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Original Research Article

Cage type and mineral nutrition had independent impact on skeletal development in Lohmann LSL-Lite pullets from hatch to 16 weeks of age

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ABSTRACT

The effects of rearing cage type and dietary Ca, available P and vitamin D_3 ($VitD_3$) on body and skeletal development were studied. A total of 3,420 Lohmann LSL-Lite day-old chicks were reared in conventional (CON) or furnished cages (FUR) to 16 wk of age. Initially, 40 and 150 chicks/cage were placed in CON and FUR and transitioned to 20 and 75 chicks/cage at 8 wk of age, respectively. Three diets: Diet 1, Diet 1.5 and Diet 2 were formulated to meet nutrient specifications with Diet 1.5 and Diet 2 containing 1.5 and 2 times more Ca, P and $VitD_3$ than Diet 1, respectively. Diets were allocated within cage type to give 6 replicates and fed in 3 feeding programs: starter, grower and developer. At 4, 12 and 16 wk of age, BW was recorded, and femur, tibia and blood samples for bone quality and related parameters. There were no interactions ($P > 0.05$) of cage type, diet and pullet age on BW, plasma Ca and inorganic P, femur and tibia morphometry, mineral density (MD), breaking strength (BS) and ash concentration (AC). Concentration of Ca, P and $VitD_3$ linearly decreased BW ($P < 0.001$), relative femur ($P = 0.010$) and tibia weight ($P = 0.013$). A quadratic increase on femur MD ($P = 0.03$) and BS ($P = 0.026$) was observed with dietary concentration of Ca, P and $VitD_3$. Femur ($P = 0.031$) was longer for CON than FUR pullets, however, femur for FUR pullets had higher ($P = 0.003$) AC. Cage had no effect ($P \geq 0.415$) femoral MD and BS. Pullets reared in FUR cages exhibited higher tibial MD ($P = 0.015$), BS ($P = 0.071$), AC ($P < 0.01$) and whole-body mineral content ($P < 0.01$). In conclusion, cage type and diets showed independent effect on femur and tibia quality with FUR pullets exhibiting enhanced indices of mineralization. Feeding pullets twice the recommended Ca, P and $VitD_3$ decreased BW, relative weight of leg bone but enhanced femoral strength with no effects on tibia attributes.

1. Introduction

In the last 7 decades, geneticists have intensely selected hens for egg productivity and feed efficiency (Preisinger and Flock 2000). This has eventually resulted in birds with lower BW and lower feed intake, but higher egg production. Moreover, the age at sexual maturity and first egg decreased (Whitehead et al., 2003) and lay persistency increased (Bain et al., 2016). Multiple factors such as early start of lay and long lay cycle increase pull out of calcium (Ca) from bones (Korver, 2020). For example, a white hen lays 340 eggs by 72 wk of age (Lohmann, 2016), based on fact that eggshell contain 2.5 g of Ca and whole-body Ca of a pullet at onset of lay is 25 g (Khanal et al., 2020a). It follows that she will draw more than 30 times her body Ca for eggshell formation over the course of lay cycle (Kim et al., 2012). An equilibrium between Ca deposition and

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release maintains bone quality and any imbalance between bone formation and resorption will result in the skeletal problems during laying. The lower bone mineral density (MD) at the onset of lay has been associated with selection for increased egg production (Whitehead, 2004). It is plausible that enriching bone quality prior to sexual maturity could be one of the approaches to improve skeletal integrity in laying hens (Khanal, 2020).

The bone quality in layer is influenced by housing and nutrition (Fleming et al., 2006). Provision of physical activities in the housing environment has been shown to influence skeletal development in pullets (Regmi et al., 2015; Casey-Trott et al., 2017a; Khanal et al., 2020a). Enriched or furnished cages (FUR) in comparison to conventional cages (CON) provide more space for movement, more opportunity for load bearing exercise such as perching, jumping etc., and support for expression of normal behavior such as scratching, perching, preening etc. Rearing pullets in FUR could be one way of enhancing bone mineralization and strength in pullets (Campbell et al., 2019) because exercise positively enhances bone development (Greene et al., 2006; Koistinen et al., 2014; Yuan et al., 2016; Patel et al., 2020). Mineral nutrition especially Ca and P plays a pivotal role in establishing and sustaining bone quality (Lukić et al., 2009; Korver, 2020). Vitamin D_3 ($VitD_3$) is intimately linked to Ca and P metabolism (Akbari Moghaddam Kakhki et al. 2019ab; Adhikari et al., 2020). Pullets fed diets with a high level of $VitD_3$ at placement to 17 wk of age had enhanced leg bone mineralization (Wen et al., 2019). Similarly, a high dose of dietary $VitD_3$ increased bone mass and strength in mice (Williamson et al., 2017). But there is limited knowledge regarding increased dietary Ca, P and $VitD_3$ during rearing on skeletal development in pullets. Our recent study (Khanal et al., 2020a) showed that pullets reared in FUR cages developed leg bones with higher bone MD and content (MC) than pullets reared in CON cages. This suggested that the cage type might potentially interact with dietary minerals on bone mineralization. However, there is dearth of knowledge on interactive effect of dietary Ca, P and $VitD_3$ and rearing cage type on skeletal development.

Modern-day commercial pullets develops 95% of skeletal frame by 12 wk of age (Whitehead 2004; Bain et al., 2016). A higher level of circulating estradiol at sexual maturity shifts bone formation from cortical to medullary (Whitehead 2004). Thus, interventions at early age could be a strategy to increase structural bone mass accretion and MD before first egg. However, there is limited information on the impact of feeding higher dietary concentration of Ca, P and $VitD_3$ at placement to sexual maturity to characterize impact on skeletal development. Several findings described impact on the dietary adjustment of minerals and VitD3 on bone quality in laying hens (Akbari Moghaddam Kakhki et al., 2019b; Khanal et al., 2019). Moreover, ongoing changes in pullets and hens housing affect feed intake, Ca and P utilization, and body and bone mineralization (Eusebio-Balcazar et al., 2018; Khanal et al., 2020b). Thus, we hypothesized that there is a possibility of interaction between rearing housing and dietary concentration of Ca, P and $VitD_3$ on skeletal development in pullets. Hence, the objective of this study was to investigate the interactive effect of rearing cage type (CON vs. FUR) and dietary Ca, P and $VitD_3$ on growth and indices of skeletal development in Lohman LSL-lite pullets at placement to 16 wk of age.

