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Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 589–594

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Cestrum diurnum leaf as a source of 1,25$(OH)_2$ Vitamin D_3 improves egg shell thickness☆

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Abstract

A continuing concern of the poultry industry is the high incidence (12%) of egg losses in the laying house due to poor egg shell quality. Calcium (Ca) homeostasis is a key factor in egg shell formation. The economy of Ca utilisation is under the control of Vitamin D_3, particularly its active metabolite 1,25-dihydroxy cholecalciferol [1,25$(OH)_2D_3$]. Supplementation of 1,25$(OH)_2D_3$ has been shown to increase specific gravity, shell thickness and shell weight of the egg. However, commercially available synthetic 1,25$(OH)_2D_3$ is very expensive. Earlier studies from our Institute [Phytochemistry 37 (1994) 677] have identified a cheap, natural and rich source of 1,25$(OH)_2D_3$ in the leaves of *Cestrum diurnum* (CD), a member of the *Solanaceae* family. In this study, CD leaves were explored as a source of 1,25$(OH)_2D_3$ in the feed of layer birds to improve the egg shell thickness. Fifteen-week-old white leghorn layers were divided into four treatments of 60 birds each and as follows: (I) normal diet with Vitamin D_3, (II) normal diet with Vitamin D_3 + CD, (III) normal diet without Vitamin D_3 and, (IV) normal diet without Vitamin D_3 + CD powder. CD leaf powder was incorporated in to the feed at 0.3% level. The experimental feeding was continued up to 72 weeks of age of the birds. Weekly food intake and daily egg production were noted throughout the experimental period and the specific gravity of the eggs, feed consumed to lay one egg and egg shell thickness were determined. Incorporation of CD leaves in the feed had the maximal effect on all the parameters studied. The feed consumed to lay one egg was 20 g less than the control group. The specific gravity of the egg was higher by 0.005, than the control egg, indicating a 5% decrease in the breakage of eggs in CD fed chicks. Also there was a significant increase ($P < 0.001$) in egg shell thickness. The data suggest that incorporation of CD leaf powder in the feed of poultry layers increased the egg shell thickness, which in turn could decrease the economic loss due to breakage of eggs.

Keywords: Cestrum diurnum; 1,25-Dihydroxy Vitamin D_3; Egg shell thickness

1. Introduction

The importance of Vitamin D_3 in poultry diets is well documented [1,2]. Vitamin D_3 is necessary for the bird to absorb, transport and utilize Ca and P. The proper utilisation of Ca in bird may result both in better egg production and increased egg shell thickness. The economy of Ca utilisation is under the control of Vitamin D particularly on its active metabolite 1,25-dihydroxy cholecalciferol [1,25$(OH)_2D_3$].

The rate of egg production decreases as the hen ages and efforts are made to minimise the fall of egg production during ageing. Not only egg production but egg shell strength also decreases towards the end of the laying period. Soares et al. [3] concluded that, with age, there appears to be a progressive deterioration in the ability of the hen's liver to hydroxylate Vitamin D_3 to 25-OH-D_3. Stevens et al. [4] stated that reduced hydroxylation of Vitamin D_3 in the liver or kidney could result in inadequate production of 1,25$(OH)_2D_3$ for minimum absorption of Ca and P for bone formation. Liver or kidney or both exhibit inadequate ability for hydroxylation, the end result is deficiency of 1,25$(OH)_2D_3$. This may explain in part the normal decline in egg shell quality and tibia strength with age.

Egg shell quality tends to deteriorate as egg production continues. It has been calculated that there would be about 12% egg losses due to poor egg shell quality [5,6].

Egg production, feed consumption were shown to increase with supplemental 1,25$(OH)_2D_3$ when Vitamin D_3 was not fed [7]. It has been shown that egg specific gravity, shell weight, percentage of shell, and egg breaking strength were significantly increased with the supplementation of

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

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doi:10.1016/j.jsbmb.2004.03.101

$1,25(OH)_2D_3$ studies have shown that the optimal dietary level of $1,25(OH)_2D_3$ for improving egg shell quality without affecting egg production was approximately 5 μg/kg and the toxic level was 7 μg/kg. It has been shown that inclusion of $1,25(OH)_2D_3$, at 5.0 μg/kg diet, not only resulted increased egg production but also in better quality of egg shell in layers [8]. The addition of $1,25(OH)_2D_3$ in the feed prevented a rapid decrease in egg production [9].

