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Case Report

Effect of Feeding a High Calcium: Phosphorus Ratio, Phosphorous Deficient Diet on Hypophosphatemic Rickets Onset in Broilers

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Abstract: Recently, a P-deficient diet caused rickets in commercial chicks within three days. This study aimed to investigate the duration of onset of rickets in chicks. Data were collected from 3–11 day old chicks raised on 88 commercial farms. Male day-old Arbor Acres Plus broilers (n = 450) were studied in three trials, with three to four treatments each. Each treatment used one of the following crumbled feeds: control feed (calcium (Ca): phosphorus (P)-1.41), slightly high Ca:P feed (SHCa:P, Ca:P-2.69), high Ca:P ratio, P deficient feed (HCa:P, Ca:P-3.08), and HCa:P feed plus 1.5% dicalcium phosphate (HCa:P + DP). Each treatment had three replicates with 15 birds each. Rickets was induced by HCa:P, and cured by HCa:P + DP, confirmed by gross anatomy, gait score, serum P concentration and growth performance. Lameness was not found in control groups, whereas, observed in the HCa:P groups as early as day 2.7 on commercial farms and day 3 in experimental farm. Serum P was reduced in HCa:P ($p < 0.01$). Bodyweight and feed intake started decreasing at day 3 on commercial farms and in all trials ($p < 0.01$). The duration of onset of hypophosphatemic rickets in broiler chicks fed HCa:P crumbled feed is approximately three days.

Keywords: lameness; broiler chicken; poultry; hypophosphatemic rickets; Ca:P ratio; phosphorus deficiency; phosphorus requirement; duration of onset; animal nutrition; animal husbandry

Citation: Xu, L.; Li, N.; Farnell, Y.Z.; Wan, X.; Yang, H.; Zhong, X.; Farnell, M.B. Effect of Feeding a High Calcium: Phosphorus Ratio, Phosphorous Deficient Diet on Hypophosphatemic Rickets Onset in Broilers. *Agriculture* **2021**, *11*, 955. https://doi.org/10.3390/agriculture11100955

Academic Editor: István Komlósi

Received: 21 August 2021
Accepted: 26 September 2021
Published: 1 October 2021

1. Introduction

The word "rickets", derived from the old English "wrikken", to twist or bend, refers to a condition of impaired mineralization of growing bones which ultimately results in their bowing and twisting [1]. In humans, rickets is most commonly attributed to vitamin D deficiency [2]. In growing poultry, rickets most often results from feed mixing errors resulting in changes in dietary vitamin D, calcium, or phosphorus (P) concentrations [3]. Calcium (Ca) and P deficiency are well-known factors affecting bone strength, bone mineralization, and growth performance [4–11]. Vitamin D plays a role in maintaining the homeostatic balance between calcium and phosphorus in birds [12], affecting bone growth and mineral deposition [13]. In addition to absolute deficiencies of Ca or P, an improper Ca:P ratio can affect growth performance [14,15], the incidence of leg abnormalities [14], gait scores, and prevalence of hock burn and footpad lesions in birds [14–17]. When feed lacks inorganic P, rickets could occur two weeks earlier in birds fed a high Ca:P diet compared to those fed a low Ca:P diet [7]. The reason may be that a high dietary Ca:P ratio decreases the bioavailability of Ca and P to sub-optimal levels [18], thereby causing a deficiency of one or both of these elements.

Hypophosphatemic rickets was reported in 33 northern China poultry farms in broiler chicks ranging from three to 11 day-olds (n ≈ 410,400). Lameness (an inability to stand, walk or run freely due to physical problems, poor gait score) is a typical characteristic of rickets. In our investigation on this case, farmers reported that the prevalence of lameness started between two to four days after accidental commencement of feeding of nutritionally deficient rations. Through our survey, we observed that the incidence of lameness rose rapidly to 70% within six days but was ameliorated within two days in birds switched to a complete ration provided by another company (data for 70,800 chicks on 6 farms). The duration of onset of rickets observed was much sooner than in previous reports which mentioned onsets at seven [7,8], seven to ten [9], and 18 days [8]. Few reports concerning an early duration of onset of rickets caused by P deficiency in poultry in recent years could be found. Thus, the conception of a slow onset of hypophosphatemic rickets in poultry may hinder the timely diagnosis of this syndrome by veterinarians and nutritionists. As early identification of a nutrient deficiency offers an opportunity to ameliorate the ill effects of a feed processing error, the early diagnosis and immediate treatment of hypophosphatemic rickets is of utmost importance [19], with irreparable economic losses resulting if this is not possible. We hypothesized that the duration of onset of hypophosphatemic rickets might be around three days after commencement of feeding the chicks a high Ca:P ratio, P deficient (HCa:P) crumbled feed.

We aimed to evaluate rickets' duration of onset in broiler chicks that fed HCa:P crumbled feed via a field survey and three trials, based on our observations concerning gross anatomy, serum P concentration, the incidence of lameness and growth performance. The purpose of this study is to provide information enabling more efficient diagnosis and management of early-onset hypophosphatemic rickets in chicks by nutritionists, quality supervisors, flock supervisors, and veterinarians.

2. Materials and Methods

2.1. Ethical Statement

The procedures followed for the field research and all trials were approved by the Institutional Animal Care and Use Committee of Yangzhou University (ethical protocol code: YZUDWSY 2017-05-09.03) and conducted according to the relevant animal welfare regulations.

2.2. Field Research

We surveyed 88 farms whose chicks had been fed feed from the same mill. Thirty-three farms (problem farms) bought feed produced on the same day and had lameness issues in their bird flocks, whereas 55 farms which bought feed produced on other dates did not have such problems. The scale of these farms varied from 3000 to 12,000 birds per flock, in total including approximately 410,400 fast-growing white feather broilers (Arbor Acres Plus (AA+) or Cobb 500 broilers), male and female sourced from seven hatcheries. All birds had been raised on plastic mesh flooring, 50–80 cm over the ground, at an approximate density of 30–35 birds/m^2 during the first week, and 20–30 birds/m^2 during the second week. Air temperature outside of the chicken houses varied between 22–35 °C, with diurnal temperature ranges of 6–11 °C during our survey in summer. Internal temperatures ranged between 30.2–34.5 °C for chicks aged between 11 and three days old, and humidity ranged between 70–84% in various chicken houses on the days on which we collected field data. Birds had free access to feed and water. We assessed gait scores, lameness incidence, body weights, feed intakes (FI) record, and gross anatomy at these commercial farms. The history of drug use was reported by farmers.

