l Comp.) on Kodak high resolution mammography films. An aluminum step wedge consisting of 16 × 0.25 mm steps was placed in a fixed position



**Fig. 1: Radiographs taken from wing of three hens (control, medium and with low calcium diet groups) to show humerus radiopacity and thickness of cortex using aluminum step-wedge. Bone radiopacity and the thickness of cortex are more in control group in compare with two other groups**

on radiographic cassette at the same level for each exposure and the radiograph was digitized by computer (Fig. 1). The final image was assessed using “Image J” software. Each image was calibrated using the software; mean pixel intensities were measured for each step by placing a square repeatedly over the image of the step wedge to measure the mean pixel intensity within each region of interest. To avoid slight changes caused by non specific pixels, the square was placed on a blank screen (no wing or step wedge) and regarded as 0 mm-Al (Aluminum thickness). A non linear

calibration curve (3rd degree polynomial) was produced. All subsequent measurements were made on each of the calibrated images based on millimeters of the aluminum equivalent (MAE). The entire diaphysis of humerus or tibiotarsus was marked using a freehand tool and mean radiographic density was measured and expressed as MAE. "Image J" software was also used to measure the cortex/diameter ratio (C/D). Calibration was performed by using a 10 mm width of the step wedge. Afterwards, bone diameters (D) were measured in the central point of each bone between the outer margins of the cortexes. The width of the medullary cavity (MC) was measured as the distance between the inner margins of the cortexes. Cortex thickness (C) and cortex/diameter ratio were measured with these formulae:

$$C_{(mm)} = D_{(mm)} - MC_{(mm)} \text{ and } C/D \text{ ratio} = C_{(mm)} / D_{(mm)}$$

### Assessment of bone mineral content

The hens were sacrificed and humeri and tibiotarsal bones were subjected to ash and calcium content measurements. Each bone was cleaned out of the muscle and dried overnight in an oven at 80°C to obtain the dry weight. Bones were defatted by submerging in di-ethyl ether for 3 h. Bones were ashed overnight in a furnace oven at 600°C and ash was weighed. The ash was digested in a mixture of 10 ml of 0.01 M HCl and 10 ml of concentrated (36%) HCl. The acid ash/slurry was made up to 50 ml using distilled water. The sample was further diluted 1:20 in distilled water (0.5 ml of sample and 9.5 ml of water): 0.05 ml of this solution was added to 4.95 ml of pybus reagent (10 g strontium chloride, 9 ml of 70% perchloric acid made up to 1000 ml using distilled water). It was then assayed for calcium content using atomic absorption spectrophotometry. Calcium content was expressed as gram per femur value (Schreiweis *et al.*, 2003).

### Statistical analysis

Data were analysed statistically by using SPSS 16 software. One way analysis of variance (ANOVA) was performed to detect the significant differences between the different groups' data.

## Results

### Bone densities

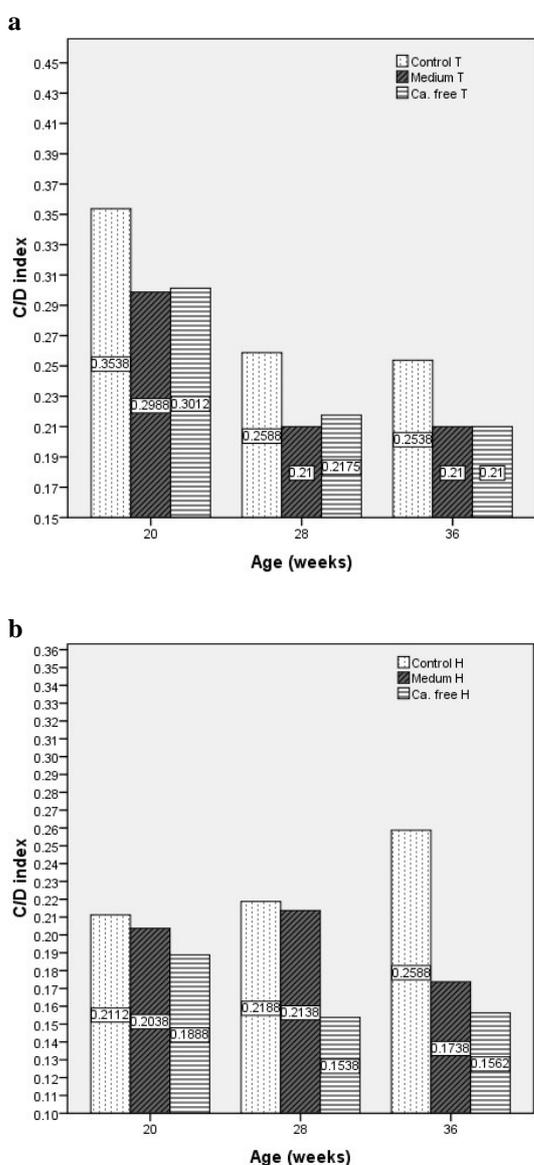
Tibiotarsal bone densities showed no significant differences at 20 and 28 weeks of age but at 36 weeks of age, they were significantly higher in the control group in comparison with the second (P=0.02) and third (P=0.007) groups. Humerus bone densities also had no significant differences at 20 and 28 weeks of age but at 36 weeks of age, they were significantly higher in the control group in comparison to the second (P=0.04) and third (P=0.004) groups (Figs. 2a, b).



