KRMIVA

GROWTH AND IMMUNE RESPONSES OF CHICKENS FED VARIOUS DIETARY CONCENTRATIONS OF CALCIUM AND PHOSPHORUS

RAST I IMUNI ODGOVOR PILIĆA HRANJENIH RAZLIČITOM KONCENTRACIJOM KALCIJA I FOSFORA

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SUMMARY

Male chicks (Ross x Ross) were obtained from a commercial hatchery and fed diets containing 0.60%, 0,80%, 1.00%, 1.20% and 1.40% calcium *ad libitum* for 42 days. Available phosphorous was kept at 0.45% in one experiment and was varied in another experiment to maintain calcium to phosphorous ratios at 2.0. Chickens were maintained free of adventitial infections and vaccinated at 10 and 18 days of age for infectious bursal disease and at 21 days of age for Newcastle disease and infectious bronchitis. Serum samples were collected at 21 and 42 days of age and assayed for specific titers by enzyme-linked immunosorbent assays (ELISA) for the three vaccines and by hemagglutination inhibition (HI) for Newcastle disease. Body weight gains, feed conversion ratios, and relative bursa weights were adversely affected at 0.60%, but not 0.80% through 1.20% calcium. In the first experiment (constant level of phosphorous at 0.45%), chickens fed 0.60% calcium had significantly greater incidence of tibial dyschondroplasia (not evaluated in second experiment). In both experiment, HI titers for Newcastle disease were significantly increased at 1.20% calcium.

INTRODUCTION

Inhibitory and stimulatory influences of dietary minerals on immune responses in several species have been described and investigated (Descotes et al., 1990; Gershwin et al., 1995; Lawrence, 1985). Metals, such as cadmium, are inhibitory whereas others, such as zinc, are stimulatory, and still others, such as lead, may be inhibitory or stimulatory, depending on species and concen-tration. Although calcium has been recognized as a second signal for activation of lymphocytes in immune responses, importance of dietary calcium for immune competence has been little investigated. We reported earlier that immune responses to Newcastle disease vaccine were improved in chickens fed 1.30% calcium (Ragland et al. 1994). The present report is concerned with the effect on immunity of calcium levels in the context of phosphorus concentration.

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MATERIALS AND METHODS

Animals. In each experiment, 225 male chickens (Ross x Ross) from a commercial hatchery were kept in battery cages with wire floors under continuous light for 42 days in rooms supplied with filtered air under positive pressure to maintain the chickens free of adventitial viral infection. Chickens were provided feed and water *ad libitum*. Body weight gains and feed consumption were measured at 21 and 42 days of age.

Experiment 1. Chickens were fed five levels of calcium (0.60%, 0.80%, 1.00%, 1.20%, 1.40%) and the same level of available phosphorus (0.45%). Composition of each diet is given in Table 1. Chickens were randomly assigned to three replicate pens for each diet with 15 chickens per pen. Chickens were vaccinated at 10 and 18 days of age for infectious bursal disease (IBD) (IBD-BLEN™, CEVA Laboratories, Overland Park, Kansas), and at 21 days of age for Newcastle disease (NCD) (Clonevac-30r®, Intervet, Milsboro, Delaware) and infectious bronchitis disease (IB) (MILDVAC-MR, Intervet). Vaccines were given by eye-drops. Serum samples were collected at 10 and 18 days of age from 10 chickens of each group and assayed by enzyme-linked immunosorbent assays (ELISA) using commercial reagents (IDEXX Laboratories, Westbrook, Maine) to ascertain the immune status from maternal antibodies and that the chickens were responding to vaccination. Antibody titers for the three vaccines were measured in serum samples collected from all chickens at 42 days of age. Immune response to Newcastle disease vaccine also was measured by hemagglutination inhibition (Bread, 1980). Relative organ weights were calculated for bursa Fabricius, thymus, and spleen from all chickens at 42 days. Serum alkaline phosphatase (EC 3.1.3.1) (Colorimetric test - Sigma procedure no. DG 1245. Sigma Diagnostics, Grünwalder Weg 30, 8024 Deisenhofen, Germany), total calcium (Hill, 1965) and total phosphorus (Kraml, 1966) were measured at 21 days (10 birds per group) and at 42 days (all chickens). Bone ash from left tibia (Deutsch, 1990) and severity of tibial dyscondroplasia (TD) (Edwards and Veltmann, 1983) were also measured.