2. Materials and methods

The experimental protocol (#3634) was reviewed and approved by the University of Guelph Animal Care Committee. This experiment took place at the University of Guelph's Arkell Poultry Research Center in Guelph, ON, Canada and birds were cared in accordance with the Canadian Council on Animal Care guidelines (CCAC 2009) and Canadian code of practice for the care and handling of pullets and laying hens (NFACC 2017).

2.1. Birds, cages and diets

A total of 3,420 Lohmann LSL-lite day-old pullets (Archer's Hatchery, ON, Canada) were placed in CON or FUR cages. The details of cage were presented in our previous publication (Khanal et al., 2020a). Briefly, the dimension (length × breadth × height) of CON (Ford Dickinson Inc., Mitchell, ON, Canada) and FUR (Farmer Automatic, Clark Ag Systems, ON, Canada) were respectively 76 cm × 71 cm × 46 cm and 239 cm × 80 cm × 75 cm with total floor space area 5,396 and 19,120 cm^2. Pullets were placed in cages based on BW, 40 and 150 birds/cage for CON and FUR cages, respectively. There were 18 cages for each cage type and the coefficient of variation of BW of chicks within the cage type was less than 2%. The spacing at placement to 8 wk of age in CON and FUR was 135 and 128 cm^2/pullet, respectively. At 8 wk of age, CON and FUR had 20 and 75 pullets/cage equivalent to 270 and 256 cm^2/pullet respectively. The spacing at 8 wk of age was doubled to that of day-old chicks in order to meet the requirements of code of practice for care and handling of pullets (NFACC 2017). The FUR had a platform (floor area = 5,950 cm^2), which increased the space per pullet to 335 cm^2. Excess pullets were transferred to the general flock at the research station. The pullet spacing for CON and FUR were similar, however, the total utilizable space in FUR was 3-fold more due to platforms and perches. The FUR cages were designed in a combi model which were meant for aviary, but the foldable doors were closed throughout this experiment so that they worked as enriched cages as previously described elsewhere (Habinski et al., 2017). The FUR cage had a feeder trough along the length of the cage floor (width = 12 cm and depth = 9 cm; 23 cm from the front wall and 52 cm from the back wall of the cage) and allowed access to feed from both sides. The water line was located behind the feeder, ran parallel to the feeder, and was equipped with 12 nipple drinkers and an additional 4 nipple drinkers over the platforms. For CON cage, a feeder with the dimension of 62 cm (length) × 8.25 cm (width) × 5.75 cm (depth) was fitted at front of the cage allowing access to the feed from one side. The water lines were in the middle of the cages with 2 nipple drinkers per cage.

The pullets were reared in a 3-phase feeding programs: starter (day old to 4 wk of age), grower (5 to 8 wk of age) and developer (9 to 16 wk of age) (Table 1). The 3 experimental diets (Diet 1, Diet 1.5 and Diet 2) in each phase met or exceeded Lohman LSL-Lite nutrient specifications (Lohmann 2016). The concentration of Ca, P and $VitD_3$ in Diet 1.5 and Diet 2 were 1.5 and 2 times higher than that of Diet 1 while keeping all other nutrients similar. Diets were prepared in crumble form.

2.2. Animal experimentation and sampling

Within cage type, the diets were allocated to give 6 replicates per diet. Although the CON and FUR cages were housed in 2 rooms, the pullets were provided with similar lighting, ventilation, temperature and humidity. The lighting program followed Lohmann LSL-Lite management guide. The relative humidity was maintained at between 50% and 60% throughout the experiment. Pullets had free access to feed and water throughout the experiment. For the first 4 d of placement, chicks were provided feed on disinfected plastic egg tray to motivate intake. From the fifth day onwards, feed was provided into their respective feeder. The feeders were hand filled with feed at 09:00 and 13:00 on daily basis.

To assess growth, pullets were weighed on cage basis at 4, 12 and 16 wk of age. At 16 wk of age, 5 pullets/cage in CON and 20 pullets/cage in FUR (approximately 25% of cage population as per

Table 1
Ingredients and chemical composition of diets.[1]

Item	Starter			Grower			Developer		
	Diet 1	Diet 1.5	Diet 2	Diet 1	Diet 1.5	Diet 2		Diet 1.5	Diet 2
Ingredients, g/kg as fed basis									
Corn	541	529	530	570	570	550	560	560	565
Soybean	306	310	317	264	272	275	170	170	180
Wheat	100	80.0	43.5	100	80.0	73.0	150	140	114
Wheat middlings	–	–	–	–	–	–	80.0	71.3	60.0
Soy oil	10.0	19.0	28.0	1.00	5.00	15.0	4.00	5.00	11.0
Limestone	18.3	24.5	32.0	18.0	26.0	33.3	17.3	25.0	32.0
Mono calcium phosphate	13.6	26.0	38.0	12.0	24.0	36.0	9.00	19.0	28.0
Salt	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50
L-Lys-78%	1.30	1.30	1.30	0.50	0.50	0.50	0.50	0.50	0.50
DL-Met	1.50	1.50	1.50	0.80	0.80	0.80	0.50	0.50	0.50
Vitamins and trace premix[2]	4.30	4.30	4.30	4.30	4.30	4.30	4.30	4.30	4.30
Vitamin A[3], mg/kg	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
Vitamin D_3[3], mg/kg	–	2.00	4.00	–	2.00	4.00	–	2.00	4.00
Calculated composition, % as is basis									
AME, mcal/kg	2.90	2.90	2.90	2.80	2.80	2.80	2.80	2.80	2.80
Linoleic acid	1.70	2.20	2.60	1.30	1.50	2.00	1.50	1.60	1.90
Crude protein	19.9	19.9	19.8	18.5	18.5	18.3	15.4	15.2	15.2
SID Met + Cys	0.69	0.69	0.68	0.60	0.59	0.58	0.51	0.51	0.50
SID Lys	1.02	1.04	1.05	0.88	0.89	0.89	0.66	0.66	0.67
Ca	1.00	1.50	2.00	1.00	1.50	2.00	0.90	1.36	1.80
Available P (av. P)	0.48	0.72	0.96	0.45	0.68	0.91	0.38	0.57	0.75
Ca-to-av. P ratio	2.10	2.10	2.10	2.20	2.20	2.20	2.40	2.40	2.40
Na	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Cl	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Vitamins, kIU/kg									
Vitamin A, premix	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.1	5.1
Vitamin A, added	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Vitamin D_3, premix	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
Vitamin D_3, added	0.0	1.0	2.0	0.0	1.0	2.0	0.0	1.0	2.0
Determined composition, % as is basis									
Dry matter	87.9	88.4	88.7	88.1	88.3	88.6	87.8	87.1	87.2
Ash	5.42	7.61	9.06	5.98	7.58	9.07	5.60	7.65	8.90
Ca	0.90	1.29	1.76	0.84	1.49	1.89	0.84	1.34	1.75
Total P	0.63	1.03	1.41	0.69	0.95	1.20	0.65	0.91	1.19

