

Article

Effects of Supplementary Genistein on Bone Development in Hy-Line Brown Pullets

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Abstract

Skeletal deterioration is a major welfare and production concern in laying hens, as substantial quantities of calcium are mobilized from bone to support eggshell formation during the laying cycle. Nutritional strategies that promote skeletal development during the pullet phase may therefore improve bone integrity later in production. The objective of this study was to evaluate the effects of dietary genistein supplementation on growth performance, bone development, and mineralization in Hy-Line Brown pullets. A total of 600 pullets were randomly assigned to four dietary treatments consisting of a control diet (0 mg/kg genistein; CON) or diets supplemented with 20 (G20), 60 (G60), or 100 mg/kg (G100) genistein from 5 to 17 weeks of age. Growth performance, bone mineral density, muscle deposition, biomechanical strength, bone ash content, and circulating bone formation biomarkers were evaluated. Pullets receiving G60 and G100 supplementation exhibited greater body weight, bone mineral density, cortical bone area, muscle weights, biomechanical strength, bone ash percentage, and circulating concentrations of BALP and P1NP compared with CON and G20 birds. Feed intake did not differ among treatments. These findings indicate that genistein supplementation, particularly at 60 and 100 mg/kg, may enhance skeletal development during the pullet rearing period. Further research is warranted to determine whether these improvements persist throughout the laying cycle and influence production, as well as the potential deposition of genistein-derived compounds in eggs.

Keywords: phytoestrogen; skeletal health; genistein; laying hen

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1. Introduction

Sustained egg production places considerable physiological demands on laying hens, particularly in relation to skeletal integrity and mineral metabolism [1–3]. As hens progress through the laying cycle, large quantities of calcium are unceasingly mobilized from the skeleton to support eggshell formation. Over time, this process can compromise bone mineralization and contribute to skeletal disorders such as osteoporosis and increased fracture susceptibility [1,2]. This advances significant welfare concerns in commercial laying systems [4–6]. Because egg formation and bone metabolism are closely interconnected processes, nutritional strategies that support mineral utilization and skeletal health may also influence productive performance [7–10].

Endocrine regulation of hormonal signaling plays an important role in these physiological processes. Circulating estrogen concentrations increase as birds approach sexual

maturity and initiate egg production. This facilitates reproductive development, calcium metabolism, and bone turnover [1]. However, estrogen levels decline later in the production cycle, a stage often associated with reduced productivity and declining skeletal quality [1,2]. Compounds capable of interacting with estrogen-related pathways may therefore influence both reproductive performance and skeletal stability.

A group of compounds receiving growing consideration in this context is soy-derived isoflavones (ISF). Isoflavones are naturally occurring phytoestrogens found primarily in legumes, including soybeans [11,12]. Among them, genistein (4,5,7-trihydroxyisoflavone; GEN) represents a major component of soybean ISF and is structurally similar to estradiol [13,14]. This similarity enables GEN to interact with estrogen receptors and influence endocrine signaling pathways, thereby exerting estrogenic or anti-estrogenic activity contingent on the physiological context [15,16]. In addition to its endocrine activity, GEN has been shown to have antioxidant, metabolic, and antimicrobial effects in various animal models [17–21].

Soybean products are widely used as protein sources in poultry diets, making ISF a naturally occurring component of many commercial rations. Previous studies suggest that dietary phytoestrogens may influence reproductive performance, growth, and production traits in livestock species [22–24]. Additionally, phytoestrogens have been associated with improved bone preservation in other species, particularly under conditions of reduced estrogen activity, such as postmenopausal women [14,25]. Genistein may explicitly contribute to skeletal health by influencing pathways involved in mineral metabolism, such as aiding in the regulation of intestinal calcium transport and modulation of bone remodeling [21,26–28]. Through estrogen receptor signaling, mechanisms that may enhance calcium utilization and support bone mineral deposition, similar to effects produced by estrogen itself [26,29]. Despite these potential benefits, the physiological responses to GEN supplementation appear to vary among studies. Differences in dosage, duration of supplementation, and the age or hormonal status of birds have been associated with these inconsistent outcomes [16,26,29].

While the effects of genistein on bone metabolism have been documented in mature and laying hens, its role during the pullet phase, when peak skeletal development occurs, remains largely unexplored [12,21,22,24,27]. This study extends existing knowledge by evaluating genistein supplementation during a critical developmental window prior to the onset of lay, where skeletal reserves are established [1]. Unlike previous work conducted during active egg production, this study isolates developmental effects on bone formation, providing insight into whether early nutritional intervention may enhance structural integrity before the physiological demands of egg laying begins.

We hypothesized that GEN supplementation would promote increased bone development in pullets. Therefore, the objective of this study was to examine the estrogenic effects of GEN on bone development in immature pullets by assessing growth responses, including performance, musculoskeletal, and serum parameters.

2. Materials and Methods

2.1. Ethics

This experiment was approved by Clemson University's Institutional Animal Care and Use Committee (protocol # AUP2020-0050).

