In Ovo Administration
of Boron Alters Bone Mineralization
of the Chicken Embryo

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ABSTRACT

It has been hypothesized that boron (B) is an essential element for animals, especially in bone metabolism. In this study, the influence of in ovo boron administration was assessed in the chicken. At 8 d of embryogenesis, carrier or B (0.1, 0.5, or 1.0 mg) was injected on to the chorioallantoic membrane of fertile eggs. At hatching, body weights were recorded and tissue samples collected. Although boron failed to alter bone mineralization, it decreased ($p < 0.05$) dried bone weight, suggesting a reduction in the bone organic matrix. Furthermore, 1 mg boron decreased ($p < 0.05$) hatchability and increased ($p < 0.05$) the height of the proliferative zone in the growth plate, indicating an unfavorable effect on bone elongation of the developing chick.

Index Entries: Boron; trace element; bone; mineralization; growth plate; chick; in ovo.

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INTRODUCTION

Since the initial classification of boron (B) as an essential element for plants (1,2), numerous attempts have been made to establish a similar relationship in animals (3–7). Boron's ubiquitousness in the environment has confounded these experiments. If boron is required in very small amounts, as has been hypothesized (3,4,8), then the amount of boron already present in the diet and water may have been sufficient for the organism, therefore negating any further physiological effects associated with boron supplementation. The inability to demonstrate a clear boron deficiency resulted in its classification as nonessential for animals.

In the early 1980s, Hunt and Nielsen suggested that a sign of boron deficiency was produced in chickens fed a diet low in boron and vitamin D (9). Subsequent studies implied that boron has a significant function in bone metabolism (8–16). Thus, the purpose of the present study was to determine if *in ovo* boron administration influences bone formation and mineralization of the chicken embryo.

MATERIALS AND METHODS

Experimental Protocol

Five hundred fertile broiler eggs (Boothe Hatchery, Gonzales, TX) were placed in a forced-ventilation incubator (Petersime model 1, Petersime Incubator Co., Gettysburg, OH) at 37.6°C with 56% relative humidity. On day 8 of embryonic development the eggs were briefly removed from the incubator and candled. Those eggs with a live embryo were randomly placed in treatment groups.

Solutions of 1000, 5000, and 10,000 ppm of boron were prepared using sodium tetraborate as the boron source (borax; $Na_2B_4O_7 \cdot 10H_2O$, Fisher Scientific, Fair Lawn, NJ). Since borax is 12.06% Na, the raising boron concentration produced an increase in the Na concentration in the injections. Therefore, three separate control groups, one for each level of boron, received saline (NaCl), which was Na-balanced on a molar basis. The superior end of each egg was wiped with 80% alcohol, a pinpoint puncture was made, and 100 μL of the appropriate solution was injected onto the chorioallantoic membrane of each egg. The eggs received 0.1 mg of B (B-0.1), 2.7 mg of NaCl (control-1), 0.5 mg of B (B-0.5), 13.6 mg of NaCl (control-2), 1.0 mg of B (B-1.0), or 27.1 mg of NaCl (control-3). The injection site was immediately sealed with paraffin and the eggs were returned to the incubator.

On the 18th day of development, the eggs were transferred to a hatcher (Petersime model H-145) where the temperature was maintained at 36.9°C with 69% relative humidity. At 21 d, hatchability was determined [(hatched eggs ÷ fertile eggs) × 100]. All the live chicks were leg-banded, weighed, and transferred to the laboratory for tissue sampling.