[1] Dietary Ca, P and vitamin D_3 at 1, 1.5 and 2.0 times Lohmann LSL-Lite recommendations.
[2] Provided per kilogram of premix: [IU] vitamin A, 1,200,000; vitamin D_3, 500,000; vitamin E, 8,000; [μg] vitamin B_{12}, 1,700; biotin, 22,000; [mg] menadione, 330; thiamine, 400, riboflavin, 860; pantothenic acid, 2,000; pyridoxine, 430; niacin, 6,500; folic acid, 220; choline, 60,000; Fe, 6,000; Cu, 1,000.
[3] Vitamin A, 1,000,000 IU/g; Vitamin D_3, 500,000 IU/g (DSM Nutritional Products, Ayr, ON, Canada), added on top of premix.

breeder recommendation) were randomly selected and individually weighed for BW uniformity assessment. At the end of the 4, 12 and 16 wk of age, 2 pullets per cage were randomly sampled, weighed and sacrificed. Before sacrifice, about 4 mL of blood sample was drawn from wing vein into lithium heparin coated BD vacutainer (BD Canada, Mississauga, ON, Canada), placed on ice and transported to the laboratory. The first pullet was then sacrificed via cervical dislocation, left femur and tibia dissected without cartilaginous head cap intact and stored at −20 °C until further analyses. The second pullet was sacrificed, whole intact body with feathers stored at −20 °C to measure body, femur and tibia MD and MC.

2.3. Sample processing and laboratory analyses

Diets were ground using a coffee grinder (KitchenAid BCG 111OB, Whirlpool Corp, Benton Harbor, MI, USA). Dry matter was determined by AOAC 930.15 method (AOAC 2005). To determine ash content, diet samples were placed in muffle furnace at 600 °C for 12 h. For determination of Ca and P concentration, feed samples were ashed and digested with a mixture of concentrated acids (5 mL HCl and 50 μL HNO_3) in a pyrex tube at 120 °C for 20 h, the mixture was then diluted with double deionized water to 100 mL and aliquots submitted for reading using inductively coupled plasma atomic emission spectrometry (ICP-AES, Varian Inc., California, USA). Femur and tibia samples were defleshed, and the length and diameter determined using a digital vernier caliper (Mastercraft tools, Guelph, ON, Canada) with accuracy of 0.01. Femoral length was taken from the tip of greater trochanter to the edge of medial condyle. Tibial length was taken from the edge of tibial plateau of medial condyle to medial malleolus. The diameter of both bones was taken on diaphysis exactly at the midpoint. The weight of wet (defleshed and fresh) and dried (100 °C for 12 h) femur and tibia was taken using a digital weighing scale with accuracy of 0.0001.

The whole body, femur and tibia MD and content were determined using dual energy X-ray absorptiometry (DEXA) as described in Khanal et al. (2020a). Briefly, the pullets were first evaluated for whole body MD and content. Then, the pullets were then necropsied to excise femur and tibia for DEXA measurements. The femur and tibia breaking strength (BS) were determined using a three-point bending test in an instron material tester after which bone ash was measured as described in our previous paper (Khanal et al., 2020a). Upon arrival in the laboratory, plasma was separated by centrifuging blood at 2,000 × *g* at 8 °C for 15 min and 0.5-mL samples submitted Animal health laboratory, University of Guelph. Total plasma Ca and P were determined using CA2 Calcium Gen.2 and PHOS2 Phosphate inorganic Ver.2. system of Cobas C311/

501 analyzer (Cobas Roche/Hitachi, Roche diagnostics GmbH, Mannheim, Germany).

2.4. Calculations and statistical analyses

The BW uniformity (BWU) was determined using individual BW of pullets (5/CON and 20/FUR) at the end of 16 wk of age. First, the average cage BW was determined, then the BW uniformity range was fixed at 10% above and below the average cage BW (i.e., 90% to 110% range). The number of pullets falling within that uniformity range were identified and BWU calculated as follows:

$$\text{BWU} = \frac{\text{Number of pullets within BWU range}}{\text{Number of pullets sample}} \times 100\%.$$

The relative femur and tibia weight were derived by dividing fresh weight with BW. The femur and tibia ash content were divided by weight (dry) to give percent ash concentration (AC).

The cage was the experimental unit and the data was subjected to Proc GLIMMIX of SAS 9.4 (SAS Inc., 2014). The model was:

$$Y_{ijkl} = \mu + a_i + b_j + c_k + ab_{ij} + bc_{jk} + ac_{ik} + abc_{ijk} + \varepsilon_{ijkl},$$

where Y_{ijkl} was the response variable, a_i was age (4, 12 or 16 wk of age), b_j was cage type (CON or FUR), c_k was diets (Diet 1, 1.5 or 2); ab_{ij} was the interaction of age and cage, bc_{jk} was the interaction of cage and diet, ac_{ik} was interaction of age and diet, abc_{ijk} was interaction of age, cage and diet, and E_{ijkl} was the error (where i, j, k, l are replications from 1 to 6). The linear and quadratic orthogonal polynomial contrasts were evaluated for diets response. The LS means were separated using Tukey test and significance was declared at $P < 0.05$, and a tendency to a pattern was declared at $0.05 \leq P \leq 0.1$.