2.2. Animal Husbandry

The study was conducted at the Morgan Poultry Center at Clemson University (Clemson, SC, USA). A total of 600 day-old Hy-Line Brown pullets, obtained from the Hy-Line North America Hatchery, Georgia (4432 County Rd 213, Mansfield, GA 30055,

USA), were utilized from 5 to 17 weeks of age following a 4-week acclimation period. Birds were randomly assigned to 20 floor pens, resulting in five replicate pens per treatment with 30 birds per pen.

All birds were housed in identical floor pens measuring approximately 4.3 m² and bedded with approximately 5 cm of fresh wood shavings. The poultry house was fully enclosed and environmentally controlled, preventing outside climatic conditions from influencing internal temperature. Each pen was equipped with a gravity feeder and an automatic waterer. Access to nest boxes and wooden perches was not provided to prevent confounding data. All treatment groups received an identical commercially formulated base diet (Table 1). The basal diets were formulated to satisfy or surpass the recommended nutrient specifications according to the breed management guidelines [30]. Birds were provided mash-form feed during the entire experimental period. The Starter 1 diet was offered from 0 to 3 weeks of age, Starter 2 from 4 to 6 weeks, the Grower diet from 7 to 15 weeks, and the Pre-lay diet from 15 to 17 weeks of age.

Table 1. Ingredient percentage and calculated nutrient analysis of 4 basal diets used in this experiment.

Ingredient	Starter 1 (%)	Starter 2 (%)	Grower (%)	Pre-Lay (%)
Corn	58.5	61.9	61.9	61.8
45% Soybean Meal	35.7	27.8	22.0	21.7
Mono-dicalcium Phosphate	1.45	1.41	1.38	2.19
Wheat Middlings	1.31	6.42	12.4	9.68
Calcium Carbonate	1.24	1.26	1.36	2.88
Soybean Oil	0.50	0.00	0.00	0.00
Salt	0.45	0.45	0.45	0.45
Choline chloride 60%	0.45	0.40	0.40	0.40
DL-Methionine	0.27	0.22	0.19	0.19
Vitamin/Mineral Premix *	0.15	0.15	0.15	0.15
L-Threonine	0.04	0.04	0.05	0.06
L-Lysine	0.00	0.03	0.05	0.53

Samples of all diets were analyzed to confirm nutrient composition. * Provimi Corporate Layer 2 with phytase (Lewisburg, OH, USA) composed of: selenium 255 ppm, zinc 6.5%, vitamin A 8,294,000 IU/kg, phytase activity 399,166.2 FTU/kg.

Environmental conditions were regulated through mechanical ventilation consisting of tunnel fans and side-wall air inlets. Industrial heating units were utilized to maintain optimal temperatures as recommended in the breeder management guide [30]. Lighting fixtures were developed through NatureDynamics (ONCE by Signify, Plymouth, MN, USA) and managed using the Interact Agriculture platform (Signify, AE Eindhoven, Netherlands). Intermittent light schedules were initiated in accordance with breeder guidelines [30].

2.3. Treatment Design

Four dietary treatments were evaluated to determine the effects of GEN supplementation during the pullet rearing period. Treatments consisted of a control diet without GEN supplementation (CON; 0 mg/kg) and three GEN-supplemented diets at 20 mg/kg (G20), 60 mg/kg (G60), or 100 mg/kg (G100). Birds were randomly allocated to treatments with five replicate pens per treatment and 30 birds per pen. All birds received the same basal diet formulated to meet nutrient requirements for pullets. For treatment groups, GEN was incorporated directly into the feed using a tiered mixing procedure, during which the compound was blended with the basal ration for approximately 10 min to ensure uniform distribution. For every 50 lbs bag of feed, mixing began by combining the supplementation dose with approximately 1 lb of feed. This mixture was then placed into 5 lbs of feed and mixed thoroughly. Lastly, this blend was added to the remaining 44 lbs of feed at a rate

of 1 pound every 2 min. Feed and water were provided ad libitum throughout the study. Dietary treatments were administered from 5 to 17 WOA.

2.4. Measurements

2.4.1. Pullet Performance

Growth performance was monitored periodically throughout the rearing period. Body weight (BW) and feed consumption were recorded at 7, 9, 13, and 17 weeks of age. At each measurement point, the quantity of feed provided to each pen and the remaining feed were documented to determine average daily feed intake per bird (ADFI). Body weight measurements obtained at these intervals were also used to estimate average daily body weight gain per bird (ADWG) across each evaluation period. Performance variables, including ADFI and ADWG, were calculated using the formulas described below.

