

## **Mechanisms and nutritional influences in skeletal development: Influence of macro- and microelements on bone formation**

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### **Abstract**

Bone is composed of crystals of hydroxyapatite, a calcium phosphate, within an organic matrix composed mainly of collagen. Long bones grow longitudinally by endochondral ossification and bones thicken by intramembranous ossification. Vitamin D, after conversion to its active metabolite 1,25-dihydroxyvitamin D (1,25-D), is an important regulatory factor for the function of genes involved in calcium metabolism and bone growth. Calcium and phosphorus homeostasis is maintained by a regulatory feedback system involving 1,25-D and parathyroid hormone. Deficiencies and/or imbalances in dietary intake of calcium, phosphorus and/or vitamin D in young birds can result in problems of bone formation and quality characterised by rickets or tibial dyschondroplasia. There is evidence that genetic improvements in broiler performance are resulting in increased requirements for these nutrients. In laying hens, the problem of osteoporosis are not caused primarily by lack of nutrients but can be partially alleviated by providing a particulate source of calcium.

### **Bone composition**

Bone is composed of a bone mineral deposited in and around an organic matrix. The matrix, secreted by osteoblasts, is composed mainly of crosslinked microfibrils of type I collagen. The bone mineral is hydroxyapatite, deposited as small hexagonal rods, 200x50 Å. It is a calcium phosphate containing calcium and phosphorus in the approximate ratio of 2.15:1 but other cations and anions can also be present in small quantities. Normal bone contains about 30% matrix and 70% mineral. These proportions are optimal for the biomechanical properties of bone; a lower proportion of matrix results in bones being brittle, a lower proportion of mineral gives softer bones. The biomechanical properties of bone change with age as a result of continued collagen crosslinking as bones mature. In poultry, there are three types of bone whose physical properties are related to their collagen matrix. Cortical bone is lamellar in nature, with concentric rings of bone based a highly regular arrangement of collagen microfibrils. Trabecular or cancellous bone is another form of structural bone but is termed a woven bone because the collagen structure is less organised on an extended scale. The third bone type is found only in laying hens. This is medullary bone, also a woven bone but with a more disorganised collagen structure and much less intrinsic strength than the other two types.

### **Mechanisms of bone growth and remodelling**

#### *Bone metabolism in the growing bird*

Bone growth occurs in poultry in two ways. Longitudinal growth in long bones of the appendicular skeleton is brought about by a process called endochondral ossification. This is based on the epiphyseal growth plate where some of the resting chondrocytes with fibroblastic phenotype in the germinal layer become committed to differentiate into proliferative chondrocytes. These cells multiply and form columns of flattish cells closely packed within an extracellular matrix, secreted by the chondrocytes, that contains a high content of type II collagen. This zone of proliferative chondrocytes is nourished by the epiphyseal blood capillaries. These cells gradually become more separated within their columns as more matrix is secreted, then they start to differentiate into a hypertrophic state. They become enlarged and more rounded and start secreting a new matrix component, type X collagen. The hypertrophic zone receives its blood supply from metaphyseal blood vessels, so there is a narrow avascular zone of prehypertrophic chondrocytes between the proliferative and hypertrophic zones. Apart

from collagen, chondrocytes secrete other matrix components such as proteoglycans and growth factors. These matrix components in turn regulate the further development of the chondrocytes.

Bone formation starts in the lower hypertrophic zone. Chondroclasts resorb matrix and fully hypertrophied chondrocytes secrete alkaline phosphatase that helps to initiate the initial formation of crystals of hydroxyapatite. The chondrocytes die by apoptosis and are reabsorbed and osteoblasts, the bone forming cells, form from precursor cells in the marrow. Osteoblasts produce both the bone matrix of fibrils of type I collagen and induce the formation of the mineral that becomes embedded within and around this matrix. Bone resorbing cells, osteoclasts, are also active in this area and bone remodelling, the coupled actions of osteoclastic resorption followed by osteoblastic bone formation, results in the development of a network of trabecular bone. This is a woven bone based on a rather irregular structure of collagen fibrils. As the bone elongates, by continued proliferation of chondrocytes at the head of the growth plate followed by hypertrophy and mineralisation at the rear, the trabecular network is largely resorbed to form the marrow cavity. This developmental process differs from mammalian bone growth in that it does not involve a secondary ossification centre.

