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Research Article
The Effect of Feeding Various Limestone Particle Sizes, Limestone Solubility and Calcium Intake on Bone Status and Shell Quality of a Commercial White Layer Strain from 18-65 Weeks of age

[1]Bingfan Zhang, [2]Jordan Weil, [2]Antonio Beita Guerra, [2]Pramir Maharjan, [2]Katie Hilton, [2]Nawin Suesuttajit and [2]Craig N. Coon

[1]802 Jinchengyuan, Fuhao Garden, 50 Fusong Road, Caoyang District, Changchun City, Jilin Province, PRC 130012, People's Republic of China
[2]Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA

Abstract

Background and Objective: The daily requirement of calcium for Commercial White laying hens has been steadily increasing for the past five decades because primary breeders have genetically annualized an increase in egg numbers. The objective of the study was to determine if Commercial White laying hens (from 18-65 week of age) require different dietary levels of soluble and insoluble calcium than older layers. **Materials and Methods:** One hundred and ninety two,18 week old, Commercial White laying hens were housed in group wire cages with four layers per unit and randomly assigned into a 2×2 factorial arrangement (2 predicted Ca intakes hen^{-1} day^{-1} and 2 limestone sources with different *in vitro* solubility) to provide 12 replicate units per treatment. Hen day egg production (HDEP), egg weight (EW), egg mass (EM), feed intake (FI), body weight (BW), specific gravity (SG), shell weight per unit surface area (SWUSA), bone ash concentration (AC) and bone breaking force (BBF) were determined throughout the 47 week feeding study. **Results:** Commercial White laying hens consuming 5 g calcium hen^{-1} day^{-1} with an *in vitro* lower solubility of 35.8% produced eggs with a significant increase in egg specific gravity and Shell Weight per Unit Surface Area (SWUSA) compared to hens consuming 3.56 g hen^{-1} day^{-1} during a 47 week production period. Bone size was not affected by solubility, Ca intake or age; however, bone ash concentration (AC) and bone breaking force (BBF) were increased from 18 week of age to ~40/50 week of age. The AC and BBF of the Commercial White laying hens decreased during the later portion of the production period (50-64 week) especially when the daily consumption was only3.56 g Ca from limestone with higher *in vitro* solubility of 49.4%. The AC and BBF showed that 3.56 g daily Ca intake with 49.4% solubility for 18-65 wk Commercial White laying hens was not sufficient to maintain bone status of hens after 50 week. **Conclusion:** The results support the idea that both osteoclastic and osteoblastic activities are present in the bone after sexual maturity. Bone strength and bone ash content of Commercial White laying hens(18-65 week) were maintained or improved by feeding hens increased quantities of a lower soluble Ca limestone to reduce Ca mobilization from medullary and cortical bone for egg shell formation. Hens (18-65 week) consuming 5 g of the lower soluble Ca limestone produced significantly higher shell quality than hens consuming 3.5 g hen^{-1} day^{-1}.

Key words: Bone status, calcium requirement, commercial white laying hens, limestone solubility, shell quality

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Corresponding Author: C.N. Coon, Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA Tel:479-575-4134 Fax: 479-575-8775

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The prevalence of inferior shell quality and broken bones are serious problems for Commercial laying hens[1,2]. It has been estimated that 13-20% of total eggs produced are cracked or lost before reaching the market[3]. Skeletal calcium is actively utilized during the shell formation. Farmer *et al.*[4] reported that utilization of skeletal calcium for shell formation may range from 28-96% depending on time and level of calcium intake. This calcium utilization is mainly from medullary bone but at the expense of cortical bone[5]. Thus, the strength and ash content of the layer bone may be reduced by mobilization of calcium from the skeleton.

Particulated limestone has been reported to produce beneficial effects on shell quality and bone status of layers[6-10] due to the longer gizzard retention and more efficient Ca utilization[11-13]. It is not clear whether layer bone status can be maintained or improved by feeding an appropriate amount and particle size of calcium carbonate due to conflicting results in the literature[14-18]. Both osteoclastic and osteoblastic activities have been reported to be present simultaneously at all times during the ovulatory cycle for layers and during shell formation, however, osteoclastic activity predominates during active shell formation and osteoblastic activity predominates when the shell gland is inactive[14,15]. Thus, bone status may be improved if available metabolic calcium can support greater bone formation (osteoblastic activity) than resorption (osteoclastic activity). In contrast, Whitehead and group[16-18] reported that such a coupling of osteoclasts and osteoblasts does not occur in reproductively active laying hens since there is no osteoid formation in hens from onset-of-lay to end-of-lay. The authors suggested trabecular bone formation ceased at the onset of production in laying hens and nutritional approaches could only maintain but not improve bone status.

