Dietary vitamin D improves performance and bone mineralisation, but increases parasite replication and compromises gut health in *Eimeria* infected broilers

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Abstract

Coccidial infections may reduce fat soluble vitamin status and bone mineralisation in broiler chickens. We hypothesised that broilers infected with *Eimeria maxima* would benefit from increased dietary supplementation with vitamin D (vitD) or with 25-OH-D_3 ($25D_3$). Male Ross 308 chickens were assigned to diets with low (L) or commercial (M) vitD levels (1000 vs 4000 IU/kg) supplemented as D_3 or $25D_3$. At d11 of age birds were inoculated with water (C) or 7000 *E. maxima* oocysts (I). Pen performance was calculated over the early (d1 - 6), acute (d7 - 10) and recovery periods (d11 - 14) post-infection (pi). At the end of each period 6 birds per treatment were dissected to assess long bone mineralisation, plasma levels of $25D_3$, calcium and phosphorus, and intestinal histomorphometry. Parasite replication and transcription of cytokines IL-10 and IFN-γ were assessed at d6 pi using quantitative PCR. Performance, bone mineralisation and plasma $25D_3$ levels were significantly reduced during infection ($P < 0.05$). M diets or diets with $25D_3$ raised plasma $25D_3$, improved performance and aspects of mineralisation ($P < 0.05$). Offering L diets compromised feed efficiency pi, reduced femur breaking strength and plasma phosphorous levels at d10 pi in I birds ($P < 0.05$). Contrastingly, offering M diets or diets with $25D_3$ resulted in higher parasite loads ($P < 0.001$) and reduced jejunal villi length at d10 pi ($P < 0.01$), with no effect on IL-10 or IFN-γ transcription. Diets with 4000 IU/kg vitD content or with $25D_3$ improved performance and mineralisation, irrespective of infection status, whilst 4000 IU/kg levels of vitD further improved feed efficiency and mineralisation in the presence of a coccidial infection.

Introduction

Coccidiosis, caused by parasites of the genus *Eimeria*, is a widespread condition which adversely impacts broiler chicken farm profitability, by reducing growth rate and feed efficiency due to anorexia[1,2] and impaired nutrient absorption[1, 3, 4]. Malabsorptive coccidiosis, caused by infection with species such as *Eimeria maxima* and *E. acervulina* which affect the small intestine, is characterized by inflammation and intestinal epithelium damage, impaired absorption of fat, calcium (Ca) and phosphorus (P)[5,6], and long bone mineralisation[7,8,9]. Our previous study has indicated that *Eimeria maxima* infection adversely impacts bone development with effects being more pronounced at later stages of infection, long after birds have recovered and caught up with the performance of their non-infected counterparts (d13 post-infection; pi)[10].

Dietary vitamin D (vitD) supply plays a critical role in bone mineralisation of broilers[11]. It may be supplied in the form of cholecalciferol (D_3) or as 25-hydroxycholecalciferol ($25D_3$). D_3 is hydroxylated to $25D_3$, primarily in the liver, and is circulated by the vitD binding protein[12]. This form is hydroxylated further, primarily in the kidneys, to the hormonally active form 1α,25-dihydroxycholecalciferol ($1,25D_3$)[13]. $1,25D_3$ regulates calcium and phosphorus metabolism mainly by enhancing intestinal calcium and phosphate absorption and renal reabsorption, whilst it also stimulates osteoclast differentiation and calcium reabsorption from the bone and promotes mineralisation of the bone matrix[12]. In addition to its skeletal effects, $1,25D_3$ acts as an immune system modulator[14] having beneficial effects in the case of infectious and autoimmune diseases[15,16,17].

To date there have been no studies specifically investigating the effects of coccidiosis on vitD status. D_3 is a relatively non-polar molecule; it is solubilized by incorporation into bile-salt micellar solutions for movement through the body and repackaged into chylomicrons for transport by the lymphatic route[19]. It has been suggested that absorption of $25D_3$ is less fat dependent than D_3, as illustrated in patients with cholestatic liver disease[20] and in patients with steatorrhea[21]. Dietary fat is digested in the small intestine in both avian and mammalian species[22]. Although fatty acids are drained directly into the portal blood system instead of the lymph and as portomicrons instead of chylomicrons in birds as opposed to mammals[23,24], malabsorptive eimerian infections are accompanied by a pronounced depression of fat digestibility[18,25] and circulating levels of fat soluble vitamins A and E[10]. VitD has been associated with immunomodulatory roles through the production of antimicrobial peptides, cytokine responses and disease outcomes[26]. A recent study has indicated that increasing dietary vitD suppplemantation as $25D_3$ supplemantation altered cytokine responses, increasing the trascription of IL-10 and reducing that of IFN-γ and IL-1b, in layer chicks infected with a mixed *Eimeria sp.* infection whilst increasing their body weight gain, but had no effect on oocyst production [27].

To the best of the authors' knowledge, this is the first study which investigates the effects of coccidiosis on vitD status and the consequences of dietary supplementation in the form of D_3 or $25D_3$ in *Eimeria* infected broilers. In the present study we used *E. maxima* to investigate the hypothesis that circulating levels of $25D_3$ would be reduced in infected chickens and that dietary supplementation with $25D_3$ would be more effective than D_3 at reducing the effect. As a result, infected birds would benefit from higher circulating levels of $25D_3$ through increased bone mineralisation, the effects being more pronounced at later points of infection when compensatory nutrient absorption occurs[5]. In addition, we investigated whether vitD supply influences parasite replication and cytokine transcription in the jejunum, the primary site of *Eimeria maxima* colonisation and replication, at the peak of parasite replication (i.e. d6 pi[28]), and on intestinal histomorphometric features which are indicative of gastrointestinal tract (GIT) damage.

Materials and methods

Birds, husbandry and feeds

All procedures were conducted under the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU for animal experiments, carried out under Home Office authorization (P441ADF04). Three hundred and thirty six male Ross 308 day-old chicks were housed in a windowless, thermostatically controlled building in 48 pens of 0.85 m^2. Pens were equipped with tube feeders and bell-drinkers, and wood shavings served as litter. Birds had *ad libitum* access to feed and water. Pen temperature was maintained according to Aviagen recommendations[29] and a lighting schedule of 23 Light:1 Darkness was applied for the first 7 days of age, switched to 18 Light: 6 Darkness for the remainder of the trial. Basal starter (d0 – 10) and grower (d11 – 25) diets were manufactured according to Aviagen nutrition specifications[30] (Table 1), to which different source and levels of vitD were added in order to formulate 4 dietary treatments (Table 2): LD_3 (low level of D_3; 1000 IU/kg), $L25D_3$ (low level of $25D_3$; 1000 IU/kg D), MD_3 (commercial level of D_3; 4000 IU/kg) and $M25D_3$ (commercial level of $25D_3$; 4000 IU/kg). The 4000 IU/kg vitD levels (M) were selected to reflect commercial practice and breeder recommendations, whereas low levels (L) have been previously shown to reduce bone mineralisation[11]. Diets were analysed for vitD_3 and $25D_3$ contents at the DSM Laboratory (Basel, Switzerland) according to previously published methodology[31] (Table 2). The starter diet was offered in crumbled form and the grower diet in pelleted form. Birds were assessed daily for potential adverse effects of our LD treatments on their locomotion capacity. No birds were euthanized due to health related disorders and coccidiosis caused anorexia and reduced weight gain according to expectations.