2.3. Trials

2.3.1. Feeds and Treatments

Three farms were selected from the surveyed farms, as representative of healthy, slightly sick, and severely sick chicks, respectively. Thirty bags (40.0 kg each) of crumbled

feed were collected from each of these farms. The feeds were classified into the following three categories, according to their nutrient levels and impact on chick's gait score: control feed, which did not cause any lameness in chicks; HCa:P feed, which caused serious lameness in chicks and exhibited a high Ca:P ratio and P deficiency; slightly high Ca:P (SHCa:P) feed, which caused slight lameness in chicks and exhibited a slightly high Ca:P ratio and P deficiency. All the feeds were composed according to the formula and nutrient levels of Table 1. In addition, 1.5% of dicalcium phosphate (DP, $CaHPO_4 \cdot 2H_2O$) was added to 100 kg of HCa:P feed to comprise the fourth (HCa:P + DP) feed. Due to a processing error, the Ca:P ratios, Ca and P concentrations were different among treatments. These values were shown in Table 2.

Table 1. Ingredients and nutrient levels of the commercial feed and the control feed for all trials.

Ingredients and Specifications [1]	Composition	Nutrient Composition	Percent
Corn	55.00	Metabolic energy (MJ/kg)	12.28
Soybean meal(CP, 47%)	25.30	CP	21.00
Wheat flour	6.00	Lysine	1.27
Peanut meal (CP, 46%)	2.00	Methionine	0.52
Dried distilled grain with solubles (DDGS)	5.00	Available total sulfur-containing amino acids (ATSAA)	0.80
Dicalcium phosphate (DP)	1.40	Available lysine	1.15
Lime stone (coarse)	1.06	Available methionine	0.47
Feather powder (CP, 80%)	1.00	Available threonine	0.77
Duck oil	0.68	Available tryptophan	0.19
Lysine (65%)	0.60	Calcium (Ca)	0.85
Threonine (50%)	0.30	Total phosphourus	0.62
Methionine (88%)	0.26	Available phosphourus (AP)	0.38
Salt	0.20	Ca/AP	2.24
Sodium bicarbonate	0.10	Salt	0.23
Choline chloride (75%)	0.10	Sodium	0.14
Premix [2]	1.00	Sodium + potassium-chloride (mEq/kg)	219.5

[1] CP, crude protein. [2] Premix supplied the following nutrients for each kg of feed: vitamin A, 1, 300 IU; vitamin D,4500 IU; vitamin E, 80 IU; vitamin K, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 9 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.02 mg; niacin,60 mg; pantothenic acid, 19 mg; folic acid, 2.5 mg; biotin 0.30 mg; Fe, 20 mg; Cu, 16 mg; Mn, 120 mg; Zn, 110 mg; I, 1.25 mg; Se, 0.30 mg. Minerals were supplied in the forms of ferrous sulfate, copper sulfate, manganese sulfate, zinc sulfate, potassium iodide, and sodium selenite, respectively, for Fe, Cu, Mn, Zn, I, and Se. Values were calculated values.

Table 2. Calcium (Ca) and phosphorus (P) levels for each treatment in trials one, two and three.

Diets [1]	Nutrients Values (Air-Dry Basis)					Nutrients Values (Dry Matter)				Ca:P	
	Moisture, %	Ca, %	TP [2], %	Phytate P,%	Available P, %	Ca, %	TP, %	Phytate P,%	NPP [2]	Ca: TP	Ca: NPP
Control	11.05	0.87	0.62	0.18	0.44	0.98	0.69	0.20	0.49	1.41	2.00
SHCa:P	10.05	1.13	0.42	0.18	0.24	1.26	0.47	0.20	0.27	2.69	4.71
HCa:P	9.96	1.11	0.36	0.18	0.18	1.23	0.40	0.20	0.20	3.08	6.17
HCa:P + DP	10.27	1.29	0.61	0.18	0.43	1.44	0.68	0.20	0.48	2.11	3.00

[1] Control, Ca and P-balanced feed which did not cause any lameness in chicks. SHCa:P, slightly high Ca:P ratio and P-deficient crumbled feed from farms with slight chick lameness issues; HCa:P, high Ca:P ratio and P-deficient crumbled feed collected from farms with severe chick lameness issues. HCa:P + DP, feed made by adding 1.5% dicalcium phosphate (DP) powder to HCa:P crumbled feed. All the feeds were composed with reference to the same formula, with phytase at 500 FTU/kg feed (air-dry basis). [2] TP: total P; NPP: non-phytate P. The NPP and Ca:P ratios were calculated values, other values were the means of three measurements (n = 3).

As AA+ broiler was more popular in this area, we selected AA+ broiler as the experimental object. Healthy male AA+ broilers from the day of hatch (135 in trials one and two, 180 in trial three) with similar body weight (BW, 41.88 $\pm$ 1.37 g) were randomly divided into three treatments, with three replicates per treatment and 15 chicks per replicate. The birds in trial one were fed a control diet, HCa:P diet or HCa:P + DP diet. One week later, birds in trial two were fed with a control diet, SHCa:P diet, or HCa:P diet. After another one week, the treatments used in both earlier trials were repeated in trial three, which

included groups of birds fed control diet, HCa:P diet, SHCa:P diet, and HCa:P + DP diet. As lameness incidence had reached over 80% by day 7 of trial one, the birds were kept only until day 7 in trials two and three.

2.3.2. Animals and Management

Chickens in all three trials were raised under the same room, with the same equipment and environmental controls, and following the AA+ broiler management guide [20]. Broiler chicks were raised in cages of dimensions 0.8 × 0.6 × 0.6 m (31 birds per m^2). Water and feed were provided ad libitum unless otherwise stated. The temperature inside the house was 33 °C on the first day and was reduced by 2 °C per week, with humidity ranging from 60–70%.