Table 1. Composition of feed for experiment 1.
Tablica 1: Sadržaj krmnih smjesa za pokus 1

Ingredients - Sirovine	Ca (%)				
	0.60	0.80	1.00	1.20	1.40
Corn - Kukuruz	55.18	54.02	52.87	51.71	50.55
Soybean meal-48 - Sojina sačma 48	37.13	37.34	37.55	37.76	37.97
Poultry fat - Peradarska mast	4.72	5.14	5.56	5.99	6.41
Dicalcium phosphate - Dikalcij fosfat	1.48	1.48	1.50	2.03	2.55
Limestone - Vapnenac	0.46	0.98	1.48	1.48	1.48
Common salt - Sol	0.40	0.40	0.40	0.40	0.40
DL-Methionine - Metionin	0.20	0.20	0.20	0.20	0.20
Coban 60	0.08	0.08	0.08	0.08	0.08
Bacitracin	0.05	0.05	0.05	0.05	0.05
Mineral premix	0.05	0.05	0.05	0.05	0.05
Vitamin premix	0.25	0.25	0.25	0.25	0.25
Composition (estimated from NRC, 1984, composition tables): - Sastav (dobiven prema NRC - 1984.)					
ME (Kcal/g)	3.20	3.20	3.20	3.20	3.20
Protein (%) - Bjelančevine (%)	23.00	23.00	23.00	23.00	23.00
Calcium (%) - Ca (%)	0.60	0.80	1.00	1.20	1.40
Available P (%) - P iskoristivi (%)	0.45	0.45	0.45	0.45	0.45
Calcium : Avail. Phos. - Ca : P iskoristivi	1.33	1.78	2.22	2.67	3.11

Experiment 2. Chickens were fed the same levels of calcium as in experiment 1 and different levels of available phosphorus (0.30%, 0.40%, 0.50%, 0.60%, 0.70%, respectively) to maintain the same calcium to phosphorus ratio in all diets (Table 2). Chickens were randomly assigned to three replicate pens for each diet with 15 chickens per pen. Chickens were immunized, serum samples collected and antibody titers measured as described for experiment 1. Relative bursal weights were measured at 42 days.

Table 2: Composition of feed for experiment 2.

Tablica 2: Sadržaj krmnih smjesa za pokus 2

Ingredients - Sirovine	Ca/P (%)				
	0.6/0.3	0.8/0.4	1.0/0.5	1.2/0.6	1.4/0.7
Corn - Kukuruz	55.84	54.23	52.62	51.00	49.39
Soybean meal-48 - Sojina sačma 48	36.97	37.26	37.55	37.85	38.13
Poultry fat - Peradarska mast	4.54	5.13	5.72	6.31	6.89
Dicalcium phosphate - Dikalcij fosfat	0.77	1.24	1.71	2.18	2.66
Limestone - Vapnenac	0.85	1.11	1.37	1.63	1.89
Common salt - Sol	0.40	0.40	0.40	0.40	0.40
DL-Methionine - Metionin	0.20	0.20	0.20	0.20	0.20
Coban 60	0.08	0.08	0.08	0.08	0.08
Bacitracin	0.05	0.05	0.05	0.05	0.05
Mineral premix	0.05	0.05	0.05	0.05	0.05
Vitamin premix	0.25	0.25	0.25	0.25	0.25
Composition (estimated from NRC, 1984, composition tables): - Sastav (dobiven prema NRC - 1984.)					
ME (Kcal/g)	3.20	3.20	3.20	3.20	3.20
Protein (%) - Bjelančevine (%)	23.00	23.00	23.00	23.00	23.00
Calcium (%) - Ca (%)	0.60	0.80	1.00	1.20	1.40
Available P (%) - P iskoristivi (%)	0.30	0.40	0.50	0.60	0.70
Calcium : Avail. Phos. - Ca : P iskoristivi	2.00	2.00	2.00	2.00	2.00

Statistical analysis. Data were analyzed first for pen effects. When none was found, data from the three replicates for each treatment were pooled and subjected to ANOVA. Multiple comparisons were made with Tukey-Kramer post hoc test. Non-parametric data (TD scores and NCD HI titers) were analyzed by Pearson's multiple Chi Square test. When a significant difference was observed, multiple comparisons were made with Tukey ANOVA for non-parametric data (Zar, 1984). Relative numbers of chickens with TD scores of 3 in the treatment groups were compared by Fisher's exact test. Newcastle disease HI titers also were transformed to log_{10} and subjected to ANOVA, using Tukey-Kramer post hoc test to compare means.