The long bones widen by a process involving intramembranous ossification. Osteoblasts develop in the perichondrium and produce spicules of bone that merge to produce a network of bone with cavities lined by osteoblasts. These cavities are gradually infilled by osteoblasts continuing to secrete concentric layers of lamellar cortical bone. At the endosteal surface, osteoclasts resorb bone so that the bone widens as an expanding ring, with bone formation on the outer surface and resorption on the inner. In the early growing period, the ring expands rapidly so that cavities do not become completely filled with bone before endosteal resorption commences but as growth slows the degree of infilling rises. During early growth there is little remodelling of bone but towards the end of the growing period secondary osteons are formed by cutting cones in which osteoclasts cut a tunnel in the bone and are followed by osteoblasts forming new concentric layers of lamellar bone. In the process of formation of primary and secondary osteons, osteoblasts become entombed within bone and differentiate into osteocytes. These cells have a network of interconnections and can regulate bone remodelling in response to biomechanical forces.

#### *Bone metabolism in hens*

Bone metabolism changes considerably with the onset of sexual maturity in the female chicken. The large rise in circulating oestrogen switches osteoblasts to forming medullary bone rather than structural bone. Medullary bone is a type of woven bone based on a very irregular structure of collagen fibrils. It forms on the surfaces of structural bone and in small particles within the marrow cavity and serves as a labile source of calcium for eggshell formation. Medullary bone is resorbed by osteoclasts during the period of shell formation and replaced by osteoblasts at times when the egg is not in the shell gland. There is thus a diurnal pattern of bone resorption and formation. During resorption, osteoclasts may also resorb structural bone so that there is a slow replacement of structural bone by medullary bone during the period that the hen is in reproductive condition.

#### **Homeostatic mechanisms**

Calcium and phosphorus homeostasis is maintained by a mechanism involving vitamin D and other hormones, principally parathyroid hormone (PTH). Vitamin D does not exert its effects directly. It is converted in the liver to 25-hydroxyvitamin D (25-D) which is then hydroxylated in the kidney to give two main forms, 1,25-dihydroxyvitamin D (1,25-D) and 24,25-dihydroxyvitamin D (24,25-D). 24,25-D may play a minor role in bone metabolism, but

is mainly an excretion route for excess vitamin D and 25-D. 1,25-D is the form that exerts the main biological effects of vitamin D, since it binds tightly to the vitamin D receptor (VDR) in the first step in gene activation. Among the vitamin D-dependent genes is calbindin, an important factor in calcium absorption and transport. Circulating calcium concentration is monitored by calcium sensing receptor in the parathyroid gland and a calcium deficiency triggers the production of PTH. This activates the renal 1-hydroxylase to synthesis 1,25-D and increase calcium absorption. Intestinal absorption of phosphorus is induced also by 1,25-D by another mechanism. In the absence of sufficient dietary calcium or phosphorus, PTH and 1,25-D can activate osteoclasts to provide calcium and phosphorus from resorbed bone. Because of the metabolic linkage between these nutrients, antihomoeostatic mechanisms may also occur causing a toxicity of one nutrient when the other is limiting. For instance, with a diet of high calcium and low phosphorus concentration, increased absorption of both nutrients can lead to hypercalcaemia. Use of excessive amounts of 1,25-D as a feed additive can also induce hypercalcaemia, especially with higher dietary concentrations of calcium (Rennie et al., 1995).

### **Nutrition and bone quality in broilers**

Correct calcium and phosphorus nutrition is vital for proper bone quality. Vitamin D is also essential, because of its role in the homeostatic mechanism for calcium and phosphorus. Vitamin D can be provided as cholecalciferol or as 25-D, which can exert all of the biological effects associated with vitamin D itself. Defects in calcium/phosphorus/vitamin D nutrition or metabolism are associated with two main abnormalities of bone development in growing birds.