2.3.3. Measurements

• Determination of Ca, P, and non-phytate P (NPP) in feeds

Control (n = 19), SHCa:P (n = 10), and HCa:P (n = 11) feed samples were randomly collected from 19 out of 55 normal farms and 21 out of 33 trouble farms. Limestone and DP samples were collected from the feed mill. A total of 2.00 kg samples were taken using a multipoint method, further reduced to 0.25 kg via quartering, before being ground to a fineness that permitted sample particles to pass through a 0.42 mm sieve. Calcium was measured via the potassium permanganate method (GB/T 6436-2018) [21]. Phytate P was measured according to Haugh and Lantzsch [22]. Total P was determined via spectrophotometry [23]. The NPP content was calculated by subtracting phytate P from total P (TP) in each feed [24]. Each sample was measured with three replicates, and their mean was taken as the final value.

• Growth performance

The bodyweight (BW) and FI were recorded on days 0, 3, and 9 in trial one, and on days 0, 3, and 7 in trials two and three. Birds and feed were weighed at 8:00 am on those days, with each cage treated as a replicate. Bodyweight gain (BWG), average daily gain (ADG), and average daily feed intake (ADFI) were calculated. The feed:gain (F:G) ratio was corrected according to mortality and calculated as FI/BWG. The growth performance of commercial farms was measured on the day of our visit. Historical growth performance was reported by farmers.

• Gait score and lameness incidence observations

Broiler's walking ability was evaluated using a three-point gait scoring scale based on that of Farhadi et al. [25]. Briefly, broilers were classified to one of three gait scores, based on the following criteria: easy, well-balanced gait without any irregularity (scored: one, normal gait); irregular, uneven strides and unbalanced gait (scored: two, slight lameness); reluctance to move, takes only a few strides before sitting (scored: three, severe lameness). These observations were made every day between 8:00 to 9:00 am in all trials, after creating a noisy environment by striking the cage with two iron bars (1 × 1 × 100 cm) at a rate of four times per second. After striking, the birds' gait was observed and scored over a five-second period. The striking and observations were repeated three times for each cage of birds, and counts of lame (score two or three) birds were recorded. Lameness incidence was calculated as the average count of lame birds/total count of observed birds × 100%. In field research, we took observations of chicken gait scores at the commercial farms based on three randomly selected pens at each farm during our visit. Lameness incidence was estimated on these farms.

• Gross anatomy

Gross anatomy was done on chicks in the field survey and all trials. Ten healthy chicks and lame chicks were randomly selected from commercial farms on the day of the survey (at 3–11 days old). Two healthy chicks from control cages and lame chickens from treatment cages were randomly selected on day 4 and the last day in all trials. A panel of

veterinarians (n = 5) from three independent companies inspected the gross anatomy of the lame chickens. Chickens were necropsied after sacrificing by cervical dislocation. The beak, femur, and metatarsus were forcefully pushed (or bent) aside using finger pressure to test for bone hardness. Diagnosis of hypophosphatemic rickets was performed according to Dinev [8] via a longitudinal tibial cut at the proximal tibiotarsus. However, inspection for lesions was performed immediately after necropsy instead of histochemical analysis due to a time pressure to judge who should compensate for the economic loss of farmers. Lesions were checked in visceral organs (heart, liver, spleen, kidney, bursal, gizzard, gut, thymus, and abdominal fat) were also performed.

• Determination of serum P

On day 9 in trial one, six severely lame birds and six healthy birds were randomly selected from the cages from control group and HCa:P group, respectively. Blood samples were collected via cardiac puncture by sterile syringe after CO_2 (30% in air) aspiration for one minute. Then birds were sacrificed by cervical dislocation. The blood samples were transferred into tubes containing a coagulation promoting agent and maintained at room temperature for two hours, before centrifuging at 1000× *g* at 4 °C for 10 min for serum collection. Serum P was determined in duplicate via phosphomolybdic acid methodology using commercial kits (Zhongsheng Beikong Bio-technology and Science Inc., Beijing, China) [26] analyzed using a Beckman Coulter AU5800 unit (Beckman Coulter Inc.; Brea, CA, USA).

2.4. Statistical Analysis

Figures were edited via OriginPro 8 SR1 (OriginLab Corporation, Northampton, MA, USA) and the mspaint program of accessories for Windows 10.0 (Microsoft corp., Beijing, China). Other data were summarized with Microsoft Excel 2016 (Microsoft corp., Beijing, China). Lameness incidence was a relative value, and it was taken as the mean data of three trials. The growth performance and nutrient levels were analyzed using SPSS statistical software, version 17.0 (SPSS Inc., Chicago, IL, USA). Homogeneity of variance and Kolmogorov-Smirnov (K-S) tests were performed to confirm the assumption of normally distributed data. Data were statistically analyzed via one-way ANOVA. The significance of differences between treatments was determined at $p < 0.05$ based on Duncan's multiple range tests. Welch's ANOVA was conducted where the data did not pass homogeneity testing.

3. Results and Discussion

3.1. Verification of Hypophosphatemic Rickets in Lame Chicks

Birds fed normal rations were clean and could walk and run freely (Figure 1A). Chicks on farms with problematic HCa:P feeds appeared lame, displaying characteristics of rickets including dirty feathers, reluctance to walk, shaking or falling during walking, or walking on their shanks/hocks, unable to reach feed and water, etc. (Figure 1A'). These gait characteristics of lame chicks were consistent with previous reports of hypophosphatemic rickets [8]. Low P diets are known to be capable of reducing tibia ash and tibia P [10,11], likely one of the main reasons for the birds' poor gait scores.