RESULTS AND DISCUSSION

Body weight gains were affected more than feed conversion efficiency (Tables 3 and 4). Body weight gains were significantly less in chickens fed 0.60% calcium for 21 days, and less in chickens fed 1.40% in experiment 1. At 42 days, body weight gains were greatest in experiment 1 when 0.80% calcium was fed, and greatest in experiment 2 when 1.40% was fed. At 42 days, feed conversion was worse when 1.40% calcium was fed. Significant differences in feed conversion were not observed in experiment 2. Poor results were anticipated for the groups fed 0.60% and 1.40% calcium, based on extensive experiences of other investigators. Optimum results were obtained at 0.80% and 1.00% calcium, the levels most often used by commercial growers. Feeding 1.20% calcium was as suitable as 0.80% and 1.00%, and we posit that this level also should be considered within an optimal level for growth performance, especially when calcium to phosphorus ratio is mantained at 2.0.

Table 3. Body weight gain and feed conversion ratio (experiment 1)

Tablica 3: Prirasti tjelesne mase i iskorištenja hrane (pokus 1)

Dietary calcium Kalcij u hrani	Body weight gain Prirasti tjelesne mase	Feed conversion ratio Iskorištenje hrane
(%)	(kg)	(gm feed/gm gain) (g hrane/g prirasta)
21 days old - 21 dan starosti		
0.60	0.752[a]	1.397
0.80	0.792[b]	1.310
1.00	0.814[b]	1.393
1.20	0.795[b]	1.443
1.40	0.742[a]	1.473
42 days old - 42 dana starosti		
0.60	2.23[ab]	1.88[a]
0.80	2.37[a]	1.78[b]
1.00	2.32[ab]	1.85[a]
1.20	2.35[ab]	1.84[a]
1.40	2.18[b]	1.94[c]

Values are means for 27 to 45 chickens per treatment group. Values in the same column with different superscript letters are different, $p \leq 0.05$.

Vrijednosti su prosjek za 27 do 45 pilića u pokusnoj skupini. Vrijednosti u samoj koloni s različitim slovima su zazličite kod $p \leq 0.05$.

Weight gains and feed efficiency are not the only parameters of concern when considering minerals in the diet. Growth and mineralization of bone are critical factors because rapidly growing chickens need adequately strong bones to support them. Without strong bones, leg problems develop and lead to economic losses. Bone ash in chickens fed 1.40% calcium for 21 days was inexplicably low, but was significantly greater after 42 days (Table 5). Differences were not detected in mean TD lesion scores, but were detected when numbers of chickens with lesion scores of 3, those that are indicative of pathologic effects, were considered (Table 5). Whereas the mean lesion scores could not be discriminated by ANOVA, they correlated with relative numbers having lesion scores of 3; 95% correlation at 21 days and 98% at 42 days. The relative numbers having lesion scores of 3 at 21 days were not different because sample size was to small. Nevertheless, correlation with relative numbers at 42 days was 91%. Not surprisingly, 0.60% calcium cannot be recommended, and no recommendation can be made on basis of bone quality among the higher doses, even 1.40% calcium.

Table 4: Body weight gain and feed conversion ratio (experiment 2).

Tablica 4: Prirasti tjelesne mase i iskorištenja hrane (pokus 2)

Dietary calcium Kalcij u hrani Ca (%)	Available Iskoristivi P (%)	Body weight gain Prirasti tjelesne mase (kg)	Feed conversion ratio Iskorištenje hrane (gm feed/gm gain) (g hrane/g prirasta)
21 days old - 21 dan starosti			
0.60	0.30	0.732[a]	1.457
0.80	0.40	0.787[b]	1.416
1.00	0.50	0.803[b]	1.376
1.20	0.60	0.806[b]	1.393
1.40	0.70	0.803[b]	1.417
42 days old - 42 dana starosti			
0.60	0.30	2.15[a]	1.93
0.80	0.40	2.24[ab]	1.85
1.00	0.50	2.34[b]	1.85
1.20	0.60	2.37[b]	1.82
1.40	0.70	2.38[b]	1.77

Values are means for 27 to 45 chickens per treatment group. Values in the same column with different superscript letters are different, $p \leq 0.05$.

Vrijednosti su prosjek za 27 do 45 pilića u pokusnoj skupini. Vrijednosti u samoj koloni s različitim slovima su zazličite kod $p \leq 0.05$.

