Effects of reducing growth rate via diet dilution on bone mineralization, performance and carcass yield of coccidia-infected broilers

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ABSTRACT Coccidiosis and rapid growth rate (GR) compromise bone mineralization in modern broilers. We tested the hypothesis that reducing GR via diet dilution during peak bone development will improve bone mineralization in both infected and uninfected broilers. A total of 384 male Ross 308 chicks were allocated to a basal grower diet (3,107 kcal/kg ME and 19.4% CP) diluted with 0, 5, 10, or 15% lignocellulose (n = 12 pens/treatment, 8 birds/pen) at day 10 of age. Prior to this, birds in each group received half the intended diet-dilution levels (day 8 to 10 of age) and a common starter diet (day 1 to 7 of age). At day 13 of age (day 0 post-infection, pi), birds were orally inoculated with either 7,000 sporulated *Eimeria maxima* oocysts (I) or water (C), forming a 4 diet-dilution level × 2 infection status factorial experiment. Performance was measured over 12 days pi and scaled to BW at infection (day 0 pi) to account for *a priori* BW differences. At day 12 pi (day 25 of age), 1 bird/pen (a total of 6 birds/treatment) was sampled to assess tibia and femur mineralization relative to BW, and carcass yield. There was no interaction ($P > 0.05$) between infection status and diet-dilution level on ADFI/BW measured over day 1 to 12 pi, or on any bone variable. ADG/BW pi decreased ($P < 0.01$) with diet dilution amongst C birds, but was statistically similar ($P > 0.05$) amongst I birds. I compared to C birds had reduced breast meat ($P < 0.05$) and eviscerated carcass yield ($P < 0.01$), femur ($P < 0.05$) and tibia ($P < 0.01$) breaking strength (BS), and femur ash weight (AW) ($P < 0.05$). Diet dilution did not affect carcass yield, but improved femur BS ($P < 0.001$), and tended to improve ($P < 0.1$) femur and tibia AW. Overall, diet dilution significantly affected femur, more than tibia, variables: relative BS, robusticity index, and ash percentage. Reducing GR affected broiler long bone mineralization to a similar degree in the presence or absence of coccidiosis.

Key words: broiler, coccidiosis, diet dilution, growth rate, bone mineralization

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INTRODUCTION

Intensive genetic selection for performance in modern broilers has increased growth rate (**GR**), such that the time required to reach 2 kg BW reduced by 3 wks between the 1950s and 2014 (Tallentire et al., 2016), but at the same time appears to have compromised skeletal development and integrity (Julian, 1998; Pratt and Cooper, 2018). Rapid GR causes rapid periosteal bone deposition, impaired mineralization, altered biomechanical properties, and radial vascular canal orientation, which increases porosity of the cortical bone (Williams et al., 2004; Pratt and Cooper, 2018). Skeletal problems, particularly those affecting leg bones, are associated with chronic pain in broilers that negatively affect their welfare (Julian, 1998; Danbury et al., 2000; Dibner et al., 2007). Moreover, their increased prevalence causes substantial financial losses due to lameness, which limits bird access to feed (Knowles et al., 2008) and increased mortality, and culling rates due to leg fractures and vertebral column abnormalities, as well as increased carcass condemnations caused by muscle haemorrhage and the presence of bone fragments in meat portions (Driver et al., 2006; Knowles et al., 2008; Pines and Reshef, 2015).

Broilers with slow compared to fast GR have lower incidence of leg disorders (Corr et al., 2003; Caplen et al., 2012; Kapell et al., 2012), better adaptation to increased mechanical load (Pitsillides et al., 1999), higher bone ash, and reduced occurrence of cortical

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bone porosity (Leterrier and Nys, 1992; Williams et al., 2004; Pratt and Cooper, 2018). Sakkas et al. (2018) reported a similar bone ash percentage (**AP**), but a higher amount of ash in proportion to body weight (**BW**) and improved femur strength for the slower growing of 2 broiler lines divergently selected for GR from the same maternal stock. Generally, studies suggest that the axial bone of modern-day fast-growing broilers remains under development throughout its lifetime, never reaching the stage of homeostasis and remodeling as seen in other vertebrates (Rath et al., 2000; Dibner et al., 2007), and factors including infectious agents, flock management system, and nutrition affect healthy bone development (reviewed in Kierończyk et al. 2017).

Bone development in broilers peaks during the first 3 wks of life (Lilburn, 1994; Williams et al., 2000). Reducing GR during this period may facilitate bone development in fast-growing broilers. GR reduction via feed restriction has been shown to improve bone mineralization and skeletal development, and impact positively on the bone quality of healthy broilers (Pratt and Cooper, 2018). Quantitative feed restriction as a means of delaying GR achieved lower bone porosity and higher mineralization (Williams et al., 2004). However, this methodology raises welfare concerns due to the association of chronic hunger with prolonged restrictions (Kyriazakis and Tolkamp, 2018). Another effective method of GR reduction is the use of qualitative feed restriction which involves diluting the diet with an inert ingredient or with feed ingredients of low nutritional value (Rezaei and Hajati, 2010; Atapattu and Silva, 2016; Xu et al., 2017). This method reduces the adverse effects of starvation or chronic hunger on broiler welfare that are associated with the quantitative restriction method.