$$ADFI = \frac{\text{Feed Offered} - \text{Feed Refused}}{\# \text{ Days} \times \# \text{ Birds}}$$

$$ADWG = \frac{\text{Finish Weight} - \text{Start Weight}}{\# \text{ Days}}$$

2.4.2. Computed Tomography Acquisition and Analysis

At 17 weeks of age, three pullets from each pen ($n = 60$ total) were humanely euthanized on-site using cervical dislocation. Immediately following euthanasia, birds were placed on ice and transported to the Godley-Snell Research Center at Clemson University for imaging. Upon arrival, each bird was prepared for computed tomography (CT) scanning. Individuals were positioned in dorsal recumbency within a V-shaped foam support cradle placed on top of a hydroxyapatite calibration phantom (QRM Quality Assurance in Radiology and Medicine, Möhrendorf, Germany). To maintain consistent body orientation during scanning, the head and legs were extended in opposite directions and secured using adhesive tape. CT images were obtained using a helical scanning protocol designed for small animals (0–10 kg). Image acquisition parameters included a slice thickness of 0.5 mm, and both bone and soft tissue reconstruction algorithms were applied to the scan data. Imaging was conducted using a Toshiba Aquilion TSX-101A 16-slice CT scanner (GE Healthcare, Chicago, IL, USA) as described by Clark-Millspaugh et al. (2026; in press with *Poultry*). Following completion of CT imaging, birds were immediately frozen for subsequent analysis.

Following image reconstruction, bone measurements were obtained using 3D Slicer software (version 5.0.3). The analysis protocol was developed in consultation with an experienced image analysis specialist and an ACVR-certified veterinary radiologist, and all image processing was conducted by a trained graduate researcher. To ensure consistency in image analysis, a repeatability assessment was conducted prior to data extraction, during which a subset of scans was analyzed in triplicate using the same segmentation protocol. This preliminary evaluation confirmed consistency in measurement procedures, and the finalized protocol was applied uniformly across all samples.

CT images were evaluated to quantify tibiotarsal bone characteristics. Using a bone window setting (WL/WW: 300/1500), the tibiotarsus was identified in transverse, sagittal, and dorsal views. The total bone length was measured in the sagittal plane, and a standardized mid-diaphyseal region of interest was selected to allow consistent comparisons among birds. Segmentation masks were then applied to isolate cortical and medullary bone using Hounsfield unit (HU) thresholds, with values ≥ 700 HU representing cortical bone and ≤ 300 HU representing medullary bone [31–33]. Non-target structures, including the fibula, were removed during segmentation.

A similar segmentation approach was used to isolate the hydroxyapatite (HA) calibration phantom, which contained three reference densities. Measurements from these standards (0, 100, and 200 mg/cm³) were used to generate a calibration curve for converting HU values to bone mineral density (BMD). Density values for cortical and medullary bone were calculated within the software and exported to spreadsheet software for further analysis, following procedures similar to those described by [34].

2.4.3. Muscle Dissection

To compare musculoskeletal anatomy across aviary systems, selected muscles and bones were dissected following CT and X-ray imaging. Birds were placed in dorsal recumbency, and the skin and feathers covering the thoracic region and limbs were removed. Muscles were identified along natural fascial planes and separated from surrounding tissues using blunt dissection.

In the left forelimb, the M. biceps brachii and M. triceps brachii were isolated and excised at their proximal origins and distal insertions near the ulna [35]. The M. pectoralis thoracicus (pectoralis major) was then removed bilaterally from its sternal origin and humeral insertion, followed by removal of the M. supracoracoideus (pectoralis minor) from the sternum and dorsal tubercle of the humerus [35,36].

For the hindlimb, the left femoral head was disarticulated from the acetabulum to remove the limb intact. Hindlimb musculature was then removed collectively to determine total hindlimb muscle mass. Following muscle dissection, the left tibiotarsus was disarticulated at its respective joints for biomechanical testing [35]. The fibula was removed, and the tibia was placed in saline for each left limb. Right limbs were excised intact and frozen for subsequent analyses.

2.4.4. Bone Biomechanical Testing

Prior to testing, the length and mid-diaphyseal diameter of each tibia were measured. The mechanical properties of the left tibiotarsus were assessed using a three-point bending procedure, performed in accordance with guidelines established by the American National Standards Institute (ANSI) for biomechanical testing of animal bone specimens [37] and followed the protocol detailed in [38]. Mechanical testing was carried out using an Instron Dynamic and Static Materials Testing System (Model 5944, Instron Corp., Canton, MA, USA) equipped with a 500 N load cell and operated using Automated Material Test System software. Custom rounded support pins and loading blades were fabricated in accordance with ANSI/ASAE S459 MAR1992 (R2017) standards for three-point bending of animal bones [37]. Mechanical loading was applied at a crosshead speed of 3 mm/min, and testing continued until the structural failure of the bone occurred. Throughout the test, load and displacement data were continuously recorded and subsequently used to calculate breaking strength (N) and bone stiffness (N/mm).

2.4.5. Bone Ash

Previously frozen whole legs were thawed under refrigeration (~4 °C) for 24 h prior to analysis. Soft tissues surrounding the right tibiotarsi and the fibulae were carefully removed. Empty ceramic crucibles were first weighed to obtain baseline measurements. The right tibiotarsi (n = 60) were then fragmented, placed into the pre-weighed crucibles, and weighed. Samples were dried at 100 °C for 1 h, cooled in a desiccator for 1 h, and reweighed. Bones were subsequently ashed at 600 °C for 6 h using a Thermolyne 30400 muffle furnace (Barnstead International, Dubuque, IA, USA). Following ashing, crucibles were returned to a desiccator for 1 h prior to obtaining final ash weights, and final ash percentages were calculated.