3. Results

3.1. Body weight and bone weight, length and diameter

There was no interaction ($P > 0.05$) between age, cage type and diet or cage type and diet on BW and femur and tibia weight (Table 2). However, the cage interacted ($P < 0.01$) with age on BW such that FUR and CON pullets had similar BW at 4 wk of age (260 vs. 266 g), but FUR pullets were heavier than CON pullets at 12 wk of age (1,008 vs. 963 g) and 16 wk of age (1,200 vs. 1,142 g). With the increasing dietary Ca, P and $VitD_3$, the BW decreased linearly ($P \leq 0.0001$). There was an interaction between age and diet on absolute femur weight ($P = 0.026$) such that femurs were similar in weight for all 3 diets at 4 and 12 wk of age but heavier at 16 wk of age for pullets fed Diet 2 (6.4 g) than for pullet fed Diet 1.5 (6.0 g) and Diet 1 (6.0 g). Also, diets interacted with cage on absolute tibia weight ($P = 0.038$) such that at 12 wk of age, tibia were heavier for Diet 1.5 (7.7 g) than Diet 2 (7.3 g) and Diet 1 (7.3 g) but at 16 wk of age, tibia were heavier for Diet 2 (8.5 g) than Diet 1.5 (7.7 g) and Diet 1 (8.0 g). There was a diet effect on relative femur and tibia weight such that increasing concentration of Ca, P and $VitD_3$ linearly increased femur ($P = 0.010$) and tibia weight ($P = 0.013$). Pullets reared in CON cages had heavier ($P = 0.024$) relative femur and tibia than FUR pullets. The relative femur and tibia weight decreased with the increase in age ($P = < 0.0001$) reflecting heavier BW as pullet aged. The relative femur weight was similar between 12 and 16 wk of age but that of tibia decreased when pullets reached to 16 wk of age. We did not observe interaction between the cage type and diets or main effects on BWU ($P = 0.758$) (Data not shown). Pullet BWU was similar ($P = 0.317$) between FUR (91.4%) and CON (88.8%) pullets and between ($P = 0.273$) Diet 1 (91.8%), Diet 1.5 (91.5%) and Diet 2 (87.2%).

There was no interaction between age, cage type and diet or cage and diet on femur and tibia length and latero-medial diameter ($P > 0.05$; Table 3). There was interaction between age and diet on tibia length ($P = 0.012$); with tibia being longer for Diet 1.5 (114 mm) than Diet 2 (109.7 mm) and Diet 1 (113.8 mm) at 12 wk of age but being longer for Diet 2 (116.3 mm) than Diet 1.5 (114 mm) and Diet 1 (113.8 mm) at 16 wk of age. Both femur ($P = < 0.0001$) and tibia ($P = < 0.0001$) elongated with the age. Femur were longer for CON pullets than for FUR pullets ($P = 0.031$). Also, the tibia tended to be longer for CON than for FUR pullets ($P = 0.063$). The femoral and tibial length and diameter were not ($P > 0.05$) affected by diets (Table 3). The age tended to interact with the cage ($P = 0.08$) on femur lateromedial diameter such that at 4 wk of age, the diameter was larger for CON pullets and larger for FUR pullets at 12 and 16 wk of age. Also, the age interacted with cage on tibia latero-medial diameter ($P = 0.02$) such that CON pullets had wider tibia (3.79 vs. 3.74 mm) at the end of 4 wk of age which later became wider for FUR pullets at 12 (6.36 vs. 6.09 mm) and 16 (6.72 vs. 6.67 mm) wk of age.

3.2. Whole body and plasma mineral content

The age, cage type and diet tended to interact ($P = 0.085$) on whole body MD (BoMD) (Table 4). The cage type interacted with the age ($P = 0.040$) on BoMD such that BoMD of CON and FUR pullets were similar at 4 (0.123 vs. 0.125 g/cm^2) and 16 (0.228 vs. 0.225 g/cm^2) wk of age, however, at 12 wk of age FUR pullets had higher (0.221 vs. 0.206 g/cm^2) BoMD than CON pullets. The BoMD increased with the age ($P = < 0.0001$); being highest at 16 wk of age. The cage type tended to affect BoMD ($P = 0.089$) such that the FUR pullets had higher BoMD than CON pullets. The BoMD showed a quadratic relationship ($P = 0.015$) with dietary concentration of Ca, P, $VitD_3$ such that BoMD was higher for pullets fed Diet 1.5 compared with Diet 1 and Diet 2. We did not observe interaction ($P = 0.694$) between age, cage and diet on body mineral content (BoMC), however, the cage type interacted with the age ($P = 0.001$) such that the BoMC was similar for FUR and CON pullets at 4 wk of age, however, it was higher for FUR pullets than CON pullets at 12 and 16 wk of age. A tendency for quadratic effect ($P = 0.077$) of diet was observed for BoMC with Diet 1.5 showing higher BoMC than either Diet 1 or Diet 2 (Table 4). We did not find any interactive effect ($P > 0.05$) of age, cage type and diet or main effects of cage type and diet on plasma concentration of the total Ca and inorganic P (Table 4). Plasma total Ca and inorganic P decreased ($P < 0.01$) as the pullets grew.