Blood Analyses

Blood (>500 μL) was obtained via cardiac puncture in heparinized syringes (Na heparin, 25 mg/100 mL). Whole blood was immediately analyzed for Ca^{2+}, Na^+, and K^+ (Chemlyte Ion Selective Analyzer, Gilford Systems, Oberlin, OH). Remaining blood samples were kept on ice and centrifuged (Model CR3000, Jouan Inc., Winchester, VA). Plasma was frozen for later analysis of Mg, total Ca, and P_i. Total plasma Ca and Mg were determined by atomic-absorption spectrophotometry (Perkin-Elmer model 4000, Norwalk, CT) (17). The analysis of plasma P_i was done using the Sigma Diagnostics procedure No. 360 UV (Sigma Diagnostics, St. Louis, MO), reading at 340 nm in a Beckman spectrophotometer model DU-6 (Beckman Instrument, Inc., Irvine CA).

Sexing

The chicks were sexed by gonad examination during sample collection.

Bone Analyses

After the chicks were killed via cervical dislocation, the legs were removed and the muscle dissected from the bone and discarded. The left tibiae were measured for length and width, fat-extracted with ether (Soxhlet apparatus) for 16 h, and dried overnight in a drying oven at 102°C. The dehydrated, defatted bones were weighed and ashed in a muffle furnace (Thermolyne Furnatol II, Thermolyne Corporation, Dubuque, IA) at 550°C for 18 h. The ash percentage of each bone was determined, and its mineral composition was assessed by atomic absorption (Ca, Mg, Na, K, Cu) and colorimetrically (P_i).

Histology

The right dissected tibiae were slit longitudinally and placed in Carson's fixative. Six tibiae from each sex and treatment were chosen at random for histological and morphometric analysis. One half of the fixed proximal tibia was dehydrated with ethyl alcohol and embedded in paraffin. Two 5-μm sections were taken from each sample and stained with hematoxylin and eosin. The growth-plate image was projected from the microscope onto paper and traced at a magnification of 14.75×, and reserve, proliferative, and hypertrophic zones were indicated. The height of each zone was then measured at three intervals by an individual unfamiliar with the treatment distribution. The average of the three measurements was used as the zone height.

Statistical Analyses

The data were statistically analyzed by the Statistical Analysis System (18) utilizing the General Linear Model (GLM) procedure for an

unbalanced analysis of variance (ANOVA) using a split-plot design (19,20). A p value of < 0.05 was considered statistically significant, and the means were partitioned by Duncan's Multiple-Range Test (21).

RESULTS

None of the variables analyzed demonstrated a significant difference attributable to sex. All data presented represent values pooled across both sexes. Boron-1.0 injection had a lower ($p < 0.05$) hatch percentage than the other boron dosages and control groups (Fig. 1). The relative body weights (body weight ÷ egg weight) × 100, were calculated in order to account for any bias introduced by egg selection. The relative body weights were similar among the treatment groups (Table 1). However, paired comparisons (GLM) showed that the relative body weight of the B-0.5 dose treatment was higher ($p < 0.05$) than its control (Fig. 2).

The left-tibiae dry weight was less ($p < 0.05$) for the B-0.5 and B-1.0 groups (Table 2). Neither the bone length nor width, nor the mineral composition (Ca, P, Mg, Na, K, Cu) of bone ash, showed a significant difference associated with treatment (Table 2 and 3). Although the percentage of bone ash was comparable for all treatments, a pattern suggesting a dose response was evident (Fig. 3). This pattern, with the ash percentage peaking at the middle dosage, was observed for the controls as well.

Although the growth-plate heights were similar among all treatment groups, the proliferative-zone height was increased ($p < 0.05$) for the B-1.0 treatment (Fig. 4). The heights of the reserve and hypertrophic zones showed no significant difference.

The results of plasma minerals are summarized in Tables 4 and 5. Plasma Ca^{2+} and K^+ were the only minerals that demonstrated a significant difference ($p < 0.05$) between treatments. The B-0.5 group had lower plasma Ca^{2+} than its control (control-2) and the B-1.0 group, and plasma K^+ was higher for the B-1.0 group than for all other treatments.