2.4.6. Blood Sampling and Analysis

Blood samples were collected at week 17 to evaluate circulating mineral and hormonal indicators associated with skeletal development. At each sampling event, three birds were randomly selected from each pen ($n = 60$), yielding 15 birds per treatment per week. Blood was obtained from the brachial vein using collection procedures found in [39,40]. Approximately 0.5 mL of whole blood was collected from each pullet and transferred into anticoagulant-treated collection tubes. Samples were subsequently placed into microcentrifuge tubes and centrifuged at 6000 rpm for 10 min at 4 °C to separate the serum fraction. Following centrifugation, serum was carefully harvested and stored at −20 °C until laboratory analyses were conducted.

Serum concentrations of bone-specific alkaline phosphatase (BALP) and procollagen type I N-terminal propeptide (P1NP) were quantified using commercially available ELISA kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China; MyBioSource, San Diego, CA, USA; respectively) according to the manufacturers' instructions. These assays use antibody-based immunoassay detection in which analyte–antibody binding is measured through a colorimetric reaction, and concentrations are calculated from absorbance values relative to a standard curve.

2.4.7. Statistical Analysis

Data were analyzed using R (version 3.3.1) with mixed-effects models fitted using the lme4 package [41,42]. For performance variables measured repeatedly across the rearing period, treatment, age, and interaction were included as fixed effects. Pen was included as a random effect to account for the experimental unit of replication and to account for repeated observations within pens over time. Serum biomarker variables were analyzed as single-time-point outcomes and were therefore not included in repeated-measures models.

For variables measured at the individual bird level at the end of the study (e.g., computed tomography bone measurements, muscle weights, biomechanical testing parameters, and bone ash percentage), treatment was included as a fixed effect, and pen was included as a random effect. For these measurements, birds were nested within pen to account for the hierarchical structure of the data.

Continuous response variables were analyzed assuming Gaussian error distributions. Least-squares means (LSMeans) were estimated, and the overall significance of fixed effects (treatment, age, and treatment $\times$ age interaction) was evaluated prior to conducting pairwise comparisons. Pairwise comparisons among treatments were conducted using Tukey's Honestly Significant Difference (HSD) adjustment implemented in the *multcomp* package [43].

Model diagnostics were conducted to verify model assumptions. For continuous outcomes, normality of residuals was evaluated using the Shapiro–Wilk test (e.g., breaking strength and stiffness). Descriptive statistics were calculated using the *psych* package, and results are reported as mean $\pm$ standard error of the mean (SEM). Statistical significance was declared at $p < 0.05$.

3. Results

3.1. Pullet Performance Results

Performance analysis revealed significant effects of genistein (GEN) supplementation on body weight (BW) and average daily weight gain (ADWG) (Table 2). At week 9, pullets in the control group (CON) exhibited lower BW compared with pullets receiving 100 mg/kg GEN (G100; $p = 0.004$). By weeks 13 and 17, CON pullets had significantly lower BW than birds receiving G20, G60, and G100 supplementation (week 13: $p = 0.002$, <0.001 , and <0.001 , respectively; week 17: $p = 0.003$, <0.001 , and <0.001 , respectively).

Table 2. Performance Data Results.

		Performance Per Bird			
		Week 7	Week 9	Week 13	Week 17
Body Weight (g)	CON	584.43 ± 18.74 ^a	787.10 ± 15.62 ^b	1187.20 ± 17.48 ^b	1442.73 ± 19.86 ^b
	G20	595.81 ± 13.26 ^a	803.41 ± 12.84 ^{ab}	1245.68 ± 15.93 ^a	1502.47 ± 18.74 ^a
	G60	607.34 ± 16.92 ^a	827.93 ± 13.71 ^{ab}	1281.54 ± 18.06 ^a	1548.99 ± 20.41 ^a
	G100	623.65 ± 20.35 ^a	852.13 ± 10.96 ^a	1288.30 ± 19.41 ^a	1596.82 ± 21.77 ^a
ADWG (g)	CON	71.22 ± 8.94 ^a	79.42 ± 3.18 ^b	68.14 ± 4.72 ^b	42.50 ± 3.88 ^b
	G20	75.85 ± 5.48 ^a	84.12 ± 4.63 ^{ab}	77.44 ± 3.57 ^a	55.91 ± 2.98 ^a
	G60	82.12 ± 11.37 ^a	90.50 ± 5.72 ^{ab}	84.50 ± 4.88 ^a	62.92 ± 4.12 ^a
	G100	92.73 ± 7.26 ^a	94.73 ± 4.11 ^a	85.16 ± 5.94 ^a	63.89 ± 4.96 ^a
ADFI (g)	CON	48.15 ± 4.86 ^a	68.63 ± 5.94 ^a	80.92 ± 1.42 ^a	89.68 ± 1.54 ^a
	G20	49.20 ± 4.21 ^a	67.98 ± 6.38 ^a	79.55 ± 1.36 ^a	89.90 ± 1.63 ^a
	G60	50.01 ± 3.68 ^a	67.10 ± 6.81 ^a	78.32 ± 1.51 ^a	90.03 ± 1.58 ^a
	G100	50.87 ± 4.74 ^a	66.45 ± 8.92 ^a	78.04 ± 1.67 ^a	90.08 ± 1.72 ^a