Experimental design and inoculations

The experiment followed a 2 × 2 × 2 factorial design with vitD level, source and infection status as the independent variables. Upon arrival chicks were randomly assigned to dietary treatment groups at one of two vitD levels (M vs L) and one of two sources of vitD activity (D_3 vs $25D_3$). At 11 days of age (d0 pi) they were further allocated to two levels of infection ((non-infected control group (C) vs infected group (I)) and were orally inoculated with a single 0.5-mL oral dose of water (C) or 7.0 x 10^3 (I) of sporulated *E. maxima* oocysts of the Weybridge strain. Each treatment group consisted of 6 replicate pens and the initial stocking density was 7 birds per pen. Pen BW was measured at placement (d0 of age) whilst bird individual body weight (BW) and pen feed intake were measured at d0, d6, d10 and d14 pi (d11, d17, d21 and d25 of age, respectively). One bird per pen with a BW close to the pen average was selected at weighing on d6, d10 and d14 pi for sampling.

Sampling

The selected birds were individually weighed before blood sampling via the wing vein and were subsequently euthanized with a lethal injection of sodium 135 – 137 pentobarbitone (Euthatal®, Merial, Harlow, United Kingdom). Blood was placed in 5 ml sodium heparin plasma tubes (BD Vacutainer, SST II Advance Plus Blood Collection Tubes - BD, Plymouth, UK). Collected samples were immediately placed on ice and centrifuged for 600 s at 1500 g at 4 °C within 1.5 h of collection. Aliquoted plasma samples were stored at -80 °C pending analyses. Following blood sampling of the selected birds at d6 pi, 6 cm of intestinal tissue were excised from the immediate region of Meckel's diverticulum, which is the mid-point of the intestinal area infected by *E. maxima*[32], opened longitudinally and digesta contents were removed. Following this, 5 cm of tissue were submerged in 7 ml bijous and 1cm proximal to the jejunum in 1.5 ml screw cap micro tubes (Thermo Scientific) filled with RNAlater® (Life Technologies; Carlsbad, CA, USA). Samples were immediately stored at -80 °C pending analyses. Additionally, 3 segments of 1 cm, one from the duodenal loop, one from the mid-jejunum (midway between Meckel's diverticulum and the end of the duodenal loop) and one from the mid-ileum (midway between Meckel's diverticulum and the ileocecal junction) were sampled from all dissected birds on d6, d10 and d14 pi, and were fixed in 10% buffered formalin for histomorphometrical assessment. Following intestinal tissue sampling, the right tibia and femur were dissected, defleshed and stored at -20 °C pending analysis in airtight sealable polyethelyne bags.

Bone analysis

Bones were thawed at 4 °C and tibia and femur length were measured with digital callipers. Subsequently, bone weight was recorded. Robusticity[33] and Seedor[34] indices were calculated using the following formulae:

$$\text{Robusticity index} = \frac{\text{bone length (mm)}}{\text{bone weight (mg)}}$$

Bones were subjected to a 3-point break test using an Instron testing machine (Instron 3340 Series Single Column-Bluehill 3) using previously employed methodology[10,11]. Broken tibias were boiled for 300 s in deionized water at 100 °C to facilitate removal of cartilage caps and bones were split in half for manual removal of the bone marrow. Following this, bones were placed in vessels containing 10 ml of acetone and 10 ml of petroleum ether (VWR) and were subjected to fat extraction in a Mars 6 Microwave Assisted Reaction System 6 (CEM, Matthews, USA) with a set temperature of 180 °C for 4800 s. Fat extracted tibias were then placed in an oven at 105 °C for 18 h and were weighted to obtain the dry defatted bone weight. Subsequently they were ashed in a Phoenix CEM ashing microwave furnace (CEM, Matthews, USA) at 850 °C for 1.5 h to obtain the ash weight (g).

Plasma levels of Ca, P and 25D$_3$

Plasma concentration of 25D$_3$ (ng/ml) was analysed using the 25-Hydroxy Vitamin D Direct EIA kit (IDS Diagnostics, Fountain Hills, AZ, USA) and plasma concentrations of Ca and P (mmol/l) were determined in an ABX Horiba Pentra 400 automatic analyser (Horiba Medical, Irvine, CA, USA) in duplicate, according to manufacturer's instructions.

Histology

Excised, formalin-fixed intestinal sections were processed according to previous used methodology, stained with hematoxylin/eosin[10]. Mounted slides were scanned (Leica SCN400, Leica, Microsystems, Germany), and images were captured using the Leica Image Viewer Software (Software version: SlidePath Gateway Client Viewer 2.0). Captured images were assessed for the determination of villus length (VL) and crypt depth (CD) using ImageScope® software (Aperio Technologies, Vista, CA, USA). Ten villi with their corresponding crypts were measured per section to obtain an estimated length, expressed in micrometres (μm).

Eimeria maxima genome copy number

To assess parasite replication we used quantitative real-time PCR to measure parasite genome copy number (GC) in tissues surrounding Meckel's diverticulum. This method supports higher throughput analysis and minimises the impact of variation related to the temporal manner of oocyst excretion[28]. The methodology was used as described previously in studies of parasite replication in chicken lines differing in growth rate[10].

RNA isolation, reverse transcription, and real-time qPCR

RNA was extracted from intestinal tissue using the Isolate II RNA Mini Kit (Bioline Reagents, United Kingdom) following the manufacturer's protocol. RNA concentration and quality was confirmed using a NanoDrop spectrophotometer (NanoDrop™ 2000, NanoDrop Products). Isolated RNA extracts were reverse-transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer's protocol and stored at -20 °C until use. Oligonucleotide primers for cytokine and reference gene transcripts were adopted from the published literature (Table 3). Standard PCR was carried out on a cDNA sample with each primer pair using MyFi Mix polymerase (Bioline, United Kingdom) as described by the manufacturer to provide template for serial dilution standard curves. Tenfold serial dilution was performed in molecular grade water to generate standard curves (10^{10} – 10^{1}) for three reference genes ((GADPH, RL13 and TATA - Binding Protein (TBP)) and for the cytokine genes of interest, IL-10 and IFN-γ. Real time qPCR was performed with amplification and detection carried out using Roche 96 LightCycler detection system (Roche, Mannheim, Germany). The qPCR was performed in a 20 µl reaction containing 2 µl of cDNA from the RT reaction, 10 µl SYBR Green PCR Master Mix (Roche, Mannheim, Germany), 0.75 µl primer (at 10 uM concentration) and 6.5 µl of RNase free water, using the following cycle: pre-incubation: 95 °C for 600 s, 3 step amplification 40 cycles at 95°C / 10s, 60°C / 10s, 72°C / 20s; Melting: 95°C / 10s, 65°C / 60s, 97°C / 1s (continuous) conditions. Each RT-PCR experiment contained triplicate no-template controls, test samples and a $\log^7$ – $\log^1$ dilution series of standard cDNA. Calculation of copy number of each qPCR target was performed according to the slope and intercept of the corresponding dilution series. Absolute gene transcription was quantified for each target test gene, followed by normalisation of their expression ratio using the geometric mean of the three reference genes.