DISCUSSION

In studies conducted by Pardue et al. (16) it was reported that *in ovo* boron administration (150 µL of 500 or 1000 ppm boron) increased embryonic (26 d) weight, tibial length, and yolk-sac internalization of the turkey embryo. At hatching (28 d), the boron-treated poults had a higher ($p < 0.05$) percentage of bone ash than the controls. Further trials (unpublished data from this lab) with turkey embryos showed a tendency for increased bone ash in boron-treated poults. This effect was not maintained throughout the growing period, and at 3 wk of age, the boron-treated turkeys tended to have less bone ash than the controls. A decrease ($p < 0.05$) in the percentage of bone K of the 1-d-old poults
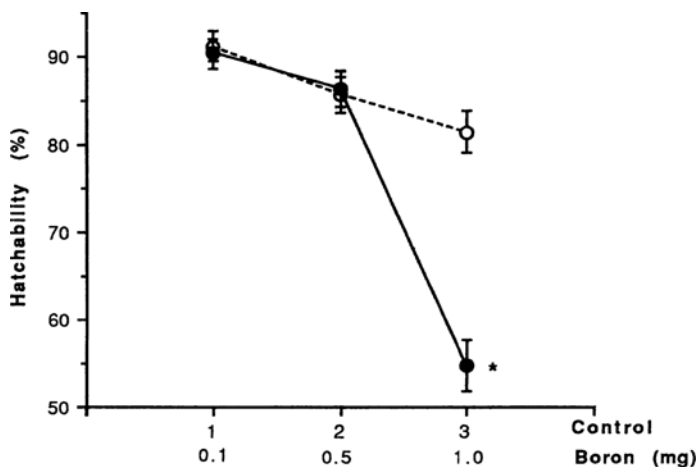
Fig. 1.   The effect of *in ovo* B administration (-●-, bor-on; ---O---, control) on egg hatchability. Each point is the mean ± SEM of at least 68 eggs. Values marked with an asterisk (*) differ significantly ($p < 0.05$) from all others.

Table 1
Effect of *in ovo* B Administration on Embryonic Growth[1]

Treatment	n	Egg weight[2]	Body weight[3]	Relative[4] body weight
		g	g	%
B-0.1	57	63.23 ± 0.59	46.65 ± 0.47	74.00 ± 0.26
Control-1	59	63.12 ± 0.61	46.51 ± 0.49	73.72 ± 0.39
B-0.5	37	62.37 ± 0.68	46.67 ± 0.54	74.84 ± 0.41
Control-2	48	62.81 ± 0.61	45.92 ± 0.45	73.40 ± 0.24
B-1.0	8	66.25 ± 1.10	49.32 ± 1.27	74.41 ± 1.26
Control-3	48	62.56 ± 0.68	46.20 ± 0.55	74.17 ± 0.45

[1]Data are mean ± SEM. Values do not differ significantly (Duncan's Multiple-range test).
[2]Egg weight at setting time.
[3]Body weight at hatching.
[4]Relative body weight = (body weight/egg weight) × 100.

treated with boron was also noted. At 3 wk of age, the bone K values were similar in both groups, whereas the bone Ca percentage was de-creased ($p < 0.05$) for the boron group. Additional studies carried out by Pardue et al. (22) further suggest that boron either administered *in ovo* (100 µL of 100 ppm) and/or supplemented in the diet (3 mg/kg diet), may influence mineral status and growth in the broiler chicken.