Performance values across treatments (g) fed a control diet (CON, 0 mg/kg) or a basal diet supplemented with Genistein (G20, 20 mg/kg; G60, 60 mg/kg; G100, 100 mg/kg). Data are displayed as LSMeans ± SEM, where superscripts of varying letters indicate significant differences ($p < 0.05$).

Similarly, treatment effects were observed for ADWG at multiple time points. At week 9, CON pullets demonstrated lower ADWG than birds receiving G100 supplementation ($p = 0.003$). At weeks 13 and 17, ADWG remained lower in CON pullets compared with G20, G60, and G100 groups (week 13: $p = 0.004$, <0.001 , and <0.001 , respectively; week 17: $p = 0.006$, <0.001 , and <0.001 , respectively). No treatment effects were detected for BW or ADWG at week 7, and average daily feed intake (ADFI) did not differ among treatments.

3.2. Bone Area and Density Results

Analysis of bone cross-sectional area and bone mineral density (BMD) revealed treatment effects for several parameters (Table 3). For total bone area, CON pullets exhibited lower values than those receiving G60 and G100 supplementation ($p = 0.003$ and <0.001 , respectively). Additionally, G20 pullets showed lower total area values than G100 pullets ($p = 0.006$). Treatment effects were also observed for total BMD. Pullets in the CON treatment had lower total BMD than birds in the G60 and G100 groups ($p < 0.001$ for both comparisons). Similarly, G20 pullets exhibited lower total BMD than pullets receiving G60 and G100 supplementation ($p = 0.009$ and 0.004 , respectively).

Table 3. CT Scan Analysis Results.

Bone Area and Density (Tibiotarsus)						
	Total Area (mm ²)	Total BMD (mg/cm ³)	Medullary Area (mm ²)	Medullary BMD (mg/cm ³)	Cortex Area (mm ²)	Cortex BMD (mg/cm ³)
CON	52.09 ± 0.42 ^c	668.96 ± 21.76 ^c	15.88 ± 0.48 ^a	138.53 ± 57.50 ^a	36.31 ± 0.61 ^c	1127.30 ± 149.78 ^c
G20	52.53 ± 0.46 ^{bc}	691.51 ± 23.14 ^c	15.69 ± 0.52 ^a	134.73 ± 54.67 ^a	36.73 ± 0.65 ^{bc}	1183.78 ± 153.62 ^{bc}
G60	53.42 ± 0.44 ^{ab}	799.14 ± 59.60 ^{ab}	14.99 ± 0.50 ^a	131.22 ± 45.81 ^a	38.90 ± 0.63 ^{ab}	1634.26 ± 218.68 ^{ab}
G100	53.82 ± 0.47 ^a	814.12 ± 57.01 ^a	14.80 ± 0.49 ^a	129.90 ± 43.72 ^a	39.43 ± 0.67 ^a	1662.95 ± 213.22 ^a

Bone cross-sectional area (mm²) and bone mineral density (mg/cm³) values across treatments fed a control diet (CON, 0 mg/kg) or a basal diet supplemented with Genistein (G20, 20 mg/kg; G60, 60 mg/kg; G100, 100 mg/kg). Data are displayed as LSMeans ± SEM, where superscripts of varying letters indicate significant differences ($p < 0.05$).

Significant differences were also detected for cortical bone measurements. Cortex area was lower in CON pullets compared with G60 and G100 pullets ($p = 0.001$ and <0.001 , respectively), and G20 pullets likewise showed lower cortex area values than those receiving

G60 and G100 supplementation ($p = 0.009$ and 0.003 , respectively). A similar pattern was observed for cortical BMD, where CON pullets had lower values than G60 and G100 pullets ($p < 0.001$ for both comparisons), and G20 pullets exhibited lower cortical BMD than birds in the G60 and G100 treatments ($p = 0.007$ and 0.003 , respectively). Significant differences in treatment were not detected for medullary bone measurements.

3.3. Muscle Deposition Results

Significant treatment effects were detected across all measured muscle parameters (Table 4). For the biceps brachii and triceps brachii muscles, CON pullets exhibited lower values than pullets receiving G60 and G100 supplementation (biceps brachii: $p < 0.001$ for both comparisons; triceps brachii: $p = 0.001$ and <0.001 , respectively). Similarly, G20 pullets had lower biceps brachii and triceps brachii weights than birds in the G60 and G100 treatments (biceps brachii: $p < 0.001$ for both comparisons; triceps brachii: $p = 0.002$ and 0.001 , respectively).