The $1,25(OH)_2D_3$ is prohibitively expensive. Attempts were made to explore inexpensive natural sources of Vitamin D_3 metabolites. Reducing cation–anion difference of diets fed just before parturition can prevent milk fever (http://www.nps.ars.usda.gov [10]). The inclusion of dried *Cestrum diurnum* leaf powder in a rachitogenic diet restored intestinal calcium binding protein (CaBP) synthesis and increased serum Ca also in the cholecalciferol deficient chick [11]. Earlier studies from our laboratory [12] have identified a cheap and rich source of $1,25(OH)_2D_3$ in one of the *Solanaceae* family plants, *C. diurnum*. They also reported increased bone weight and density in experimental rats fed dry leaves of *C. diurnum*. In this study, the efficacy of dried leaves of *C. diurnum* were explored as a cheap and natural source of $1,25(OH)_2D_3$ in the feed of layers.

2. Materials and methods

Three hundred and sixty white leghorn layers of 15 weeks old (about 3 weeks before the start of laying eggs) were taken and divided into six treatments of 60 birds each (10 replicates of 6 birds) using randomised block design. The dried *C. diurnum* leaf powder was mixed properly and fed. The different groups (I) normal diet, (II) normal diet + 0.15% CD, (III) normal diet + 0.3% CD, (IV) normal diet without Vitamin D_3, (V) normal diet without Vitamin D_3 + 0.15% CD, (VI) normal diet without vitamin, D_3 + 0.3% CD. The birds were given water and feed ad libitum.

The date of the start of egg laying of each bird was noted. The daily feed intake of six birds and eggs laid by each bird was recorded. Eggs were collected on 26th, 27th and 28th day of each laying period (28 days) for the estimation of various parameters in eggs. The experiment was carried out 58 weeks. Feed consumption for each replicate group per treatment was recorded for each laying period during the experiment. The efficiency of feed utilisation for each treatment was measured as the amount of feed consumed per dozen eggs produced using the formula: feed conversion = kg of feed consumed/dozen of eggs.

The body weights were recorded individually first at the beginning of the experiment, i.e.15th week latter 20th, 28th, 40th, 60th and 72nd week. The body weight gains were computed using the data. Daily egg production records were maintained for each 28-day period for entire 12 such laying periods of the experiment. Percentage egg production was obtained by dividing the number of eggs laid by total number of hens multiplied by 100.

Eggs were collected during the last 3 days of each laying period from each treatment and weighed. Egg specific gravity was measured based on the principle of Archimedis.

The eggs were broken washed free of any adhering albumin. Then they were dried in a hot air oven at 100 °C for 6 h and were weighed. Egg shell thickness was measured with Dial Thickness Gage Mitutoyo, Japan with an accuracy of 0.01 mm. The thickness was determined at three different parts of the egg shell (an equator and each end of the egg