Table 5. Tibial bone ash and tibial dyschondroplasia (TD), (experiment 1)

Tablica 5: Pepeo tibije i tibijalna dishondroplasia (TD), (pokus 1)

Dietary calcium Kalcij u hrani (%)	Ash Pepeo (%)	Mean Score Prosječno bodova	Tibial Dyschondroplasia Tibijalna dishondroplasia No. Chickens with TD Scores of 3./Number examined Broj pilića s TD bodovima od 3/broj pretraga
21 days old - 21 dan starosti			
0.60	30[ab]	1.1	3/7
0.80	31[a]	1.0	2/8
1.00	33[a]	0.0	0/7
1.20	31[a]	1.0	2/6
1.40	26[b]	0.2	0/9
42 days old - 42 dana starosti			
0.60	31[a]	1.1	8/28[a]
0.80	34[ab]	0.4	3/29[ab]
1.00	36[b]	0.2	0/37[bc]
1.20	34[ab]	0.4	3/29[ab]
1.40	35[b]	0.0	0/30[bc]

Values are means for 27 to 45 chickens per treatment group. Values in the same column with different superscript letters are different, $p \leq 0.05$.

Vrijednosti su prosjek za 27 do 45 pilića u pokusnoj skupini. Vrijednosti u samoj koloni s različitim slovima su zazličite kod $p \leq 0.05$.

Relative bursal weight may correlate with immune status, but is not a reliable parameter. Whereas bursal weights were different among treatment groups at 21 days in experiment 1, they did not follow a pattern that could be explained biologically (Table 6). Furthermore, there were no differences at 42 days. No differences were observed in experiment 2 (data not shown). Level of calcium did not affect bursal weights in our earlier report (Ragland et al., 1994).

Table 6: Relative bursa, thymus and spleen weights (gm organ weight/ gm body weight X 100) (experiment 1).

Tablica 6: Relativne težine burze, timusa i jetre (g težine organa/g težine tijela x 100) (pokus 1)

Dietary calcium (%)	Bursa	Thymus	Spleen - Slezena
21 days old - 21 dan starosti			
0.60	0.24[a]	0.50	0.11
0.80	0.34[b]	0.51	0.11
1.00	0.34[b]	0.55	0.09
1.20	0.24[a]	0.59	0.10
1.40	0.26[ab]	0.56	0.10
42 days old - 42 dana starosti			
0.60	0.15	0.50	0.14
0.80	0.16	0.51	0.14
1.00	0.17	0.44	0.14
1.20	0.16	0.50	0.14
1.40	0.17	0.52	0.16

Values are means for 27 to 45 chickens per treatment group. Values in the same column with different superscript letters are different, $p \leq 0.05$.

Vrijednosti su prosjek za 27 do 45 pilića u pokusnoj skupini. Vrijednosti u samoj koloni s različitim slovima su zazličite kod $p \leq 0.05$.

Measurement of vaccinal titers for all three vaccines by ELISA did not detect differences among treatment groups in either experiment (Tables 7 and 8). This was result of high coefficients of variation. In our earlier report, we detected differences in response to NCD, using the ELISA, in spite of high coefficients of variation. In one experiment, the difference was significant at $p \leq 0.08$, not 0.05. The commercial ELISA were designed for evaluating status of flocks, for which they are adequate, but it is clear that these assays are not sufficiently precise for most scientific investigations. Better tests must be developed for further investigations in this field. On the other hand, the HI test for NCD did discriminate among treatment groups (Tables 7 and 8). In experiment 1, HI titers were significantly greater when 1.20% and 1.40% calcium were fed, and in experiment 2, they were greater when 0.80% to 1.20% calcium was fed. For this and other reasons, vide supra, 1.40% calcium cannot be recommended. Even though the ELISA for NCD could not be separated by ANOVA, the means were consistent with our earlier report. Moreover, they correlated with the HI results; 62% in experiment 1 and 78% in experiment 2.

It is well known that minerals are easily absorbed from the gut. They are distributed throughout the body by a large number of proteins that have binding sites for calcium. Whereas zinc is known to be necessary for immune function, we are just beginning to appreciate the importance of calcium. Ratios of various minerals in the diet have been shown to be important in the physiological effects of the minerals in a number of systems, and one should anticipate the same may occur in the immune system. Mechanisms of action are poorly understood and open to speculation. The mechanism of calcium could be mediated through protein kinase C, which is activated by calcium. Protein kinase C has been associated with activation of immune cells (Hengel, 1991). Flux of intracellular calcium is known to be a major second signal in activation. Two of the thymic hormones, both present in chickens, have metals associated with them. Avian thymic hormone is a parvalbumin having two

EF hands for calcium binding (Brewer et al., 1989), and thymulin requires zinc for activity (Bach et al., 1977). In addition, Radulescu (1995) has found metal-binding domains for calcium and zinc finger-like sequences in the constant regions of immunoglobulin light and heavy chains.

Table 7: Vaccinal titers for Newcastle disease (NCD) by enzymelinked immunosorbent assys (ELISA) and hemagglutinin inhibition (HI), and vaccinal titers for infectious bronchitis (IB) and infectious bursal disease (IBD) measured by ELISA at 42 days of age (experiment 1).