Coccidiosis penalizes bone mineralization by limiting intestinal absorption and utilization of vital bone minerals (Turk, 1973, 1978; Sakkas et al., 2018), as well as increasing bone resorption in broilers (Akbari Moghaddam Kakhki et al., 2018). Our recent study showed that significant effects of malabsorptive coccidiosis on bone mineralization are delayed until the later stages (beyond 12 D) of infection (Sakkas et al., 2018). This suggests that previous studies may have underestimated the adverse effect of coccidiosis on broiler bone mineralization as they typically assess bone mineralization within the first 6 D post-infection (**pi)** when productive performance is grossly impaired (Blake and Tomley, 2014).

In the present study, we aimed to investigate whether a diet dilution-induced reduction in early GR (during weeks 2 and 3 of age), would improve bone mineralization of broilers in the presence, as in the absence, of coccidiosis. We diluted grower diets with lignocellulose, an inert substance with high water-holding capacity (**WHC**) and no added nutritive value to the feed, to limit the nutrient intake and consequently ADG. The hypothesis was that GR reduction would improve parameters of bone mineralization in broilers infected with coccidiosis as in their uninfected counterparts.

MATERIALS AND METHODS

Animals and Husbandry

A total of 384 male Ross 308 chicks were raised from hatch until 25 days old. They were housed in 48 rectangular 0.84 m^2 pens situated in a thermostatically controlled building. Each pen was equipped with a tube feeder and a bell drinker offering birds *ad libitum* access to feed and water throughout the experimental trial. Wood shavings to a depth of 5 cm were used as litter. Routine husbandry procedures were carried out. All experimental procedures complied with the UK Animals (Scientific Procedures) Act 1986, were approved by Newcastle University Animal Welfare and Ethical Review Body (AWERB), and carried out under Home Office authorization (P441ADF04).

Experimental Design, Diets, and Inoculation

The experiment had a 4 × 2 factorial arrangement with 4 feeding treatments and 2 infection statuses. Upon arrival, broilers were allocated to a conventional starter diet until 7 D of age. A basal grower diet with 3,107 kcal/kg ME and 19.4% CP was diluted at graded levels, i.e., 0, 5, 10, or 15%, of Arbocel RC Fine lignocellulose (JRS PHARMA, Rosenberg, Germany) to formulate 4 experimental diets (R0, R1, R2, and R3, respectively); R0 (undiluted) being the basal feed (Table 1). The energy to nutrient ratios of the diets were maintained due to the inert nature of the diluent. Arbocel is a natural lignocellulose produced from fresh spruce trees (*Picea* species) by JRS PHARMA and is characterized by its high WHC. It was used as the feed diluent because of its inert nature and high WHC, which was expected to cause a reduction in feed intake and hence GR at the levels of inclusion in the diet. An adaptation period was incorporated from day 8 to 10 of age, during which chicks within each diet group were assigned to half of the predetermined dilution level, before allocating them to the dietary treatments at 10 D of age. All diets were offered as a coarse mash passed through a 5 mm screen. At 13 D of age (day 0 pi), birds were orally inoculated with a single 0.5 mL dose of water containing either 0 (control; **C**) or 7,000 (infected; **I**) sporulated *Eimeria maxima* oocysts of the Weybridge laboratory reference strain, using 1 mL syringes. The inocula were prepared at the Royal Veterinary College, University of London, using a previously described method (Pastor-Fernández et al., 2018). Each treatment group was replicated in 6 pens.

Sampling

Performance. Birds and feed weight were measured at days 0, 6, and 12 pi to evaluate the growth performance of birds during the early (day 1 to 6 pi) and the late (day 7 to 12 pi) stages of infection, and the entire period (day 1 to 12 pi) of infection.

Table 1. Ingredients and chemical composition (%) of the starter (day 0 to 7) and grower (day 8 to 25) diets offered to birds.

Ingredients (%)	Starter Basal	Grower Basal (R0)	Low lignocellulose (R1)	Medium lignocellulose (R2)	High lignocellulose (R3)
Ground maize	10.0	10.0	9.5	9.0	8.5
Ground wheat	51.5	53.9	51.2	48.5	45.8
Soybean meal (48% CP)	26.0	23.0	21.8	20.7	19.5
Arbocel (lignocellulose)	-	-	5	10	15
Full fat soya	5.0	5.0	4.75	4.5	4.25
Limestone	1.25	1.25	1.19	1.13	1.06
L-Lysine HCL	0.4	0.3	0.29	0.27	0.26
DL-Methionine	0.4	0.35	0.33	0.32	0.3
L-Threonine	0.15	0.15	0.14	0.14	0.13
Soya oil	3.00	3.50	3.33	3.15	2.98
Monocalcium phosphate	1.50	1.25	1.19	1.13	1.06
Salt	0.25	0.25	0.24	0.23	0.21
Sodium bicarbonate	0.15	0.15	0.14	0.14	0.13
Premix	0.4	0.4	0.4	0.4	0.4
Titanium dioxide	–	0.5	0.5	0.5	0.5
Nutrient composition (%)[1]					
ME (kcal/kg) (calculated)	3,059	3,107	2,940	2,796	2,629
Crude protein	21.4	19.4	18.5	17.6	16.9
Crude fiber	2.9	2.3	4.5	7.2	10.7
Ether extract (oil A)	5.55	6.59	6.13	5.79	5.53
Total oil (oil B)	–	7.32	6.86	6.51	6.30
Acid detergent fiber	–	3.25	5.61	7.96	11.60
Neutral detergent fiber	–	8.3	11.5	15.7	21.9
Acid detergent Lignin	–	0.48	1.39	2.45	3.23

[1]Analyzed nutrient composition (%) unless otherwise stated.

The nutrient composition was in accordance with Aviagen nutrient specifications (Aviagen, 2014), but the 4 grower diets contained 0, 5, 10, and 15% lignocellulose supplemented at the expense of wheat and soybean meal.

