The Expression of Calbindin in Chicks that Are Divergently Selected for Low or High Incidence of Tibial Dyschondroplasia[1]

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ABSTRACT Three experiments were conducted with broiler chicks that were divergently selected for low or high incidence of tibial dyschondroplasia (LTD and HTD, respectively) to determine if the expression of intestinal calbindin-28 kD mRNA and protein differed between the 2 strains. In addition, levels of intestinal vitamin D receptor mRNA and plasma thyroid hormone concentrations were also examined. In experiment 1, LTD and HTD chicks were fed a corn-soybean meal diet that was adequate in all nutrients except cholecalciferol (D_3), which was titrated to 5 or 40 μg/kg diet in a completely randomized 2 × 2 factorial arrangement. At 4 and 8 d of age, HTD chicks fed 5 μg of D_3/kg of diet had a lower ($P < 0.05$) expression level of calbindin-28 kD mRNA than the LTD chicks fed the same diet. At 4 and 8 d of age, HTD chicks fed 5 μg of D_3 had the lowest intestinal expression of calbindin-28 kD protein. Expression of vitamin D receptor mRNA did not differ for broiler strains at either level of D_3 supplementation. In experiment 2, there was no significant difference in the expression of calbindin-28 kD mRNA or vitamin D receptor mRNA between day-of-hatch LTD, HTD, and commercial broiler chicks. Experiment 3 was similar in design to the first experiment except that the birds were fed for 18 d. Calbindin-28 kD and vitamin D receptor mRNA expression levels at 18 d were similar to those observed in experiment 1. Plasma triiodothyronine and free-triiodothyronine concentrations were greater for LTD chicks, regardless of dietary D_3 supplementation levels. These results suggest that divergent selection of broilers for LTD or HTD alters the physiological response to nutritionally inadequate levels of dietary D_3.

(*Key words*: broiler chick, calbindin, thyroid hormone, tibial dyschondroplasia, vitamin D receptor)

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INTRODUCTION

In a normally developing long bone such as the tibiotarsus, chondrocytes of the epiphyseal growth plate are organized in columns. The resting chondrocytes of the proximal epiphysis divide into proliferating cells that progressively mature into proliferating, prehypertrophic, and hypertrophic chondrocytes, which eventually develop into endochondral or metaphyseal bone (Leach and Gay, 1987; Farquharson and Jefferies, 2000). Alterations to this normal process can lead to several bone diseases such as Ca rickets, P rickets, or tibial dyschondroplasia (TD). The characteristic feature of a TD lesion is the presence of a nonmineralized, amorphous area of avascular cartilage near the metaphysis where hypertrophic chondrocytes have arrested development, possibly leading to premature apoptotic cell death (Orth and Cook, 1994; Praul et al., 1997; Farquharson and Jefferies, 2000).

Tibial dyschondroplasia is a crippling bone disease that is often observed in rapidly growing poultry. Poultry that develop TD have a characteristic bowlegged and unstable gait, are less likely to consume feed and water or move away from more aggressive birds, and have reduced growth rates and a higher incidence of morbidity and mortality. As reviewed by Orth and Cook (1994), the complex etiology of TD remains unclear; however, several factors may be involved such as genetic selection for growth rate, exposure to aflatoxin, and feeding diets that are deficient in cholecalciferol, contain excessive chloride, or have an imbalance of dietary Ca and P.

Increasing the ratio of dietary Ca:P decreases the incidence and severity of TD in broiler chicks (Edwards and Veltmann, 1983; Roberson et al., 1993; Elliot and Edwards, 1994, 1997; Elliot et al., 1995; Rennie et al., 1995; Edwards, 2000). Likewise, dietary supplementation with cholecalciferol, 1-α- hydroxycholecalciferol, and 1,25-dihydroxy-

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Abbreviation Key: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HTD = high incidence of tibial dyschondroplasia; LTD = low incidence of tibial dyschondroplasia; TD = tibial dyschondroplasia; T_3 = 3,5,3′-triiodothyronine; 1,25-$(OH)_2D_3$ = 1,25-dihydroxycholecalciferol; vitamin D_3 = cholecalciferol.

cholecalciferol [1,25-$(OH)_2D_3$] can also lower the incidence and severity of TD (Elliot and Edwards, 1994, 1997; Elliot et al., 1995; Rennie et al., 1995; Roberson and Edwards, 1996; Edwards, 2000) by increasing Ca and P absorption and reabsorption from the intestine and the kidney, respectively. An increase in active transport of Ca across epithelial cells of the duodenum has been linked, in part, to a Ca/cholecalciferol (vitamin D_3)-dependent protein, calbindin-28 kD (Cross et al., 1986; Theofan et al., 1987; Cross and Peterlik, 1988, 1991; Sechman et al., 1996). Intestinal expression of calbindin-28 is enhanced by D_3 and 1,25-$(OH)_2D_3$ (Cross et al., 1986; Theofan et al., 1987; Cross and Peterlik, 1988, 1991; Sechman et al., 1996). In addition, other investigators (Cross et al., 1986; Cross and Peterlik, 1988, 1991; Sechman et al., 1996) have reported that an increase in the expression of calbindin-28 kD caused by 1,25-$(OH)_2D_3$ can be further enhanced by addition of thyroid hormones (thyroxine and 3,5,3′-triiodothyronine). These results suggest that an increase in calbindin-28 kD synthesis within the duodenum may be linked to differences in the serum concentration of 1,25-$(OH)_2D_3$ or thyroid hormone.