Calculations and Statistics

The calculation of sample size was performed using software G*power (vs 3.1). Based on results of previous studies[10,11], we determined that we needed 10 replicates for the interaction between level of vitD supply and Infection status to achieve 80 % power at a significance level of 0.05 for tibia ash % at the end of the grower period. Since we lacked experimental data on the effect of source of dietary vitD supply or on its interactive effects with vitD level and infection it was not possible to estimate the required sample size to investigate these two- and three-way interactions. We employed a greater sample size than the one indicated by the power analysis (12 instead of 10 replicate pens) to investigate the two-way interaction between level and infection. Under the hypothesis that in the presence of infection circulating levels of vitamin D would be severely depressed and that source would be a critical factor, we estimated that the currently employed sample size of 6 replicate pens would suffice to investigate the three-way interactions amongst main factors. All statistical analyses were conducted in SAS 9.4 (SAS Institute, Cary, NC). For all statistical assessments pen was considered the experimental unit and all variables were analysed with vitD level, source and infection status as main effects and their interactions with PROC GLM. Pen data included average BW pre-infection (d11 of age) and at the end of the experiment (d25 of age), daily feed intake (ADFI; g/d), average daily gain (ADG; g/d) and feed conversion ratio (FCR) calculated over the pre-infection period (d0 – 11 of age) and over the early (d0 – 6 pi), acute (d7 – 10 pi), recovery periods (d11 – 14 pi), and overall period pi (d0 – 14 pi). Tibia and femur bone breaking strength (BBS; N) as well as ash (g) were calculated as a proportion of the bird BW (kg) prior to dissection. Single timepoint data deriving from one bird per pen dissected on d6, d10 or d14 pi included circulating plasma levels of $25D_3$, Ca and P, bone parameters and histological measurements, as well as parasite GC and mRNA transcription levels of IFN-γ and IL-10 at d6 pi. Uninfected birds were excluded from the model for *E. maxima* GC and IL-10 expression levels since both were below the level of detection. For all statistical procedures, the normality of the residuals was assessed with the Shapiro-Wilk test. Predicted *E maxima* GC, cytokine transcription levels and plasma levels of $25D_3$ were log-transformed to normalise residual distribution. When significant differences were detected, treatment means were separated and compared by the Tukey's multiple comparison test. Significance was determined at $P < 0.05$. All values are expressed as model-predicted least square means along with their pooled SEM.

Results

Performance

No significant difference was detected in chick BW at placement between treatment groups (average 43.5 g; SEM = 0.41; $P > 0.1$). The main effects of vitD level, vitD source and infection on performance variables over the periods pre- and post-infection are presented in Table 4.VitD level significantly interacted with infection for FCR ($P < 0.05$) over the overall period pi (d0 – 14 pi), being the highest in infected birds on LD diets (Figure 1). There were no other two or three-way interactions between vitD level, vitD source and infection status for on broiler growth performance parameters. At d0 pi (d11 of age) bird BW, ADG and ADFI were significantly higher for birds on M diets ($P < 0.05$) than birds on L diets. Infection significantly reduced ADFI and ADG, and increased FCR over the early, acute and overall periods pi ($P < 0.0001$), whilst performance of C and I birds was similar ($P > 0.1$) over the recovery period. Birds on M diets had significantly higher final BW (d25 of age) and ADG over the early and acute periods ($P < 0.05$) and lower FCR ($P < 0.05$) over the early, acute and overall periods pi than birds on L diets. Birds on $25D_3$ diets had significantly higher final BW and ADG over the acute, and overall period pi and lower FCR over the overall period pi ($P < 0.05$) than birds on D3 diets.

Bone variables

The main effects of vitD level, vitD source and infection on bone variables over the timepoints pi are presented in Tables 5 and 6 and all significant interactions are presented in Figures 2 and 3. VitD level and infection interacted for femur BBS at d10 pi ($P < 0.005$) as I birds at the low level of supplemantation had reduced BBS in comparison to all other treatment groups (Figure 2). In addition, vitD level, source and infection significantly interacted ($P < 0.01$) for ash weight at d14 pi with I birds on the LD_3 treatment displaying the lowest values (Figure 3). There were no other two or three-way interactions between factors for any of the bone variables. Femur and tibia Seedor indices were significantly decreased ($P < 0.05$) at all timepoints pi and Robusticity index ($P < 0.05$) was significantly increased at d6 and d14 pi, in response to infection. Infection significantly reduced tibia ash (%) at all timepoints ($P < 0.01$, $P < 0.0001$, $P < 0.0001$ at d6, 10 and 14 pi, respectively). On the other hand, tibia ash weight was significantly reduced only at d14 pi ($P < 0.0001$). Femur BBS was affected on d6 pi ($P < 0.0001$) and on d14 pi ($P < 0.001$), whilst tibia BBS was affected on d10 ($P < 0.05$) and d14 pi ($P < 0.0001$). Offering commercial levels of vitD (M) supply significantly improved both Seedor and Robusticity indices of the femur on d10 pi ($P < 0.05$), but did not affect the tibia. At the same time it

increased femur BBS on d10 ($P < 0.05$) and tibia BBS on d6 pi ($P < 0.01$). Although tibia ash weight was not affected by the level of vitD supply, tibia ash % was significantly ($P < 0.05$) increased at d10 and d14 pi. The source of vitD supply significantly affected the tibia Seedor index at d6 pi and Robusticity index at d14 pi ($P < 0.05$). There was no significant effect of source of vitD supply on BBS. However, birds receiving $25D_3$ achieved significantly higher tibia ash values at d6 and d10 pi ($P < 0.05$) than birds receiving D_3. Finally, $25D_3$ significantly increased tibia ash % at d10 and d14 pi ($P < 0.05$).

Plasma levels of Ca, P and 25D$_3$

The main effects of vitD level, vitD source and infection on plasma levels of Ca, P and $25D_3$ over the timepoints pi are presented in Table 7 and all significant interactions are presented in Figures 4 and 5. There were no significant three-way interactions between factors on plasma levels of Ca, P and $25D_3$. VitD level and infection interacted ($P < 0.05$) for P level at d10 pi with I birds on the L diets having significantly lower values than C birds on L and M diets (Figure 4A). VitD source and infection interacted ($P < 0.05$) for Ca levels at d10 pi with I birds on the $25D_3$ treatment, achieving significantly higher values than C birds on the same dietary treatment (Figure 4B).VitD level interacted with vitD source for $25D_3$ levels ($P < 0.0001$) at d10pi; they were similar for MD_3 and $L25D_3$ diets and significantly higher ($P < 0.0001$) than LD_3 and lower ($P < 0.0001$) than $M25D_3$ diets (Figure 5A). Furthermore, vitD level and infection interacted for $25D_3$ levels on d10 pi ($P < 0.05$), being similar for LD_3 uninfected and MD_3 infected birds and singifincatly higher ($P < 0.0001$) than LD_3 infected birds and significantly lower ($P < 0.0001$) than MD_3 uninfected birds (Figure 5B). There were no other two-way interactions between factors for any of the plasma variables. Infection significantly reduced levels of Ca and P only at d6 pi (both $P < 0.0001$). The level of vitD supply significantly affected Ca levels ($P < 0.05$) on d6 and d10 pi, with birds on L diets having lower values. On the other hand, vitD level did not affect P at any of the timepoints. Source did not affect the level of Ca or P, at any of the 3 timepoints. Plasma levels of $25D_3$ were significantly affected at d6, d10 and d14 pi by vitD level ($P < 0.0001$), source of vitD supply ($P < 0.0001$) and infection status ($P < 0.0001$); being significantly higher at all timepoints in birds on $25D_3$ treatments than birds on D_3 treatments, at high levels than low levels of vitD supply and in C than I birds.

Histology

The main effects of vitD level, vitD source and infection on plasma levels of histomorphometric measurements pi are presented in Table 8 and all significant interactions are presented in Figures 6-8. There were no significant three-way interactions between factors on histological measurements. VitD level and source interacted on jejunal VL at d10 pi ($P < 0.01$); being significantly higher in birds on LD_3 treatments than birds on MD_3 treatments (Figure 6A). Furthermore, jejunal VL:CD ratio was

singnificantly higher in LD_3 birds than MD_3 birds at d14 pi ($P < 0.05$; Figure 6B). VitD level and infection interacted for jejunal VL at d10 pi with I birds on high vitD treatments having significantly lower values than all other treatment groups ($P < 0.01$; Figure 7). Source and infection interacted for ileal VL ($P < 0.05$) and VL: CD ratio ($P < 0.01$), at d6 pi being significantly higher for uninfected $25D_3$ birds ($P < 0.05$) than infected birds receiving either D_3 or $25D_3$ (Figures 8A and 8B, respectively). There were no other two- or three-way interactions between factors for any of the histomorphometric measurements. At both d6 and d10 pi, infection significantly decreased duodenal VL ($P < 0.0001$ and $P < 0.001$, respectively), increased CD ($P < 0.0001$) and reduced VL:CD ratio ($P < 0.0001$). At d14 pi effects persisted only on CD ($P < 0.05$) and VL:CD ratio ($P < 0.01$). The same direction of effects, on the same days, was observed for histomorphometric measurements of the jejunum and the ileum, albeit the ileal VL:CD ratio was significantly affected only at d6 pi ($P < 0.0001$) (Table 8). VitD level significantly affected duodenal VC ratio at d14 pi, with birds on LD treatments having higher values ($P < 0.05$). On the other hand, $25D_3$ treatments had significantly higher CD at d6 pi in comparison to D_3 treatments ($P < 0.05$).