Fecal Sampling for Oocysts Count. Polyethylene sheets were placed over the wood shavings of each pen for 90 min daily from day 4 to 10 pi to obtain excreta samples for enumerating coccidia oocysts per gram (**OPG**). After that, approximately 10 g of pooled feces from each pen was collected in screw cap pots and stored at 4°C pending OPG determination.

Carcass. One bird per pen weighing close to the pen average was selected for carcass yield evaluation at day 12 pi. Birds were euthanized with a lethal injection of sodium pentobarbitone (Euthatal, Merial Harlow, United Kingdom), and then eviscerated. The weight of the eviscerated carcass and portions including breast meat, wings, thigh, and drumstick were measured using a digital scale. Following carcass evaluation, the right femur and tibia were removed, defleshed, and stored in airtight individually labeled polyethylene bags at −20°C pending evaluation.

Sample Analysis

Fecal OPG Count. Fecal OPG was estimated using the modified McMaster technique (Kaufmann, 2013). After thorough mixing of feces, a 3 g sample was removed for OPG determination and the remaining fecal material was freeze-dried to estimate dry matter (**DM**) content. Sampled material for OPG determination was mixed with 42 mL of water and then passed through a sieve. The suspension formed was transferred to a glass tube, centrifuged at 1,500 rpm for 2 min at room temperature, the supernatant was carefully siphoned off, and then the underlying pellet was vortexed until resuspended. After that, 10 mL of saturated sodium chloride solution was added, the suspension was mixed thoroughly, and a sample taken from the center of the tube was carefully transferred to the chambers of a McMaster counting slide. Slides were left to stand for 10 min to allow the oocysts to rise to the top of the slide, before being read at ×100 magnification. Values obtained were further expressed per unit gram of fecal DM content to obtain "OPG fecal DM."

Bone Assay. Bones were thawed at 4°C overnight and placed at room temperature for 1, h before further defleshing of adhering soft tissues. Length and width, at the center of the diaphysis, were measured for tibia and femur using a digital caliper, and then the weight of each bone was measured using an analytical balance. The bones were subjected to a 3-point break test using an Instron testing machine (Instron 3340 Series, Single Column-Bluehill, Norwood) to determine breaking strength (**BS**) in Newtons (**N**). The testing support consisted of an adjustable 2-point block jig, spaced at 30 mm for both tibia and femur bones. The crosshead descended at 30 mm/min until a break was determined by measuring a reduction in force of at least 5%. After BS assessment, tibia and femur ash weight (**AW**, g) and AP were determined using a previously described method (Oikeh et al., 2019).

Calculations and Statistics

All statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC). The pen was used as the experimental unit for all statistical assessments

except post-mortem evaluation, which was conducted using 1 bird per pen (6 birds/treatment combination). Values obtained for ADFI (g/d) and ADG (g/d) were expressed relative to BW (g) at infection to account for *a priori* differences in BW between groups. Carcass yield (%) was obtained by expressing the weight of eviscerated carcass and portions including breast meat and thigh plus drumstick as a percentage of live BW at dissection (day 12 pi). Breast meat and thigh plus drumstick were further expressed as a percentage of eviscerated carcass weight to obtain part yield. Tibia and femur BS, length and width (mm), and AW measured at day 12 pi were expressed in proportion to BW at dissection. Robusticity index and AP were calculated for both long bones using the prescribed formula (Riesenfeld, 1972). Pen relative performance and bone data generated from sampled birds were analyzed with dietary treatment and infection status as fixed factors using a general linear model. Fecal OPG data was further analyzed using the repeated measures mixed procedure (PROC MIXED). The model contained diet and day as the factors and the 2-way interaction between diet and day. Linear and quadratic responses to diet dilution were determined using orthogonal polynomial contrasts for all variables. Treatment means were compared by the Tukey's multiple comparison test, which maintains the desired alpha levels provided the model assumptions such as normality and homogeneity of residuals are met. For assessing the normality of the studentized residuals, the Shapiro–Wilk test was used, and non-normalized data, such as OPG were log-transformed. Significance was determined at $P < 0.05$ and tendency at $0.05 < P < 0.1$. Furthermore, allometric scaling relationships between tibia (Y) length, width, weight, and AW, and their corresponding measurements for femur (X) bone were determined for broilers in all the treatment groups, using reduced major axis linear regression. This method accounts for variations in both X and Y axes (Rayner, 1985; Sokal and Rolf, 1995). All regression analyses were performed on natural log-transformed data to establish the allometric equation:

$$\text{LogY (tibia variable 1)} = \text{Loga} + \text{b LogX(femur variable 1)}$$

Then the difference between slope (b) derived from R0 vs. R1, 2 or 3 treatments was assessed separately based on infection status (i.e., control and infected) for each variable using the prescribed formula (Andrade and Estévez-Pérez, 2014). The difference between slopes derived from R0 in control vs. R0 to 3 in infected broilers for length, width, weight, and AW were also calculated using the prescribed formula:

$$\frac{\text{b1} - \text{b2}}{\sqrt{\text{SEb1}^2 + \text{SEb2}^2}}$$

where b1 and b2 represent the individual slopes, and SEb1 and SEb2 were the respective standard errors of the slopes.

RESULTS

Performance Variables

Results on growth performance are presented for the early, late, and entire periods pi (Tables 2 and 3).

BW Pre-Infection. Broilers had similar BW ($P > 0.05$) at the beginning of the adaptation period, day 8 of age (data not shown). Diet dilution led to a reduction ($P < 0.05$) in the BW of R1, 2, and 3 compared to R0 broilers by approximately 7, 9, and 15%, respectively (estimated from the main effect of diet on BW day 0 pi, Table 2) at the point of infection, day 13 of age.