Through genetic selection of commercial broiler chicks, Wong-Valle et al. (1993) established 2 genetic lines of broiler chicks that have low (LTD) or high (HTD) incidence of TD. The heritability of TD in the HTD line was 0.437 (Wong-Valle et al., 1993). Although the LTD and HTD strains have been used in several studies to investigate dietary factors that may alleviate or exacerbate TD (Mitchell et al., 1997a,b), there has been limited investigation into the potential biochemical differences between these 2 strains of broilers. Therefore, 3 experiments were conducted to test 2 hypotheses: that the expression of intestinal calbindin-28 kD mRNA, calbindin-28 kD protein, and vitamin D receptor mRNA differs between broiler chicks divergently selected for low or high incidence of TD and that plasma thyroid hormone concentrations between the strains reflect differences in the incidence of TD.

MATERIALS AND METHODS

Experiment 1

The NRC nutrient values for ingredients (NRC, 1994) were used to formulate a corn-soybean meal basal diet that contained soybean oil as the fat source. The basal diet met or exceeded the NRC nutrient requirements for poultry from 0 to 21 d of age, except for vitamin D_3. Crystalline vitamin D_3 (250 mg/vial)[3] was carefully diluted with 10 mL of ethanol and then diluted with 190 mL of propylene glycol to produce a vitamin D_3 stock solution with a concentration of 1 mg/mL. This solution was then added to the basal diet at 5 μg D_3/kg of diet (200 IU/kg diet) to create treatment 1 or 40 μg D_3/kg diet to create treatment 2.

In a completely randomized 2 × 2 factorial arrangement, day of hatch, mixed-sex chicks from each TD line[4] were wing-banded, weighed, and randomly allotted to 1 of the 2 dietary treatments. Each treatment was replicated 6 times, and each replicate pen contained 4 birds. Throughout the experiment, chicks were given free access to water and mash-feed, brooded in thermostatically controlled Petersime[5] starter batteries with raised wire floors, and reared on a 24-h lighting schedule. The starter batteries were kept in an environmentally controlled room (22°C), where fluorescent lights outside and inside the batteries were covered with plastic sleeves[6] to eliminate ultraviolet light exposure and any possible de novo vitamin D_3 synthesis by the chick (Edwards et al., 1994; Elliot and Edwards, 1997; Mitchell et al., 1997b).

On d 4 and 8 of experiment 1, two randomly selected chicks from each pen were killed by cervical dislocation. The proximal duodenum of each bird was immediately excised to obtain epithelial cells for future calbindin-28 kD mRNA and vitamin D receptor mRNA Northern blot analyses and calbindin-28 kD Western blot analysis. The Institutional Animal Care and Use Committee of the University of Georgia approved all animal procedures.

Experiment 2

This experiment was conducted to determine if differences in calbindin-28 kD mRNA expression existed between the LTD and HTD broiler lines at the time of hatch. Intestinal scrapings from 24 chicks per strain were collected from the LTD and HTD strains as well as from a Ross 308 commercial broiler strain (control). Intestinal cells were pooled from 2 birds of each line (n = 12) for RNA extraction and future Northern blot analysis for calbindin-28 kD and the vitamin D receptor.

Experiment 3

This experiment was done to determine if chicks from the LTD and HTD lines differed in plasma thyroid hormone concentrations. The experimental design for this experiment was very similar to experiment 1, except that the birds were maintained on the dietary treatments for 18 d. Furthermore, in addition to utilizing chicks from the LTD and HTD lines, chicks from a commercial broiler strain (Ross 308) were used as a control. Each treatment contained 4 pens of 6 birds per pen in a completely randomized 3 × 2 factorial arrangement. On d 18, chicks were weighed, and a heparinized blood sample was obtained from each bird by cardiac puncture. Chicks were then killed by cervical dislocation. Intestinal scrapings from the proximal duodenum of 2 randomly selected chicks from each pen of the LTD and HTD lines were collected and pooled for RNA extraction. After blood and tissue samples were taken, the right tibia of each chick was then scored for the incidence and severity of cholecalciferol-

[3]Sigma Chemical Co., St. Louis, MO.
[4]Auburn University, Auburn, AL.
[5]Petersime Incubator Co., Gettysburg, OH.
[6]Arm-a-Lite Thermoplastic Processes, Sterling, NJ.

dependent rickets (Long et al., 1984) and TD (Edwards and Veltmann, 1983). The left tibia of each chick was banded for identification and removed for future determination of percentage of tibia ash on a dry, fat-free basis (AOAC, 1995).