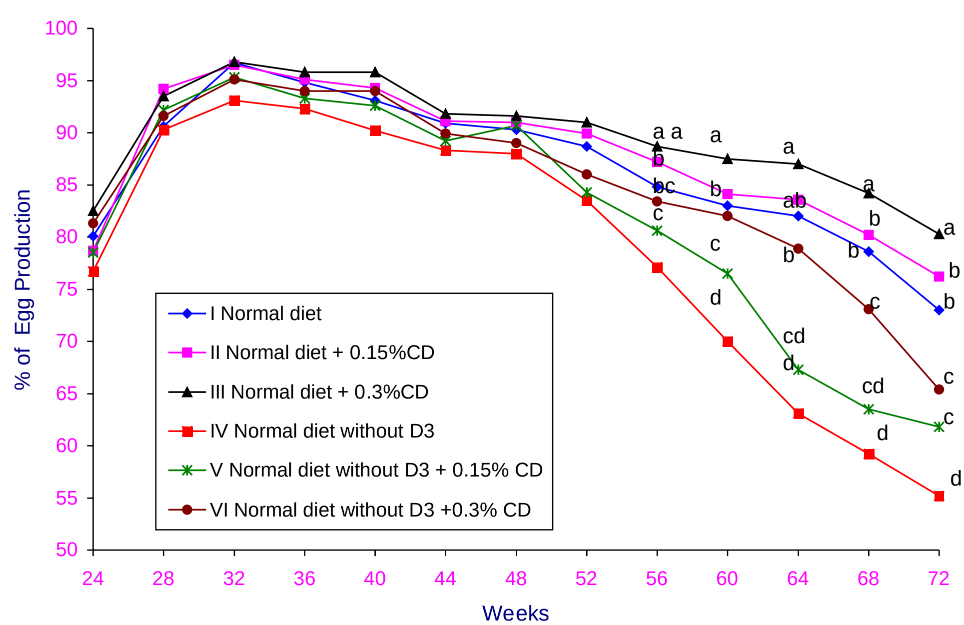

Fig. 1. Effect of *C. diurnum* (CD) leaf powder feeding on egg production in layers. Values are mean ± S.E. of 60 determinations. Values bearing different superscripts are significantly different between the groups $P < 0.05$ (ANOVA) at a given time point.

shell) according to Abe et al. [18]. The diet was prepared as per NRC [13].

2.1. Statistical analysis

The data was analysed by analysis of variance (ANOVA) and using Duncan's multiple range test [14].

3. Results

3.1. Egg production

There was a gradual increase in egg production in all the groups up to 40th week and the egg production varied from 90.2 in layers fed Vitamin D_3 deficient diet to 95.8 in layers fed normal diet incorporated with 0.3% *C. diurnum* (Fig. 1). From 44th week onwards the egg production decreased gradually to as low as 55% in layers fed Vitamin D_3 deficient diet where as in layers fed normal diet with 0.3% *C. diurnum* decrease was prevented and was at

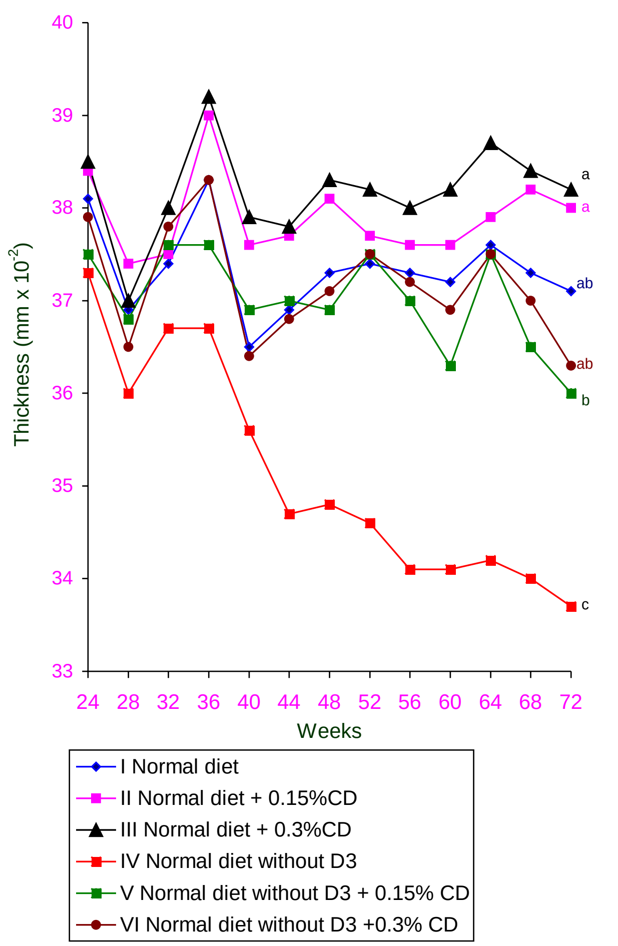

Fig. 2. Effect of *C. diurnum* leaf powder feeding on egg shell thickness (mm $\times$ 10^{-2}) in layers. Values are mean ± S.E. of 60 determinations. Values bearing different superscripts are significantly different between the groups at $P < 0.05$ (ANOVA).

Table 1
Effect of *C. diurnum* leaf powder feeding on FCR (feed consumed in gms to lay 12 eggs) in layers