Hypophosphatemic rickets was further identified in the gross anatomy of chicks (Figure 1B–F,B'–F'). The bone structure of chicks on commercial farms was affected by problematic feed as early as the 4th day. Healthy chicks' bones were strong, well calcified, and well formed (Figure 1B–F); Bones in lame chicks fed the HCa:P feeds were pliable, elastic, and tended to be bent (Figure 1B'–F'). Lame chicks had "S"-shape soft ribs (Figure 1B'), swollen and cylinder-like costochondral junctions (Figure 1C'), elastic bones in metatarsus (Figure 1D') and beak; poorly calcified tibia proximal tibiotarsus with visible hollows (dark red parts) (Figure 1E'), and malleable femur (no fractures after pushing outwards (Figure 1F). Consistent with the P-deficient chicks in the case reports of Gröne et al. and Dinev [3,8] and the experiment of Shao et al. [7], these chicks had rickets lesions in the form of pliable bones, bent ribs, swollen costochondral junctions, and non-calcified tibia

proximal tibiotarsus growth plates. The visible hollows at the tibia proximal tibiotarsus were consistent with the lesions observed in P-deficient chicks in Shao et al. [7].

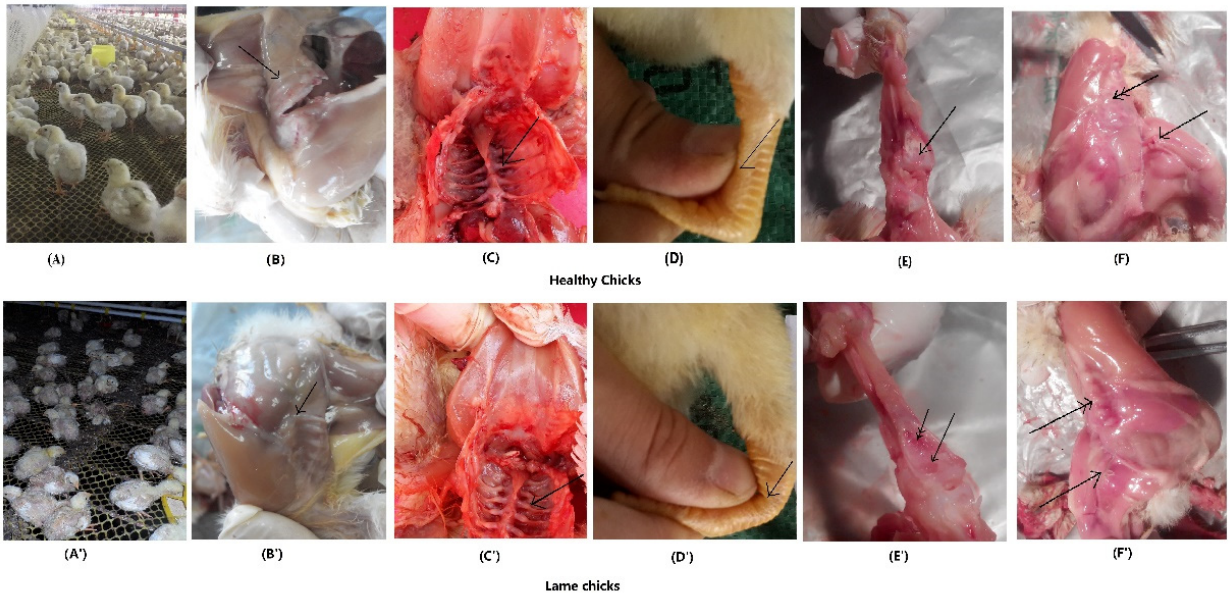

Figure 1. Rickets characteristics apparent in gait features and gross anatomy of 4–11 day-old broiler chicks caused by high-calcium (Ca): phosphorus (P) ratio, P-deficient (HCa:P) crumbled feed. Pictures show changes in walking characteristics (**A**/**A′**), ribs (**B**/**B′**), costochondral junctions (**C**/**C′**), metatarsus (**D**/**D′**), tibia proximal tibiotarsus (**E**/**E′**), and femur (**F**/**F′**).

The commercial feeds which caused the severe lameness in chicks (HCa:P feeds) contained higher (all $p < 0.01$) levels of Ca, Ca:P and Ca:NPP ratios, and lower ($p < 0.01$) levels of TP and NPP compared to the normal feeds that did not cause lameness issues (Table 3). We discovered that during the production of the defective feed, limestone was erroneously substituted for a proportion of the DP in the DP bin. Consistent with the feed processing error and low dietary P level, the concentration of serum P in severely lame chicks (gait score = 3) was only 38% of that of healthy chicks (gait score = 1) on day 9 ($p < 0.01$, Figure 2) in trial one. Low blood P concentration is an indication of hypophosphatemic rickets [8]. Consistent with our study, Li et al. [26] and Zhang et al. [10] reported that the serum P was decreased by dietary P deficiency. The primary reason for low serum P may be due to the high Ca:NPP ratio, as serum P levels were not affected when a balanced Ca:NPP ratio was maintained in a low-P diet [26].

Table 3. Calcium (Ca), phosphorus (P) levels and Ca:P ratios in commercial feeds from field research (air-dry basis).

Variables [1]	Defective Feeds from Commercial Farms [2]			Pooled SEM	*p* Values
	Normal Feeds	SHCa:P	HCa:P		
Ca, %	0.89 [a]	1.03 [b]	1.07 [b]	0.02	<0.01
TP, %	0.59 [a]	0.54 [b]	0.39 [c]	0.02	<0.01
Ca: TP	1.52 [a]	1.94 [b]	2.72 [c]	0.09	<0.01
NPP, %	0.41[a]	0.36 [b]	0.21 [c]	0.02	<0.01
Ca: NPP	2.19 [a]	2.99 [b]	5.03 [c]	0.21	<0.01

[1] TP: total P; NPP: non-phytate P. [2] Feed samples from commercial farms were classified into normal feeds (n = 19), high-Ca:P ratio, P-deficient (HCa:P, n = 10) feeds, and slightly high Ca:P (SHCa:P, n = 11) feeds, based on chicks fed these feeds being healthy, severely lame or slightly lame. The average moisture content of the feeds was 10.33%. Each sample value given is the mean of three measurements. [a–c] Means within a row without common superscripts differ significantly ($p < 0.01$).

Based on our studies in both commercial and the experimental farm, we confirmed that a high Ca:P ratio, P deficient crumbled feed was the cause of hypophosphatemic rickets identified in the broilers in this case, as attested by feed processing records, extremely

low dietary P levels in feeds and serum, high Ca:P ratios in feeds, and the poor gait characteristics (lameness) and bone development observed.