All boron treatments received the same amount of Na as their re-spective controls, and the osmolarity of the treatments was comparable.
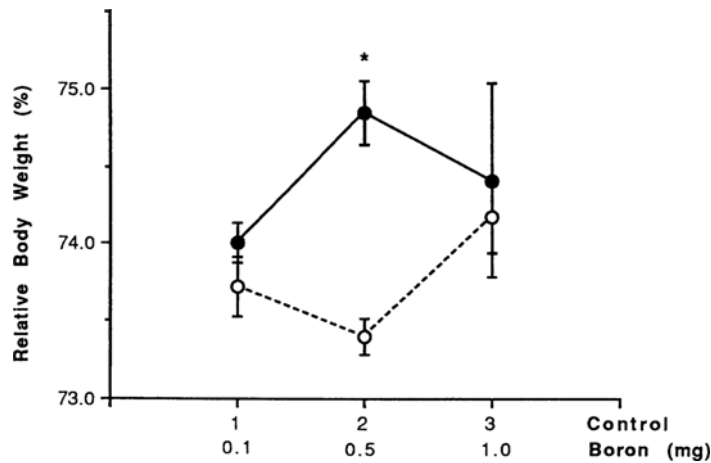
Fig. 2. The effect of *in ovo* B administration (-●-, boron; ---○---, control) on embryonic growth. Points are the mean ± SEM for 57 chicks for B-0.1, 37 chicks for B-0.5, eight chicks B-1.0, 59 chicks for control-1, 48 chicks for control-2, and 48 chicks for control-3. The asterisk (*) indicates significant difference ($p < 0.05$) between B-0.5 and control-2 when paired comparison were done.

Table 2
Effect of *in ovo* B Administration on Tibial Characteristics
of One Day Old Chicks[1]

Treatment	n	Weight[2]	Length	Width	Ash
		mg	mm	mm	%
B-0.1	57	73.38 ± 0.89[a]	31.6 ± 0.1[a]	1.8 ± 0.0[a]	31.45 ± 0.30[a]
Control-1	56	74.08 ± 0.91[a]	31.5 ± 0.1[a]	1.8 ± 0.0[a]	30.89 ± 0.37[a]
B-0.5	37	67.15 ± 0.94[b]	31.3 ± 0.2[a]	1.8 ± 0.0[a]	31.66 ± 0.36[a]
Control-2	46	74.10 ± 1.01[a]	31.6 ± 0.1[a]	1.8 ± 0.0[a]	31.27 ± 0.38[a]
B-1.0	8	65.30 ± 2.92[b]	31.6 ± 0.3[a]	1.8 ± 0.1[a]	30.95 ± 1.61[a]
Control-3	47	73.73 ± 1.08[a]	31.6 ± 0.2[a]	1.8 ± 0.0[a]	30.27 ± 0.44[a]

[1]Data are mean ± SEM. Means followed by a different superscript letter within a column differ significantly ($p<0.05$; Duncan's Multiple-range test).
[2]Dried, defatted left tibia.

It is unlikely that the B-1.0 effect on hatchability was a result of either the Na concentration or the osmolarity of the injected solution. The possibility of a Na–B interaction affecting hatchability remains to be explored.

Boron, along with other nutritional factors, such as manganese, biotin, and riboflavin, has been known to cause embryo malformations and poor hatchability (23–25). Boron toxicity displays the same teratogenicity as does riboflavin deficiency. It has been suggested that boron and riboflavin form a water-soluble complex (26) that is readily

Table 3
Effect of *in ovo* B Administration on Tibial Mineral Composition
of One Day Old Chicks[1,2]

Treatment	Ca	P	Mg	Na	K	Cu
B-0.1	30.33±0.41 n=55	29.89±1.68 n=14	0.76±0.01 n=58	4.04±0.20 n=13	1.70±0.02 n=57	0.16±0.01 n=56
Control-1	31.03±0.41 n=52	26.34±1.25 n=13	0.74±0.01 n=58	3.73±0.21 n=13	1.66±0.02 n=57	0.16±0.00 n=58
B-0.5	30.60±0.38 n=36	28.19±1.66 n=11	0.77±0.01 n=37	4.30±0.28 n=11	1.70±0.03 n=37	0.15±0.01 n=37
Control-2	30.03±0.46 n=47	26.88±2.61 n=10	0.76±0.01 n=48	3.92±0.31 n=8	1.70±0.02 n=49	0.16±0.01 n=49
B-1.0	30.99±1.26 n=7	27.92±2.88 n=5	0.72±0.04 n=8	4.26±0.12 n=4	1.70±0.07 n=8	0.15±0.01 n=8
Control-3	30.69±0.29 n=47	28.39±1.56 n=9	0.75±0.01 n=49	4.18±0.18 n=9	1.70±0.02 n=49	0.17±0.00 n=49