Plasma Analysis and RIA

Heparinized blood samples were centrifuged for 10 min at 3,000 × *g*. Plasma from each sample was separated into 2 volumes. One volume was used for an immediate determination of ionized plasma Ca.[7] The other volume was frozen at –20°C for future assays of thyroid hormone.

Plasma concentrations of thyroxine, 3,5,3′-triiodothyronine (T_3), and free T_3 were determined by using RIA kits.[8] The RIA were performed following the manufacturers instructions. The R^2 value for each of the standard curves was greater than 0.99.

SDS-PAGE and Western Immunoblot Analysis

For the Western immunoblot analyses, intestinal cells (~100 mg) were scraped from the proximal portion of the duodenum and briefly sonicated in 200 μL of lysis buffer containing protease inhibitors (5 m*M* HEPES, pH 7.5; 0.5 m*M* EDTA; 5 μg/mL pepstatin A; 10 μg/mL leupeptin, 0.5 m*M* AEBSF; 5 μg/mL aprotinin; 250 m*M* sodium fluoride; and 1.0 *M* NaCl). The cell lysate was centrifuged at 15,000 × *g* for 15 min at 4°C, and the supernatant fraction was recovered. For each sample, 40 μg of cell lysate protein was electrophoretically separated using a 12.5% SDS-polyacrylamide gel (8 × 10 × 0.15 cm gel) and electroblotted onto a polyvinylidene fluoride membrane using Towbin's buffer (Towbin et al., 1979). Protein determinations were performed using the Lowry procedure (Lowry et al., 1951), and bovine serum albumin was used as a standard.

Immunostaining was performed using procedures previously described (Compton et al., 2001). The polyvinylidene fluoride membranes were incubated at room temperature for 2 h with a 1:1,250 dilution of mouse, anti-chicken calbindin-28 kD monoclonal antibody.[3] After a washing step, the membranes were incubated at room temperature for 1.5 h with a 1:8,000 dilution of goat, anti-mouse IgG alkaline phosphatase conjugate.[3] After a washing step, the membranes were treated with a fluorogenic substrate,[9] and autoradiography was used to visualize calbindin-28 kD expression.

The membranes were stripped after the calbindin-28 kD immunostaining and then immunostained with a goat, anti-sea urchin monoclonal α-tubulin antibody that has high specificity for chicken α-tubulin.[3] The membranes were stripped at 50°C for 30 min with gentle agitation in a buffer containing 0.1 mol/L 2-meraptoethanol, 0.07 mol/L SDS, and 0.0625 mol/L Tris-HCl, pH 6.7. Before calculation of the relative calbindin-28 kD expression, α-tubulin expression was used to correct the calbindin-28 kD density values for equality of loading and transfer for each blot. Specifically, the sample of the blot with the largest α-tubulin density value was divided by the α-tubulin density value of each of the other samples of the blot to obtain a correction factor for each sample. This correction factor was then used to multiply the calbindin-28 kD density values for each sample to normalize them for loading and transfer differences. Then the relative level of calbindin-28 kD expression was determined for the samples of each blot by calculating the signal intensity for each sample relative to the strongest calbindin signal, which was assigned a value of 1.