3.3. Femur and tibia mineral density and breaking strength

There was no interaction ($P > 0.05$) of age, rearing cage type and age or cage type and diet on femur and tibia MD, breaking strength (BS) and ash concentration (AC) (Table 5). The femur and tibia MD increased ($P > 0.01$) from 4 to 12 wk of age, however, was similar ($P > 0.05$) for 12 and 16 wk of age. The femur MD was similar for FUR and CON pullets, but tibia MD was higher for FUR (approximately 6.2%) than CON pullets. The diet had a quadratic effect on femur MD ($P = 0.03$) with Diet 1.5 showing the least femur MD than other diets but there was no diet effect ($P = 0.478$) on tibial MD. Age affected ($P < 0.001$) femur and tibia BS such that strength increased from 4 to 12 wk of age but was similar between 12 and 16 wk of age (Table 5). Cage had no effect ($P = 0.607$) on femur BS, however, tibia BS of FUR pullets tended to be stronger than that of CON pullets ($P = 0.076$). Diet had a quadratic effect ($P = 0.026$) on femur BS with Diet 2 having the highest and Diet 1.5 having the lowest femur BS,

Table 2
Effect of rearing cage type and dietary concentration of Ca, P and vitamin D_3 on BW and femur and tibia weight in Lohmann LSL-Lite pullets from d 0 to 16 wk of age ($n = 6$).

Age, wk	Cage	Diet[1]	BW	Absolute weight, g		Relative weight, g/kg BW	
				Femur	Tibia	Femur	Tibia
4	CON	Diet 1	269.1	1.49	2.24	5.57	8.39
		Diet 1.5	269.1	1.58	2.24	5.87	8.33
		Diet 2	260.9	1.66	2.43	6.36	9.36
	FUR	Diet 1	263.3	1.52	2.25	5.80	8.55
		Diet 1.5	259.6	1.50	2.22	5.80	8.57
		Diet 2	258.4	1.58	2.24	6.14	8.71
12	CON	Diet 1	968.3	5.44	7.16	5.62	7.31
		Diet 1.5	970.5	5.64	7.69	5.82	7.93
		Diet 2	950.9	5.46	7.37	5.75	7.76
	FUR	Diet 1	1023.9	5.56	7.56	5.44	7.40
		Diet 1.5	1009.2	5.64	7.33	5.59	7.65
		Diet 2	993.1	5.20	7.39	5.24	7.44
16	CON	Diet 1	1,154.0	6.17	8.31	5.34	7.19
		Diet 1.5	1,141.4	6.08	7.96	5.33	6.99
		Diet 2	1,131.8	6.61	8.72	5.84	7.71
	FUR	Diet 1	1,219.2	6.01	7.80	4.93	6.40
		Diet 1.5	1,194.3	5.99	7.56	5.02	6.33
		Diet 2	1,182.5	6.36	8.19	5.38	6.92
SE			8.26	0.164	0.22	0.22	0.29
Main effects							
Age, wk							
4			263.4^{c}	1.55^{c}	2.27^{c}	5.92^{a}	8.65^{a}
12			986.0^{b}	5.49^{b}	7.48^{b}	5.57^{b}	7.60^{b}
16			1,170.5^{a}	6.20^{a}	8.09^{a}	5.31^{b}	6.92^{c}
SEM			3.37	0.06	0.09	0.09	0.12
Cage							
CON			790.7^{a}	4.46	6.01	5.72^{a}	7.89^{a}
FUR			822.6^{b}	4.37	5.88	5.48^{b}	7.55^{b}
SEM			2.75	0.05	0.07	0.074	0.09
Diet							
Diet 1			816.3^{a}	4.36	5.88	5.45^{b}	7.55^{b}
Diet 1.5			807.3^{ab}	4.40	5.90	5.57^{ab}	7.63^{ab}
Diet 2			796.3^{b}	4.48	6.06	5.78^{a}	7.98^{a}
SEM			3.75	0.06	0.09	0.09	0.12
P-values							
Age			<0.001	<0.001	<0.001	<0.001	<0.001
Cage			<0.001	0.293	0.216	0.024	0.016
Diet							
Linear			<0.001	0.245	0.194	0.010	0.013
Quadratic			0.797	0.846	0.536	0.663	0.367
Cage × Diet			0.493	0.569	0.745	0.547	0.482
Age × Cage			<0.001	0.758	0.054	0.323	0.122
Age × Diet			0.281	0.026	0.038	0.163	0.285
Age × Cage × Diet			0.936	0.952	0.954	0.961	0.798

CON = conventional cage (76 cm × 71 cm × 46 cm); FUR =Furnished cage (239 cm × 80 cm × 75 cm) outfitted with platforms and terraces to increases opportunities for load bearing exercises (e.g. jumping, perching, flying) (Khanal et al., 2020a).
[a-c] Means (main effect) within a column assigned different superscripts differ, $P < 0.05$.
[1] Dietary Ca, P and vitamin D_3 at 1, 1.5 and 2.0 times Lohmann LSL-Lite recommendations.

however Diet 1.5 and Diet 1 had similar ($P > 0.05$) femoral BS. Diets had no effect ($P = 0.231$) on tibia BS. The cage type interacted ($P = 0.007$) with age on the AC of femur such that the femur of FUR pullets (41.0%) had higher AC than that of CON pullets (38.0%) at 4 wk of age whilst the femur AC of pullets was similar ($P > 0.05$) at 12 (32.7% and 32.5%) and 16 wk of age (32.2% vs. 31.6%). Diet linearly increased ($P = 0.022$) femur AC. The tibia AC decreased ($P < 0.01$) with pullets' age. Diet had no effect ($P > 0.05$) on tibia AC, however, cage had effect ($P = < 0.0001$) such that the tibia AC of FUR pullets was higher than that of CON pullets (36.83% vs. 35.19%).

4. Discussion

The continuous genetic selection has changed body composition and nutritional requirements of modern layers. Simultaneously, in response to consumer and legislation pressure, the egg industry is in the process of transitioning from conventional to alternative housing systems in some jurisdictions (Ochs et al., 2018). Unfortunately, little emphasis has been given on pullet rearing, and as highlighted by Widowski and Torrey (2018), rearing pullets in a housing environment system that matches production environment is critical. Thus, nutritional studies in pullets carried out decades ago when hens were reared in conventional cages and produced relatively fewer eggs might no longer be applicable to pullets reared in alternative housing. There appears to be a disconnection between previously established and actual requirement of dietary Ca and P, especially regarding optimal skeletal development in pullets reared in alternative housing (e.g. furnished cages). Thus, the present study investigated the interactive effects of cage type and dietary concentration of Ca, P and $VitD_3$ on growth and skeletal development during the pullet phase.