Parasite replication

E. maxima GC were not affected by the interaction between vitD level and source. However, they were significantly affected by both vitD level *(*$P < 0.0007$) and vitD source ($P < 0.0001$); birds on MD_3 had higher parasite burdens than birds on LD diets (11.5 vs 11.1; SEM = 0.08) and birds receiving $25D_3$ had higher parasite burdens than birds receiving D_3 (11.6 vs 11.0; SEM = 0.08).

IFN-γ and IL-10 mRNA levels

Both IFN-γ and IL-10 were not affected by vitD level ($P = 0.800$ and $P = 0.721$, respectively), vitD source ($P = 0.998$ and $P = 0.488$, respectively) or their two -way interaction ($P = 0.737$ and $P = 0.488$, respectively). Gene expression of IFN-γ was significantly upregulated by infection ($P < 0.0001$) and it was not affected by the two-way interaction with level ($P = 0.726$) and source ($P = 0.904$), or their three-way interaction ($P = 0.940$).

Discussion

In a previous study using the same host-parasite model, *E. maxima* infection reduced bone mineralisation both in fast and slow growing broiler lines[10]. In the present study we assessed whether offering differing dietary levels of vitD (4000 vs 1000 IU/kg), and/or different forms ($25D_3$ instead of D_3) would alleviate the effects of infection on performance and bone mineralisation in fast growing broilers. We also assessed parasite-related aspects of the infection through cytokine expression and parasite GC at peak parasite replication. The basis of the hypothesis was that fat soluble vitamin status is impaired during coccidiosis, which in turn may further aggravate a marginal vitD deficiency and that $25D_3$ may be absorbed in a more fat-independent manner, being more potent in mediating vitD activity.

Consistent with previous findings[10], infection penalised performance of infected chickens during the early and acute periods of infection, but it was identical to that of uninfected birds during the recovery period. Gastrointestinal damage occured across all segments of the small intestine around peak parasite replication[28], the effects being more pronounced and persisting longer in the proximal and mid-intestine, which is the predilection site for *E. maxima*[35-37]. Compensatory ileal villi development took place as described previously in similar studies with the same parasite[38], but not at the acute stage of infection (d6 pi). In terms of bone mineralisation, the effects of infection were present throughout the pi period for both femur and tibia with both showing inferior robusticity and seedor indices. Femur BBS responded to infection earlier than tibia BBS, which could be attributed to the faster mineralisation rate of the former in comparison to the latter at initial stages of broiler growth[39]. Despite the fact that the proportion of tibia ash to BW at dissection was constant for uninfected birds throughout d17 - 25 post hatch[40], this was not the case for infected birds where a progressive decrease was noted. By d14 pi infected birds matched the growth rates of their non infected counterparts, but their tibias carried 14 % less ash (g). Moreover, tibia ash % was severely depressed at all timepoints, being more pronounced at d10 pi but persisting at d14 pi. These results bear significance considering that although ADG was comparable between infected and uninfected birds over the recovery period, the BW of infected birds was significantly lower, indicating that proportionally more stress was applied to their long bones.

Consistent with our hypothesis, vitD status was impaired in response to infection with *E. maxima*. Infection reduced levels of $25D_3$ across the pi period, reaching the lowest levels on d10 pi. Studies in mammalian species suggest that some storage occurs in the liver, adipose and muscle tissues [41-43]. Furthermore, stores can be released slowly in periods of vitD deficiency raising plasma $25D_3$ levels; the rate of release being higher when subjected to a negative energy balance[41,42,44]. Although there is no information on vitD storage and kinetics in avian species, these reserves are depleted within a week in the

absence of dietary supply in minipigs[41]. Our results suggest that within a few days of coccidian challenge systemic circulating $25D_3$ levels become severely depressed. At d6 pi levels of plasma Ca and P, and bone mineralisation, were penalised; likely due to their reduced absorption as a result of gastrointestinal tissue damage. However, homeostasis of both Ca and P was attained later during infection, while penalties on vitD concentration and bone mineralisation persisted throughout.

The results of feed analysis suggested that the amount of dietary $25D_3$ was consistently lower than D_3, in both the starter and grower diets. The reason for this disrepancy is likely analytical in nature i.e related with the methodology for estimating $25D_3$ contents rather than associated with feed mixing. Ultimately, $25D_3$ status was significantly higher for birds receiving the $25D_3$ than the D_3 diets. Therefore, results presented in the current study can be interpreted with confidence. Overall, plasma $25D_3$ levels were significantly increased by higher vitD supplementation and by offering $25D_3$ as the source of vitD activity in both uninfected and infected birds. The interaction between level and source indicates that offering $25D_3$ is more efficient than D3 in raising its concentration and is consistent with previous reports in chickens where serum or plasma concentrations of the metabolite were assessed[11,45-47]. Although there was no formal interaction between level, source and infection on circulating levels of $25D_3$, at d10 pi when effects of infection were maximized, $25D_3$ levels were similar between MD_3 and $L25D_3$ birds suggesting a better absorption efficiency for dietary $25D_3$ (Figure 5a). On the other hand, vitD supply interacted with infection status for levels of $25D_3$ at d10 pi, being significantly depressed in L infected birds but maintained in M infected birds to similar levels as L uninfected birds. Infected birds on low vitD diets also showed inferior FCR across the pi period, and had the lowest femur BBS and circulating P levels on the same day pi. The effect of vitD on phosphate absorption is thought to be mediated via the saturable transcellular mechanism as increased levels of NaPiIIb in the brush border membrane have been measured in response to 1,25D3 treatment of patients with renal failure and in vitD deficient rats[48,49]. The only formal interaction between level, source and infection was detected at d14pi for ash (g) where LD_3 infected birds showed the lowest ash (g) overall. Collectively, these results indicate that a low vitD supply penalised bone development in infected chickens, with the greatest impact at later stages of infection when offered in the form of D_3. On the other hand, although dietary $25D_3$ was more efficient for maintaining vitD status, it did not offer additional benefits in the presence of infection. Previous studies involving increased dietary supply of Ca[50] and P[8] have been unsuccessful in improving bone mineralisation in coccidiosis infected birds while phytase supplementation has limited efficacy[51, 52]. It is apparent that there are limitations in the capacity of infected birds to compensate for penalties imposed on their bone development, at least within the time period studied.

Final BW was improved by both vitD level and source, but vitD level affected ADG only over the pre-infection period while vitD level only during the post infection period and FCR was affected only during the post infection period by both vitD level and source. Although performance responses to vitD supply are typically present when offering suboptimal levels of Ca and P supply, our results are consistent with previously published studies[45,53] and suggest that vitamin D requirements of broilers for growth functions may remain high throughout the grower period. On the other hand, increasing vitD supplementation, or offering $25D_3$, improved all markers of bone mineralisation effects were not consistent across sampling points. Nonetheless, tibia ash% which is the most important marker of bone mineralisation, was significantly increased by d10 and d14 pi when offering commercial levels of vitD or in the form of $25D_3$. These results show that benefits from increased vitD supply on bone mineralisation extend beyond the starter period and are in agreement with a recently published study evaluating effects of vitD supply in fast growing broiler lines[11]. A higher level of vitD supply also increased plasma concentration of Ca but not of P. Although this could have occurred due to increased bone resorption or enhanced Ca and/or P absorption, ultimately bones were more mineralised promoting mineralisation of the bone matrix [12,54, 55]. The efficiency of Ca absorption is low in vitD deficient animals[56] and has been related to transcellular and the paracellular absorption mechanisms[57,58].