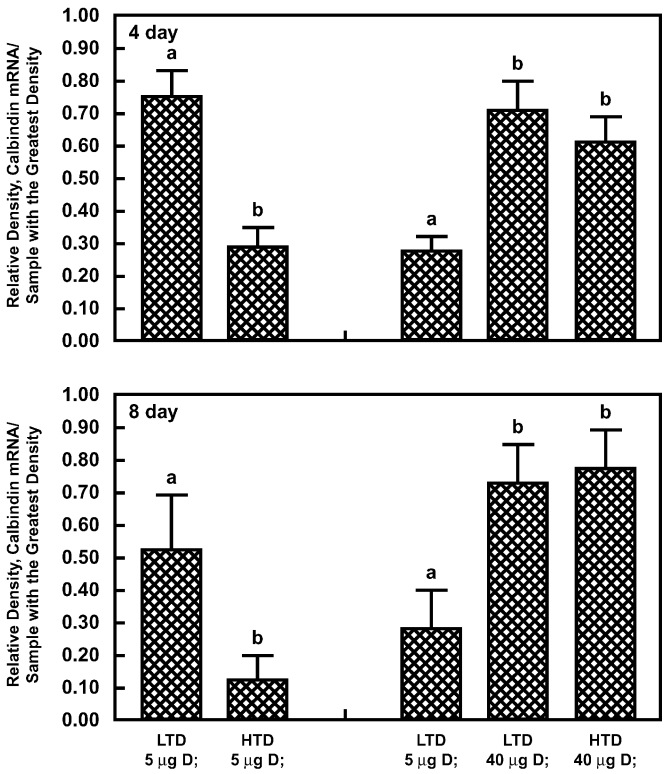

FIGURE 1. The relative density of intestinal calbindin-28 kD mRNA in broiler chicks selected for a low or high incidence of tibial dyschondroplasia (LTD and HTD, respectively) fed 5 or 40 μg cholecalciferol (D_3)/kg diet for the 4- and 8-d experiments (experiment 1). Values are means ± SEM, n = 6 replicate pens of 2 birds each. In order to accurately quantitate the density of the samples from the chicks fed 40 μg D_3/kg diet, film exposure had to be 24 h or less; however, for the samples from the HTD chicks fed 5 μg D_3/kg diet to be visible, film exposure had to be 48 h or greater. Therefore, the mRNA expression of calbindin-28 kD in the HTD chicks fed 5 μg D_3/kg diet could only be calculated relative to the LTD chicks fed the same level of D_3. [a,b]Means with different letters within a panel of the figure differ; $P < 0.05$.

RNA Extraction and Northern Blot Analyses

Total RNA was extracted from intestinal cells collected from 2 birds for each sample using a guanidine isothiocy-

[7]Section N-31, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.
[8]ICN Pharmaceuticals, Diagnostic Division, Costa Mesa, CA.
[9]CDP-Star, Applied Biosystems, Bedford, MA.

TABLE 1. Effects of dietary cholecalciferol on growth, feed utilization, and plasma Ca concentrations in commercially available (control) broiler chicks and in broiler chicks genetically selected for a low or high incidence of tibial dyschondroplasia (experiment 3)[1]

Broiler strain	Cholecalciferol (μg/kg diet)	BWG[2] (g/chick)	FI (g/chick)	G/F	Plasma Ca (mg/100 mL)
Control	5	446[a]	621[a]	0.716[a]	10.7[ab]
Low TD		334[bc]	477[b]	0.700[ab]	10.5[b]
High TD		311[bc]	477[b]	0.652[c]	10.5[b]
Control	40	405[ab]	591[a]	0.683[abc]	11.1[a]
Low TD		330[c]	495[b]	0.667[bc]	11.0[a]
High TD		354[c]	548[ab]	0.647[c]	10.9[ab]
PSEM		13.4	12.1	0.007	0.09
Main effect means					
Cholecalciferol					
5		364	525	0.689[a]	10.6[b]
40		363	545	0.666[b]	11.0[a]
PSEM		16.5	20.9	0.008	0.11
Broiler strain					
Control		426[a]	607[a]	0.700[a]	10.9
Low TD		332[b]	486[b]	0.684[a]	10.8
High TD		333[b]	512[b]	0.650[b]	10.7
PSEM		23.3	29.6	0.012	0.15
ANOVA					
R^2		0.58	0.55	0.59	0.46
Source of variation		Probability > F			
Treatment		0.0052	0.0087	0.0039	0.0338
Broiler strain		0.0009	0.0018	0.0018	0.5413
Cholecalciferol		0.9886	0.4259	0.0247	0.0015
Broiler strain × cholecalciferol		0.2241	0.2576	0.4236	0.8568

[a–c]Values within a column with different superscripts differ; $P < 0.05$.

[1]Values are means ± SEM, n = 4 replicate pens of 6 chicks.

[2]BWG = body weight gain; FI = feed intake; G/F = body weight gain divided by food consumption; PSEM = pooled standard error of the mean; TD = tibial dyschondroplasia.

nate/phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA (25 μg/sample) was run on an agarose/formaldehyde gel and then transferred to a nylon membrane as previously described (Davis and Johnson, 1998). Chicken calbindin-28 kD (Hunzicker, 1986), vitamin D receptor[10] (Lu et al., 1997), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Davis and Johnson, 1998) cDNA clones were prepared and labeled with ^{32}P for Northern blot analysis as previously described (Davis and Johnson, 1998). The order of hybridizations was calbindin-28 kD, GAPDH, and the vitamin D receptor. Blots were stripped of the previously hybridized probe before being hybridized with the subsequent probe as previously described (Chen and Johnson, 1996). Hybridization and densitometry procedures also followed those described previously (Davis and Johnson, 1998). For each experimental duration, there were 2 Northern blots with replicate samples from each treatment being split evenly between the 2 blots. The 2 blots were hybridized at the same time and exposed to the same film. Before calculation of relative calbindin-28 kD and vitamin D receptor mRNA levels, GAPDH mRNA expression was used to correct the calbindin-28 kD and vitamin D receptor density values for equality of RNA loading and transfer for each blot. Specifically, the sample of the blot with the largest GAPDH density value was divided by the GAPDH density value of each of the other samples of the blot to obtain a correction factor for each sample. This correction factor was then used to multiply the calbindin-28 kD and vitamin D density values for each sample to correct for loading and transfer differences. Then the relative mRNA expression for calbindin-28 kD was determined for the samples of each blot by calculating the signal intensity for each sample relative to the strongest calbindin signal, which was assigned a value of 1. Relative vitamin D receptor mRNA expression was calculated in the same manner.