Relative BW, ADG, and ADFI Post-Infection. There was a significant interaction ($P < 0.05$) between diet and infection status for ADFI relative to BW (ADFI/BW) during the late stages of infection, relative BW at day 12 pi, and ADG relative to BW (ADG/BW) during the early, late, and entire periods pi. During the late period of infection, the diluted diets (R1 to 3) had statistically similar effects ($P > 0.05$) on ADFI/BW of C and I birds, while the I compared to the C birds receiving undiluted, R0, diet had a lower ADFI/BW ($P < 0.05$) (Table 3). On the other hand, relative BW at day 12 pi and ADG/BW reduced ($P < 0.05$) with increasing diet dilution amongst C birds, while values were statistically similar ($P > 0.05$) amongst the I birds.

Performance at any time point was significantly impaired ($P < 0.001$) by infection. Diet dilution affected ADFI/BW only during the early stage of infection ($P < 0.05$); R3 compared to R0 broilers had significantly higher ADFI/BW, while intake levels relative to BW were statistically similar for R0 to 2, as well as R2 and 3 broilers (Table 3). Furthermore, diet dilution reduced ($P < 0.05$) BW at day 6 and 12 pi and relative BW at day 12 pi followed a statistically linear reduction ($P < 0.001$). The effect of diet dilution on ADG/BW was statistically significant ($P < 0.05$) during the early, late, and entire periods pi. Birds receiving the R3 compared to the R0 diet had significantly reduced ADG/BW while R1, R2, and R3 birds had statistically similar ADG/BW.

Oocyst Excretion

The effects of diet dilution on OPG are presented in Figure 1. There were no oocysts detected in the control pens. The repeated measurements analysis on daily oocyst excretion revealed no significant interaction ($P > 0.05$) between diet and day pi for OPG fecal DM output. Diet dilution did not affect ($P > 0.05$) excreted oocysts calculated per gram of fecal DM content from day 4 to 10 pi (Figure 1). However, there was a linear decrease ($P < 0.01$) in oocyst output with increasing levels of diet dilution for OPG fecal DM at days 7, 8, and 9 pi. Day affected ($P < 0.05$) OPG fecal DM (4.62 vs. 5.24 vs. 4.37 vs. 3.55, respectively; SEM = 0.0973) for day 6 to 9 pi; values were highest at day 7 pi compared to the other days pi.

Table 2. Effect of diet diluted with 0, 5, 10, or 15% lignocellulose and infection status in broiler chicken orally inoculated with 0 (control) or 7,000 sporulated oocysts of *E. maxima* (infected) at day 13 post hatch on body weight and relative body weight at infection (day 0 pi), and during the early (day 6 pi) and late (day 12 pi) phases post-infection (pi).

		Body weight (g)			Relative body weight (g/g)	
Infection × diet		Day 0 pi	Day 6 pi	Day 12 pi	(Day 6/day 0)pi	(Day 12/day 0)pi
Control	R0	351	707	1178	2.04	3.47[a]
	R1	340	625	982	1.97	3.27[a,b]
	R2	326	654	1063	1.95	3.17[a-c]
	R3	302	555	927	1.89	2.96[b-d]
Infected	R0	356	617	979	1.80	2.90[c,d]
	R1	317	568	877	1.78	3.03[b-d]
	R2	330	565	868	1.78	2.81[d]
	R3	297	525	804	1.81	2.81[d]
SEM		13.7	24.5	36.5	0.029	0.068
Main effect						
Infection						
Control		330	636	1022	1.96	3.22
Infected		325	569	898	1.80	2.89
SEM		6.8	12.2	18.2	0.015	0.034
Diet						
R0		354[a]	666[a]	1080[a]	1.92	3.18
R1		329[a,b]	605[a,b]	995[a,b]	1.87	3.15
R2		328[a,b]	592[b]	928[b,c]	1.87	2.99
R3		300[b]	545[b]	836[c]	1.85	2.89
SEM		9.7	17.3	25.8	0.021	0.048
		Probabilities				
Diet × infection		0.682	0.579	0.329	0.096	**0.024**
Infection		0.626	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Diet		**0.004**	**<0.001**	**<0.001**	0.123	**<0.001**
Diet (linear)		**<0.001**	**<0.001**	**<0.001**	0.489	**<0.001**
Diet (quadratic)		**0.025**	**<0.001**	**<0.001**	0.982	0.885

[a-d]Means in a column with different superscript differ significantly ($P < 0.05$)
R0, 1, 2, and 3 represent 0, 5, 10, and 15% lignocellulose-diluted diets, respectively.
Relative BW is BW divided by BW at infection.

Carcass Evaluation

The main effects of diet dilution and infection status on carcass variables are presented in Table 4. There was no interaction between diet and infection status for carcass variables measured in this study. Infection reduced ($P < 0.001$) relative eviscerated carcass and breast meat yield, but did not affect ($P > 0.05$) relative thigh plus drumstick yield, breast meat, and thigh plus drumstick part yield at day 12 pi. Diet dilution did not affect ($P > 0.05$) relative eviscerated carcass, breast, and thigh plus drumstick yields at day 12 pi. There was no significant effect ($P > 0.05$) of diet dilution on breast meat and thigh plus drumstick part yield, i.e., when expressed as a percentage of eviscerated carcass weight. Furthermore, increasing diet dilution caused a linear decrease in all carcass variables measured at day 12 pi, while the reduction in relative eviscerated carcass yield followed both a quadratic and linear pattern (Table 4).