Table 4. Muscle Deposition.

	Muscle Weight (g)				
	Bicep Brachii	Triceps Brachii	Pectoralis Major	Pectoralis Minor	Leg Muscle Group
CON	3.36 ± 0.07^b	2.33 ± 0.10^b	104.55 ± 1.78^b	48.23 ± 0.79^b	123.12 ± 1.75^b
G20	3.42 ± 0.08^b	2.37 ± 0.11^b	105.96 ± 1.86^b	48.71 ± 0.84^b	124.63 ± 1.88^b
G60	3.96 ± 0.06^a	2.69 ± 0.11^a	112.85 ± 2.01^a	51.69 ± 0.82^a	131.25 ± 2.45^a
G100	4.02 ± 0.09^a	2.73 ± 0.12^a	114.30 ± 1.93^a	52.11 ± 0.80^a	132.74 ± 2.41^a

Muscle deposition (g) values across treatments fed a control diet (CON, 0 mg/kg) or a basal diet supplemented with Genistein (G20, 20 mg/kg; G60, 60 mg/kg; G100, 100 mg/kg). Data are displayed as LSMeans $\pm$ SEM, where superscripts of varying letters indicate significant differences ($p < 0.05$).

A similar pattern was observed for the pectoralis major and pectoralis minor muscles. Pullets in the CON treatment had lower muscle weights than those in the G60 and G100 groups (pectoralis major: $p = 0.003$ and 0.001 , respectively; pectoralis minor: $p = 0.005$ and 0.002 , respectively). Likewise, G20 pullets exhibited lower pectoralis major and minor values than birds receiving G60 and G100 supplementation (pectoralis major: $p = 0.004$ and 0.002 , respectively; pectoralis minor: $p = 0.007$ and 0.003 , respectively). Analysis of the leg muscle group showed a similar response, with CON and G20 pullets displaying lower values than G60 and G100 pullets (CON: $p = 0.002$ and 0.001 ; G20: $p = 0.003$ and 0.002 , respectively). No significant differences were detected between CON and G20 or between G60 and G100 treatments.

3.4. Bone Biomechanical Testing Results

Significant treatment effects were detected for breaking strength, stiffness, and maximum bending moment (Table 5). For breaking strength and stiffness, pullets in the CON and G20 treatments exhibited lower values than those receiving G60 and G100 supplementation (breaking strength: CON $p < 0.001$ for both comparisons; G20 $p < 0.001$ for both comparisons; stiffness: CON $p = 0.002$ and 0.001 ; G20 $p = 0.003$ and 0.002 , respectively). Similarly, maximum bending moment followed the same pattern, with CON and G20 pullets displaying lower values than G60 and G100 pullets (CON: $p = 0.018$ and 0.012 ; G20: $p = 0.024$ and 0.017 , respectively). No significant differences were detected between CON and G20 or between G60 and G100 treatments.

Table 5. Bone Biomechanical Testing Results.

Bone Biomechanical Testing			
	Failure Load (N)	Stiffness (N/mm)	Max. Bending Moment (N·m)
CON	220.02 ± 8.15 ^b	240.59 ± 18.95 ^b	660.14 ± 25.22 ^b
G20	224.31 ± 8.42 ^b	244.72 ± 19.36 ^b	664.88 ± 26.01 ^b
G60	305.84 ± 10.31 ^a	321.47 ± 12.45 ^a	728.96 ± 32.47 ^a
G100	309.52 ± 10.78 ^a	325.12 ± 12.92 ^a	734.18 ± 33.10 ^a

Bone biomechanical values [failure load (N), stiffness (N/mm), and Maximum Bending Moment (N·m)] across treatments fed a control diet (CON, 0 mg/kg) or a basal diet supplemented with Genistein (G20, 20 mg/kg; G60, 60 mg/kg; G100, 100 mg/kg). Data are displayed as LSMeans ± SEM, where superscripts of varying letters indicate significant differences ($p < 0.05$).

3.5. Bone Ash Percentage Results

Analysis of bone mineral content revealed significant treatment effects for bone ash percentage (Table 6). Pullets in the CON and G20 treatments exhibited lower ash% than pullets receiving G60 and G100 supplementation (CON: $p = 0.004$ and 0.002 ; G20: $p = 0.008$ and 0.005 , respectively). No significant differences were detected between CON and G20 or between G60 and G100 treatments.

Table 6. Bone Ash% Results.

Bone Mineral Content	
	Ash Percentage (%)
CON	50.85 ± 3.55 ^b
G20	53.63 ± 4.58 ^b
G60	66.58 ± 3.03 ^a
G100	68.96 ± 5.78 ^a

Bone mineral content values across treatments fed a control diet (CON, 0 mg/kg) or a basal diet supplemented with Genistein (G20, 20 mg/kg; G60, 60 mg/kg; G100, 100 mg/kg). Data are displayed as LSMeans ± SEM, where superscripts of varying letters indicate significant differences ($p < 0.05$).