Group	Treatment	Age of the birds in weeks						
		24	32	40	48	56	64	72
I	Normal diet	1563.7 ± 47.03	1209.4 ± 20.80	1462.0 ± 21.12	1567.0 ± 32.90	1586.1 ± 41.16	2014.7 ± 97.29[a]	2044.2 ± 155.01[ab]
II	Normal diet + 0.15% CD	1613.9 ± 65.55	1186.1 ± 9.19	1422.9 ± 22.05	1587.0 ± 22.75	1599.0 ± 29.97	1806.0 ± 67.84[bc]	1782.5 ± 26.30[b]
III	Normal diet + 0.3% CD	1516.1 ± 31.52	1222.3 ± 9.19	1449.3 ± 22.29	1611.4 ± 31.05	1595.0 ± 66.68	1794.2 ± 49.81[b]	1804.8 ± 54.11[b]
IV	Normal diet without D_3	1640.2 ± 155.6	1224.0 ± 20.98	1493.8 ± 32.73	1602.4 ± 41.86	1752.6 ± 104.32	2042.0 ± 82.76[ab]	2482.5 ± 375.55[a]
V	Normal diet without D_3 + 0.15% CD	1536.2 ± 38.31	1258.4 ± 39.88	1529.0 ± 61.23	1648.4 ± 61.23	1775.7 ± 118.00	2167.7 ± 86.39[a]	2176.5 ± 146.75[ab]
VI	Normal diet without D_3 + 0.3% CD	1533.9 ± 83.40	1251.3 ± 22.02	1453.6 ± 28.38	1555.7 ± 42.20	1671.1 ± 46.00	1910.3 ± 63.07[bc]	1839.0 ± 108.67[b]

Values are mean ± S.E. of 60 determinations. Values bearing different superscripts are significantly different between the groups at $P < 0.05$ (ANOVA).

Table 2
Effect of *C. diurnum* leaf powder feeding on egg weight (g) in layers

Group	Treatment	Age of the birds in weeks							
		24	32	40	48	56	60	64	72
I	Normal diet	49.5 ± 0.94	52.4 ± 0.42^{a}	56.5 ± 0.43^{a}	57.4 ± 0.66^{a}	58.5 ± 0.57^{ab}	60.6 ± 0.45^{a}	62.3 ± 0.76^{ab}	62.0 ± 0.35^{a}
II	Normal diet + 0.15% CD	49.6 ± 0.95	52.8 ± 0.43^{a}	56.3 ± 0.35^{a}	58.0 ± 0.54^{a}	58.8 ± 0.45^{ab}	60.7 ± 0.31^{a}	62.2 ± 0.57^{ab}	62.5 ± 0.37^{a}
III	Normal diet + 0.30% CD	49.8 ± 0.55	53.2 ± 0.50^{a}	56.6 ± 0.31^{a}	58.3 ± 0.59^{a}	59.9 ± 0.47^{a}	61.4 ± 0.46^{a}	62.7 ± 0.22^{a}	62.8 ± 0.26^{a}
IV	Normal diet without D_3	48.6 ± 0.70	51.2 ± 0.48^{b}	56.1 ± 0.45^{a}	56.9 ± 0.67^{b}	57.1 ± 0.76^{b}	58.8 ± 0.74^{b}	60.6 ± 0.72V	60.7 ± 1.35^{b}
V	Normal diet without D_3 + 0.15% CD	48.7 ± 0.39	53.1 ± 0.51^{a}	57.1 ± 0.47^{a}	57.5 ± 0.95^{a}	58.7 ± 0.90^{ab}	60.8 ± 0.58^{a}	61.5 ± 0.39^{ab}	62.5 ± 0.52^{a}
VI	Normal diet without D_3 + 0.30% CD	48.2 ± 0.56	52.7 ± 0.37^{a}	56.9 ± 0.35^{a}	57.9 ± 0.55^{a}	58.6 ± 0.46^{ab}	60.8 ± 0.65^{a}	61.1 ± 0.44^{ab}	62.8 ± 0.32^{a}

Values are mean ± S.E. of 60 determinations. Values bearing different superscripts are significantly different between the groups at $P < 0.05$ (ANOVA).