Bone Evaluation

The main effect of diet dilution and infection status on long bone variables at day 12 pi are presented in Table 5. There were no interactions ($P > 0.05$) between diet and infection for tibia or femur bone variables measured.

Relative Linear Growth of Long Bone and Robusticity Index. Infection increased ($P < 0.05$) tibia length and femur width expressed as a proportion of BW at dissection, an artifact of the reduced BW pi. Diet dilution caused a significant linear increase ($P < 0.05$) in tibia and femur length and width. Femur robusticity index of R3 and R2 compared to R0 broilers was higher ($P < 0.05$). Femur, but not tibia, robusticity increased linearly ($P < 0.05$) with an increase in dilution level. Other effects were not significant ($P > 0.05$).

Breaking Strength in Proportion to BW. Infection significantly reduced ($P < 0.05$) femur and tibia BS. On the other hand, diet dilution increased ($P < 0.001$) femur, but not tibia BS for R3 in comparison to R0 and R1 birds (Table 5). Also, increasing diet dilution level caused a quadratic increase ($P < 0.001$) in femur and tibia BS relative to BW; R0 broilers had the lowest value of 17.0 N/g.

Ash Percentage and Ash in Proportion to BW. Infection did not affect ($P > 0.05$) femur and tibia AP, and tibia AW in proportion to BW (**ash/BW**) at day 12 pi. However, it significantly reduced ($P < 0.05$) femur ash/BW. Diet dilution tended to increase

Table 3. Effect of diet diluted with 0, 5, 10, or 15% lignocellulose and infection status in broiler chicken orally inoculated with 0 (control) or 7,000 sporulated oocysts of *E. maxima* (Infected) at day 13 post hatch on average daily gain (ADG) and average daily feed intake (ADFI) post-infection (pi).

		Early stage (day 1–6 pi)		Late stage (day 7–12 pi)		Post-infection (day 1–12 pi)	
Infection × diet	Diet	ADG (g/d)/ BW day 0 pi (g)	ADFI (g/d)/ BW day 0 pi (g)	ADG (g/d)/ BW day 0 pi (g)	ADFI (g/d)/ BW day 0 pi (g)	ADG (g/d)/ BW day 0 pi (g)	ADFI (g/d)/ BW day 0 pi (g)
Control	R0	0.170[a]	0.223	0.225[a]	0.329[a]	0.197[a]	0.276
	R1	0.149[b]	0.226	0.198[a,b]	0.318[a,b]	0.176[b]	0.272
	R2	0.149[b]	0.236	0.187[b,c]	0.321[a,b]	0.168[b,c]	0.278
	R3	0.145[b,c]	0.246	0.172[b-d]	0.317[a,b]	0.159[b-d]	0.281
Infected	R0	0.126[d]	0.182	0.170[b-d]	0.266[c]	0.148[c,d]	0.224
	R1	0.124[d]	0.189	0.181[b-d]	0.298[a-c]	0.152[c,d]	0.244
	R2	0.120[d]	0.194	0.159[c,d]	0.282[b,c]	0.139[d]	0.238
	R3	0.128[c,d]	0.211	0.153[d]	0.308[a-c]	0.140[d]	0.259
SEM		0.0038	0.0060	0.0063	0.0096	0.0044	0.0073
Main effect							
Infection							
Control		0.153	0.233	0.195	0.321	0.175	0.277
Infected		0.124	0.194	0.166	0.288	0.145	0.241
SEM		0.0019	0.0030	0.0031	0.0096	0.0022	0.0037
Diet							
R0		0.148	0.202[b]	0.197	0.298	0.173	0.250
R1		0.137	0.208[b]	0.189	0.307	0.164	0.258
R2		0.134	0.215[a,b]	0.173	0.302	0.153	0.258
R3		0.136	0.229[a]	0.163	0.312	0.149	0.270
SEM		0.0027	0.0043	0.0044	0.0068	0.0031	0.0052
				Probabilities			
Diet × infection		**0.004**	0.941	**0.019**	**0.043**	**0.007**	0.198
Infection		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Diet		**0.011**	**0.001**	**<0.001**	0.468	**<0.001**	0.066
Diet (linear)		0.929	0.646	0.289	0.966	0.576	0.882
Diet (quadratic)		0.961	0.995	0.999	0.999	0.999	0.999

Values expressed in proportion to body weight at infection (BW day 0 pi).
[a-d]Means in a column with different superscript differ significantly ($P < 0.05$).
R0, 1, 2, and 3 represent 0, 5, 10, and 15% lignocellulose-diluted diets, respectively.

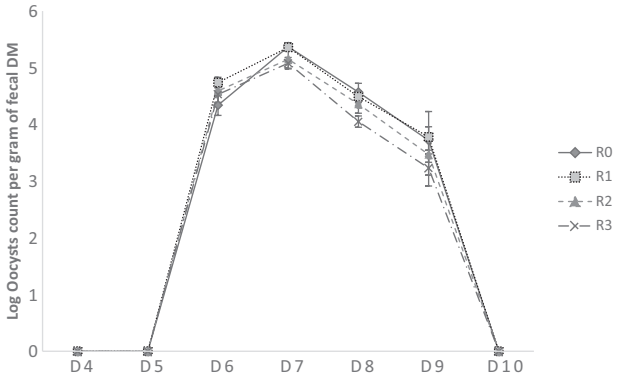

Figure 1. Effects of diet diluted with 0, 5, 10, or 15% lignocellulose in broiler chickens orally inoculated with 7,000 sporulated oocysts of *E. maxima* at day 13 post-hatch on excreted oocysts per gram of dry fecal matter from day 4 to 10 post-infection (pi). R0, 1, 2, and 3 represent 0, 5, 10, and 15% lignocellulose-diluted diets, respectively. Statistical analysis was conducted using the repeated measures mixed procedure with diet and day as the factors and their 2-way interaction.