The birds were in large groups and the feeders were in the middle of floor for FUR cages causing excessive feed spillage due to scratching and foraging behavior. For this reason, we could not monitor feed intake. Pullets fed diets with higher concentration of

Table 3
Effect of rearing cage type and dietary concentration of Ca, P and vitamin D_3 on length and diameter of femur and tibia in Lohmann LSL Lite pullets from d 0 to 16 wk of age ($n = 6$).

Age, wk	Cage[1]	Diet[2]	Length, mm		Latero-medial diameter at mid, mm	
			Femur	Tibia	Femur	Tibia
4	CON	Diet 1	45.86	63.41	4.18	3.75
		Diet 1.5	47.36	63.15	4.16	3.69
		Diet 2	46.44	62.93	4.26	3.93
	FUR	Diet 1	45.76	62.59	4.11	3.78
		Diet 1.5	45.14	61.93	4.04	3.74
		Diet 2	45.48	61.89	4.12	3.69
12	CON	Diet 1	75.90	109.72	6.52	5.93
		Diet 1.5	76.75	111.13	6.47	6.24
		Diet 2	75.59	108.18	6.39	6.12
	FUR	Diet 1	75.25	109.89	6.65	6.42
		Diet 1.5	76.72	110.75	6.71	6.37
		Diet 2	74.37	108.27	6.65	6.29
16	CON	Diet 1	79.26	113.68	7.00	6.75
		Diet 1.5	78.30	115.79	6.73	6.59
		Diet 2	80.13	117.45	7.06	6.82
	FUR	Diet 1	77.64	114.01	6.93	6.67
		Diet 1.5	78.19	112.34	6.87	6.64
		Diet 2	78.97	115.35	7.09	6.69
SE			0.87	1.06	0.12	3.75
Main effects						
Age, wk						
4			46.01[c]	62.65[c]	4.14[c]	3.76[c]
12			75.76[b]	109.64[b]	6.56[b]	6.22[b]
16			78.75[a]	114.74[a]	6.95[a]	6.69[a]
SEM			0.35	0.43	0.04	0.04
Cage						
CON			67.29[a]	96.15	5.86	5.53
FUR			66.39[b]	95.20	5.91	5.59
SEM			0.29	0.35	0.04	0.03
Diet						
Diet 1			66.61	95.53	5.90	5.55
Diet 1.5			67.08	95.83	5.83	5.54
Diet 2			66.83	95.66	5.93	5.59
SEM			0.35	0.43	0.04	0.04
P-values						
Age			<0.001	<0.001	<0.001	<0.001
Cage			0.031	0.063	0.432	0.335
Diet			0.633	0.887	0.353	0.767
Linear			0.666	0.837	0.676	0.556
Quadratic			0.416	0.657	0.168	0.669
Cage × Diet			0.934	0.463	0.809	0.530
Age × Cage			0.896	0.395	0.080	0.025
Age × Diet			0.193	0.012	0.391	0.530
Age × Cage × Diet			0.622	0.793	0.944	0.597

[a-c] Means (main effect) within a column assigned different superscripts differ, $P < 0.05$.
[1] CON, conventional cage (76 cm × 71 cm × 46 cm); FUR, furnished cage (239 cm × 80 cm × 75 cm) outfitted with platforms and terraces to increases opportunities for load bearing exercises (e.g. jumping, perching, flying) (Khanal et al., 2020a).
[2] Dietary Ca, P and vitamin D_3 at 1, 1.5 and 2.0 times Lohmann LSL-Lite recommendations.

Ca, P and $VitD_3$ had lower BW which may have been be associated with depressed feed intake due to Ca. Although Ca concentrations in the present study was not that high, Kim et al. (2017) reported a decrease in feed intake and growth in broilers fed high dietary Ca. It has been demonstrated that high (3.0%) dietary Ca leads to metabolic alkalosis (Guo et al., 2008) leading to depressed feed intake and thus growth. Wideman et al. (1985) reported mortality of up to 12% in pullets fed a diet with higher Ca (3.25%). Higher dietary Ca has also been shown to decrease amino acids digestibility in broilers (Amerah et al., 2014). It is thus possible growth depression seen in the present study could have been linked to limitation in nutrient bioavailability. Recently, Wen et al. (2019) reported that feeding pullet as high as 8,000 IU of $VitD_3$/kg had no effect on growth. Thus, it is likely that, the level of VitD3 was not linked to growth depression observed in the present study. Furnished cage enhanced pullet growth in the present study extending previous observations in pullets reared in furnished vs. conventional cages (Casey-Trott et al., 2017b; Li et al., 2019; Khanal et al., 2020a). The enhanced BoMD of FUR pullets was related to higher bone MD because a large proportion of the minerals are deposited in the bones. The enhanced BoMD of FUR pullets agreed with our previous study (Khanal et al., 2020a). The higher BoMC of FUR pullets was attributable to heavier body, and higher BoMD. The BoMD and BoMC showed a quadratic relationship with dietary Ca, P and $VitD_3$ concentration. This could probably be due to lighter BW linked to lower feed intake as Ca increased as previously discussed. A marked fluctuation in the plasma concentration of Ca and inorganic P might negatively affect many physiological and biochemical functions in the body (Marks et al., 2010). As blood minerals are tightly regulated; this could be the reason why we observed similar plasma Ca and P levels even when birds were provided with higher Ca, P and $VitD_3$ in diets. Moreover, as alluded to, perhaps depressed feed

Table 4
Effect of age of rearing cage type and dietary concentration of Ca, P and vitamin D_3 on whole body and plasma minerals in Lohmann LSL-Lite pullets from d 0 to 16 wk of age ($n = 6$).