[1]Data are mean ± SEM. Values do not differ significantly (Duncan's Multiple-range test).
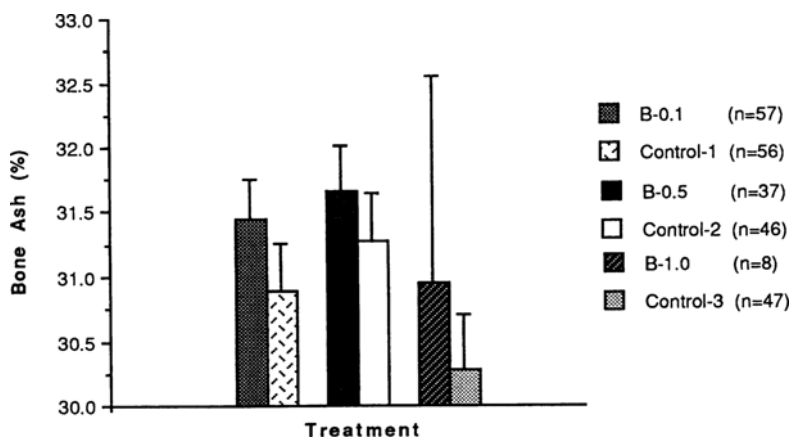[2]Percent mineral in bone ash.

Fig. 3.   The effect of *in ovo* B administration on bone mineralization. Values are mean ± SEM. Means do not differ significantly.

excreted, depleting the body of riboflavin. Furthermore, the addition of riboflavin was found to lower the incidence of malformations caused by boric acid in avian embryos (23). In recent studies, Rossi and coworkers (27) observed that riboflavin would not ameliorate the negative effects associated with high B (320 ppm) in broilers' diets.

Landauer (24) injected 2.5 mg of boric acid or 3.5 mg of sodium borate (0.4 mg B) into the yolk sac. The B-1.0 eggs in the present study
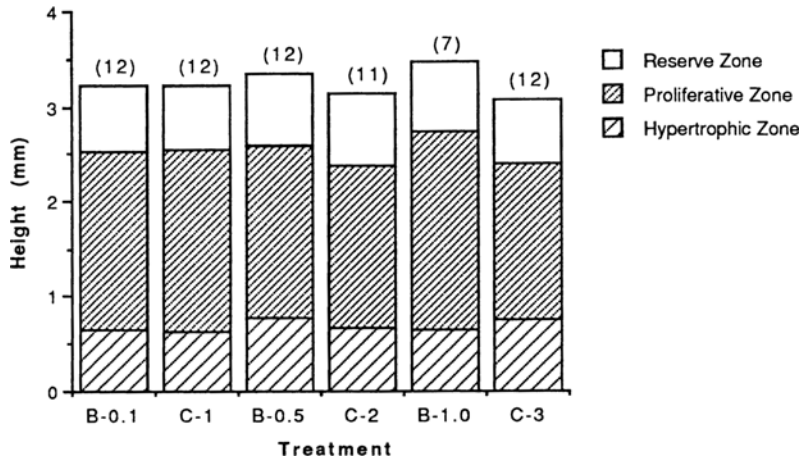
Fig. 4.   The effect of *in ovo* B administration on the height of
the epiphyseal plate zones. The height of the growth-plate, re-
serve, and hypertrophic zones does not differ significantly. The
proliferative zone is longer ($p < 0.05$) with B-1.0 treatment than in
the corresponding control (C-3).