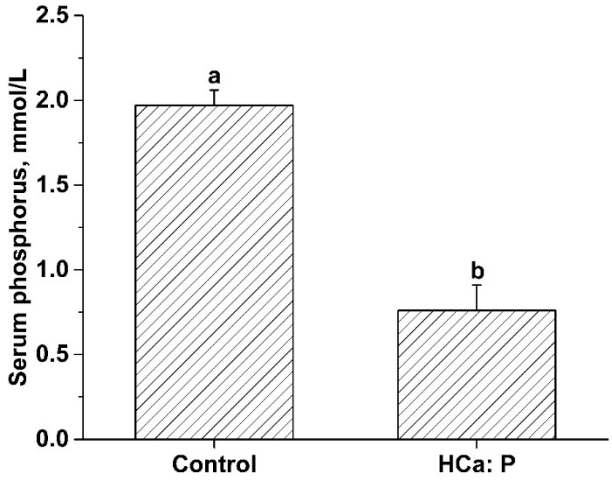

Figure 2. Effect of high-calcium (Ca): phosphorus (P) ratio and P-deficient crumble (HCa:P) feed on serum P of broiler chicks in trial one. Control, Ca and P-balanced feed which did not cause chick lameness. HCa:P, high Ca:P ratio, P-deficient crumbled feed from farms with severe chick lameness issues. $^{a–b}$ Means on the different columns without common superscript, differ significantly ($p \leq 0.01$). Data were showed in the form of means + SD.

3.2. Determination of the Duration of Onset of Hypophosphatemic Rickets

The changes in bone structure began to be affected by the feed as early as the 4th day in all trials, similar to the observations on the commercial farms. Low dietary NPP levels is a cause of bone abnormalities, leading to significantly decreased tibia ash, mineral, Ca and P content [5,24], and reduced bone density and bone strength in broilers [24]. Dietary P levels and the ratio of available P (AP) are important factors affecting bone mineralization, bone strength, gait score, and rickets in broiler chickens [14–18]. The rapidity of impact reflected the greater impact of a Ca:P imbalanced diet on tibia bone mineral density and ash content, and therefore, bone-breaking strength, compared with a Ca:P balanced, P-deficient diet [26]. This may be because a high dietary Ca:AP ratio causes the formation of insoluble Ca-P complexes in the intestines [27], reducing the true digestibility of Ca [17], increasing Ca excretion, and decreasing plasma P concentration [16], thus leading to poor tibial mineralization [10,11]. In our study, no consistent difference was observed in lesions of visceral organs (heart, liver, spleen, kidney, bursal, gizzard, gut, thymus, and abdominal fat) between healthy chicks and lame chicks. Data collected from our survey also showed that various drugs (antibiotics, antiviral drugs, Chinese herbal medicines, mycotoxin adsorbent, etc.) were used to treat this problem. However, no curative effect was observed. Thus, infectious disease was excluded from the factors that affect P digestion and absorption. As we did not make anatomical observations on day 3, whether significant differences would be observable at that point remains to be seen.

There was a strong correlation between lameness (gait score) and rickets based on our anatomical observations at commercial and experimental farms. From our survey on farmers, the duration of onset of rickets was 2.70 ± 0.69 day in chicks based on lameness (gait scores, 2 and 3) surveyed at ages of 6.93 ± 2.73 d. On commercial farms feeding HCa:P feeds, the average incidence of lameness was 74.11% after the 6th day. Lameness was not found in the control groups, whereas, it was observed in the HCa:P groups as early as day 4, day 3, and day 4 in trials one, two and three, respectively. The average lameness incidence in the HCa:P group rose to be about 71% and 79% on the 6th and 7th day in three trials; and reached 93% and 100% on the 8th and 9th day in trial one (Figure 3). Adding DP in HCa:P feed resulted in a lameness incidence between 0 to 11% in trials one and three. However, as we visually identified lameness, the possibility that some birds had

developed pathological changes but were nonetheless continuing to move normally cannot be excluded. We recommend that more sensitive methods, such as PCR, histochemical methods or immunohistochemical methods, be adopted in future for further study of the early course of rickets in chicks. Compared with commercial farms, the onset was later (4 or 3 days vs. 2.7 days) in all three trials. The higher stocking density of intensive commercial farms might represent another factor aggravating leg problems [28]. In addition, on the commercial farms, the temperatures were between 30 and 34 °C, and the humidity was 70–84%; these are high for 6- to 11-day-old birds and may have aggravated problems stemming from P deficiency syndrome. Based on gait score and lameness incidence in all trials, the duration of onset of hypophosphatemic rickets was as early as three days after commencement of feeding HCa:P diets.

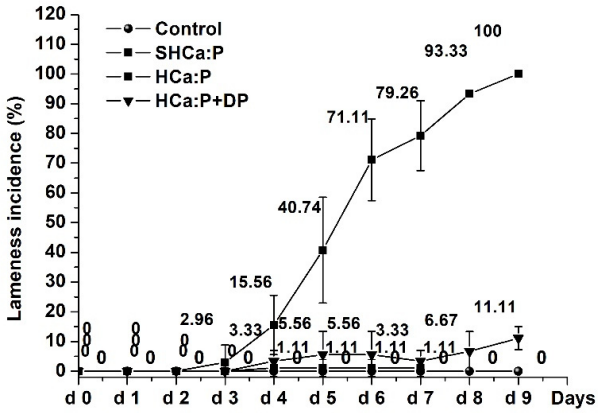

Figure 3. Lameness incidence in broiler chicks caused by high-calcium (Ca): phosphorus (P) ratio and P-deficient crumbled feeds in trials one to three (C). Control, Ca and P-balanced feed which did not cause chick lameness (n = 9 at days 0 to 7; n = 3 at days 8 and 9); HCa:P, high Ca:P ratio, P-deficient crumbled feed from farms with severe chick lameness issues (n = 9). SHCa:P, slightly high Ca:P ratio, P-deficient crumbled feed from farms with slight chick lameness issues (n = 6); HCa:P + DP, feed made by mixing 1.5% dicalcium phosphate (DP) powder into HCa:P crumbled feed (n = 6 at days 0 to 7; n = 3 at days 8 and 9). Lameness incidence is calculated from mixed data in trials one to three and given as mean ± SD.