Statistical Analyses

Analysis of variance for the three experiments was completed using the general linear models procedure of SAS software (SAS, 1999). Tukey's multiple-comparison procedure (Neter et al., 1990) was used to detect significant differences among diets and strain of birds for Northern and Western Immunoblot data. Significant differences among pen means between broiler strains for all other measured parameters were detected using Duncan's multiple range test (Steel and Torrie, 1980). Differences were considered significant when $P < 0.05$.

[10]A generous gift from H. DeLuca, University of Wisconsin, Madison, WI.

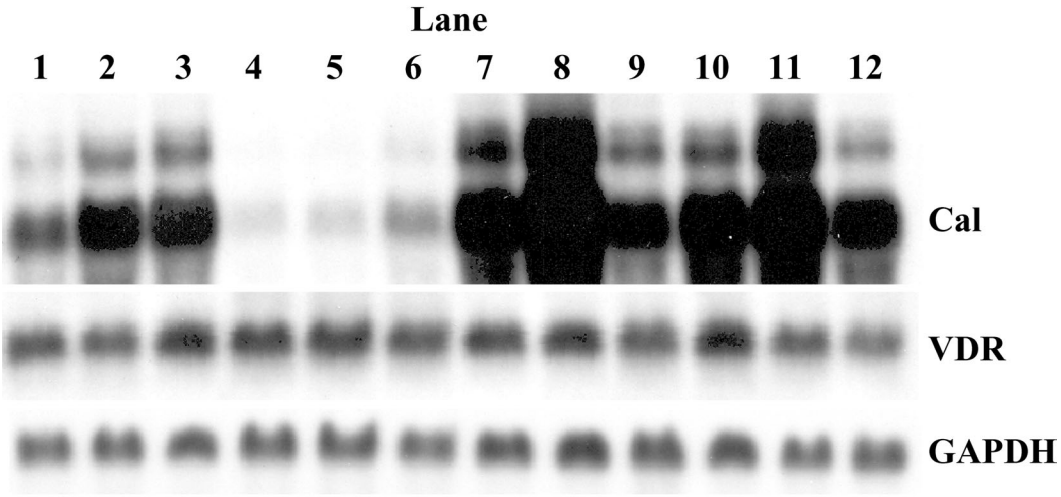

FIGURE 2. Autoradiograms from the Northern analysis of calbindin-28 kD and vitamin D receptor, showing 3 of the 6 replicate samples from broiler chicks selected for a low (LTD) or high (HTD) incidence of tibial dyschondroplasia fed 5 or 40 μg D_3/kg diet for the 4-d experiment (experiment 1). Total RNA (25 μg) was loaded for each sample. Samples obtained from the LTD and HTD genetic lines of broilers fed 5 μg D_3/kg diet are in lanes 1 to 3 and 4 to 6, respectively, and samples obtained from the LTD and HTD genetic lines of broilers fed 40 μg D_3/kg diet are in lanes 7 to 9 and 10 to 12, respectively. The film exposure times were 50, 22, and 3 h for the calbindin-28 kD, vitamin D receptor, and GAPDH Northern blots, respectively. Cal = calbindin-28 kD; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; VDR = vitamin D receptor.

RESULTS

Experiment 1

There were no differences in feed consumption and BW gain for the 4- or 8-d experimental period (data not shown) between the TD strains at either dietary level of D_3 supplementation.

When 5 μg of D_3 was supplemented to the diet, LTD chicks had greater expression of calbindin-28 kD mRNA (Figures 1 and 2) and protein (Figures 3 and 4) than the HTD broiler chicks for the 4- and 8-d experiments. Chicks fed 40 μg of D_3 had greater intestinal expression of calbindin-28 kD mRNA than those fed 5 μg of D_3. Intestinal expression of calbindin-28 kD protein, however, was equal for chicks fed 40 μg of D_3 and the LTD chicks fed 5 μg of D_3 (Figure 3).

After quantification by densitometry and correction for RNA loading and transfer with GAPDH, the mean ± SEM