The present experiment was conducted to determine if feeding a higher level of calcium and a larger particle size (or lower solubility)limestone can maintain or improve the shell quality and the bone status in Commercial White laying hens during the laying period from 18-65 week.

MATERIALS AND METHODS

A total of 192 Commercial White Leghorn-type pullets, 18 weeks of age, were housed four birds to a cage. Each cage was randomly assigned to 2×2 factorial treatments (two Ca dietary levels: 3.5 and 5.0% from 18-34 week and 3.2 and 4.4% from 34-65 week of age; two sizes of limestone) with 12 replicates each. The 3.5 and 3.2% Ca levels are referred to as 3.5 g Ca daily intake level and the 5.0 and 4.4% Ca levels are referred to as 5.0 g Ca daily intake level for the rest of the context. The 3.5 g Ca intake level equates to an actual Ca intake of 3.33, 3.52 and 3.56 g hen^{-1} day^{-1} and the 5.0 g Ca intake level equates to an actual Ca intake of 4.76, 4.84 and 4.89 g hen^{-1} day^{-1} at ages: 18-34, 34-50 and 50-62 week, respectively.

The experimental diets were corn-soybean meal formulated to contain 17% protein, 2890 kcal ME kg^{-1} and 46% available phosphorus and all the other nutrients were kept at or above the recommended levels[19] after the inclusion of limestone. Hens received feed *ad libitum* throughout the experiment. The photoperiod was initially set at L:D = 13:11 h. A weekly 30 min increase in light was provided until 15 h day^{-1} and then a 15 min weekly increase until 16 h day^{-1} light period was obtained.

Egg production was recorded daily. Feed consumption was calculated every four wk. Egg weight was measured biweekly. Egg specific gravity, shell weight, shell weight per unit of surface area[20] (SWUSA) and body weight were determined every eight wk. The shell of each egg was dried in an oven at 40°C until a constant weight was obtained. The Ca content of the basal diet and limestone was analyzed by atomic absorption spectrophotometer before mixing.

Four hens from each treatment were sacrificed at 20, 30, 40, 50, 60 and 64 week of age. Right tibia bones were removed and stored at -20°C until tested for the various bone parameters. The bone volume was taken by the weight change in water method[21]. Briefly, tibia bones were weighed in the air and in the water. The weight change equals to the weight of water replaced by the bone and the volume was calculated by assuming water specific gravity of 1.0 g/cm^3. Ash weight of the bone was obtained after ashing at 600°C for 24 h. Bone ash concentration was calculated by the formula of bone ash weight divided by its volume. Bone breaking force was measured by an Instron Testing Machine (Model 1122; Canton, MA 02021). Tibia bones were supported by a fulcrum with 8 cm width. A probe with 1.4 cm length and 0.3 cm at the base was attached to a 500 kg load cell with a crosshead speed of 200 mm min^{-1}.

Limestone used in the study was from the same commercial source (ILC Resources, Des Moines, Iowa) and was screened into two different sizes. The mean size of the Shell and Bone Builder Blend larger particle was 2856 microns and the smaller particle Unical-S limestone product had a mean particle size of 450 microns. The limestones were measured by laser diffraction instead of sieve screen analysis. Solubility was the basis of evaluating limestone quality in present research because of previous research[7] showing solubility produced a higher correlation to shell quality and bone status compared to particle size measurements. Limestone solubility was

determined using both the Weight Loss Method (WLM)[22] and a modified WLM (MWLM)[23]. The MWLM method consisted of utilizing 400 mL beakers and adding 200 mL of 2 N HCl. A beaker with hydrochloric acid was warmed for 15 min in a 42°C water bath oscillating at 80 Hz. Approximately 2 g limestone sample was poured into the beaker. After 10 min in the oscillating water bath, the hydrochloric acid containing solubilized limestone and non-solubilized limestone was filtered over a pre-weighed Whatman ashless filter paper with excess deionized water. The remaining non-solubilized limestone sample was weighed after drying in a 70°C oven for 10 h or until constant weight was reached. Solubility was expressed as the percentage weight loss of the limestone.