In the present study offering a higher level of vitD, or replacing with $25D_3$, associated with a higher degree of parasite replication. Likewise, a higher degree of gastrointestinal damage was observed with higher levels of vitD activity. In the presence of infection offering MD_3 diets evoked greater jejunal VL than LD_3 diets at d10pi, and $25D_3$ diets resulted in smaller ileal VL and VCR at d6pi than D_3 birds. Regardless, intestinal transcription of IFN-γ and IL-10 was not differentially affected by dietary vitD supply. *Eimeria maxima* evokes a complex cytokine response characterized by increased production of Th1 pro-inflammatory cytokines such as IL-1b, IL-6, IL-8, IL-17, and IFN-γ in the small intestine, as well as Th2 anti-inflammatory cytokines such as IL-4, IL-10[35,59,60]. In particular, increased IFN-γ mRNA levels are thought to associate with antigen-specific resistance to coccidiosis, promoting Th1 cell production, whilst preventing Th2 cell production[36,61], balanced by IL-10[62]. Elevated IL-10 mRNA levels have been described in susceptible compared to resistant broiler chicken lines[62], whilst dietary fed oral antibody to chicken IL-10 prevents growth depression due to a mixed *Eimeria* spp. infection[63]. On the other hand, $1,25D_3$ may support conversion of naïve T cells into T regulatory cells, which produce IL-10 and TGF-β that inhibit the expression of pro-inflammatory cytokines such as IFN-γ and IL-17[64] and to upregulate IL-10 production in macrophages[18,65]. Previous research has shown that increased supplementation of $25D_3$, above 2000 IU/kg of feed, in white Leghorn chicks infected with a mixed *Eimeria spp.* resulted in smaller penalties on their ADG similar to the present study[27]. However, decreased IL-1β and increased IL-10 transcripts were detected in the cecal tonsils. It is possible that in the present study a delayed upregulation of IFN-γ, or an earlier upregulation of IL-10, rather than variation in

their absolute levels at the peak of the infection may have affected parasitological outcomes and degree of GIT damage. Further investigation of the immune response at earlier stages of infection is required to elucidate the observed effects. In addition, outcomes may differ according to the parasite species in question; *E. maxima* in particular, induces a strong pro-inflammatory response as opposed to the more balanced Th1/Th2 phenotype which characterizes infections with *E. acervulina* and *E. tenella*[35]. Furthermore, differential effects may be observed in regards to vitamin D status in single or mixed eimerian sp. infections, which are known to occur in practice[66] depending on the species present; *E. acervulina* and *E.maxima* significantly decrease fat soluble vitamin status[10,67] as they both affect regions of the small intestine where fat absorption occurs[22], while species such as *E. tenella* which affect the ceca have milder effects[68]. Future studies should investigate the magnitude of reduction in bone mineralization and vitamin D status over time when infecting with different species and under different infection pressures, as it has been previously shown that effects may be dose dependent[69].

Interestingly, parasitological and histological findings did not corroborate performance outcomes. It has been previously shown that a higher vitD status results in increased fractional rate of synthesis and increased breast muscle yield in broilers[45]. Therefore, the reduced FCR observed in high vitD fed infected broilers could be attributed to their increased vitD status and their improved ability to accrete body protein in the presence of infection[45]. The lack of an interactive effect of source of vitD supply and infection status on performance variables indicates that vitD source is less critical than level of level of vitD supply under these experimental conditions.

In conclusion, the present study shows that an *E. maxima* infection penalizes broiler chicken performance, bone mineralisation and vitD status, whilst a low vitD supply seems to aggravate the adverse effects of infection. In contrast, a higher vitD supply resulted in higher parasite loads and compromised gut architecture in the absence of adverse effects on performance variables. Transcription of IL-10 and IFN-γ was unaffected. Additional studies are needed to elucidate the effects of vitD supply on immune responses over time in different host/pathogen systems.

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Conflict of interest

None

Authorship

The authors' responsibilities were as follows–PS, IK: designed the research; 2. IO, PS and DPB and SS: conducted the research; DPB: provided essential materials; PS and IO: analysed the data; PS, IO, IK, DPB: wrote the manuscript; PS and IK: had primary responsibility for final content; IK was responsible for grant management; all authors have read and approved the final manuscript.