#### *Rickets*

Deficiencies of calcium, phosphorus and/or vitamin D result in rickets in young birds. This results in delayed and poor calcification of bones. Growth plates are enlarged and the organic matrix of bone is undermineralised, resulting in low bone ash contents. Bones are soft and rubbery and other defects, such as beading of ribs, are also apparent. The morphology of the growth plate can be used to distinguish between the rickets caused by calcium/vitamin D deficiency or phosphorus deficiency. In the former, there is a considerable increase in the thickness of the proliferative zone, and the chondrocytes lose their ordered, columnar arrangement. In phosphorus deficiency rickets, the proliferative zone is relatively normal in size and structure but, instead, there is a considerable increase in the size of the hypertrophic zone and a delay in mineralisation of this zone. Rickets of both types can be caused by dietary deficiency or an imbalance in the proportions of calcium and phosphorus. Thus an excess of one can induce a deficiency of the other. Rickets can also occur with diets that are not overtly inadequate in calcium/phosphorus/vitamin D and in these cases the problem is thought to be caused by infections that impair nutrient absorption (malabsorption). The growth plate abnormalities can lead to bone growth deformity, resulting ultimately in clinical lameness. However, the lameness may not become apparent until some time after the occurrence of the, perhaps transient, lesion. Thus, when examining the dietary history of flocks showing lameness problems, it is necessary to consider all diets the birds may have been fed, not just the diet at the time of observation of the lameness.

#### *Tibial dyschondroplasia (TD)*

TD is characterized by an accumulation or prehypertrophic chondrocytes resulting in a thickened layer of avascular cartilage between the proliferative and hypertrophic zones. This occurs primarily in the proximal tibia, hence the name. The problem is caused by an impairment of the differentiation process that converts proliferative into hypertrophic

chondrocytes, but the underlying mechanistic cause of this is not yet properly understood. There are many cellular and other abnormalities within the lesion, but there is difficulty in distinguishing between cause and effect.

The size of the lesion can vary, from a small focal lesion to a large lesion occupying the whole width of the growth plate. The lesion can start forming during the first week of life of the broiler and may reach its greatest size at about 3-4 weeks before regressing. Large lesions can result in abnormal angulation of the proximal tibia and leg deformity. Several nutritional factors can influence the occurrence of TD. A low ratio of calcium:phosphorus can increase the incidence of TD, but there is no indication that a correct balance of these nutrients will necessarily prevent the condition (Edwards and Veltmann, 1983). Other nutrients, including monovalent cations and anions (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>) can also alter the incidence, but the only nutrients that have so far been shown to completely prevent TD are vitamin D and its metabolites. 1,25-D is particularly effective in the dose range 5 to 10 mg/kg (Edwards, 1989; Rennie et al., 1993), but 25-D has also shown to be effective, though at higher dose rates (75-250 mg/kg) (Rennie and Whitehead, 1996). Lower doses of these nutrients can also decrease the severity of lesions and this may be sufficient to decrease the occurrence of clinical lameness. More recent findings (Whitehead et al., 2004) indicate that vitamin D itself, when fed at high dose rates, can reduce or prevent the occurrence of TD, as shown below.

TD also occurs in turkeys, though it shows several important differences from broiler TD. The age of development is later in turkeys, with peak incidence occurring around 10 weeks of age. The cellular characteristics of the lesion appear to be the same as in broilers but the causative mechanism may differ, because dietary calcium/phosphorus and vitamin D metabolites are ineffective in affecting the incidence. The consequences of the lesion are much less severe in turkeys, where the condition is not usually associated with bone deformity or lameness.

#### *Calcium and phosphorus requirements*

The current NRC (1994) requirements for broilers for calcium and non-phytate phosphorus are 1.0 and 0.45% respectively for starter and 0.9 and 0.35% for finisher diets. These nutrients are approximately in the ratio of 2.2:1, particularly for the starter diets, that corresponds with the bone mineral ratio. Requirements for these nutrients have traditionally been established on the basis of measurements of bone quality, usually bone ash content which is depressed at suboptimal levels of either nutrient. Evidence from recent experiments has suggested that the requirements may be changing in response to genetic improvements in broiler growth rate. Studies on TD have suggested that better bone calcification was obtained if dietary calcium/available phosphorus was increased above 2.2/1 (Roberson et al., 1993). More recently, Williams et al. (2000a) compared the proportions of calcium and phosphorus at different ages in two strains of broilers, one a very modern fast growing selected strain, the other an older, slower growing control strain. The results suggest that although the 2.2/1 ratio was broadly appropriate for the control strain, the ratio was higher during the early stages of growth of the selected strain. Morphological studies confirmed that the cortical bone of the selected line was less well mineralised and more porous. It appeared that the bone was growing so fast that bone-forming osteoblasts were being replaced by bone-resorbing osteoclasts before bone had become fully mineralised. These observations suggest that the mechanisms of bone formation and the composition of the bone are changing in response to continued selection for fast growth of broilers and this may result in altered nutrient requirements.