Statistical analysis: Data was analyzed using the general linear models (GLM) and regression procedures of statistical analysis software (SAS)[24]. Statistical significance was based on a 5% probability level. Duncan's range test was used to separate the means of each variable. The average measurements for each variable were used in analyzing data. Hens that were going through natural molting during the experiments were excluded from the analysis. Limestone solubility instead of source and particle size was used in data analysis. It has been reported that regressed shell quality and bone parameter traits for layers relates better to limestone Ca solubility than limestone particle size[7]. All procedures regarding the use of live animals in this study were carried out in accordance with the Animal Use Protocol 03008, which was approved by the University of Arkansas Institutional Animal Care and Use Committee.

RESULTS AND DISCUSSION

The *in vitro* solubility was determined to be 35.8 and 49.4% using MWLM[23] and 11.4 and 15.2% using WLM[22] for the two limestone sources, respectively. However, the solubility treatments are referred to those determined using MWLM hereafter unless specified.

No significant effects of Ca dietary level and solubility on egg production ($p > 0.05$) were found. There was no significant interaction ($p > 0.05$) effect of calcium level and limestone solubility on egg production (Table 1).

The effects of solubility and Ca dietary level on egg weight were not significant ($p > 0.05$). Egg weight increased as hens aged for all the treatments (Table 1). No treatment effects on egg mass ($p > 0.05$) were found although the egg mass was lower for hens fed 3.5 g Ca hen^{-1} day^{-1} compared with those fed 5.0 g Ca hen^{-1} day^{-1} at the end of the production period (Table 1).

Feed intake was not affected by the solubility and level of dietary Ca before hens reached 50 week of age ($p > 0.05$). From 50-62 week of age feed intake was lower for hens fed 3.5 g Ca hen^{-1} day^{-1} for both of the two solubility treatments compared with those fed 5.0 g Ca hen^{-1} day^{-1} ($p < 0.05$). The reduction in feed intake of the low Ca intake group was likely due to less egg mass produced by this group. The feed intake was also increased with age (Table 1).

Body weight increased with age. More weight gain was found in the early stage of lay. The body weight change after 50 week of age was very small. Body weight was not significantly ($p > 0.05$) affected by solubility and Ca intake (Table 1).

Table 1: Composition and analysis of the experimental diets

	Percentage	
Ingredients	3.5 Ca% diet	5.0 Ca% diet
Corn	64.70	56.40
Soybean meal, 47% Crude protein	23.90	25.40
DL-methionine	0.09	0.01
Limestone	8.20	12.10
Salt	0.18	0.19
Vitamin mixture1	0.04	0.04
Mineral mixture2	0.04	0.04
Choline chloride, 50%	0.13	0.13
Vegetable oil	1.01	3.82
Mono-potassium phosphate/mono-sodium phosphate	0.30	0.30
Calculated composition		
ME (kcal kg^{-1})	2890.00	2890.00
Crude protein	17.00	17.00
Non-phytate P	0.46	0.46
Analyzed composition		
Calcium	3.46	4.94

[1]Vitamin mixture provides in milligrams per kilogram of diet; Vitamin A: 5,500 IU, Vitamin E: 25 IU, Menadione: 1.45 mg, Cholecalciferol: 1,100 IU, Riboflavin: 5.4 mg, Pantothenic acid: 23 mg, Nicotinic acid: 55 mg, Vitamin B12: 9.9 mg, Vitamin B6: 9.5 mg, Thiamine: 5.4 mg, Folic acid: 1.8 mg, Biotin: .28 mg, [2]Trace mineral mixture provides in milligrams per kilogram of diet: Mn: 68, Zn: 61, Fe: 120, Cu: 7, I: .7, Se: .3

Egg shell quality was influenced by solubility and Ca intake. Highest egg specific gravity was observed for hens fed 5.0 g Ca hen^{-1} day^{-1} with the lower soluble limestone source (35.8% *in vitro* solubility) at early age (22-38 weeks). For both of the two solubility groups (35.8 and 49.4%), the higher Ca intake (5.0 g Ca hen^{-1} day^{-1}) produced higher specific gravity compared to the lower Ca intake (3.5 g Ca hen^{-1} day^{-1}) at later stages of lay (46-62 week of age). Skrivan *et al.*[25] did not evaluate solubility of limestone but found that young ISA Brown layers (24-36 week) and older ISA Brown layers (56-68 week) both increased shell quality (shell weight, shell thickness and shell Ca) when fed all limestone with particle size ranging from 0.8-2.0 mm compared to feeding both ages of layers fine particle limestone (<0.5 mm). The researchers did not find that large particulate limestone improved the shell breaking strength for the younger or older hens. Pelicia *et al.*[26] reported that 58 week old Hisex Brown commercial layers in a 4×3 factorial design fed four levels of calcium (3,3.5, 4 and 4.5%) and 3 particle size distributions (100% fine, 50% fine and 50% coarse and 30% fine and 70% coarse) for 84 day produced no interaction effects on shell quality or performance however the researchers showed that hens fed 4.5% calcium increased the shell weight per unit surface area. The researchers suggested the highest calcium level may be reducing the *in vivo* limestone solubility and producing a slower calcium release and better calcium utilization as previously suggested by Gordon and Roland[27]. In the present study, hens fed either the 3.5 g or 5 g Ca intake hen^{-1} day^{-1} with diets containing limestone that is lower in solubility consistently produced eggs with higher specific gravity than those fed diets with higher limestone solubility. Similar results were also observed for SWUSA (Table 2). These results confirm the earlier findings that the particulate limestone is superior to pulverized limestone for shell quality in laying hens[6-10].