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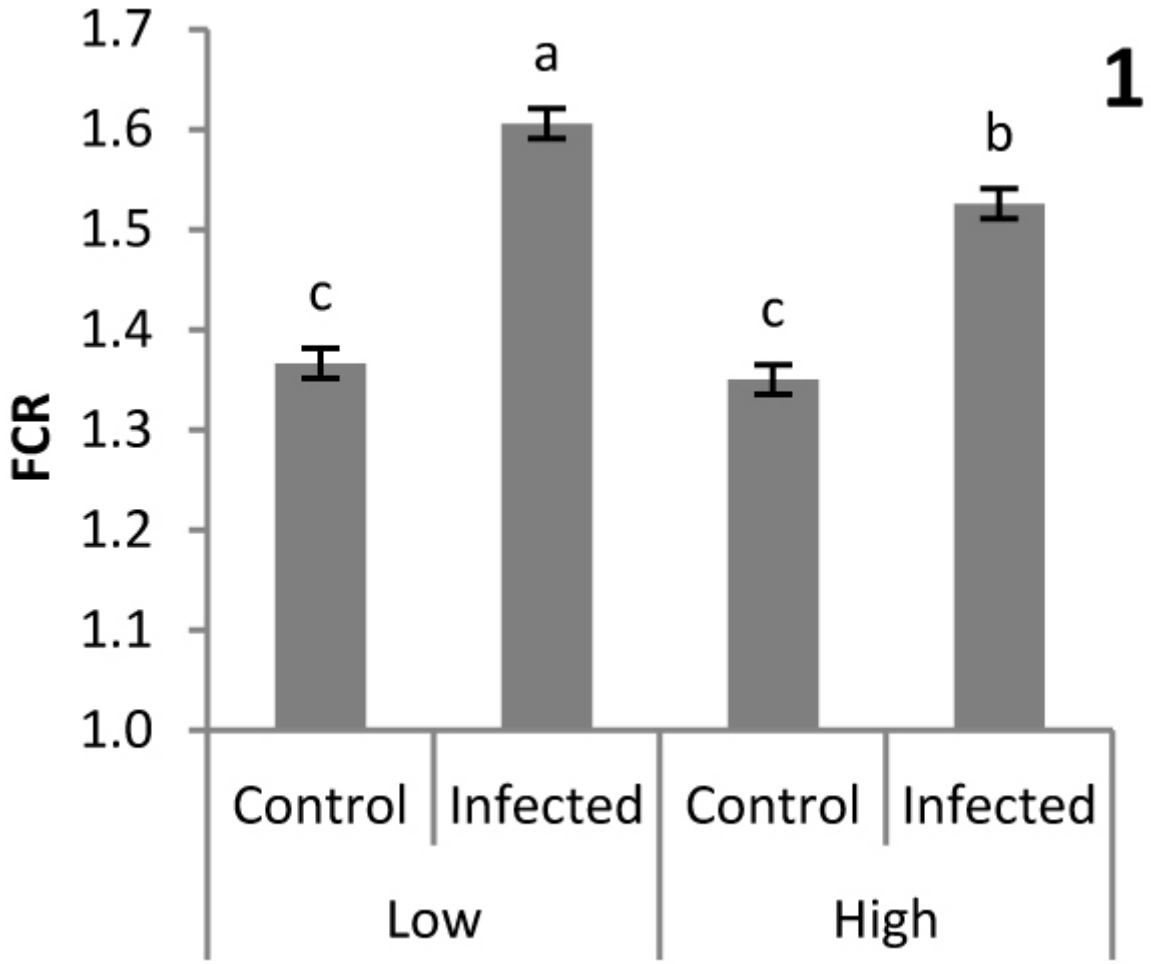
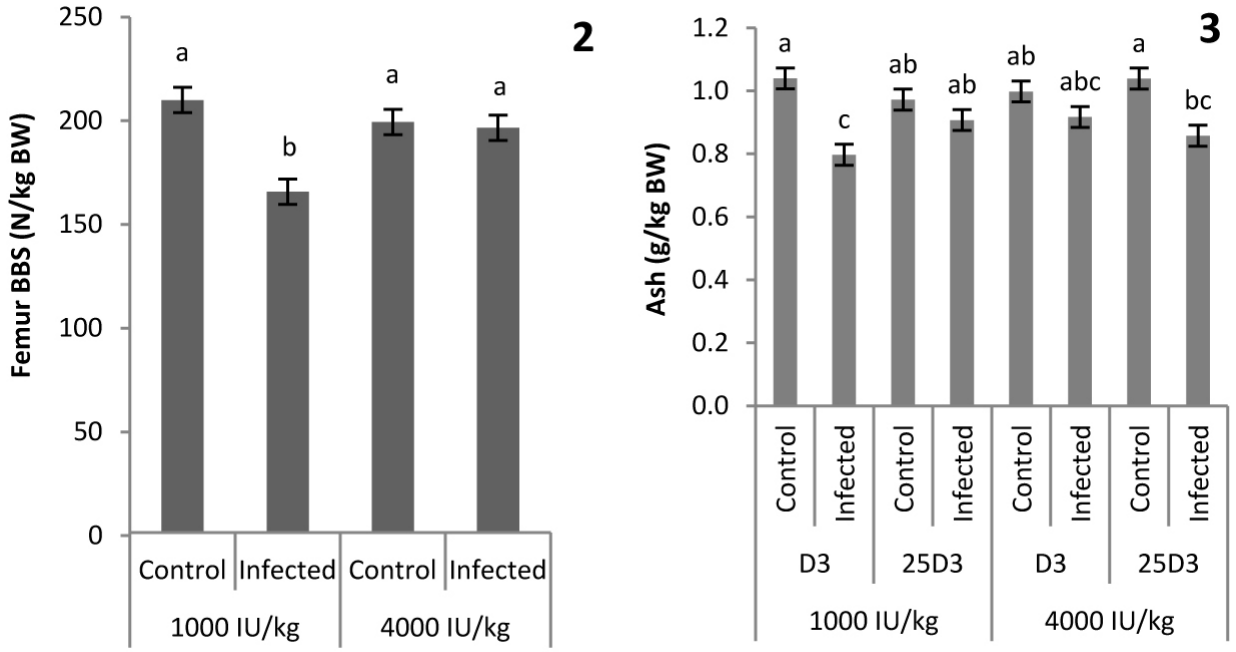
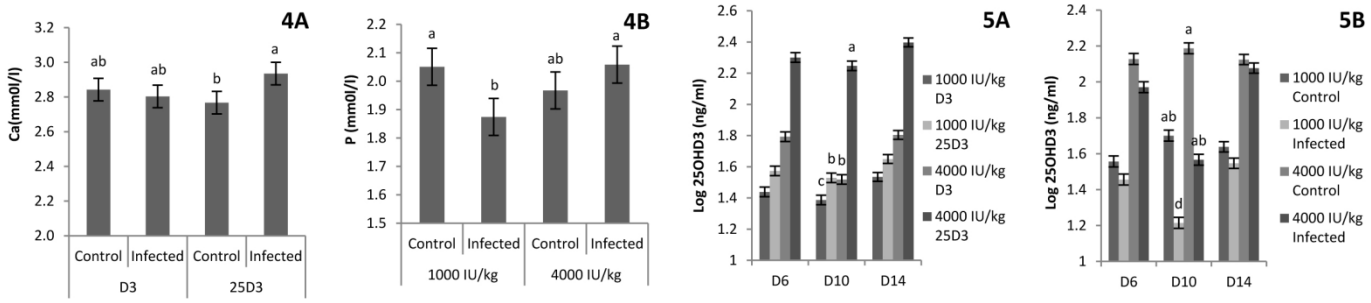


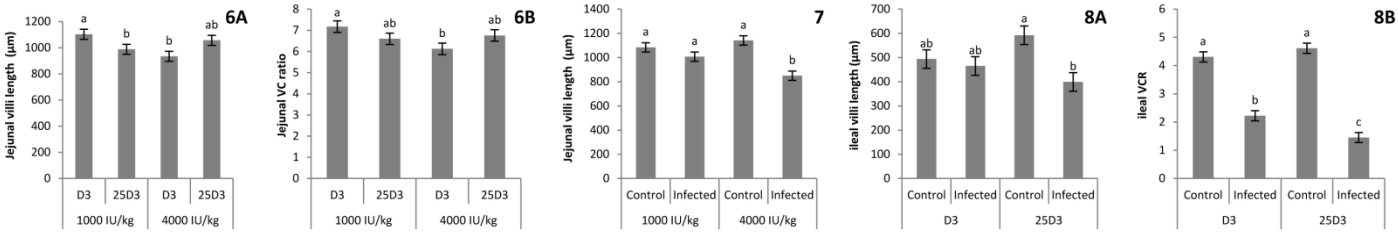
Figure 1. Significant interaction between vitamin D level (1000 or 4000 IU/kg) and infection status (Control or Infected with 0 or $7x10^3$ sporulated oocysts of *E. maxima* at d11 post hatch) on feed conversion ratio (FCR) of broiler chicken over the course of infection (d1 – 14 pi) (*P* = 0.039). [a,b] Mean values with unlike superscript letters were significantly different (P < *0.05*).



Figures 2 and 3. Interactive effects of main factors vitamin D level (1000 or 4000 IU/kg), source of vitamin D supply ($25D_3$ or D_3) and infection status (Control or Infected with $7x10^3$ sporulated oocysts of *E. maxima* at d11 post hatch) on bone variables of broiler chicken. Significant interactions between vitamin D level and infection on femur bone breaking strength (BBS) (P = 0.002) at d10 pi **(2)** and between vitamin D level, source of vitamin D supply and infection on ash weight (g) expressed as a proportion of body weight at dissection (g/kg BW) ($P = 0.005$) at d14 pi **(3)**. ^{a,b,c} Mean values with unlike superscript letters were significantly different ($P < 0.05$).



Figures 4 and 5. Interactive effects of main factors vitamin D level (1000 or 4000 IU/kg), source of vitamin D supply ($25D_3$ or D_3) and infection status (Control or Infected with $7x10^3$ sporulated oocysts of *E. maxima* at d11 post hatch) on plasma parameters of broiler chicken. Significant interactions between source of vitamin D supply and infection status on plasma Ca concentration (mmol/l) (P = 0.040) **(4A)** and between vitamin D level (1000 IU/kg or 4000 IU/kg) and infection on plasma P concentration (mmol/l) (P = 0.046) **(4B)** at d10 pi. Significant interactions between vitamin D level and source ($P < 0.0001$) **(5A)** and between vitamin D level and infection (P = 0.033) on log transformed circulating levels of vitamin 25-OH-D3 (ng/ml; 25D3) **(5B)** at d10 pi. [a,b,c] Mean values with unlike superscript letters were significantly different ($P < 0.05$).