Subsequent studies have given further insight into this matter. An experiment was carried out in which histological characteristics of tibial growth plates were compared at 14 days in fast

growing broilers fed diets containing different amounts of calcium and available phosphorus (Williams et al., 2000b). Changes associated with hypocalcaemic and hypophosphataemic rickets and TD were seen with the different diet combinations, but the combinations associated with the most normal growth plate morphology centred on a diet content of 1.2% calcium and 0.45% phosphorus. This finding thus suggests that the calcium requirement of the latest fast growing broiler strains is increasing for the early stages of growth and that dietary content of calcium and calcium:phosphorus ratio needs to be higher than current recommendations for starter diets.

Requirements for calcium and phosphorus during the finisher period can be assessed on the basis of bone composition at the end of the finisher period; there is virtually no carry over effect of starter nutrition on bone composition because the fast growth of bones means that all bone formed during the starter period has been replaced completely by the end of the finisher period. Requirements have usually been assumed to be lower than for starting broilers, as suggested by NRC (1994) but a recent report by Bar et al. (2003) suggests that requirements for a modern strain may be higher than earlier estimates, up to 1.0% calcium and 0.48% non-phytate phosphorus.

#### *Microelements*

Some metal elements (e.g. Mg, Zn, Sr, Pb, Al) can be incorporated into bone hydroxyapatite structure in place of Ca in amounts depending upon the circulating concentrations (i.e. low Ca or high element concentrations will increase incorporation). Normally these elements do not affect bone quality but toxicities can cause problems: for instance excessive incorporation of Sr weakens bone structure, of Al suppresses osteoblasts and mineralization. Other elements, including B, Cd, Si and V have also been shown to affect bone development in extreme situations

Other metals can affect the bone growth plate. Mn deficiency results in chondrodystrophy and slipped tendon. Cu deficiency weakens collagen structure through inhibition of lysyl oxidase and crosslinking. High dietary levels of Cu or Zn can alleviate the effect of fusarochromanone, a mycotoxin, in inducing TD, as can supplemental Mo in alleviating cystine-induced TD. However, there is no indication that these minerals can influence normal spontaneous TD.

Among the anions, F<sup>-</sup> can be incorporated into bone. This inhibits the action of osteoclasts in resorbing bone and can increase bone strength. However, the response is dose-dependent because feeding higher concentrations of F<sup>-</sup> results in increases in hydroxyapatite crystal size and lower bone strength. Supplemental F<sup>-</sup> may also improve hen bone quality through greater medullary bone formation, but a practical role for F<sup>-</sup> in improving poultry bone quality has not yet been established.

The general conclusion from these observations is that minor element nutrition has little impact upon bone characteristics under normal practical nutritional conditions.

#### *Vitamin D*

The broiler vitamin D requirement proposed by NRC (1994) is 200 IU/kg. However, there are widely differing requirement values reported from individual studies. One study (Whitehead, 1996) showed that providing 800 rather than 400 IU/kg gave significantly higher growth and plasma 25-hydroxyvitamin D (25-D) concentration. Other studies (Edwards et al., 1994; Mitchell et al., 1997) confirm that the requirement is in the order of 800 to 1000 IU/kg as judged by bone ash and performance.