Growth performance is one of the variables most sensitive to P deficiency, by comparison with Ca deficiency alone or simultaneous Ca and P deficiency [7,10]. Farmers responded that feed intake was lower than broiler performance objectives [29,30] at two days after feeding the P deficient commercial feed, and the growth rate was obviously lower since the third day. The BW, BWG, ADG, FI, and ADFI were decreased by HCa:P feed as early as day 3 in trial one ($p \leq 0.05$, Table 4), trial two ($p < 0.01$, Table 5) and trial three ($p < 0.01$, Table 6). This result is consistent with a previous study which demonstrated that both BW and BWG were sensitive to dietary Ca and P deficiency [4]. However, effects on the F:G ratio were not apparent until day 9 in trial one ($p = 0.05$), day 7 ($p = 0.03$) in trial two, and day 3 ($p \leq 0.01$) in trial three. Similarly, the onset time of lameness had a difference of one day among trials. The inconsistency may be due to different batches of chicks and the change of environmental conditions caused by different experiment times. Consistent with our result, F:G is not very sensitive to low dietary phosphorous by comparison with FI and ADG [11]. The BW and FI at day 3 worsened increasingly as the Ca:TP ratio increased from 1.41 to 2.69 and 3.08 in our trials. Consistent with our study, FI and BW or ADG reduced in broilers fed with low P [7,15] diets or both low P and high Ca:P ratio diet [7]. Adding DP improved the growth performance harmed by HCa:P feed in both trial one (e.g., $ADG_{day\ 0–9}$ and $ADFI_{day\ 3–9}$, all $p \leq 0.05$) and trial three (e.g., $ADG_{day\ 0–3}$, $ADFI_{day\ 0–3}$, F: G $day_{0–7}$, all $p \leq 0.01$). Data of growth performance suggested that the day of onset of P deficiency was three days, earlier than noted in the previous reports [4,11,15]. The earlier changes in FI and growth rates in our study may reflect the more severe lack of NPP (0.18–0.24%),

high Ca (about 1.23%) and high Ca:TP ratio (>2.69) resulted from a replacement of DP by limestone. Hu et al. suggested that a dietary Ca:TP ratio of 1.00–1.25 is optimal in terms of maximizing digestion of Ca and P while maintaining tibia strength and growth performance characteristics [18]. Based on growth performance in all trials, the duration of onset of hypophosphatemic rickets was as early as three days after commencement of feeding HCa:P diets.

Table 4. Effect of high-calcium (Ca): phosphorus (P) ratio, P-deficient (HCa:P) crumbled feed on growth performance in 0–9 day-old broiler chicks during trial one.

Variables [1]	Items [1]	Treatments [2]			Pooled SEM	*p* Value
		Control	HCa:P	HCa:P + DP		
BW, g/bird	$BW_{day\ 0}$	40.33	40.60	40.57	0.65	0.99
	$BW_{day\ 3}$	81.93 ^{a}	73.07 ^{b}	77.93 ^{ab}	1.52	0.03
	$BW_{day\ 9}$	181.37 ^{b}	164.23 ^{b}	182.90 ^{a}	3.29	<0.01
BWG, g/bird	$BWG_{day\ 0–3}$	41.60 ^{a}	32.47 ^{b}	37.37 ^{ab}	1.51	0.01
	$BWG_{day\ 3–9}$	99.43 ^{ab}	91.17 ^{b}	104.97 ^{a}	2.39	0.03
	$BWG_{day\ 0–9}$	141.03 ^{b}	123.63 ^{b}	142.33 ^{a}	3.13	<0.01
ADG, g/bird/d	$ADG_{day\ 0–3}$	13.87 ^{a}	10.82 ^{b}	12.46 ^{ab}	0.50	0.02
	$ADG_{d\ 3–9}$	16.57 ^{ab}	15.20 ^{b}	17.50 ^{a}	0.40	0.03
	$ADG_{day\ 0–9}$	15.67 ^{a}	13.74 ^{b}	15.81 ^{a}	0.35	<0.01
FI, g/bird	$FI_{day\ 0–3}$	45.57 ^{a}	39.30 ^{b}	44.43 ^{ab}	1.23	0.05
	$FI_{d\ 3–9}$	164.40 ^{a}	139.50 ^{b}	169.53 ^{a}	5.06	<0.01
	$FI_{d\ 0–9}$	209.83 ^{a}	178.73 ^{b}	213.40 ^{a}	6.11	<0.01
ADFI, g/bird/d	$ADFI_{day\ 0–3}$	15.19 ^{a}	13.10 ^{b}	14.81 ^{ab}	0.41	0.05
	$ADFI_{day\ 3–9}$	27.40 ^{a}	23.25 ^{b}	28.27 ^{a}	0.84	<0.01
	$ADFI_{day\ 0–9}$	23.32 ^{a}	19.86 ^{b}	23.71 ^{a}	0.68	<0.01
F:G	$F:G_{day\ 0–3}$	1.10	1.21	1.20	0.04	0.48
	$F:G_{day\ 3–9}$	1.65 ^{a}	1.53 ^{b}	1.61 ^{ab}	0.02	0.05
	$F:G_{day\ 0–9}$	1.49	1.45	1.50	0.02	0.47

[1] BW, body weight, BWG, body weight gain; ADG, average daily gain, FI, feed intake; ADFI, average daily feed intake. F:G, feed:gain ratio. [2] Control, Ca and P-balanced feed which did not cause chick lameness. HCa:P, high Ca:P ratio, P-deficient crumbled feed collected from farms whose chicks had severe lameness issues. SHCa:P, slightly high Ca:P ratio, P-deficient crumbled feed from farms whose chicks had slight lameness issues; HCa:P + DP, feed made by mixing 1.5% dicalcium phosphate (DP) powder into HCa:P crumbled feed. $^{a–b}$ Values within a row without common superscripts differ significantly ($p \leq 0.05$).