The bone volume was not affected by solubility, Ca intake or age ($p>0.05$). This indicates that the size of bone changes little (if any) during the laying period. However bone ash concentration expressed as the ash weight per unit of bone

Table 2: The effect of age, Ca intake and *in vitro* solubility on egg mass, egg weight, egg production, feed intake and body weight for 18-65 week old Commercial White layers

Age (weeks)				
Solubility (%)	Ca intake (g)	18-34	34-50	50-62
Egg production (%)				
35.8	3.5	77.5000	91.5000	84.5000
	5.0	76.6000	91.5000	87.9000
49.4	3.5	74.1000	91.8000	85.0000
	5.0	73.6000	92.7000	86.6000
SEM		2.9100	1.5400	2.5200
Egg weight (g)				
35.8	3.5	50.8000	57.9000	61.1000
	5.0	51.5000	58.3000	61.7000
49.4	3.5	51.0000	58.2000	61.8000
	5.0	50.8000	58.6000	62.6000
SEM		0.6342	0.5429	0.7212
Egg mass (g day^{-1} hen^{-1})				
35.8	3.5	39.9000^{a}	52.8000	52.1000
	5.0	40.1000^{a}	53.2000	54.0000
49.4	3.5	38.7000^{ab}	53.4000	52.5000
	5.0	38.0000^{b}	54.2000	54.2000
SEM		0.8453	0.7807	1.4210
Feed intake (g day^{-1} hen^{-1})				
35.8	3.5	95.1000	108.7000	107.7000^{b}
	5.0	96.1000	109.5000	114.1000^{a}
49.4	3.5	94.5000	110.0000	110.6000^{ab}
	5.0	95.0000	111.5000	112.6000^{a}
SEM		1.4200	1.7500	2.0100
Body weight (g)				
35.8	3.5	1544.0000	1741.0000	1772.0000
	5.0	1538.0000	1754.0000	1781.0000
49.4	3.5	1551.0000	1753.0000	1760.0000
	5.0	1535.0000	1761.0000	1806.0000
SEM		19.2000	20.3000	25.4000

^{a,b}Means within each column and variable with no common letters were significantly different ($p<0.05$)

volume was increased from 20 week of age until 40-50 week of age. Thereafter, hens from all the Ca treatments maintained their bone status except hens fed 3.5 g Ca intake with diets containing pulverized limestone (smaller particle size) with higher solubility showed a small decrease in bone ash concentration from the 40 weeks period through 60 weeks of age. Bone breaking force showed a similar trend except the hens fed 5 g Ca day^{-1} with diets containing higher soluble limestone source also decreased in breaking strength during the period from 40 weeks through 60 weeks of age (Table 3). These present findings indicate that bone ash and strength increases after sexual maturity up to 40-50 week of age prior to showing a plateau. Whitehead and Wilson[16], Whitehead[17] and Whitehead and Fleming[18] have reported that hens actively producing eggs are incapable of forming trabecular bones. Whitehead and Wilson[16], Whitehead[17]and Whitehead and Fleming[18] also suggests that medullary bone contributes very little to bone strength. The increase in bone breaking strength and bone ash from 20-50 week of age found in the present study may indicate that trabecular bone was still being formed during this production period or the increase in bone ash concentration was only from medullary bone formation. The present data supports the findings of Taylor and Belanger[14] and Miller[15] that both osteoclastic and osteoblastic activities are present simultaneously during the ovulatory cycle and during shell formation but that osteoclastic activity (bone resorption) predominates during active shell formation and osteoblastic activity (bone formation) predominates when the shell gland is inactive. Thus the Ca reserve in skeletal bones can be replenished when the shell gland is inactive and Ca is available from digestive tract. The research indicates that bone strength and bone ash content of laying hens can be improved by supplying hens with sufficient Ca intake from lower soluble large particle size calcium carbonate source so that the rate of bone formation exceeds the rate of bone reabsorption. The bone ash concentration for hens fed 3.5 g dietary Ca day^{-1} hen^{-1} from diets containing limestone with higher solubility (49.4%) tended to decrease during the last 1/3 of laying period (>50 weeks of age) while hens in the other groups kept bone ash concentration relatively constant for the same duration. The highest bone ash concentration for all production periods was determined for hens fed 5.0 g Ca day^{-1} hen^{-1} (Table 4).