Table 3
Effect of *C. diurnum* leaf powder feeding on specific gravity in layers

Group	Treatment	Age of the birds in weeks							
		24	32	40	44	48	56	64	72
I	Normal diet	1.087 ± 0.0033	1.091 ± 0.0006^{a}	1.088 ± 0.0007^{bc}	1.088 ± 0.0007^{a}	1.087 ± 0.0009^{b}	1.089 ± 0.0008^{ab}	1.086 ± 0.0010^{b}	1.083 ± 0.0004^{b}
II	Normal diet + 0.15% CD	1.087 ± 0.0011	1.092 ± 0.0007^{a}	1.090 ± 0.0003^{a}	1.090 ± 0.0008^{a}	1.089 ± 0.0009^{ab}	1.090 ± 0.0007^{a}	1.087 ± 0.0006^{b}	1.087 ± 0.0007^{a}
III	Normal diet + 0.30% CD	1.089 ± 0.0011	1.093 ± 0.0010^{a}	1.091 ± 0.0008^{a}	1.091 ± 0.0008^{a}	1.090 ± 0.0012^{a}	1.091 ± 0.0005^{a}	1.090 ± 0.0007^{a}	1.088 ± 0.0006^{a}
IV	Normal diet without D_3	1.089 ± 0.0009	1.089 ± 0.0016^{b}	1.084 ± 0.0008^{d}	1.084 ± 0.0009^{b}	1.083 ± 0.0012^{c}	1.082 ± 0.0007^{c}	1.078 ± 0.0006^{c}	1.074 ± 0.0007^{d}
V	Normal diet without D_3 + 0.15% CD	1.090 ± 0.0008	1.091 ± 0.0007^{a}	1.085 ± 0.0008^{c}	1.088 ± 0.0006^{a}	1.087 ± 0.0008^{b}	1.087 ± 0.0008^{b}	1.084 ± 0.0010^{b}	1.081 ± 0.0006^{c}
VI	Normal diet without D_3 + 0.30% CD	1.089 ± 0.0011	1.092 ± 0.0005^{a}	1.087 ± 0.0006^{bc}	1.088 ± 0.0007^{a}	1.087 ± 0.0008^{b}	1.089 ± 0.0007^{ab}	1.085 ± 0.0004^{b}	1.083 ± 0.0003^{b}

Values are mean ± S.E. of 60 determinations. Values bearing different superscripts are significantly different between the groups at $P < 0.05$ (ANOVA).

80.3%. The drop in egg production (55%) was significantly ($P < 0.05$) less in Vitamin D_3 deficient diet fed layers compared to layers fed normal diet. Though there was a fall in percentage of egg production in layers fed without Vitamin D_3 diet along with 0.15 and 0.3% *C. diurnum* but it was significantly different ($P < 0.05$) compared to Vitamin D_3 deficient diet fed group. Even in the layers where the normal diet fed along with 0.15 and 0.3% *C. diurnum*, the fall of egg production was significantly ($P < 0.05$) less compared to layers fed normal diet indicating the positive impact of *C. diurnum* leaf incorporation in diet on egg production.

3.2. Age at sexual maturity

The time of first lay of eggs by hens has been taken as an indication of sexual maturity of the layers. The results indicate that the age at sexual maturity was 135 days in layers fed Vitamin D_3 deficient diet compared to layers fed normal with 0.3% *C. diurnum* (132 days). In layers fed normal diet without Vitamin D_3 with 0.15 and 0.3% *C. diurnum*, the age at sexual maturity was 134 and 133 days, respectively. However, there was no difference between layers fed normal and normal with 0.15% *C. diurnum*. Even though there was no statistical significance between 135 and 132 days but it has a tremendous practical implication on poultry industry, since the farmer will get the benefit of early egg laying.

The feed conversion ratio (FCR) (Table 1) varied from 1516.1 g in layers fed Vitamin D_3 with 0.3% *C. diurnum* leaf to 1640.2 g in layers fed diet without Vitamin D_3 at the end of 24th week. There was a gradual increase in FCR from 32nd week onwards until the experiment was completed. There was a significant ($P < 0.05$) difference in FCR of normal diet fed layers when compared to Vitamin D_3 deficient diet fed group at the end of 72nd week. The FCR of the layers fed Vitamin D_3 deficient diet had significantly higher FCR compared to layers fed Vitamin D_3 with or without *C. diurnum*. The FCR of layers fed normal diet with 0.3% *C. diurnum* leaf had significantly ($P < 0.05$) lower when compared to layer fed with normal diet.

3.3. Egg weight

The data on egg weight is presented in Table 2. The egg weight gradually increased as the age of the bird increased from 24 to 72 weeks. The egg weight of the layers fed Vitamin D_3 deficient diet had significantly ($P < 0.05$) lower weight compared to all other treatments from 48th to 72nd week.

3.4. Egg specific gravity

The egg specific gravity (Table 3) is ranged from 1.087 in layers fed normal diet to 1.090 in layers fed Vitamin D_3 deficient diet at the end of 24th week. The specific gravity of the egg of layers fed normal diet with 0.15 and 0.3% *C. diurnum* were significantly ($P < 0.05$) higher compared to the specific gravity of the eggs of hens fed Vitamin D_3 deficient diet with 0.15 and 0.3% *C. diurnum* at the end of 72nd week.