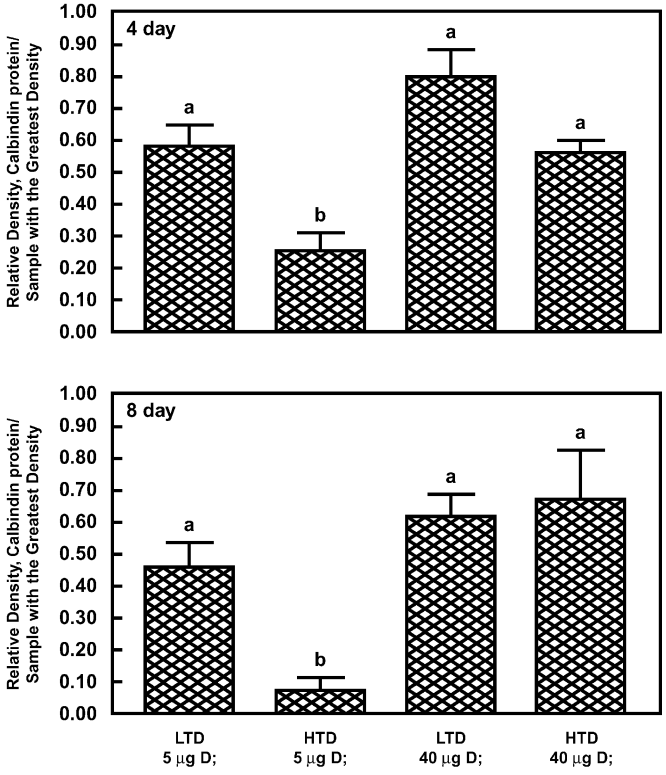

FIGURE 3. Relative density of intestinal calbindin-28 kD protein in broiler chicks selected for low (LTD) or high (HTD) incidence of tibial dyschondroplasia fed 5 or 40 μg cholecalciferol (D_3)/kg diet for the 4- and 8-d experiments (experiment 1). Values are means ± SEM, n = 6 replicate pens. [a,b]Means with different letters within a panel of the figure differ; $P < 0.05$.

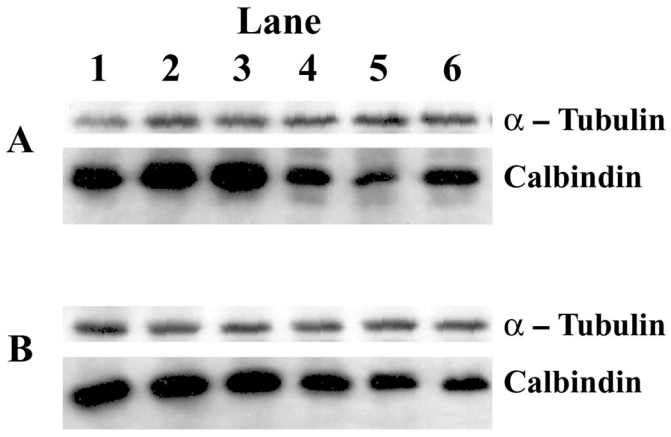

FIGURE 4. Autoradiograms from Western blot analysis of calbindin-28 kD, showing 3 of the 6 replicate samples from broiler chicks selected for a low (LTD) or high (HTD) incidence of tibial dyschondroplasia fed 5 or 40 μg cholecalciferol (D_3)/kg diet for the 4-d experiment (experiment 1). For each sample, 40 μg of protein was loaded. Samples obtained from the LTD and HTD genetic lines of broilers fed 5 μg D_3/kg diet are in lanes 1 to 3 and 4 to 6 of panel A, respectively, and samples obtained from the LTD and HTD broilers fed 40 μg D_3/kg diet are in lanes 1 to 3 and 4 to 6 of panel B, respectively.

TABLE 2. The incidence and severity of tibial dyschondroplasia and cholecalciferol (D_3)-dependent rickets in commercially available (control) broiler chicks and in broiler chicks genetically selected for low or high incidence of tibial dyschondroplasia (experiment 3)[1]

Broiler strain	Cholecalciferol (μg/kg diet)	Score[2]		Incidence		Tibia ash (%)
		TD[3]	D_3 rickets	TD (%)	D_3 rickets (%)	
Control	5	0.282^{c}	0.558^{ab}	23.3^{bc}	35.0^{ab}	39.2^{bc}
Low TD		1.000^{b}	0.300^{abc}	45.0^{ab}	15.0^{bc}	38.4^{cd}
High TD		2.082^{a}	0.750^{a}	73.3^{a}	44.2^{a}	37.3^{d}
Control	40	0.000^{c}	0.050^{c}	0.0^{c}	5.0^{c}	41.6^{a}
Low TD		0.082^{c}	0.000^{c}	8.3^{c}	0.0^{c}	40.9^{ab}
High TD		1.200^{b}	0.200^{bc}	50.0^{ab}	10.0^{c}	40.9^{ab}
PSEM		0.116	0.084	5.97	4.0	0.6
Main effect means						
Cholecalciferol						
5		1.122^{a}	0.536^{a}	47.2^{a}	31.4^{a}	38.3^{b}
40		0.428^{b}	0.083^{b}	19.4^{b}	5.0^{b}	41.1^{a}
PSEM		0.142	0.103	7.3	4.9	0.3
Broiler strain						
Control		0.141^{b}	0.304^{ab}	11.7^{b}	20.0^{ab}	40.4^{a}
Low TD		0.541^{b}	0.150^{a}	26.7^{b}	7.5^{b}	39.6^{ab}
High TD		1.641^{a}	0.475^{b}	61.7^{a}	27.1^{a}	39.1^{b}
PSEM		0.200	0.145	10.3	6.9	0.4
ANOVA						
R^2		0.82	0.53	0.67	0.64	0.70
Source of variation		Probability > F				
Treatment		0.0001	0.0120	0.0007	0.0014	0.0003
Broiler strain		0.0001	0.1103	0.0004	0.0344	0.1060
Cholecalciferol		0.0005	0.0013	0.0041	0.0002	0.0001
Broiler strain × cholecalciferol		0.2319	0.6615	0.7608	0.3683	0.5813

[a–c]Values within a column with different superscripts differ; $P < 0.05$.