Table 5. Effect of high-calcium (Ca): phosphorus (P) ratio, P-deficient (HCa:P) crumbled feed on growth performance in 0- to 7- day-old broiler chicks during trial two.

Variables	Items [1]	Treatments			Pooled SEM	*p* Value
		Control	SHCa:P	HCa:P		
BW, g/bird	$BW_{day\ 0}$	42.33	42.67	42.7	0.09	0.19
	$BW_{day\ 3}$	91.13 ^{a}	84.20 ^{bc}	80.67 ^{c}	1.71	<0.01
	$BW_{day\ 7}$	194.60 ^{a}	171.27 ^{b}	142.37 ^{c}	7.65	<0.01
BWG, g/bird	$BWG_{day\ 0–3}$	48.80 ^{a}	41.53 ^{b}	37.97 ^{b}	1.75	<0.01
	$BWG_{day\ 3–7}$	102.87 ^{a}	87.07 ^{b}	61.70 ^{c}	6.29	<0.01
	$BWG_{day\ 0–7}$	102.87 ^{a}	87.07 ^{b}	61.7 ^{c}	6.30	<0.01
ADG, g/bird/d	$ADG_{day\ 0–3}$	16.27 ^{a}	13.84 ^{b}	12.66 ^{b}	0.58	<0.01
	$ADG_{day\ 3–7}$	14.70 ^{a}	12.44 ^{b}	8.81 ^{c}	0.90	<0.01
	$ADG_{day\ 0–7}$	11.43 ^{a}	9.67 ^{b}	6.86 ^{c}	0.70	<0.01
FI, g/bird	$FI_{day\ 0–3}$	45.70 ^{a}	41.03^{b}	38.17 ^{c}	1.12	<0.01
	$FI_{d\ 4–7}$	136.270^{a}	123.97 ^{b}	99.00 ^{c}	5.56	<0.01
	$FI_{d\ 0–7}$	181.63 ^{a}	165.00 ^{b}	137.13 ^{c}	6.58	<0.01
ADFI, g/bird/d	$ADFI_{day\ 0–3}$	15.23 ^{a}	13.68 ^{b}	12.72 ^{c}	0.37	<0.01
	$ADFI_{day\ 3–7}$	34.07 ^{a}	31.00 ^{b}	24.75 ^{c}	1.39	<0.01
	$ADFI_{day\ 0–7}$	25.95 ^{a}	23.57 ^{b}	19.59 ^{c}	0.94	<0.01

Table 5. *Cont.*

Variables	Items [1]	Treatments			Pooled SEM	*p* Value
		Control	SHCa:P	HCa:P		
F:G	$F:G_{day\ 0–3}$	0.94	0.99	1.01	0.02	0.32
	$F:G_{day\ 3–7}$	1.32	1.43	1.62	0.06	0.07
	$F:G_{day\ 0–7}$	1.20 [a]	1.29 [ab]	1.38 [b]	0.03	0.03

[1] Control, Ca and P-balanced feed which did not cause chick lameness. HCa:P, high Ca:P ratio, P-deficient crumbled feed collected from farms whose chicks had severe lameness issues. SHCa:P, slightly high Ca:P ratio, P-deficient crumbled feed from farms whose chicks had slight lameness issues; HCa:P + DP, feed made by mixing 1.5% dicalcium phosphate (DP) powder into HCa:P crumbled feed. [a–c] Values within a row without common superscripts differ significantly ($p \leq 0.05$).

Table 6. Effect of high-calcium (Ca): phosphorus (P) ratio, P-deficient (HCa:P) crumbled feed on growth performance in 0- to 7-day-old broiler chicks during trial three.

Variables [1]	Items	Treatments				Pooled SEM	*p* Value
		Control	SHCa:P	HCa:P	HCa:P + DP		
BW, g/bird	$BW_{day\ 0}$	42.13	42.3	42.7	42.47	0.09	0.16
	$BW_{day\ 3}$	89.67 [a]	89.63 [a]	81.33 [b]	89.47 [a]	1.14	<0.01
	$BW_{day\ 7}$	191.3 [a]	181.77 [b]	155.27 [c]	187.4 [a]	4.31	<0.01
BWG, g/bird	$BWG_{day\ 0–3}$	47.53 [a]	47.33 [a]	38.63 [b]	47.00 [a]	1.18	<0.01
	$BWG_{day\ 3–7}$	101.63 [a]	92.13 [c]	73.93 [d]	97.93 [b]	3.29	<0.01
	$BWG_{day\ 0–7}$	149.17 [a]	139.47 [b]	112.57 [c]	144.93 [ab]	4.36	<0.01
ADG, g/bird/d	$ADG_{day\ 0–3}$	15.84 [a]	15.78 [a]	12.88 [b]	15.67 [a]	0.39	<0.01
	$ADG_{day\ 3–7}$	25.41 [a]	23.03 [b]	18.48 [c]	24.48 [a]	0.49	<0.01
	$ADG_{day\ 0–7}$	21.31 [a]	19.92 [b]	16.08 [c]	20.71 [ab]	0.62	<0.01
FI, g/bird	$FI_{day\ 0–3}$	45.07 [a]	45.8 [a]	39.93 [b]	44.23 [a]	0.74	<0.01
	$FI_{day\ 3–7}$	138.4 [a]	125 [b]	105.1 [c]	130.53 [b]	5.06	<0.01
	$FI_{day\ 0–7}$	183.47 [a]	170.8 [b]	145.03 [c]	174.77 [b]	6.11	<0.01
ADFI, g/bird/d	$ADFI_{day\ 0–3}$	15.02 [a]	15.27 [a]	13.31 [b]	14.74 [a]	0.25	<0.01
	$ADFI_{day\ 3–7}$	34.6 [a]	31.25 [b]	26.28 [c]	32.63 [b]	0.95	<0.01
	$ADFI_{day\ 0–7}$	26.21 [a]	24.40 [b]	20.72 [c]	24.97 [b]	0.63	<0.01
F:G	$F:G_{day\ 0–3}$	0.97 [a]	0.97 [a]	1.03 [b]	0.94 [a]	0.01	0.01
	$F:G_{day\ 3–7}$	1.37	1.36	1.42	1.33	0.01	0.13
	$F:G_{day\ 0–7}$	1.23 [a]	1.22 [a]	1.29 [b]	1.21 [a]	0.01	<0.01

[1] Control, Ca and P-balanced feed which did not cause chick lameness. HCa:P, high Ca:P ratio, P-deficient crumbled feed collected from farms whose chicks had severe lameness issues. SHCa:P, slightly high Ca:P ratio, P-deficient crumbled feed from farms whose chicks had slight lameness issues; HCa:P + DP, feed made by mixing 1.5% dicalcium phosphate (DP) powder into HCa:P crumbled feed. [a–c] Values within a row without common superscripts differ significantly ($p \leq 0.05$).