Tablica 7: Titar vakcinacije newkastelske bolesti (NKB) putem enzime-linked immunosorbent assay (ELISA), hemaglutinin inhibicije (HI) i vakcinalni titar za infekcijozni bronhitis (IB) i infekcioznu burzalnu bolest (IBB) mjereno metodom ELISA sa 42 dana starosti (pokus 1)

Dietary Ca (%)	NCD - NKB		IB	IBD - IBB
Kalcij u hrani (%)	ELISA*	HI**	ELISA*	ELISA*
0.60	4600	1.34[a]	3900	1300
0.80	4000	1.24[a]	700	800
1.00	6600	1.37[a]	700	1100
1.20	6800	1.42[b]	1200	1500
1.40	4500	1.41[b]	600	1300

* Values are mean titers, rounded to mearest hundred, for 27 to 30 chickens per treatment group.

** Values are geometric mean titers ($\log_{10}$) for 27 to 30 chickens per treatment group.

Values with different letter superscripts are different, $p \leq 0.05$.

* Vrijednosti su prosječni titar, okruglo blizu stotine, za 27 do 30 pilića po tretitanoj skupini.

** Vrijednosti su geometrijski srednji titar ($\log_{10}$) za 27 do 30 pilića po tretiranoj skupini.

Vrijednosti s različitim slovima označena na vrhu su različita kod $p \leq 0.05$.

Table 8: Vaccinal titers for Newcastle disease (NCD) by enzymelinked immunosorbent assay (ELISA) and hemagglutinin inhibition (HI), and vaccinal titers for infectious bronchitis (IB) and infectious bursal dieseas (IBD) measured by ELISA at 42 days of age (experiment 2).

Tablica 8: Titar vakcinacije newkastelske bolesti (NKB) putem enzime-linked immunosorbent assay (ELISA), hemaglutinin inhibicije (HI) i vakcinalni titar za infekcioni bronhitis (IB) i infekcioznu burzalnu bolest (IBB) mjereno metodom ELISA sa 42 dana starosti (pokus 2)

Dietary Ca (%)	Available - Iskoristivi	NCD - NKB		IB	IBD - IBB
Kalcij u hrani (%)	P (%)	ELISA*	HI**	ELISA*	ELISA*
0.60	0.30	5000	1.19[b]	3900	1300
0.80	0.40	7600	1.23[a]	700	800
1.00	0.50	8600	1.25[a]	700	1100
1.20	0.60	9400	1.30[a]	1200	1500
1.40	0.80	8500	1.21[b]	600	1300

* Values are mean titers, rounded to mearest hundred, for 27 to 30 chickens per treatment group.

** Values are geometric mean titers ($\log_{10}$) for 27 to 30 chickens per treatment group.

Values with different letter superscripts are different, $p \leq 0.05$.

* Vrijednosti su prosječni titar, okruglo blizu stotine, za 27 do 30 pilića po tretitanoj skupini.

** Vrijednosti su geometrijski srednji titar ($\log_{10}$) za 27 do 30 pilića po tretiranoj skupini.

Vrijednosti s različitim slovima označena na vrhu su različita kod $p \leq 0.05$.

SAŽETAK

Muški pilići (Ross x Ross), porijeklom iz komercijalnog valilišta, 42 dana bili su hranjeni *ad libitum* hranom, koja je sadržavala 0.6, 0.8, 1.0, 1.2 i 1.4% kalcija. Razina fosfora je u jednom pokusu bila 0.45%, a u drugom se razlikovala, kako bi omjer kalcija i fosfora bio 2.0. Pilići su cijepljeni u dobi od 10 i 18 dana protiv infekciozne bolesti burze i u dobi od 21 dan protiv Newcastleske bolesti i infekcioznog bronhitisa. Uzorci seruma su prikupljeni u dobi od 21 i 42 dana i specifični titar za tri cjepiva je utvrđen pomoću enzyme-linked immunosorbent assays (ELISA) te pomoću inhibicije hemaglutinacije (HI) za Newcastle-sku bolest. Dodatak 0.6% kalcija hrani je imao negativan utjecaj na prirast, iskoristivost hrane i relativnu težinu burze, dok 0.8% i 1.2% kalcija nisu negativno utjecali. U prvom pokusu (stalna razina fosfora od 0.45%), kod pilića hranjenih hranom s 0.6% kalcija, dyschondroplasia tibiae je bila znatno češća (u drugom pokusu nije praćeno). U oba pokusa, HI titar za Newcastlesku bolest je bio povišen u grupi, čija hrana je sadržavala 1.20% kalcija.