Age, wk	Cage[1]	Diet[2]	Whole body mineral		Plasma mineral, mmol/L	
			Density, g/cm^2	Content, g	Total Ca	Inorganic P
4	CON	Diet 1	0.120	3.14	2.42	2.02
		Diet 1.5	0.122	2.91	2.31	2.21
		Diet 2	0.125	2.99	2.27	2.11
	FUR	Diet 1	0.126	2.97	2.28	1.98
		Diet 1.5	0.135	3.15	2.36	2.05
		Diet 2	0.115	2.74	2.42	1.98
12	CON	Diet 1	0.197	15.60	2.53	1.62
		Diet 1.5	0.219	17.88	2.52	1.56
		Diet 2	0.202	16.63	2.59	1.68
	FUR	Diet 1	0.221	19.68	2.55	1.62
		Diet 1.5	0.224	19.90	2.54	1.70
		Diet 2	0.217	18.67	2.48	1.70
16	CON	Diet 1	0.231	19.15	1.78	0.95
		Diet 1.5	0.229	20.09	1.87	1.00
		Diet 2	0.223	20.60	1.75	1.02
	FUR	Diet 1	0.219	22.11	1.94	1.05
		Diet 1.5	0.227	22.99	1.90	1.04
		Diet 2	0.228	22.15	1.79	1.02
SE			0.006	0.69	0.075	0.07
Main effects						
Age, wk						
4			0.124^c	2.98^c	2.34^b	2.12^a
12			0.213^b	18.06^b	2.53^a	1.71^b
16			0.226^a	21.18^a	1.84^c	1.07^c
SEM			0.002	0.280	0.030	0.029
Cage						
CON			0.185	13.22^b	2.23	1.57
FUR			0.190	14.93^a	2.25	1.57
SEM			0.002	0.230	0.02	0.02
Diet						
Diet 1			0.186	13.78	2.25	1.54
Diet 1.5			0.193	14.48	2.25	1.59
Diet 2			0.185	13.96	2.21	1.58
SEM			0.002	0.280	0.03	0.02
P-values						
Age			<0.001	<0.001	<0.001	<0.001
Cage			0.089	<0.001	0.515	0.918
Diet			0.051	0.188	0.660	0.394
Linear			0.865	0.644	0.433	0.293
Quadratic			0.015	0.077	0.646	0.385
Cage × Diet			0.921	0.341	0.980	0.770
Age × Cage			0.040	0.001	0.491	0.094
Age × Diet			0.781	0.568	0.744	0.579
Age × Cage × Diet			0.085	0.694	0.236	0.733

[a–c] Means (main effect) within a column assigned different superscripts differ, $P < 0.05$.
[1] CON, conventional cage (76 cm × 71 cm × 46 cm); FUR, furnished cage (239 cm × 80 cm × 75 cm) outfitted with platforms and terraces to increases opportunities for load bearing exercises (e.g. jumping, perching, flying) (Khanal et al., 2020a).
[2] Dietary Ca, P and vitamin D_3 at 1, 1.5 and 2.0 times Lohmann LSL-Lite recommendations.

intake and digestibility may have depressed bioavailability of these minerals.

The skeletal frame of the modern-day commercial layers is almost fully developed by 12 wk of age (Whitehead 2004). As expected, the absolute femur and tibia weight increased with age. The higher relative femur and tibia weight for Diet 2 compared with Diet 1 was due to lighter BW. Cage and diet did not affect leg bone length and diameter. Similar length and diameter in femur and tibia could be attributed to genetics as it has dominant factor on bone morphology relative to nutrition and housing (Fleming et al., 2006). The bone mineralization geared up during grower phase (4 to12 wk of age). This was evidenced by a significant increase in femur and tibia MD from 4 to 12 wk of age and leveling off between 12 and 16 wk of age. The femur MD was similar for FUR and CON pullets. The cage type might not have imposed a strong strain to femur as it does to tibia, and this could have been because of anatomical positioning of femur. Rearing pullets in FUR enhanced tibial MD which was in line with other findings (Regmi et al., 2015; Casey-Trott et al., 2017a; Campbell et al., 2019). Other findings showed that bones retained higher minerals in layers housed in furnished cages (Tactacan et al., 2009; Regmi et al., 2016; Casey-Trott et al., 2017b; Neijat et al., 2019).

Bones assessed in the present study reacted to dietary Ca, P and $VitD_3$ concentrations differently; femur showed a quadratic relation whereas tibia was indifferent. Also, bone responded to cage type differently, cage type highly influenced tibia bone mineralization but not the femur. The MD is one of the strongest contributors to bone breaking strength (Hester et al., 2004; Topoliński et al., 2012). The higher tibia BS in FUR pullets was associated to higher physical activity. The larger the spacing for birds, the stronger the tibia (Li et al., 2019). Extended restriction on exercise results to low long bone mass and poor biomechanical properties (Shipov et al., 2010; Aguado et al., 2015). Load bearing has been shown to increase bone mass, enhance microstructure, MD and BS

Table 5
Effect of rearing cage type and dietary Ca, P and vitamin D_3 on bone mineralization and strength in Lohmann LSL-Lite pullets from d 0 to 16 wk of age ($n = 6$).