($P < 0.1$) femur and tibia ash/BW, and femur AP for R3 in comparison to R0–2 birds (Table 5). Tibia AP was not affected by diet dilution ($P > 0.1$), while tibia and femur AP increased in a quadratic manner with diet dilution level in this study.

Allometric Scaling of Tibia vs. Femur. Results of differences between the slopes derived from linear regressions of the tibia (Y) vs. femur (X), (i.e., comparing the dietary treatments) are shown for length, width, weight, and AW in the appendix. There were no significant differences ($P > 0.05$) between the slope for R0 vs. R1, 2, or 3 amongst the control or the infected birds. Also, the difference between the slope for R0 in control vs. R0, 1, 2, or 3 in the infected broilers was not statistically significant ($P > 0.05$) for the variables above (see appendix).

DISCUSSION

In the present study, we utilized lignocellulose-diluted grower diets to reduce the GR of a widely used rapid-growing broiler genotype during the second and third weeks of life, at peak bone development (Williams et al., 2000). We expected that slowing down early GR during the above period would impact positively on bone development of uninfected broilers (Knowles et al., 2008; Shim et al., 2012; Pratt and Cooper, 2018) and coccidia-infected birds. We anticipated significant improvement in BS, AW, and AP, i.e., markers of bone mineralization, amongst control broilers receiving the diluted (R1 to 3) compared to the undiluted (R0) feed.

Table 4. Main effects of diet diluted with 0, 5, 10, or 15% lignocellulose and infection status in broiler chicken orally inoculated with 0 (control) or 7,000 sporulated oocysts of *E. maxima* (infected) at day 13 post-hatch, on carcass yield (%) at day 12 post-infection (pi).

	Eviscerated/ BW (%)	Breast muscle/ BW (%)	Thigh + drumstick /BW (%)	Breast muscle/ eviscerated (%)	Thigh + drumstick/ eviscerated (%)
Main effect					
Infection					
Control	63.1	20.6	17.8	32.6	28.1
Infected	61.3	19.4	17.4	31.7	28.4
SEM	0.38	0.33	0.18	0.44	0.32
Diet					
R0	62.6	20.7	17.8	33.0	28.4
R1	62.5	20.3	17.9	32.5	28.5
R2	62.4	19.8	17.4	31.7	27.9
R3	61.3	19.3	17.2	31.5	28.1
SEM	0.54	0.46	0.26	0.63	0.45
			Probabilities		
Infection	**0.001**	**0.015**	0.178	0.149	0.547
Diet	0.268	0.201	0.279	0.330	0.723
Diet (linear)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.017**
Diet (quadratic)	**0.003**	0.989	0.376	0.658	0.999

Eviscerated/BW = percentage of the eviscerated carcass to live body weight; breast muscle/BW = percentage of breast muscle weight to live body weight; thigh + drumstick/BW = percentage of thigh plus drumstick weight to live body weight; breast muscle/eviscerated = percentage of breast muscle weight to eviscerated carcass weight; thigh + drumstick/eviscerated = percentage of thigh plus drumstick weight to eviscerated carcass weight.

R0, 1, 2, and 3 represent 0, 5, 10, and 15% lignocellulose-diluted diets, respectively.

Genetic selection for reduced GR improved bone mineralization in our previous study, with effects persisting in the presence of infection (Sakkas et al., 2018). Hence, we expected a similar result amongst broilers in the case of an artificial diet-induced reduction in GR.

We measured oocyst excretion solely to establish the occurrence of *E. maxima* infection in this study. However, the OPG results suggested a linear reduction in *E. maxima* parasite burden at increasing levels of diet dilutions from day 7 to 9 pi. This inverse relationship between parasite burden and diet dilution persisted after accounting for the water content, which also increased with diet dilution, in the droppings of infected birds. The reduction in OPG at increasing levels of dilution may be ascribed to the increasing bulkiness of the feed and the indigestible nature of the diluent, which resulted in greater fecal excretion.

The observed effects of coccidiosis on productive performance were in line with our previous study (Sakkas et al., 2018). Also, the reduction in GR induced by the diluted diets was as expected pre-infection, i.e., from day 8 to 13 of age. The diluent, lignocellulose, only contributed to bulkiness, but not to the nutritive value of the diet (Zeitz et al., 2018). We could not achieve graded diet-induced GR amongst the infected birds over the infection period (day 1 to 12 pi), as we did for the control birds. This was because of the differences in the magnitude of anorexia in the infected broilers (Table 3).

Anorexia was previously hypothesized to be a host defense or disease-coping strategy (Kyriazakis et al., 1998; Kyriazakis, 2014). The present study provides further evidence for this hypothesis showing that broilers receiving a poor (diluted) diet exhibit a lesser reduction in voluntary feed intake (anorexia) compared to broilers receiving a balanced (undiluted) diet following coccidiosis infections. A physiological explanation for this lies in the inextricable link suggested between the immune system and anorexia (Kyriazakis et al., 1996; Kyriazakis, 2014). Activation of the immune system following pathogen infection, with the associated upregulation of cytokines, has been implicated as the primary cause of anorexia (Van Niekerk et al., 2016).