Figures 6-8. Interactive effects of main factors vitamin D level (1000 or 4000 IU/kg), source of vitamin D supply ($25D_3$ or D_3) and infection status (Control or Infected with $7x10^3$ sporulated oocysts of *E. maxima* at d11 post hatch) on histological parameters of broiler chicken. Significant **i**nteractions between vitamin D level and source of vitamin D supply on jejunal villi length at d10 pi (P = 0.004) **(6A)** and on jejunal villi length to crypt depth ratio (VCR) at d14 pi (P = 0.008) **(6B)**. Significant interactions between vitamin D level and infection on jejunal villi length at d10 pi (P = 0.008) **(7).** Significant interactions between vitamin D source and infection status on ileal villi length (P = 0.022) **(8A)** and ileal villi length to crypt depth ratio (VCR) (P = 0.005) **(8B)** at d6 pi. [a,b] Mean values with unlike superscript letters were significantly different ($P < 0.05$).

Tables

Table 1. Ingredient and analysed chemical composition of the starter (d0–10) and grower (d11–25) basal diets offered to chickens.

Item	Starter	Grower
Ingredient (%)		
Wheat	47.9	51.6
Corn	10	10
Soybean meal (48% CP)	32	25.3
Soybean full fat	4.0	7.0
Soy crude oil	1.84	2.32
Dicalcium phosphate	1.82	1.60
Limestone	0.77	0.67
Vitamin and mineral premix	0.40	0.40
DL methionine	0.33	0.30
L-Lysine	0.27	0.25
Sodium bicarbonate (27 %)	0.21	0.20
Sodium chloride (39 %)	0.19	0.20
L-Threonine	0.14	0.12
Choline chloride (60 %)	0.05	0.05
L-Valine	0.03	0.02
Nutrient composition (%) *		
ME (kcal/kg) (calculated)	3,000	3,100
Crude protein	23.1	21.37
Crude fat	5.03	4.87
Crude fibre	2.39	2.13
Ash	5.43	4.83
Calcium	1.03	0.80
Phophorus	0.74	0.62
Available phosphorus (calculated)	0.48	0.44
Sodium	0.18	0.15
Manganese (mg/kg)	218.2	168.8

* The nutrient composition was in accordance with Aviagen nutrient Specifications [25] apart from vitamin D source and level of supply.

Table 2. Analysed D_3 and $25D_3$ content (IU/kg of feed) of the 4 dietary treatments: LD_3 (low level of D_3; 1000 IU/kg), $L25D_3$ (low level of $25D_3$; 1000 IU/kg D), MD_3 (commercial level of D_3; 4000 IU/kg) and $M25D_3$ (commercial level of 25D3; 4000 IU/kg). NA=not applicable.

Vitamin D supplementation level		D_3		$25D_3$	
		Starter	Grower	Starter	Grower
Low	D_3 (LD_3)	1560	1020	NA	NA
	$25D_3$ ($L25D_3$)	NA	NA	844	652
High	D_3 (MD_3)	4910	4520	NA	NA
	$25D_3$ ($M25D_3$)	NA	NA	2828	2720

Table 3. Oligonucleotides used for quantitative RT-PCR

cDNA Target	Primer Sequence (5'-3')		Accession no.*	Annealing temp (°C)	Efficiency (%)
	Forward	Reverse			
28S[70]	GGCGAAGCCAGAGGAAACT	GACGACCGATTTGCACGTC	AH001604	61	0.97
GAPDH[71]	TGTGACTTCAATGGTGACAGC	GCTATATCCAAACTCATTGTCATACC	NM_204305	55	0.97
TATA-BP[72]	TAGCCCGATGATGCCGTAT	GTTCCCTGTGTCGCTTGC	D83127	58	0.99
IFN-γ[73]	GTGAAGAAGGTGAAAGATATCATGGA	GCTTTGCGCTGGATTCTCA	Y07922	59	1.00
IL-10[61]	CATGCTGCTGGGCCTGAA	CGTCTCCTTGATCTGCTTGATG	AJ621614	59	0.99

*Genomic DNA sequence (NCBI GenBank)

Table 4. Main effects of level, source of vitamin D supply and *Eimeria* infection status on chicken performance over the period pre-infection (d0-11 days of age) and over the early (d0 - 6), acute (d6 - 10), recovery (d10 - 14), and overall periods (d0 - 14) post infection. Chickens orally inoculated with 0 (Control) or $7x10^3$ sporulated *E. maxima* oocysts (Infected) at d11 post hatch (d0 pi)**.**

	Body weight post hatch (g)		Average daily gain (g/d)					Average daily feed intake (g/d)					Feed conversion ratio				
Period	Pre-infection	Post-infection	Pre-infection	Post -infection				Pre-infection	Post –infection				Pre-infection	Post-infection			
Days	d11 of age (d0pi)	d25 of age (d14pi)	d0-11 of age	d0-6	d6-10	d10-14	d0-14	d0-11 of age	d0-6	d6-10	d10-14	d0-14	d0-11 of age	d0-6	d6-10	d10-14	d0-14
Level																	
1000 (IU/kg)	434	1570	35.5	64.8	77.9	109	80.7	36.4	91.5	116	160	122	1.11	1.43	1.55	1.47	1.48
4000 (IU/kg)	444	1608	36.4	67.2	82.3	108	82.4	40.4	91.1	117	158	122	1.11	1.37	1.47	1.47	1.43
Source																	
D_3	437	1563	35.8	65.5	77.1	106	79.9	39.7	90.6	113	158	121	1.11	1.40	1.54	1.49	1.47
$25D_3$	441	1614	36.1	66.5	83.2	110	83.1	40.0	92.0	119	159	123	1.09	1.41	1.48	1.45	1.44
Infection																	
Control	-	1729	-	76.2	102	107	91.8	-	96.9	134	158	130	-	1.27	1.32	1.49	1.36
Infected	-	1448	-	55.8	57.9	110	71.2	-	85.7	97.8	159	114	-	1.54	1.70	1.45	1.57
SEM	3.6	12.8	0.31	0.84	1.53	1.82	0.79	0.319	1.06	2.00	1.90	1.10	0.0001	0.014	0.027	0.017	0.010
								Probabilities									
Level	**0.041**	**0.042**	**0.038**	0.056	**0.047**	0.638	0.128	**0.037**	0.783	0.714	0.506	0.800	0.225	**0.004**	**0.035**	0.934	**0.003**
Source	0.501	**0.008**	0.415	0.409	**0.008**	0.096	**0.007**	0.501	0.379	0.068	0.650	0.105	0.059	0.952	0.075	0.087	**0.018**
Infection	-	**<0.001**	-	**<0.001**	**<0.001**	0.242	**<0.001**	-	**<0.001**	**<0.001**	0.716	**<0.001**	-	**<0.001**	**<0.001**	0.135	**<0.001**

Table 5. Main effects of level, source of vitamin D supply and *Eimeria* infection status on chicken femur and tibia Seedor and Robusticity indices at d6, d10 and d14 post infection (pi). Chickens orally inoculated with 0 (Control) or $7x10^3$ sporulated *E. maxima* oocysts (Infected) at d11 post hatch (d0 pi)

	Femur						Tibia					
	Robusticity index*			Seedor index†			Robusticity index*			Seedor index†		
Days pi	d6	d10	d14	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level												
1000 (IU/kg)	3.35	3.34	3.36	78.5	90.0	109	3.99	4.00	4.08	83.4	99.8	120
4000 (IU/kg)	3.34	3.26	3.31	79.7	96.9	113	4.00	3.98	4.04	84.5	104.1	123
Source												
D_3	3.34	3.31	3.36	78.5	91.5	110	4.01	3.98	4.10	82.3	100.8	110
$25D_3$	3.35	3.29	3.31	79.8	95.5	112	3.98	4.00	4.03	85.5	103.0	122
Infection												
Control	3.29	3.28	3.30	82.3	101.3	119	3.96	3.97	4.01	87.3	108.9	131
Infected	3.39	3.32	3.37	76.0	85.6	103	4.03	4.00	4.12	80.5	94.9	110
SEM	0.018	0.022	0.022	0.98	2.02	1.7	0.017	0.019	0.021	1.01	1.73	2.1
						Probabilities						
Level	0.715	**0.017**	0.068	0.392	**0.019**	0.100	0.695	0.437	0.173	0.438	0.085	0.331
Source	0.711	0.439	0.072	0.362	0.169	0.598	0.227	0.491	**0.026**	**0.028**	0.375	0.364
Infection	**<0.0001**	0.163	**0.025**	**<0.0001**	**<0.0001**	**<0.0001**	**0.005**	0.385	**0.001**	**<0.0001**	**<0.0001**	**<0.0001**