Table 4
Effect of *in ovo* B Administration on Plasma Calcium
of One Day Old Chicks[1,2]

Treatment	Ionized Ca	Bound Ca[3]	Total Ca
B-0.1	1.40±.02[ab] n=14	0.89±0.18[a] n=6	1.64±0.20[a] n=13
Control-1	1.41±.02[ab] n=11	0.70±0.19[a] n=4	1.35±0.18[a] n=13
B-0.5	1.32±.03[b] n=7	0.58±0.21[a] n=3	1.26±0.15[a] n=11
Control-2	1.45±.03[a] n=7	0.53±0.23[a] n=6	1.64±0.23[a] n=10
B-1.0	1.45±.05[a] n=4	1.01±0.39[a] n=4	2.12±0.45[a] n=5
Control-3	1.37±.03[ab] n=8	0.84±0.21[a] n=7	1.88±0.25[a] n=9

[1]Data are mean ± SEM. Means followed by different superscript letters within a
column differ significantly ($p<0.05$; Duncan's Multiple-range test).
[2]Expressed in mmol/L.
[3]Bound Ca = Total Ca - Ionized Ca.

Table 5
Effect of *in ovo* B Administration on Plasma Minerals
of One Day Old Chicks[1,2]

Treatment	K^+	Na^+	P_i	Mg
B-0.1	3.7 ± 0.1^b n=56	144 ± 1^a n=50	1.9 ± 0.1^a n=14	0.7 ± 0.0^a n=12
Control-1	3.8 ± 0.1^b n=58	144 ± 0^a n=53	1.7 ± 0.1^a n=13	0.6 ± 0.0^a n=13
B-0.5	3.9 ± 0.1^b n=34	144 ± 1^a n=30	1.6 ± 0.1^a n=10	0.6 ± 0.0^a n=11
Control-2	3.9 ± 0.1^b n=48	145 ± 1^a n=44	1.7 ± 0.2^a n=8	0.7 ± 0.1^a n=9
B-1.0	4.8 ± 0.3^a n=7	143 ± 2^a n=7	1.8 ± 0.3^a n=5	0.7 ± 0.1^a n=5
Control-3	3.9 ± 0.1^b n=47	143 ± 1^a n=40	2.1 ± 0.1^a n=7	0.7 ± 0.0^a n=8

[1]Data are mean ± SEM. Means followed by different superscript letters within a column differ significantly ($p<0.05$; Duncan's Multiple-range test).
[2]Expressed in mmol/L.

received a dose 2.5 greater than those in Landauer's study. Landauer reported a hatchability of 19%,Z compared with 54% with the B-1.0 treatment obtained in the present study. Since the dead embryos in the present study were not examined, the authors can not attest to the existence of malformations. However, Landauer reported only 36% normal chicks among those hatched, with the remaining showing some degree of defect. Although there was no teratogenicity among the B-1.0 chicks, boron's toxicity was evidenced by the decreased hatchability of that group.

The decrease in dry bone weight observed in the B-0.5- and B-1.0-treated embryos, and the absence of an effect on bone mineralization, as indicated by the percentage of bone ash, suggest that boron decreased the amount of organic material in bone, although the inorganic portion was not changed. Further studies are required to examine the organic composition of bone from boron-treated embryos to determine if the organic matrix is affected.

Table 3 depicts a lack of difference in the mineral composition of the bone ash for the boron and control groups, indicating that bone mineralization was normal.

Growth-plate morphology provides a mean of assessing the growth of bone, with its enlargement being a characteristic of rickets (*28*) and

vitamin D deficiency (29,30). The effect of vitamin D deficiency on rickets development appears to be indirect, by altering serum Ca and P levels (29,30). Jande and Dickson (29) demonstrated this effect by supplementing vitamin D deficient chicks with vitamin D or Ca. They observed that, in spite of the deficiency in vitamin D, Ca supplementation decreased the proliferative-zone height and corrected the cell disarrangement associated with vitamin D deficiency in chicks (29). However, the calcification zone was poorly calcified, which was attributed to a considerable decrease in serum P (29).