Attaining a similar degree of GR reduction in both coccidiosis-infected and uninfected broilers in the current study could have been a logical reason for expecting corresponding commensurate effects on their bone mineralization. Unfortunately, this was not the case due to the statistically similar level of anorexia amongst the infected broilers. Moreover, we found no comparable studies in the bone literature that has successfully induced a graded reduction in GR of coccidiosis-infected broilers to test this hypothesis. On the use of GR reduction as a management tool for reducing the occurrence of skeletal disorders, the suggested optimum timing economically is during the second week of life (Robinson et al., 1992). Although this method of mitigating skeletal disorders delays the attainment of market weight by 2 or 3 D (Robinson et al., 1992), it should be considered in light of the substantial economic loss associated with poor skeletal development and the welfare issues that they raise (Driver et al., 2006; Knowles et al., 2008; Pines and Reshef, 2015).

There was no evidence from the present study to suggest that a reduction in early GR improved bone mineralization to a higher degree in uninfected than in coccidiosis-infected broilers. This was the case even though the experimental diets induced graded levels of

Table 5. Main effects of diet diluted with 0, 5, 10, and 15% lignocellulose (R0 to 3, respectively) and infection status in broiler chicken orally inoculated with 0 (control) or 7,000 sporulated oocysts of *E. maxima* (infected) at day 13 post-hatch on markers of femur and tibia bone development at day 12 post-infection (pi).

	Femur						Tibia					
Main effect	Breaking strength/BW (N/g)	Length/BW (mm/cg)	Width/BW (mm/cg)	Robusticity index	Ash/BW (mg/g)	Ash percentage	Breaking strength/BW (N/g)	Length/BW (mm/cg)	Width/BW (mm/cg)	Robusticity index	Ash/BW (mg/g)	Ash percentage
Infection												
Control	19.6	5.98	0.652	3.51	0.776	50.4	25.0	8.01	0.586	4.17	1. 02	50.4
Infected	18.0	6.36	0.692	3.49	0.741	50.2	20.8	8.45	0.607	4.18	0.973	50.2
SEM	0.52	0.134	0.0131	0.021	0.0016	0.38	0.74	0.172	0.0115	0.030	0.0029	0.47
Diet												
R0	17.0^{b}	5.50^{c}	0.623^{b}	3.41^{b}	0.758	50.3	22.8	7.37^{c}	0.559^{b}	4.17	1.00	50.4
R1	17.3^{b}	5.97^{b,c}	0.649^{b}	3.50^{a,b}	0.746	49.2	23.0	8.02^{b,c}	0.564^{b}	4.19	0.94	49.7
R2	19.2^{a,b}	6.48^{a,b}	0.673^{b}	3.56^{a}	0.746	50.2	22.2	8.61^{a,b}	0.606^{a,b}	4.21	0.97	49.5
R3	20.6^{a}	6.73^{a}	0.743^{a}	3.53^{a}	0.814	51.3	23.8	8.91^{a}	0.656^{a}	4.15	1.08	51.4
SEM	0.58	0.175	0.0169	0.027	0.0028	0.50	1.07	0.225	0.0153	0.040	0.003	0.63
						Probabilities						
Infection	**0.033**	0.055	**0.037**	0.571	**0.045**	0.689	**0.001**	0.082	0.202	0.928	0.187	0.769
Diet	**<0.001**	**0.001**	**<0.001**	0.010	0.064	0.076	0.737	**0.001**	**<0.001**	0.752	0.058	0.149
Diet (linear)	**<0.001**	**<0.001**	**0.002**	**0.008**	0.948	**<0.001**	0.055	**<0.001**	**0.005**	0.987	0.943	**<0.001**
Diet (quadratic)	**<0.001**	0.453	0.781	0.141	0.947	**<0.001**	**<0.001**	0.182	0.726	0.445	0.857	**<0.001**

[a-c]Means in a column with different superscript differ significantly ($P < 0.05$).

R0, 1, 2, and 3 represent 0, 5, 10, and 15% lignocellulose-diluted diets, respectively.

GR reduction amongst the control broilers during the period of examination (day 1 to 12 pi), while graded GR only lasted until the onset of anorexia (data not shown) and thereafter remained similar for the infected birds despite the different diet dilution levels.

A differential rate of mineralization between tibia and femur in growing broilers had been reported previously (Applegate and Lilburn, 2002); Sakkas et al. (2018) also supported this suggestion. Femur strength is crucial for gait stability and bearing the heavy weight of fast-growing broilers because of its position in the skeleton (Marks and Popoff, 1988; Chinsamy and Elzanowski, 2001). In modern broilers, the forward shift in the center of gravity due to increased breast muscle places specific demands on femur integrity (Paxton et al., 2014). Therefore, the finding that diet dilution increased femur strength significantly at a point (day 12 pi) when penalties of an *E. maxima* infection are maximized is a remarkable novel discovery from the current study.

Other studies have investigated the effects of reducing broiler GR on markers of leg bone quality and mineralization using various methods. A 50% restriction in feed intake (compared to consumption rate in *ad libitum* fed birds), but the same level of Ca and P as *ad lib* fed birds, caused a significant increase in tibia ash content and lower porosity at day 42 of age (Williams et al., 2004). Sequential feeding with low and high-lysine diets during the first and second half of the day, starting from day 2 to 12 of age, increased activity levels and improved leg condition at day 42 of age (Bizeray et al., 2002). Offering a low-energy diet did not affect bone quality (Leterrier et al., 1998). A light schedule of 12L: 12D improved bone ash compared to 20L: 4D (Brickett et al., 2007). Although the above studies induced an artificial reduction in GR, it is unknown whether the methods employed in the above studies may ameliorate the effects of coccidiosis on bone mineralization.