Age, wk	Cage[1]	Diet[2]	Mineral density, g/cm^2		Breaking strength, N		Ash, %	
			Femur	Tibia	Femur	Tibia	Femur	Tibia
4	CON	Diet 1	0.100	0.095	111.42	99.72	36.93	37.40
		Diet 1.5	0.065	0.092	113.45	98.04	38.18	37.87
		Diet 2	0.107	0.080	124.62	102.31	39.05	37.87
	FUR	Diet 1	0.077	0.102	109.93	104.43	39.77	38.71
		Diet 1.5	0.056	0.083	109.62	94.58	42.24	41.06
		Diet 2	0.084	0.098	119.83	93.94	41.17	38.57
12	CON	Diet 1	0.112	0.149	178.24	193.29	32.48	32.79
		Diet 1.5	0.108	0.144	173.24	193.01	32.17	32.39
		Diet 2	0.119	0.147	197.64	207.49	32.98	33.22
	FUR	Diet 1	0.114	0.161	173.64	215.92	30.62	34.80
		Diet 1.5	0.113	0.156	176.54	205.66	32.30	35.08
		Diet 2	0.121	0.162	200.40	209.83	33.95	35.37
16	CON	Diet 1	0.117	0.146	182.92	204.48	31.60	34.92
		Diet 1.5	0.122	0.152	174.26	205.07	32.18	35.20
		Diet 2	0.114	0.153	172.03	215.64	31.22	35.05
	FUR	Diet 1	0.120	0.152	180.07	214.87	32.46	35.69
		Diet 1.5	0.116	0.154	156.40	216.48	33.06	35.90
		Diet 2	0.129	0.167	181.20	237.86	32.65	36.33
SE			0.011	0.007	9.25	9.04	0.88	0.71
Main effects								
Age, wk								
4			0.082^b	0.092^b	114.81^b	98.83^b	39.56^a	38.58^a
12			0.114^a	0.153^a	183.28^a	205.32^a	32.42^b	33.94^c
16			0.119^a	0.154^a	174.48^a	215.73^a	32.19^b	35.51^b
SEM			0.004	0.002	3.77	3.69	0.36	0.29
Cage								
CON			0.108	0.129^b	158.65	169.41	34.04^b	35.19^b
FUR			0.103	0.137^a	156.40	177.06	35.36^a	36.83^a
SEM			0.004	0.002	3.08	3.01	0.29	0.23
Diet								
Diet 1			0.107	0.134	156.03^{ab}	173.02	33.91	35.72
Diet 1.5			0.097	0.130	150.58^b	168.85	35.02	36.25
Diet 2			0.112	0.135	165.95^a	177.85	35.17	36.07
SEM			0.003	0.004	1.77	1.69	0.08	0.02
P-values								
Age			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cage			0.415	0.015	0.607	0.076	0.003	<0.001
Diet			0.065	0.478	0.017	0.231	0.044	0.430
Linear			0.381	0.973	0.066	0.357	0.022	0.399
Quadratic			0.030	0.226	0.026	0.149	0.324	0.323
Cage × Diet			0.952	0.378	0.723	0.866	0.533	0.523
Age × Cage			0.122	0.660	0.906	0.238	0.007	0.262
Age × Diet			0.289	0.378	0.263	0.541	0.338	0.422
Age × Cage × Diet			0.886	0.808	0.779	0.830	0.600	0.668

[a-c] Means (main effect) within a column assigned different superscripts differ, $P < 0.05$.
[1] CON, conventional cage (76 cm × 71 cm × 46 cm); FUR, furnished cage (239 cm × 80 cm × 75 cm) outfitted with platforms and terraces to increases opportunities for load bearing exercises (e.g. jumping, perching, flying) (Khanal et al., 2020a).
[2] Dietary Ca, P and vitamin D_3 at 1, 1.5 and 2.0 times Lohmann LSL-Lite recommendations.

(Fleming et al., 2006; Enneking et al., 2012; Yuan et al., 2016; Patel et al., 2020). The FUR cages provided increased activity level and load bearing because of large utilizable space for pullet and availability of multiple perches and platforms.

Calcium and P are absorbed via 2 pathways: transcellular and paracellular. Transcellular is an active diffusion pathway and vitamin D_3 facilitate it. The paracellular is a passive diffusion, the most dominant and vitamin D_3 independent process of Ca absorption in the jejunum and ileum (Adedokun and Adeola 2013). The higher availability of Ca and P in the duodenum and jejunum, and higher availability of Vitamin D_3 at the same time could facilitate more Ca and P absorption through both paracellular and transcellular pathway. Eventually, this higher absorption of Ca and P could support the higher body and bone mineral mineralization. Keshavarz (1987) reported higher tibia ash and Ca in the pullets fed diets with higher Ca (3.5%) (14 to 20 wk of age) without adverse effects on growth and metabolism. In a study by Venäläinen et al. (2006), tibia ash, Ca and P content increased curvilinearly with increasing available P in broiler chickens. The same study showed no diet effect on tibial breaking strength even though the bone ash content were significantly different. Wen et al. (2019) reported that increasing dietary $VitD_3$ concentration (from 1,680 to 8,348 IU/Kg) significantly increased the tibia MD (from 0.195 to 0.205 g/cm^2) in pullets. The present study showed a significant increase in body and bones MD of pullets fed a diet with higher Ca, P and $VitD_3$.

The load bearing activities had different impact on tibia and femur. The physical activity influences bone matrix turnover. The increased mechanical loading enhances the formation of collagen I fiber (Tzaphlidou 2008). The microarchitecture of bone affects its strength and the loading affects the microarchitecture, bone mass and size during normal aging (Tzaphlidou 2008). The FUR cage enhances physical activity and loading, and this contributes to increased tibia breaking strength. Perching enhances the shank volume, width and the thickness of trabecular bone (Hughes et al., 1993). The bone mass, bone area and bone muscles of tibia increased significantly for pullets at 12 wk of age when pullets were

reared in cages provided with perches (Enneking et al., 2012). As evaluated by microcomputed tomography, load bearing increased site specific increment in tibial mineral content in mice (Fritton et al., 2005). At 16 wk of age, dietary Ca, P and $VitD_3$ and enriched cage affected tibial MD in the current study.

5. Conclusion

Interventions for enhancing skeletal development during pullet rearing could be one of the strategies to optimize bone quality prior to sexual maturity. Cage type did not interact with dietary Ca, P, and $VitD_3$ on many indices of skeletal development. However, the main effects were significant in some response criteria. Cage type had a dominant effect on body and leg bone mineralization. Femur showed greater response to nutrition and tibia showed greater response to the cage type. Most of the bone quality parameters were comparable when Ca, P, $VitD_3$ of pullet diet increased to 1.5 times the specification but markedly enhanced when increased to 2 times. Understanding how the nutrition and housing (or cage type) affect the development of collagen fibre and their mineralization is a potential area of exploration in future studies. A more detailed study is warranted on mineral partitioning in the body and bone in the context of increased dietary Ca, P and $VitD_3$ in alternative housing. Studies on how the bone development evolve from embryonic stage to the end-of-lay could give a comprehensive knowledge for enhancing skeletal integrity in modern high yielding hens.

Author contributions

Tanka Khanal: Conceptualization, Data generation and curation, Writing-Original draft preparation; **Gregoy Y. Bedecarrats**: Reviewing and editing; **Elijah G. Kiarie**: Conceptualization, Supervision, Validation, Writing-Reviewing and Editing, Grant holder.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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