The lengthening of the growth plate in rickets is caused primarily by an increase in the proliferative and/or hypertrophic zones (28,31). The zone affected varies among species and with the nutrient deficiency involved, namely Ca or P (32). The increase in the zone of proliferation is not associated with an augmentation in cell division, but with cellular hypertrophy and an accumulation of matrix because of the inability of the chondrocytes to mature (29,32,33). This delay in cell conversion has been associated with Ca in several ways. Since intracellular acquisition of Ca occurs in the upper regions of the zone of hypertrophy, a Ca deficiency has been associated with this delayed chondrocyte maturation (33). However, it has also been suggested that Ca, through inhibition of the growth of perforating epiphyseal vessels, alters growth-plate morphology (32). It has also been proposed by Arsenault and colleagues (34) that the presence of "anti-invasive factor(s)" in the cartilage matrix inhibits angiogenesis, suggesting the possibility of matrix calcification in turn suppressing the "anti-invasive factor(s)," hence permitting vascular invasion of metaphysis (34).

The increase in the proliferative-zone height for the B-1.0 treatment may be indicative of the inability of chondrocytes to mature in consequence of a possible boron interference with normal bone calcification (28). Boron may be interacting with Ca and/or vitamin D. If boron interacts with Ca, it may cause an apparent Ca deficiency, leading to an increase in the proliferative zone of chicks. Boron interaction with vitamin D may be twofold. If B antagonizes vitamin D, then a Ca deficiency could follow, with the resulting increase in the proliferative zone. Yet, if B is synergistic to vitamin D (9,35), then the changes in growth-plate morphology would reflect signs of vitamin D toxicity, which also include an enlarged proliferative zone (36). The two conditions can easily be separated by morphometric analysis of cell size and cell number. The increase in the proliferative zone caused by vitamin D deficiency is characterized by the enlargement of the chondrocytes, whereas vitamin D toxicity presents an increase in chondrocyte proliferation.

Based on these results, future studies with boron should include an element analysis for the growth plate as well as morphometric analysis of its cell size and number.

SUMMARY

In ovo boron administration influenced the development of the chicken embryo. There appears to be a dose–response effect, with B-0.5 promoting an increase in relative body weight ($p < 0.05$). One milligram of boron was detrimental to egg hatchability and to bone elongation ($p < 0.05$). Further, B-0.5 and -1.0 dosages decreased ($p < 0.05$) the dried bone weight, suggesting a reduction in the bone organic matrix composition. The results imply a possible involvement of boron in bone metabolism. However, additional research needs to be conducted to ascertain whether boron's effect on bone is achieved by competing with Ca utilization in bone mineralization, or whether, by decreasing bone organic matrix, bone calcification is altered.

ACKNOWLEDGMENTS

REFERENCES

1. H. Agulhon, *Ann. Inst. Pasteur* **34**, 321 (1910).
2. K. Warington, *Ann. Bot.* **37**, 629 (1923).
3. E. Hove, C. A. Elvehjem, and E. B. Hart, *Am. J. Physiol.* **127**, 689 (1939).
4. E. Orent-Keiles, *Proc. Soc. Exp. Biol. Med.* **44**, 199 (1940).
5. J. D. Teresi, E. Hove, C. A. Elvehjem, and E. B. Hart, *Am. J. Physiol.* **104**, 513 (1944).
6. J. T. Skinner, and J. S. McHargue, *Am. J. Physiol.* **143**, 385 (1945).
7. R. H. Follis, *Am. J. Physiol.* **150**, 520 (1947).
8. F. H. Nielsen, *Trace Elements in Human and Animal Nutrition*, W. Mertz, ed., Academic, FL, 1986, pp. 415–463.
9. C. D. Hunt and F. H. Nielsen, *Trace Element Metabolism in Man and Animals*, J. McC. Howell, J. M. Gawthorne, and C. L. White, eds., Springer-Verlag, Berlin, Heidelberg, New York, 1982, pp. 597–600.
10. F. H. Nielsen, C. D. Hunt, L. M. Mullen, and J. R. Hunt, *FASEB J.* **1**, 394 (1987).
11. M. A. Bock, M. Powey, and M. Ortiz, *FASEB J.* **4**, A520 (1990).
12. M. Behen, *Am. Health* **Sep.** 130 (1989).
13. R. E. Newnham, *Trace Element Metabolism in Man and Animals*, J. McC. Howell, J. M. Gawthorne, and C. L. White, eds., Springer-Verlag, Berlin, Heidelberg, New York, 1982, pp. 400–402.
14. M. A. Elliot and H. M. Edwards, *Proceedings of The Southern Poultry Science Society*, Eleventh Meeting, Atlanta, GA, 1990, p. 14.
15. T. F. Brown, M. E. McCormick, D. R. Morris, and L. K. Zeringue, *Nutr. Res.* **9**, 503 (1989).