3.6. Bone Mineralization Results

Analysis of bone mineralization biomarkers revealed significant treatment effects (Table 7). For BALP concentrations, pullets in the CON group exhibited lower values than those in the G20, G60, and G100 treatments ($p = 0.002$, <0.001 , and <0.001 , respectively). Additionally, G20 pullets showed lower BALP concentrations than pullets receiving G60 and G100 supplementation ($p = 0.018$ and 0.011 , respectively). No significant differences were detected between the G60 and G100 treatments.

Table 7. Bone Mineralization Data Results.

Bone Mineralization		
	BALP (pg/mL)	P1NP (ng/mL)
CON	222.68 ± 6.88 ^c	7.88 ± 1.12 ^c
G20	279.63 ± 9.52 ^b	13.88 ± 1.35 ^b
G60	301.84 ± 10.1 ^a	15.96 ± 1.41 ^a
G100	305.47 ± 9.87 ^a	16.34 ± 1.39 ^a

Bone mineralization values [BALP (pg/mL) and P1NP (ng/mL)] across treatments fed a control diet (CON, 0 mg/kg) or a basal diet supplemented with Genistein (G20, 20 mg/kg; G60, 60 mg/kg; G100, 100 mg/kg). Data are displayed as LSMeans ± SEM, where superscripts of varying letters indicate significant differences ($p < 0.05$).

A similar pattern was observed for P1NP concentrations. Pullets in the CON treatment had lower P1NP values than those in the G20, G60, and G100 groups ($p = 0.004$, <0.001 , and <0.001 , respectively). Furthermore, G20 pullets exhibited lower P1NP concentrations than

pullets receiving G60 and G100 supplementation ($p = 0.029$ and 0.021 , respectively), with no differences detected between the G60 and G100 treatments.

4. Discussion

The present study evaluated the influence of dietary GEN supplementation during the pullet phase on growth performance, skeletal development, and bone mineralization in Hy-Line Brown pullets. Overall, the findings indicate that GEN supplementation, particularly at 60 and 100 mg/kg, enhanced several indicators of skeletal development. These indicators consisted of bone mineral density, cortical bone area, bone biomechanical strength, and bone mineralization biomarkers. These responses suggest that GEN may positively influence bone formation during the pullet rearing period.

Growth performance results showed that pullets receiving GEN supplementation exhibited greater body weight and average daily weight gain compared with control birds beginning at week 9 and continuing through week 17. However, no differences were observed in feed intake, indicating that improvements in body weight were not attributable to increased feed consumption. Instead, these results may reflect improved nutrient utilization or metabolic efficiency associated with GEN supplementation, although additional research with direct supporting evidence is needed to verify these associations. Previous studies have reported that phytoestrogens can influence metabolic pathways and growth performance in poultry and other livestock species [22–24]. Previous studies have suggested that genistein may influence physiological processes involved in growth and tissue development [15,16]. Therefore, the enhanced body weight gain observed in the present study may be associated with the biological effects of genistein on growth-related processes during the pullet phase; however, the underlying mechanisms were not evaluated in the present study. Enhanced body weight gain observed in the present study may therefore be associated with growth-related physiological processes during the pullet phase. In addition, GEN-supplemented pullets exhibited greater muscle weights; however, because these birds also had greater overall body weights, it is difficult to determine whether the observed differences reflect specific effects on muscle deposition or are associated with increased body size. Therefore, further studies are needed to clarify the relationship between GEN supplementation, muscle development, and overall growth.

In addition to improvements in growth performance, GEN supplementation significantly increased bone cross-sectional area and bone mineral density of the tibiotarsus. These effects were particularly evident in pullets receiving the higher supplementation levels (G60 and G100), which exhibited greater cortical area and cortical BMD compared with control birds. Bone mineral density is a critical indicator of skeletal strength and structural integrity in laying hens, particularly because the skeleton serves as a major reservoir of calcium required for eggshell formation during the laying cycle [1]. Strategies that promote bone mineralization prior to the onset of egg production are therefore considered important for establishing adequate skeletal reserves and reducing the risk of osteoporosis later in life [2,3]. The increased cortical bone development observed in GEN-supplemented pullets suggests that dietary genistein supplementation may support bone mineral deposition before the physiological demands of egg production begin.

The positive effects of GEN on bone density observed in this study are consistent with findings reported in other species, where genistein supplementation has been associated with improved bone mineralization and reduced bone loss [14,25]. Previous studies have reported associations between genistein supplementation and changes in osteoblast activity, osteoclast function, and intestinal calcium absorption [26,28]. Several mechanisms have been proposed to explain the influence of genistein on bone metabolism, including potential effects on pathways involved in bone remodeling and mineral deposition [29]. However,

these mechanisms were not directly evaluated in the present study. These previously proposed mechanisms may provide possible explanations for the improvements in bone mineral density observed in the present study; however, further research is required to determine the biological pathways involved.