This duration of onset of rickets was earlier than in the previous reports, which identified onset durations such as 7 [7,8], 7–10 [9], and 18 days [8]. One reason for this may be a difference in diagnosis methods: the authors of those reports based their judgements on the incidence of rickets on observations of anatomy and serum P rather than gait score changes or growth performance. In recent years, developments in poultry breeding have produced broilers with faster growth rates and higher nutrient requirements, which may contribute to more rapid symptom onset under conditions of P deficiency [26].

The dietary level of AP was 0.28–0.34% in feed (assuming a 0.1% improvement due to phytase). The minimal AP recommendation for broilers chicks is 0.45% [29] and 0.48% [30] of feed, respectively, by breeding companies. The level of dietary NPP (0.21% in feed) was much lower than the recommendation of 0.45 by NRC standard [31] or 0.39% by Liu et al. [24]. A Ca:AP ratio at 2.0 or Ca:NPP ratio at 2.22 is recommended for broiler chicks by breeding companies [29,30] or NRC [31]. In the present case, Ca:NPP ratios were over 2.22, at 4.71 and 6.17, respectively, in the SHCa:P and HCa:P feeds. Consistent with our observations, high Ca:TP ratios caused rickets in growing rheas in a case reported by Gröne et al. [3]. Conversely, when P concentration is low, a feed with a balanced Ca:P

ratio resulted in better growth performance and bone structure than feed with high Ca:TP ratios [7,15]. In future, the use of more accurate methods to determine phytic acid and other inositol phosphate contents will be necessary to provide more exact recommendations for safe dietary doses of NPP in broiler feeds [32]. The P is largely mobilized by the yolk sac membrane during 12–17 days of incubation, and its content is very low as the chicken's embryo prepares to hatch [33]. The P leftover in ducks' yolk sac at 3 days before the hatch is only about 13.1% of the first incubation day [34]. During fasting, newly hatched broilers had normal activities during the first 58 h, absorbing about 70% of the nutrients in the yolk sac, but the birds appear dull and lack vitality from the third day [35]. We observed that severe lame birds could not reach feed and water freely. The thirsty and starvation further aggravated rickets. Therefore, a shortage of P in the yolk sac, a dietary P deficiency and an imbalanced Ca:P ratio might be the main reasons for the early onset of hypophosphatemic rickets in chicks in our study.

Physiological reasons for the early onset of rickets may involve the following factors. An unbalanced Ca:P and P deficiency in feed led to a low P concentration in blood circulation (Figure 2), which in turn caused a poor development of bone structures (Figure 1B′–F′). The leg bones were too soft (Figure 1B′–F′) to support the standing and walking of birds, leading to early onset of rickets and high lameness incidence (Figure 3). The poorly developed bones, e.g., sternum (Figure 1C′) was soft and small, and not enough to support the attachment of heavy muscles, leading to poor growth performance (Tables 4–6). The P is a component of creatine phosphate and adenosine triphosphate (ATP). Depletion of high-energy phosphate may be one reason for the hypotonia of rickets [36]. Dietary P deficiency slows down the ATP synthesis in muscles, leading to myopathy at the early stage [37]. So, a lack of energy (e.g., ATP) may obstacle movement and muscle deposit in chicks. Phosphorus is also a critical component of nucleotides (DNA and RNA), phospholipids in membranes, and phosphorylated intermediates in cellular signaling [38]. Thus, P deficiency may lead to early rickets in broilers partly via impairing these molecules.

4. Conclusions

In conclusion, crumbled feed with a high Ca:P ratio and deficient in P can cause hypophosphatemic rickets in broiler chicks as early as 3 days. Lameness incidence rose to over 70% after chicks feed a high Ca:P ratio and P deficient feed for six days. Several variables, including the dietary P level and Ca:P ratio, and chicks' growth performance, the incidence of lameness, and bone development are helpful in the early diagnosis of hypophosphatemic rickets in broilers. The current multifactorial nature of diagnosis complicates this task, and in future, the development of more specific and sensitive single methods allowing early, rapid, and accurate diagnosis of hypophosphatemic rickets caused by high Ca:P ratios and P deficiency of various degrees, would be of great practical value to poultry breeders.

Author Contributions: Data collection, animal trials, L.X.; writing—original draft preparation, N.L. and L.X.; data analysis, X.W. and X.Z.; writing—review and editing, M.B.F. and Y.Z.F.; funding acquisition, H.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the earmarked fund for Jiangsu Agricultural Industry (Broiler) Technology System (JATS[2021]183), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and the 2018 Deputy General Manager Program for Science and Technology of Jiangsu Province's "innovation and entrepreneurship plan" (FZ20180271).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Experimental Animal. The animal trials were approved by the Animal Care and Use Committee of Yangzhou University (protocol code YZUDWSY 2017-05-09.03, approved on 9 May 2017).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available on reasonable request from the corresponding author.

Acknowledgments: Special thanks are given to Lu S., Shi W., Liu H., Liu G., Li Z., and Yan W for their support and assistance. The authors are grateful to the faculties from QA, QC, feed production, and R and Day in the anonymous company for their help in the study. The authors thank the A.W.E.S.O.M.E. faculty group in the College of Agriculture and Life Sciences at Texas A and M University for assistance with editing the manuscript. Initial group financial support was through NSF ADVANCE Institutional Transformation Award 1008385, with continued support from the college.

Conflicts of Interest: The authors declare no conflict of interest.

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