[1]Values are means ± SEM, n = 4 replicate pens of 6 chicks.

[2]Scores for the severity of tibial dyschondroplasia and cholecalciferol dependent rickets were assigned based on visual observation of the epiphyseal growth plate using a numeric scale from 0 (no defects) to 3 (severe defects).

[3]TD = tibial dyschondroplasia; D_3 = cholecalciferol; PSEM = pooled standard error of the mean.

relative vitamin D receptor mRNA density values at 4 d were 0.83 ± 0.08 and 0.68 ± 0.05 for the LTD chicks and 0.73 ± 0.05 and 0.77 ± 0.06 for the HTD chicks fed 5 and 40 μg of D_3/kg diet, respectively. At 8 d, the relative vitamin D receptor mRNA values were 0.86 ± 0.03 and 0.81 ± 0.05 for the LTD chicks and 0.80 ± 0.04 and 0.91 ± 0.04 for the HTD chicks fed 5 and 40 μg of D_3/kg diet, respectively. There were no differences in the relative expression of the mRNA for the vitamin D receptor between the LTD and HTD strains or between the 2 dietary levels of D_3 (Figure 2).

Experiment 2

After quantification by densitometry and correction for RNA loading and transfer with GAPDH, the mean ± SEM relative calbindin-28 kD mRNA density values at day of hatch were 0.569 ± 0.08, 0.444 ± 0.09, and 0.458 ± 0.11 for the Ross 308 (control), LTD, and HTD strains, respectively. The mean relative vitamin D receptor mRNA expression values were 0.68 ± 0.08, 0.91 ± 0.05, and 0.70 ± 0.07 for the Ross 308 (control), LTD, and HTD strains, respectively. There were no differences in the mRNA expression of calbindin-28 kD or the vitamin D receptor among the 3 strains of broilers.

Experiment 3

Compared to commercial broiler chicks, LTD and HTD broiler chicks consuming the basal diet supplemented with 5 or 40 μg of D_3 had lower BW gains (Table 1). Plasma Ca concentrations were greater in chicks fed the basal diet that was supplemented with 40 μg of D_3 than in chicks fed the basal diet that was supplemented with 5 μg of D_3 (Table 1).

As expected, chicks from the HTD strain exhibited a higher incidence and severity of TD than the LTD chicks (Table 2). Furthermore, chicks fed a vitamin D_3-deficient diet (5 μg D_3/kg diet) had increased incidence and severity of TD and D_3 rickets and a lower percentage of tibia ash than chicks fed a diet adequate in vitamin D_3 (40 μg D_3/kg diet; Table 2).

The plasma concentrations of T_3 and free T_3 were not different for the control and HTD strains, but the concentrations of both forms of T_3 were significantly elevated in the LTD strain compared with the other 2 strains of birds, regardless of dietary D_3 concentration (Table 3). Plasma concentrations of thyroxine tended to decrease as dietary supplementation of D_3 increased (Table 3).

The expression pattern of the mRNA for calbindin-28 kD and the vitamin D receptor between the LTD and

TABLE 3. Plasma thyroid concentrations of commercially available (control) broiler chicks and in broiler chicks genetically selected for either a low or high incidence of tibial dyschondroplasia (experiment 3)[1]

Broiler strain	Cholecalciferol (μg/kg diet)	Thyroid hormone T_4 (μg/dL)	Thyroid hormone T_3 (ng/dL)	Thyroid hormone Free T_3 (pg/mL)
Control	5	2.22[a]	172.8[b]	8.03[c]
Low TD		2.07[a]	265.3[a]	10.73[a]
High TD		2.01[ab]	173.9[b]	8.12[c]
Control	40	2.13[a]	168.7[b]	7.61[c]
Low TD		1.80[b]	226.9[a]	9.42[b]
High TD		1.82[b]	176.1[b]	8.10[c]
PSEM		0.07	13.8	0.40
Main effect means				
Cholecalciferol				
5		2.10[a]	204.0	8.96
40		1.92[b]	190.6	8.38
PSEM		0.04	7.9	0.23
Broiler strain				
Control		2.18[a]	170.7[b]	7.82[b]
Low TD		1.94[b]	246.1[a]	10.08[a]
High TD		1.91[b]	175.0[b]	8.11[b]
PSEM		0.05	9.7	0.29
ANOVA				
R^2		0.62	0.70	0.70
Source of variation		Probability > F		
Treatment		0.0023	0.0003	0.0004
Broiler strain		0.0023	0.0001	0.0001
Cholecalciferol		0.0050	0.0930	0.2475
Broiler strain × cholecalciferol		0.4954	0.2932	0.3063

[a–c]Values within a column with different superscripts differ; $P < 0.05$.