Results from bone biomechanical testing further support the structural improvements observed in CT-based bone measurements. Pullets receiving GEN at 60 and 100 mg/kg exhibited greater breaking strength, stiffness, and maximum bending moment compared with birds in the CON and G20 treatments. Bone biomechanical properties reflect the ability of bone to resist mechanical forces and are strongly influenced by bone geometry and mineral composition [44,45]. The improved mechanical strength observed in GEN-supplemented birds, therefore, indicates that the increases in bone mineral density were accompanied by functional improvements in skeletal integrity.

Similarly, bone ash% was greater in pullets receiving the higher GEN supplementation concentrations, further confirming increased mineral deposition in the skeletal structure. Bone ash measurements provide a direct estimate of bone mineral content and are commonly used as indicators of skeletal mineralization in poultry research [46,47]. The higher ash values observed in G60 and G100 pullets correspond with the improvements in cortical bone density and biomechanical strength identified in the present study, suggesting that GEN supplementation enhanced mineral incorporation into bone tissue.

Changes in circulating biomarkers of bone formation also supported these findings. Pullets receiving GEN supplementation exhibited higher concentrations of BALP and P1NP, two biomarkers commonly associated with osteoblastic activity and collagen formation during bone remodeling [48,49]. BALP is an enzyme produced by osteoblasts during bone formation, while P1NP reflects the synthesis of type I collagen, a major component of the bone matrix. Elevated concentrations of these markers in the supplemented groups indicate increased bone formation activity relative to CON birds. These biochemical indicators provide additional evidence that GEN supplementation may have stimulated bone development during the pullet growth period.

Interestingly, responses to GEN supplementation appeared to plateau at the higher inclusion levels. Although G60 and G100 pullets consistently demonstrated greater values across skeletal parameters compared with the CON and G20 treatments, no significant differences were detected between the G60 and G100 groups for most measurements. This pattern suggests that GEN may exert dose-dependent effects up to a certain threshold, beyond which additional supplementation may not produce further improvements in skeletal development. Similar dose-dependent responses have also been reported in studies evaluating phytoestrogen supplementation in other animal models [26,29].

Importantly, the present study contributes to the existing body of literature by demonstrating that genistein supplementation during the pullet phase, prior to the onset of reproductive calcium demands, can enhance skeletal development. Because bone mineral reserves become increasingly mobilized once egg production begins, strategies that improve bone mineralization during early development may help mitigate skeletal deterioration later in the laying cycle [1,2]. The improvements in bone density, biomechanical strength, and bone formation biomarkers observed in this study suggest that GEN supplementation may represent a potential nutritional strategy for supporting skeletal development during the pullet phase. This distinguishes developmental effects from those observed during the laying period and suggests that early-life nutritional strategies may play a critical role in establishing long-term skeletal resilience.

5. Study Limitations

Several limitations warrant consideration when interpreting the results of this study. The lack of longitudinal assessment during the laying period limits the ability to determine whether the observed improvements in bone characteristics are sustained and whether they translate into a meaningful reduction in fracture risk during egg production. Furthermore, key performance and physiological parameters, such as nutrient digestibility, feed conversion ratio (FCR), and circulating concentrations of growth-related hormones, including insulin-like growth factor-1 (IGF-1) and growth hormone, were not evaluated. The absence of these measures limits insight into the mechanistic basis of the observed responses and their potential implications for production efficiency. In addition, although the genistein supplementation levels evaluated in the present study were selected based on previous reports demonstrating physiological and skeletal responses to dietary genistein or related isoflavones in poultry [12,21,27,28], the economic feasibility and practical applicability of these supplementation levels under commercial production conditions were not assessed. Additionally, the study did not evaluate potential side effects or trade-offs associated with genistein supplementation. Given its endocrine activity, genistein may influence reproductive development, timing of sexual maturity, or hormonal balance, which were not assessed in the present study. As a result, potential impacts on the onset of lay or reproductive performance remain unclear. Future investigations incorporating extended follow-up and comprehensive physiological profiling are warranted to better define the long-term and functional effects of genistein supplementation.

6. Conclusions

The present study indicates that dietary GEN supplementation during the pullet rearing period was associated with improved skeletal development in Hy-Line Brown pullets. Birds receiving higher levels of GEN (60 and 100 mg/kg) exhibited improvements in several indicators of bone health, including bone mineral density, cortical bone area, biomechanical strength, bone ash content, and circulating biomarkers associated with bone formation. These findings suggest that GEN may be associated with bone remodeling during a critical developmental stage prior to the onset of egg production. Skeletal reserves established during the pullet phase play an important role in supporting bone integrity throughout the laying cycle. Nutritional strategies that promote bone development before the onset of lay may help mitigate skeletal deterioration later in production. However, the present study evaluated pullets only during the rearing period, and further studies are needed to determine whether the effects observed during rearing persist into the laying period. Collectively, these findings suggest that GEN may be a potential nutritional strategy for supporting skeletal health in pullets and warrant further investigation.

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