3.5. Egg shell thickness

The egg shell thickness (Fig. 2) was 37.3×10^{-2} mm in layer fed Vitamin D_3 deficient diet and it was 38.5×10^{-2} mm in layers fed normal diet with 0.3% *C. diurnum* at the end of 24th week. There was a decrease in egg shell thickness as the hen aged from 24 to 72 weeks. The egg shell thickness was significantly ($P < 0.001$) lower in layers fed Vitamin D_3 deficient diet compared to all other treatments from 40th weeks onwards. The decrease was more significant at the end of 72 weeks. The egg shell thickness was significantly ($P < 0.01$) higher in layers fed normal diet either with 0.15 or 0.30% *C. diurnum* compared to layers fed deficient diet with 0.15 or 0.3% *C. diurnum*.

4. Discussion

Present study brought out some new aspects like egg production maintenance beyond peak egg laying period by the *C. diurnum* leaf powder fed to the layers. *Cestrum diurnum* leaf powder feeding could bring a positive change in age at sexual maturity as evidenced in laying eggs by the layers 2–3 days earlier to the other treatments. Dunnington and Seigel [15] demonstrated that the onset of sexual maturity does not occur at the same age for all pullets in commercial leghorn flock. Thus, some layers begin laying eggs at 17–19 weeks of age (early maturing) and normal maturing were between 19 and 24 where as late maturing age layers lays the eggs during 24–28 weeks of age. The preponement of egg laying by 2–3 days in *C. diurnum* fed birds is interesting. It must be noted that all the birds used in the experiment belong to the early mature variety. So the effect of early laying observed is, therefore, could be only due to the *C. diurnum* effect. Yet another interesting observation is that the egg production reached to a level of about 92% in all the groups, but remained 80% at 72 weeks, only with *C. diurnum* treatment while the layers fed Vitamin D_3 deficient diet dropped rapidly 55%. There was a drop to 68% even in layers, which received Vitamin D_3. Even this drop could be prevented by the addition of *C. diurnum* leaf powder in the feed of poultry. Carlos and Edwards [9] study also shown increase in egg production by supplementing $1,25(OH)_2D_3$. If we compute the additional eggs gained by preventing the drop due to the inclusion of *C. diurnum* leaf powder in the layer feed would result in extra eggs of about 25 per bird for the entire egg laying period of a hen. Tsang et al. [16] demonstrated that decreased egg production is related to the ageing process in the layers. Though present study also on par with literature with respect to the age but the egg production drop was prevented to a larger extent up to 72 weeks in *C. diurnum* leaf powder fed layers suggests that $1,25(OH)_2D_3$ maintains the egg production at higher levels. This may be due to increased intestinal Ca transport by the intestine since calcium absorption is dependent upon $1,25(OH)_2D_3$ levels [17]. Thus, increased Ca transport reflected both in serum Ca, egg shell thickness and in tibia strength required for the regulation of Ca absorption and provide adequate Ca for the egg shell [18].

Feed conversion ratio is another important parameter which bring impact in the commercial layers. Present study has shown a good response in FCR in layers fed *C. diurnum* leaf powder. Tsang et al. [16] also observed that feed consumption progressively decreased with increased dietary $1,25(OH)_2D_3$. Probably, *C. diurnum* leaf which contain enough $1,25(OH)_2D_3$ levels must have resulted in lesser consumption of feed. However, they still maintained the body weights higher than the rest of the groups. This clearly suggest improvement in the feed efficiency by the addition of *C. diurnum* leaf powder in the poultry feed.

Thus, increased Ca transport effectively brought the egg shell calcification in *C. diurnum* leaf fed layers both in thickness as well as in total egg shell calcium content. This was further borne out in the egg specific gravity values where *C. diurnum* leaf powder fed layers laid eggs with higher specific gravity. It is reported that under the optimal dietary regimen the mean improvement in specific gravity was 0.004. A change in egg specific gravity from 1.080 to 1.085 reduced the percentage of cracked eggs from 7.5 to 2.4%, i.e. approximately a 1% reduction of cracked eggs per 0.001 unit improvement of specific gravity. Based on this relationship, an estimated 14 times reduction in cracked eggs can be expected by feeding layers with *C. diurnum* leaf powder. There will be no residue problem because $1,25(OH)_2D_3$ does not get into the egg from hen plasma in any appreciable amounts [16].