[1]Values are means ± SEM, n = 4 replicate pens of 6 chicks.

[2]PSEM = pooled standard error of the mean; TD = tibial dyschondroplasia; T_4 = thyroxine; T_3 = 3,5,3′-triiodothyronine.

HTD chicks in this experiment was the same as it was for experiment 1 (data not shown).

DISCUSSION

Since the mid-1990s, the LTD and HTD lines have been maintained as 2 separate flocks, and no further genetic selection for TD incidence has occurred. Even though there has been an absence of genetic selection pressure for TD in these lines, they still exhibit a significant difference in the incidence and severity of TD while their relative growth rate remains equal. The HTD and LTD lines were originally selected from Ross genetic stock. The closest available genetic representative of this original stock is the Ross 308 broiler that was utilized in experiments 2 and 3. Clearly, the LTD line no longer has a lower incidence or severity of TD compared to the Ross 308 line, and LTD and HTD broilers grow at a slower rate than the Ross 308 broilers. The increased consumption of food by the Ross 308 broilers compared with the LTD and HTD broilers fed the same diets also resulted in a greater intake of cholecalciferol. The lower intake of cholecalciferol by the LTD broilers might have contributed to the observation that these broilers did not have a lower incidence or severity of TD compared to the Ross 308 broilers.

When the LTD and HTD chicks were fed a deficient level of dietary vitamin D_3 (5 μg D_3/kg diet), the LTD chicks expressed a higher level of the calbindin-28 kD transcript at 4, 8, and 18 d of age. This increased level of calbindin-28 kD expression in the LTD chicks was associated with decreased severity of TD at 18 d of age compared with the HTD birds. The differential response of the 2 genetic lines to dietary vitamin D_3 appeared to be related to a developmental process, because endogenous levels of calbindin expression in 1-d-old chicks were similar between the 2 strains. It is conceivable that the higher calbindin-28 kD mRNA expression observed with LTD chicks might have been related to the elevated plasma levels of T_3 present in this strain.

Cross et al. (1986) and Cross and Peterlik (1988, 1991) reported that Ca absorption was higher when chick intestinal segments were cosupplemented with 1,25-$(OH)_2D_3$ and T_3 than when only 1,25-$(OH)_2D_3$ was supplemented to the intestinal segment cultures. In a subsequent study, Sechman et al. (1996) reported that exogenous administration of T_3 alone had no effect on calbindin-28 kD expression, whereas administration of 1,25-$(OH)_2D_3$ increased in vivo expression of chick intestinal calbindin-28 kD protein. When T_3 and 1,25-$(OH)_2D_3$ were co-injected, Sechman et al. (1996) observed a synergistic increase in intestinal calbindin-28 kD expression beyond what was observed for chicks solely injected with 1,25-$(OH)_2D_3$.

In the current study the significantly higher plasma levels of T_3 and free T_3 found in LTD chicks compared with HTD chicks may account for the higher intestinal levels of calbindin-28 kD mRNA and protein expression

detected in these chicks. It is hypothesized that the increased level of calbindin-28 kD expression detected in the LTD chicks protected them from TD when dietary vitamin D_3 was marginal. Because HTD chicks had plasma T_3 and free T_3 concentrations equivalent to those of the Ross 308 chicks, it is unlikely that T_3 was directly involved in the higher incidence of TD observed in these birds.

There were no differences in the intestinal mRNA expression of the vitamin D receptor between the LTD and HTD lines. This finding was somewhat surprising as Berry et al. (1996) reported that commercial broiler chicks exhibiting TD had reduced levels of the vitamin D receptor in the hypertrophic chondrocytes of the tibia and that the receptors had a lower affinity for 1,25-$(OH)_2D_3$. Additionally, levels of vitamin D receptor were found to be significantly lower in the duodenal mucosa of chicks with a high incidence of TD compared to chicks with a low incidence of TD (Soares et al., 1990). The lack of difference in vitamin D receptor mRNA expression in the LTD and HTD lines used in the current research agrees, however, with a previous report that demonstrated vitamin D receptor protein expression was not different between the pedigreed LTD and HTD lines (Mitchell et al., 1997a).

In summary, when the LTD and HTD lines of broilers were initially established, it is conceivable that the LTD line was actually being selected for increased levels of plasma T_3 and free T_3. Thus, the high levels of T_3 might enhance expression of calbindin-28 kD and make the LTD broilers less susceptible to marginal dietary levels of vitamin D_3. High levels of T_3 are also likely to have positive effects on chondrocytes of the developing tibia. However, it is unclear why the HTD line of broilers had an elevated incidence of TD even when they are fed a diet adequate in vitamin D. The HTD birds did not seem to have an endogenous deficiency in plasma thyroid hormone levels or in the intestinal expression of vitamin D receptor and calbindin-28 kD. Further research is needed to determine why the incidence and severity of TD are so high in the HTD line of broilers and could provide valuable insight into the etiology of this disease that has a significant monetary impact on the broiler industry.

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