Fig. 2: Radiographic densities of tibiotarsal (a) and humeri bones (b) in three groups

### C/D ratios

Cortex/diameter ratios of tibiotarsus were significantly higher in the first group compared to the second and third groups at 20, 28 and 36 weeks of age. No significant differences were seen between C/D ratios of the second and third groups ( $P \leq 0.05$ ). Significant differences were seen at 28 weeks of age between humerus C/D ratios of the first and third ( $P=0.043$ ) and second and third ( $P=0.030$ ) groups. At 36 weeks of age C/D ratios were significantly higher in the first group compared to the second ( $P=0.001$ ) and third ( $P < 0.001$ ) groups (Figs. 3a, b).



**Fig. 3:** Cortex/diameter ratio in different groups in tibiotarsal (a) and humeri (b) bones

### Ash content

Ash content measurement showed no significant differences between the three evaluated groups at 20 and 28 weeks of age for both humerus and tibiotarsus, but it was significantly higher in the first group in comparison to the second ( $P=0.028$ ) and third ( $P=0.004$ ) groups for tibiotarsus and second ( $P=0.018$ ) and third ( $P=0.019$ ) groups for humerus at 36 weeks of age (Table 1).

### Calcium content

Calcium contents were not significantly different between the three evaluated groups at 20 and 28 weeks of age for both humerus and tibiotarsus. At 36 weeks of age, calcium content was significantly higher in the first group compared with the second ( $P=0.023$ ) and third ( $P=0.001$ ) groups for tibiotarsus. The same statistical differences were seen between all three groups for humerus ( $P=0.013$  and  $P=0.020$  for the second and third groups, respectively) (Table 2).

### Discussion

Other research groups have compared radiographic findings with bone mineral contents for two different types of bones; medullary and pneumatic, the first of which is the main source of calcium during laying period for shell formation and is mainly found in the leg bones while the second one is a hollow inner cavity bone type and is mainly found in humerus. A significant amount of shell calcium comes from resorbed medullary bone, thus there is a surge in osteoclastic resorption during the shell formation period. However, osteoclasts are not specific to medullary bone and resorption can also occur at exposed structural bone surfaces, which explains the osteoporotic structural bone loss. After the egg is laid, medullary bone regenerates and may also replace the lost structural bone. Progressive loss of structural bone during the laying period is characteristic of osteoporosis and results in weakening of the skeleton and increased fractures (Whitehead, 2004).

In our study, no significant changes of measured bone radiographic densities during

**Table 1: Ash contents (percent) of tibiotarsus and humeri bones in different groups (mean±SD)**

	Humerus			Tibiotarsus		
	Control	Medium Ca.	Low Ca.	Control	Medium	Low Ca.
1st stage	61.29±2.99	58.71±4.09	58.46±1.29	45.70±2.29	43.12±1.69	44.99±3.45
2nd stage	56.17±7.76	57.45±3.55	53.87±6.97	38.56±3.57	36.37±2.69	35.86±2.19
3rd stage	55.16±1.14	51.67±3.27	51.70±3.20	38.19±2.18	35.03±2.79	33.85±2.98

**Table 2: Calcium content (percent) of tibiotarsus and humeri in different groups (mean±SD)**

	Humerus			Tibiotarsus		
	Control	Medium	Low Ca.	Control	Medium	Low Ca.
1st stage	52.03±2.89	53.52±7.39	53.71±4.80	48.60±5.88	52.99±5.02	49.79±3.02
2nd stage	41.97±1.78	40.52±1.59	40.30±1.80	41.15±2.11	41.22±1.82	40.27±1.33
3rd stage	40.53±1.97	38.27±1.14	38.44±1.76	42.95±3.36	40.26±1.31	38.64±1.17

the first and second steps have been seen, probably because of two concurrent events: thinning of cortex and formation of medullary bone. In other words, although structural bone content declines during the laying period and medullary bone is as radiodense as structural bone (Whitehead, 2004), the accumulation of medullary bone means that total bone content may remain constant or even increase over the laying period (Fleming *et al.*, 1998; Whitehead, 2004). Decrease of bone radiographic densities during the third stage of evaluation might be due to severe depletion of cortical bone; much more than what can be hidden because of the increase of medullary bone content. It can be concluded that radiographic measurement of bone densities is a valuable diagnostic tool for avian osteoporosis only in later stages of the disease.

Cortex/diameter ratio is an important and practical index that can be used to assess bone health. Cortex/diameter ratio has previously been measured in human and animals for different reasons. Bone diseases causing decrease in bone density can be detected in early stages by knowing normal C/D ratios in bones in different animals (Hiney *et al.*, 2004; Sorouri *et al.*, 2007; Raayat Jahromi *et al.*, 2008). Decrease of this index during the disease process relates to cortical thinning, which may be due to cortex mineral depletion in order to make medullary bone during laying period. It seems to start in tibiotarsus during the initial stages of the disease and progress to humeri in later stages. Progressive decrease in C/D ratio makes it a reliable parameter for

evaluation of avian osteoporosis.

No significant changes of ash and calcium contents during the first two steps of samplings seem to be due to concurrent thinning of cortex and medullary bone formation. As decrease of these two parameters has been seen during the last stage of the sampling, measurement of ash and calcium contents seems to have lower sensitivity for detection of osteoporosis i.e. they decrease significantly just during the last stages of avian osteoporosis.

Results of this study show that radiographic evaluation of bone density is valuable just in progressed osteoporosis, while C/D index can be used for diagnosis of osteoprotic bones in earlier stages.

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