Furthermore, the conclusion of Leterrier et al. (1998) that limiting GR using a low-energy diet did not affect bone quality was based only on tibia variables. In this study and our previous experiment (Sakkas et al., 2018) we found evidence to support different mineralization rates for tibia and femur bones (Applegate and Lilburn, 2002), which Leterrier et al. (1998) did not consider in their study. Moreover, they also observed that the slower-growing broilers due to low-energy diets were more than 6-fold (3.1% vs. 19.9%) less predisposed to varus-valgus deformities than their faster-growing counterparts (Leterrier and Nys, 1992). This, therefore, suggests that slowing GR via low-energy diet did have a potentially positive effect on leg bone in that study, and perhaps on one or more of the other long bones not examined. A subsequent investigation (Bruno et al., 2000) revealed that about 15% reduction in dietary energy intake, during the second week of life only, improved humerus weight and density without affecting the same variables in tibia or femur bones. Restriction in protein intake also affected femur, but not tibia or humerus width (Bruno et al., 2000). The results above underscore the possibility that a single factor may have differential effects between long bones (Applegate and Lilburn, 2002), as was observed for GR on tibia and femur strength in the present study.

In conclusion, coccidial infection penalized some markers of long bone mineralization in modern fast-growing broilers, while a reduction in GR via diet dilution improved bone mineralization and development to a similar degree for both coccidiosis-infected and uninfected broilers. Although delaying GR by diet dilution imposes economic constraints in the intensive broiler sector due to the additional days needed to reach market weight, it should be considered as a means to improve skeletal integrity and broiler welfare. Although in the present study we utilized lignocellulose, alternative feed ingredients with a high WHC may also be utilized with the aim to reduce overall feed costs in markets when they are available and where bird welfare is valued more (Sakkas et al., 2019). Collectively, the markers of long bone mineralization evaluated in this study suggest a more pronounced effect of artificial GR reduction on improving femur compared to tibia mineralization.

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AUTHOR DISCLOSURES

I. Oikeh, P. Sakkas, J. Taylor, I. Giannenas, D. P. Blake and I. Kyriazakis declare no conflicts of interest.

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APPENDIX

Difference between regression slopes of tibia (Y-axis) vs. femur (X-axis) at day 12 pi for length (mm), width (mm), weight (g), and ash weight (g) in broiler chicken orally inoculated with 0 (control) or 7,000 sporulated oocysts of *E. maxima* (infected) at day 13 post hatch, and receiving diet diluted with 0, 5, 10, and 15% lignocellulose (R0 to 3, respectively). Regression was done using log-transformed values.

Tibia (Y) vs Femur (X)	Diets	Comparing R1–3 with R0				Comparing infected (R0–3i) with control R0 (R0c)				
		Slope (diff)	SE (diff)	t-stat	*P*	Diets	Slope (diff)	SE (diff)	t-stat	*P*
Length										
Control	R1-R0	−0.523	0.879	−0.595	0.574	R0i–R0c	−0.0311	0.955	0.0325	0.975
	R2-R0	−0.0912	0.751	−0.121	0.907	R1i–R0c	−0.0351	0.876	0.0401	0.969
	R3-R0	−0.130	1.03	−0.126	0.904	R2i–R0c	0.320	1.09	0.293	0.779
Infected	R1-R0	−0.00404	0.714	−0.00565	0.996	R3i–R0c	−0.290	1.38	0.210	0.840
	R2-R0	0.352	0.932	0.377	0.716					
	R3-R0	−0.259	1.22	−0.213	0.837					
Width										
Control	R1-R0	1.11	0.975	1.14	0.287	R0i–R0c	0.623	0.546	1.141	0.287
	R2-R0	−0.200	0.436	−0.458	0.659	R1i–R0c	−0.157	0.387	0.406	0.696
	R3-R0	−0.271	0.604	−0.448	0.666	R2i–R0c	0.00669	0.576	0.0116	0.991
Infected	R1-R0	−0.780	0.497	−1.57	0.155	R3i–R0c	−0.0261	0.399	0.0655	0.949
	R2-R0	−0.616	0.686	−0.898	0.395					
	R3-R0	−0.649	0.509	−1.27	0.238					
Weight										
Control	R1-R0	0.566	0.837	0.676	0.518	R0i–R0c	−0.0648	1.59	0.0408	0.968
	R2-R0	0.633	0.907	0.698	0.505	R1i–R0c	0.811	0.826	0.982	0.355
	R3-R0	0.543	0.939	0.578	0.579	R2i–R0c	1.18	0.973	1.21	0.262
Infected	R1-R0	0.876	0.985	0.889	0.400	R3i–R0c	0.710	1.01	0.701	0.503
	R2-R0	1.24	1.13	1.09	0.305					
	R3-R0	0.775	1.17	0.661	0.527					
Ash Weight										
Control	R1-R0	−0.0179	0.827	−0.0217	0.983	R0i–R0c	0.340	0.722	0.471	0.650
	R2-R0	0.190	0.499	0.382	0.713	R1i–R0c	0.155	0.519	0.299	0.773
	R3-R0	0.423	0.722	0.585	0.574	R2i–R0c	0.702	0.578	1.21	0.259
Infected	R1-R0	−0.185	0.496	−0.374	0.718	R3i–R0c	0.361	0.445	0.811	0.441
	R2-R0	0.362	0.556	0.652	0.533					
	R3-R0	0.0213	0.422	0.0505	0.961					