16. S. L. Pardue, T. W. Odom, N. King, and H. W. Sampson, *FASEB J.* **3**, A1072 (1989).
17. *Analytical Methods for Atomic Absorption Spectrophotometry, 0303–0152.* Perkin-Elmer, Norwalk, CT, 1982.
18. SAS Proprietary Software Release 6.03, SAS Institute, Cary, NC, 1987.
19. R. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics*, McGraw-Hill, New York, 1960.
20. D. C. Montgomery, *Design and Analysis of Experiments*, John Wiley, New York, 1984, pp. 379–391.
21. D. B. Duncan, *Biometrics* **11**, 1 (1955).
22. S. L. Pardue, S. H. Frappier, C. Garza, N. King, and T. W. Odom, *Poult. Sci.* **68** *(Suppl. 1)*, 196, (1989).
23. W. Landauer, *J. Exp. Zool.* **120**, 469 (1952).
24. W. Landauer, *Proc. Soc. Exp. Biol. Med.* **82**, 633 (1953).
25. H. R. Moseley and W. Landauer, *Fertility and Hatchability of Chicken and Turkey Eggs*, L. W. Taylor, ed., John Wiley, New York, 1949, pp. 244–337.
26. D. V. Frost, *J. Biol. Chem.* **145**, 693 (1942).
27. A. F. Rossi, S. M. Bootwalla, and R. D. Miles, *Proceedings of The Southern Poultry Science Society*, Eleventh Meeting, Atlanta, GA, 1990, p. 35.
28. R. M. Leach and C. V. Gay, *J. Nutr.* **117**, 784 (1987).
29. S. S. Jande, and I. R. Dickson, *Acta Anat.* **108**, 463 (1980).
30. P. H. Long, S. R. Lee, G. N. Rowland, and W. M. Britton, *Avian Dis.* **28**, 933 (1984).
31. J. P. Iannotti, *Orthop. Clin. North Am.* **21**, 1 (1990).
32. D. L. Lacey, and W. E. Huffer, *Am. J. Pathol.* **109**, 288 (1982).
33. P. H. Long, S. R. Lee, G. N. Rowland and W. M. Britton, *Avian Dis.* **28**, 921 (1984).
34. A. L. Arsenault, W. L. Hunter, and A. B. Hodsman, *Behavior of the Growth Plate*, H. K. Uhthoff, and J. T. Wiley, eds., Raven, New York, 1988, pp. 309–316.
35. C. D. Hunt and F. H. Nielsen, *Trace Elements in Man and Animals*, vol. 6, L. S. Hurley, C. L. Keen, R. B. Lönnerdal, and R. B. Rucker, eds., Plenum, New York, 1988, pp. 275–276.
36. M. Silbermann, N. Mirsky, K. von der, Mark, and J. Bernheim, *Acta Anat.* **124**, 93 (1985).