A complication in establishing the vitamin D requirement is the interaction of vitamin D with a number of nutrients. The effects of imbalances of calcium and phosphorus in increasing the need for vitamin D have been known for many years. Thus Waldroup et al. (1965) showed

that vitamin D requirement was increased markedly, up to almost 2000 IU/kg, when diets contained imbalances or deficiencies of calcium and phosphorus. More recently, information has been presented showing effects of elevated levels of vitamins A and E in depressing vitamin D status (Aburto et al., 1988a; 1988b). The effect of vitamin A was apparent at practical dietary levels, with an increase from 1500 to 15000 IU/kg of vitamin A resulting in an approximately 3-fold increase in the vitamin D requirement. The effect of vitamin E on vitamin D requirement was of more apparent at dietary levels of vitamin E considerably above meaningful nutritional levels. In contrast, exposure of birds to UV light can almost abolish a need for dietary vitamin D.

In view of these uncertainties over the vitamin D requirement, we have recently reassessment reassessed in 2 experiments vitamin D requirements under different combinations of calcium, phosphorus and vitamin A concentrations (Whitehead et al., 2004). The first experiment was carried out to make comparisons at 14 and 42 days. It had a factorial design with 4 concentrations of vitamin D (200, 800, 5000 and 10000 IU/kg) x 2 concentrations of vitamin A (8000 and 15000 IU/kg) x 2 concentrations of calcium and available phosphorus. The results of experiment 2 are shown in Table 1. There were no overall effects of vitamin A concentration, nor any interactions between vitamin A and vitamin D or Ca/avP concentrations so the results presented are for the vitamin A treatments combined. At 14 days, liveweight was significantly higher with 10000 IU vitamin D/kg with both Ca/avP combinations than for other concentrations of vitamin D. Tibia ash and breaking strength were both higher with 5000 and 10000 IU vitamin D/kg than for the lower concentrations of vitamin D. At 42 days, liveweight was significantly lower but tibia strength was significantly higher with 200 IU vitamin D/kg than for the other vitamin D treatments. TD incidence was high at the two lower vitamin D concentrations but very much lower at the two higher concentrations. These findings on vitamin A contrast with the previous findings of Aburto et al. (1988a; b) but suggest that variations in vitamin A concentration within the conventional practical range do not affect vitamin D status or requirements in broilers.

Results from the second experiment up to 14 days involving the adding graded levels of vitamin D to diets of different calcium/phosphorus are given in Table 2. They show that 200 IU vitamin D/kg was clearly inadequate for growth and tibia ash and breaking strength. Higher dietary vitamin D levels could not overcome the most severe calcium/phosphorus imbalance, but dietary vitamin D levels above 5000 IU/kg could optimise performance and bone characteristics with less severe imbalances. TD incidence was affected considerably by dietary Ca/P but was also highly dependent upon vitamin D concentration.

These results suggest that the vitamin D requirements of young broilers can be quite high and that responses in performance and bone characteristics can occur to dietary concentrations above 5000 IU/kg at both conventional and abnormal dietary calcium and phosphorus concentrations or ratios up to 14 days. Requirements for vitamin D may be lower in older broilers, but the data indicate that 200 IU vitamin D/kg is still clearly inadequate for growth. The very strong tibia breaking strength of birds fed this amount on vitamin D may indicate that, though smaller, the bones were well mineralised. This study was the first to show a high dependence of TD incidence on dietary vitamin D concentration

The relatively high vitamin D requirements for young broilers observed in this study contrast with previous, lower estimates and it is relevant to consider why. The high requirements may have been related to the particular conditions of the experiment, though these were not thought to be unusual. Alternatively, the results could indicate that the latest strains of broilers have higher requirements, perhaps as a result of changing bone growth characteristics and increasing calcium requirements, as discussed in the previous section. Conventional practical vitamin D supplements are normally in the range 3000-5000 IU/kg but, in view of the current

findings, it would seem prudent to supplement at the higher end of this range or above if regulations permit, especially in broiler starter diets.