Thus, *C. diurnum* plant leaves can be used in the poultry to bring a great impact in egg production, shell thickness, specific gravity. *Cestrum diurnum* leaf, a natural product for $1,25(OH)_2D_3$, is a cheap source and can be utilised by drying the leaf for supplementing in the poultry for benefiting the farmer effectively.

Acknowledgements

We acknowledge Drs. M. Raghunath and V.S. Rao for their suggestions in preparing the manuscript.

References

[1] H.F. Deluca, The vitamin D system in the regulation of calcium and phosphorous metabolism, Nutr. Res. 37 (1979) 161–192.
[2] J.H. Soares Jr., Calcium metabolism and its control—a review, Poultry Sci. 63 (1984) 2075–2083.
[3] J.H. Soares Jr., C.M. McLonghlin, M.R. Swerdel, E. Bossard, Effect of hydroxy vitamin D metabolites on the mineralization of eggshell and bones, in: Proceedings of the Margyland Nutrition Conference, College Park, MD, 1976, pp. 85–92.
[4] V.I. Stevens, R. Blair, R.E. Salmon, Influence of maternal vitamin D3 carry-over on kidney 25-hydroxy vitamin D3-1-hydroxylase activity, Poultry Sci. 63 (1984) 765–774.
[5] J. Cox, How much is egg breakage costing you? Pourtry Trib. 10 (1967) 46.
[6] Roland, Recent Advances in Eggshell Quality, in: Proc. Maryland Nutr. Cond. Paper, 1976, pp. 19–22.
[7] T.J. Frost, D.A. Poland Sr., G.G. Urtawale, Influence of vitamin D3, 1α-hydroxy vitamin D3 and 1,25-dihydroxy vitamin D3 on eggshell quality, tibiastrengh and various production parameters in commercial laying hens, Poultry Sci. 69 (1990) 2008–2016.
[8] D.M. Kaetzel Jr., J.W. Soares Jr., Effect of cholecalciferol steroids on bone and eggshell calcification in Japanese quail, J. Nutr. 109 (1979) 1601–1608.
[9] A.B. Carlos, D.H.M. Edward Jr., The effects of 1,25-dihydroxy cholecalciferol and phytase on the natural phytate phosphorous utilization by buying hens, Poultry Sci. 77 (1998) 850–858.
[10] http://www.nps.ars.usda.gov, Using Solanum glaucophyllum as a Source of $1,25(OH)_2D_3$ to Prevent Hypocalcemia, 2003.
[11] R.A. Wasserman, L.C. Krook, M.R. Hughes, M.R. Haussler, Studies on the 1α25 dihydroxy cholecalciferol-like activity in a calcinogenic plant, *Cestrum diurnum* in the chick, J. Nutr. 106 (1976) 457–465.
[12] T.P. Prema, N. Raghuramulu, Free vitamin D3 metabolites in *Cestrum diurnum* leaves, Phytochemistry 37 (1994) 677–681.
[13] NRC, Nutrient Requirements of Poultry, 9th revised ed., National Academy Press, Washington, DC, 1994.
[14] K. Visweswara Rao, Biostatistics: a Manual of Statistics Methods for Use in Health, Nutrition and Anthropology, Jaypee Brothers Medical Publishers Ltd., New Delhi, 1996.
[15] E.A. Dunnington, P.B. Seigel, Age and body weight at sexual maturity in female white legworm chickens, Poultry Sci. 63 (1984) 828–830.
[16] C.P.W. Tsang, A.A. Grunder, N. Roberto, Optimal dietary level of 1α25-dihydroxoy cholecalciferol for eggshell quality inlaying hens, Poultry Sci. 69 (1990) 1702–1712.
[17] A.D. Bar, U. Dubrov, U. Eizner, S. Hurwitz, Calcium binding protein and kidney 25-hydroxy cholecalciferol-1-hydroxylase activity in Turkey poults, J. Nutr. 108 (1978) 1501–1507.
[18] E. Abe, H. Morikawa, T. Masumusa, M. Sugahara, M. Kubota, T. Suda, Disorders of cholecalciferol a metabolism in old egg-laying hens, J. Nutr. 112 (1982) 436–446.