*Robusticity index = ((bone length (mm)) / (bone weight (mg))1/3

†Seedor index = ((bone weight (mg)) / (bone length (mm))

Table 6. Main effects of level, source of vitamin D supply and *Eimeria* infection status on chicken femur and tibia bone breaking strength (BBS, N) and tibia ash (g) expressed as a proportion of body weight (BW, kg) and on tibia ash percentage (%) at d6, d10 and d14 post infection (pi). Chickens orally inoculated with 0 (Control) or $7x10^3$ sporulated *E. maxima* oocysts (Infected) at d11 post hatch (d0 pi)

	Femur BBS (N/kg of BW)			Tibia BBS (N/kg of BW)			Tibia ash (g/kg BW)			Tibia ash (%)		
Days pi	d6	d10	d14	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level												
1000 (IU/kg)	188	167	140	204	205	186	0.997	0.985	0.929	50.7	50.9	50.4
4000 (IU/kg)	198	182	146	230	215	196	1.040	0.999	0.953	51.6	51.9	52.0
Source												
D_3	192	171	139	212	204	189	0.989	0.957	0.938	50.7	50.9	50.6
$25D_3$	194	178	147	223	217	193	1.048	1.027	0.944	51.6	51.8	51.8
Infection												
Control	205	179	152	220	221	211	1.013	1.003	1.012	51.9	53.0	52.3
Infected	181	169	133	214	199	171	1.024	0.981	0.870	50.3	49.8	50.0
SEM	4.3	4.5	3.5	5.7	6.1	5.1	0.0167	0.0185	0.0166	0.35	0.28	0.36
						Probabilities						
Level	0.105	**0.028**	0.206	**0.002**	0.241	0.166	0.072	0.595	0.316	0.066	**0.021**	**0.003**
Source	0.681	0.289	0.110	0.179	0.145	0.630	**0.018**	**0.011**	0.799	0.070	**0.030**	**0.030**
Infection	**<0.0001**	0.124	**<0.001**	0.430	**0.010**	**<0.0001**	0.660	0.400	**<0.0001**	**0.002**	**<0.0001**	**<0.0001**

Table 7. Main effects of level, source of vitamin D supply and *Eimeria* infection status on chicken plasma Ca and P concentration (mmol/l) and log transformed plasma levels of 25-OH-D_3 (ng/ml) at d6, d10 and d14 post infection (pi). Chickens orally inoculated with 0 (Control) or $7x10^3$ sporulated *E. maxima* oocysts (Infected) at d11 post hatch (d0 pi)

	Ca (mmol/l)			P (mmol/l)			Log 25-OH-D_3 (ng/ml)		
Days pi	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level									
1000 (IU/kg)	2.56	2.79	2.75	2.05	1.96	1.98	1.51	1.46	1.59
4000 (IU/kg)	2.66	2.89	2.77	2.08	2.01	2.05	2.05	1.88	2.10
Source									
D_3	2.61	2.82	2.74	2.05	1.95	1.97	1.62	1.45	1.67
$25D_3$	2.61	2.85	2.79	2.08	2.02	2.06	1.94	1.88	2.02
Infection									
Control	2.81	2.81	2.73	2.20	2.01	2.04	1.84	1.94	1.88
Infected	2.40	2.87	2.80	1.93	1.97	1.99	1.71	1.39	1.81
SEM	0.032	0.034	0.028	0.047	0.046	0.036	0.022	0.023	0.020
				Probabilities					
Level	**0.040**	**0.040**	0.593	0.730	0.442	0.161	**<0.0001**	**<0.0001**	**<0.0001**
Source	0.861	0.564	0.185	0.675	0.296	0.090	**<0.0001**	**<0.0001**	**<0.0001**
Infection	**<0.0001**	0.194	0.109	**<0.001**	0.512	0.376	**<0.001**	**<0.0001**	**0.019**

Table 8. Main effects of level, source of vitamin D supply and *Eimeria* infection status on chicken intestinal morphology. Chickens orally inoculated with 0 (Control) or $7x10^3$ sporulated *E. maxima* oocysts (Infected) at d11 post hatch ((d0 post infection, (pi)).

	Duodenum								
	Villi length (μm)			Crypt depth (μm)			Villi length: Crypt depth		
Days pi	d6	d10	d14	d6	d10	d14	d6	d10	d14
Level									
1000	1617	1880	2234	263	223	197	7.81	8.62	11.6
4000	1590	1802	2181	276	239	204	7.49	7.96	10.7
Source									
D_3	1578	1867	2215	267	238	204	7.56	8.23	11.0
$25D_3$	1629	1814	2200	272	229	197	7.74	8.34	11.3
Infection									
Control	2009	1987	2216	170	199	190	11.99	10.18	11.7
Infected	1198	1695	2199	369	269	211	3.31	6.39	10.6
SEM	40.9	50.6	44.0	9.0	7.5	5.6	0.272	0.278	0.246
Probabilities									
Level	0.644	0.280	0.397	0.327	0.352	0.355	0.418	0.100	**0.024**
Source	0.379	0.462	0.810	0.675	0.386	0.418	0.644	0.768	0.498
Infection	**<0.0001**	**<0.001**	0.788	**<0.0001**	**<0.0001**	**0.012**	**<0.0001**	**<0.0001**	**0.002**
	Jejunum								
Level									
1000	835	995	1182	230	212	190	4.75	5.03	6.44
4000	839	1045	1186	236	203	175	4.85	5.40	6.89
Source									
D_3	866	1022	1214	236	211	184	4.94	5.10	6.68
$25D_3$	808	1019	1154	229	203	181	4.66	5.34	6.65
Infection									
Control	1069	1112	1208	139	172	165	7.69	6.52	7.42
Infected	605	928	1160	326	242	200	1.91	3.92	5.91
SEM	31.5	27.1	32.5	10.4	8.1	6.6	0.167	0.154	0.191
Probabilities									
Level	0.916	0.198	0.934	0.684	0.476	0.106	0.681	0.096	0.108
Source	0.202	0.927	0.197	0.631	0.448	0.711	0.234	0.275	0.918
Infection	**<0.0001**	**<0.0001**	0.290	**<0.0001**	**<0.0001**	**<0.001**	**<0.0001**	**<0.0001**	**<0.0001**
	Ileum								
Level									
1000	479	556	663	189	132	136	3.10	4.25	4.93
4000	497	583	649	186	139	146	3.20	4.23	4.53
Source									
D_3	496	575	671	206	135	142	3.03	4.33	4.83
$25D_3$	479	563.5	642	169	137	141	3.26	4.14	4.64
Infection									
Control	543	519	645	121	124	133	4.46	4.20	4.89
Infected	432	619	667	253	148	149	1.84	4.27	4.57
SEM	24.4	19.9	27.7	10.1	4.7	5.5	0.128	0.144	0.181
Probabilities									
Level	0.605	0.345	0.709	0.834	0.308	0.216	0.579	0.930	0.124
Source	0.636	0.679	0.463	**0.015**	0.703	0.885	0.208	0.350	0.467
Infection	**0.003**	**0.001**	0.575	**<0.0001**	**0.001**	**0.041**	**<0.0001**	0.731	0.212