#### *B-Vitamins*

Deficiencies of B vitamins most commonly affect the epiphyseal growth plate. Impaired supply of essential nutrients slows the rate of chondrocyte proliferation and rate of endochondral growth of leg bones. However, appositional growth is usually relatively unaffected, so the net effect is bones of almost normal thickness but considerably shortened and showing a narrow proliferative zone in the growth plate. This condition is called chondrodystrophy. The impaired longitudinal growth may result deformity of the condyles, giving the hock joint a thickened appearance and allowing the gastrocnemius tendon to slip causing an abnormal rotation of the joint of 90 or even 180° in severe cases. This condition is often referred to as 'perosis' but more correctly as 'slipped tendon'. Chondrodystrophy is caused especially by deficiencies of nicotinic acid, biotin, pyridoxine (particularly at high protein intakes), folic acid and choline. Riboflavin deficiency can occasionally give the condition, but curled toe paralysis is more common and is caused by nerve degeneration. Pyridoxal phosphate is required, along with copper, for activity of lysyl oxidase, an enzyme involved in collagen crosslinking. Pyridoxine deficiency has been shown to result in bone of normal mineral composition but lower matrix content and less crosslinked collagen. This resulted in bone with much poorer breaking strength (Masse et al., 1996). Chondrodystrophic conditions can occur in the absence of overt vitamin deficiency caused by infections such as mycoplasma that can impair the supply of nutrients to the bone growth plate. In commercial practice, simple deficiencies of B-vitamins seldom occur, so chondrodystrophy and related conditions are rarely seen now.

#### *Other vitamins*

Vitamin K is involved in the post-transcriptional carboxylation of several bone and matrix proteins (e.g. osteocalcin, GLA protein). However, the importance of vitamin K for poultry bones under practical conditions has not been established. Vitamin C is required for the synthesis of bone and cartilage matrix collagen and is also a component of the renal enzyme system synthesizing 1,25-D. Under normal conditions, endogenous vitamin C synthesis is adequate for matrix and bone quality. However, there are some reports that supplemental vitamin C may be useful against leg abnormalities in heat stress conditions, some cases of rickets and TD (in synergism with 1,25-D).

#### **Nutrition and bone quality in layers**

Osteoporosis is the main bone-related problem in hens. It arises from the continual loss of structural bone during the laying period, which results in the bones becoming increasingly fragile and susceptible to fracture. It is not caused primarily by a lack of calcium, because the daily intake of calcium is normally sufficient to meet the needs of eggshell formation. Instead, osteoclast action in the resorption of medullary bone during the period of eggshell formation also resorbs some structural bone that is not replaced by subsequent osteoblast action. Provided the diet contains sufficient total calcium to meet the needs of the bird, the most effective nutritional means of minimizing, but not preventing, structural bone loss appears to be providing the calcium source in particulate form, either as oystershell or limestone granules (Guinotte and Nys, 1991; Rennie et al., 1997; Fleming et al., 1998). This probably acts by providing a nutritional source of calcium later into the period of eggshell formation, thus decreasing the need for bone resorption. 1,25-D is used as a treatment for human postmenopausal osteoporosis but is ineffective in laying hen osteoporosis (Rennie et al., 1997).

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**Table 1. Liveweight, tibia breaking strength and TD incidence in broilers fed diets containing different concentrations of vitamin D, calcium, available phosphorus and vitamin A (experiment 1)**

| Trait                               | Dietary |         | Vit A(IU/kg) | Dietary vitamin D (IU/kg) |                   |                   |                    |
|-------------------------------------|---------|---------|--------------|---------------------------|-------------------|-------------------|--------------------|
|                                     | Ca (%)  | avP (%) |              | 200                       | 800               | 5000              | 10000              |
| Liveweight at 14 days (g)           | 1.00    | 0.45    | 8000/15000   | 302 <sup>a</sup>          | 298 <sup>a</sup>  | 307 <sup>a</sup>  | 327 <sup>b</sup>   |
|                                     | 0.80    | 0.35    | 8000/15000   | 315 <sup>a</sup>          | 311 <sup>a</sup>  | 316 <sup>a</sup>  | 336 <sup>b</sup>   |
| Liveweight at 42 days (kg)          | 1.00    | 0.45    | 8000/15000   | 1.95 <sup>a1</sup>        | 2.35 <sup>b</sup> | 2.40 <sup>b</sup> | 2.38 <sup>b</sup>  |
|                                     | 0.80    | 0.35    | 8000/15000   | 1.73 <sup>a2</sup>        | 2.35 <sup>b</sup> | 2.33 <sup>b</sup> | 2.46 <sup>b</sup>  |
| Tibia breaking strength at 14 d (N) | 1.00    | 0.45    | 8000/15000   | 59.5 <sup>a</sup>         | 80.4 <sup>b</sup> | 92.1 <sup>c</sup> | 101.3 <sup>c</sup> |
|                                     | 0.80    | 0.35    | 8000/15000   | 61.0 <sup>a</sup>         | 78.1 <sup>b</sup> | 90.9 <sup>c</sup> | 93.5 <sup>c</sup>  |
| Tibia breaking strength at 42 d (N) | 1.00    | 0.45    | 8000/15000   | 500 <sup>a1</sup>         | 362 <sup>b</sup>  | 379 <sup>b</sup>  | 376 <sup>b</sup>   |
|                                     | 0.80    | 0.35    | 8000/15000   | 448 <sup>a2</sup>         | 395 <sup>ab</sup> | 345 <sup>c</sup>  | 376 <sup>bc</sup>  |
| TD incidence at 14 days (%)         | 1.00    | 0.45    | 8000/15000   | 78 <sup>a</sup>           | 39 <sup>b</sup>   | 4 <sup>c</sup>    | 4 <sup>c</sup>     |
|                                     | 0.80    | 0.35    | 8000/15000   | 88 <sup>a</sup>           | 51 <sup>b</sup>   | 6 <sup>c</sup>    | 8 <sup>c</sup>     |

Within a trait and comparison, values followed by different letters (in a row) or numbers (in a column) differ significantly (P<0.05)

**Table 2. Liveweight and tibia breaking strength and TD incidence at 14 days in broilers fed diets containing different concentrations of vitamin D, calcium and available phosphorus (experiment 2)**

| Trait                       | Dietary |         | Dietary vitamin D (IU/kg) |                    |                    |                     |
|-----------------------------|---------|---------|---------------------------|--------------------|--------------------|---------------------|
|                             | Ca (%)  | avP (%) | 200                       | 800                | 5000               | 10000               |
| Liveweight (g)              | 0.80    | 0.35    | 295 <sup>a2</sup>         | 297 <sup>a2</sup>  | 303 <sup>a1</sup>  | 351 <sup>b2</sup>   |
|                             | 0.80    | 0.50    | 308 <sup>a2</sup>         | 307 <sup>a2</sup>  | 339 <sup>b2</sup>  | 349 <sup>b2</sup>   |
|                             | 1.30    | 0.35    | 257 <sup>a1</sup>         | 258 <sup>a1</sup>  | 303 <sup>b1</sup>  | 261 <sup>a1</sup>   |
|                             | 1.30    | 0.50    | 301 <sup>a2</sup>         | 306 <sup>a2</sup>  | 343 <sup>b2</sup>  | 333 <sup>b2</sup>   |
| Tibia breaking strength (N) | 0.80    | 0.35    | 36.4 <sup>a1</sup>        | 44.5 <sup>a2</sup> | 61.4 <sup>b2</sup> | 76.1 <sup>c2</sup>  |
|                             | 0.80    | 0.50    | 47.9 <sup>a2</sup>        | 59.6 <sup>b3</sup> | 94.7 <sup>c3</sup> | 83.0 <sup>d23</sup> |
|                             | 1.30    | 0.35    | 26.8 <sup>a1</sup>        | 30.2 <sup>a1</sup> | 44.6 <sup>b1</sup> | 43.3 <sup>b1</sup>  |
|                             | 1.30    | 0.50    | 54.2 <sup>a2</sup>        | 65.4 <sup>b3</sup> | 89.0 <sup>c3</sup> | 88.9 <sup>c3</sup>  |
| TD incidence (%)            | 0.80    | 0.35    | 78 <sup>a</sup>           | 84 <sup>a</sup>    | 22 <sup>b</sup>    | 0 <sup>b</sup>      |
|                             | 0.80    | 0.50    | 79 <sup>a</sup>           | 78 <sup>a</sup>    | 52 <sup>b</sup>    | 20 <sup>b</sup>     |
|                             | 1.30    | 0.35    | 4                         | 0                  | 4                  | 0                   |
|                             | 1.30    | 0.50    | 40 <sup>a</sup>           | 39 <sup>a</sup>    | 8 <sup>b</sup>     | 0 <sup>b</sup>      |

Within a trait, values followed by different letters (in a row) or numbers (in a column) differ significantly (P<0.05).