Table 3: The effect of age, limestone *in vitro* solubility and Ca intake on shell weight per unit surface area (SWUSA)and egg specific gravity

Age (week)			
Solubility (%)	Ca intake (g)	22-38	46-62
Specific gravity			
35.8	3.5	1.0839^{b}	1.0774^{b}
	5.0	1.0851^{a}	1.0813^{a}
49.4	3.5	1.0825^{b}	1.0749^{c}
	5.0	1.0827^{b}	1.0802^{a}
SEM		0.0006	0.0008
SWUSA (mg/cm^2)			
35.8	3.5	77.5600^{ab}	74.9700^{ab}
	5.0	78.4400^{a}	76.3800^{a}
49.4	3.5	76.5200^{b}	74.2100^{b}
	5.0	77.5100^{ab}	75.9400^{ab}
SEM		0.6100	0.7300

$^{a-c}$Means within each column and variable with no common letters were significantly different (p<0.05)

Table 4: The effect of dietary Ca intake, limestone *in vitro* solubility and hen age on bone parameters for 18-65 week Commercial White layers

Ca dietary intake (g h^{-1} day^{-1})	Solubility (%)	Age (week)	Bone volume (cm^3)	Bone ash concentration (mg ash/cm^3)	Bone breaking force (kg)
3.5	35.8	20-30	6.7600	411.90^{e}	9.8600^{f}
		40-50	6.9200	452.20^{cd}	11.1500^{bcd}
		60-64	6.8200	460.20^{c}	11.1600^{bcd}
	49.4	20-30	7.0400	413.20^{e}	10.3900^{def}
		40-50	7.0200	451.50^{cd}	11.2600^{bc}
		60-64	7.0000	429.50^{de}	10.7300^{cde}
5.0	35.8	20-30	7.2100	410.50^{e}	10.5400^{cdef}
		40-50	6.8900	492.30^{a}	12.1000^{a}
		60-64	6.8300	484.80^{ab}	11.8200^{ab}
	49.4	20-30	6.9200	420.30^{e}	10.1800^{ef}
		40-50	7.1700	474.70^{abc}	12.1500^{a}
		60-64	6.7300	465.40^{bc}	11.1700^{bcd}
SEM			0.3609	8.5853	0.2875
Source of variance		----------Probabilities----------			
Ca intake (Cal)		NS	0.00000	0.0009	
Source (S)		NS	0.00478	NS	
Age		NS	0.00000	0.0000	
Cal×S		NS	0.03440	0.0493	
Cal×Age		NS	NS	NS	
S×Age		NS	NS	NS	
Cal×S×Age		NS	0.0268	NS	

$^{a-f}$Means within each column and variable with no common letters were significantly different (p<0.05), NS: No significant effect (p>0.05)

The present research agrees with previous research by Zhang *et al.*[6] and Cheng and Coon[7] who reported that bone ash concentration and bone breaking force positively respond to a higher intake of calcium compared to calcium intake required for egg shell quality.

CONCLUSION

In conclusion, 18-65 week old Commercial White laying hens fed 3.54 g Ca hen^{-1} day^{-1} from a low soluble limestone (35.8%) containing larger particles of calcium carbonate was adequate for egg production, egg shell quality and for maintaining bone status throughout the production period. The same 3.54 g Ca hen^{-1} day^{-1} intake from a smaller particle highly soluble limestone source (49.4%) was not adequate to maintain bone status after 50 weeks of age during the 18-65 week production period. The research reported herein does not provide a validation that osteoblastic activity occurs after sexual maturity to increase trabeculae bone but the bone status (bone breaking strength and bone ash concentration) increased after sexual maturity through 50 wk of age and then plateaued or decreased depending upon Ca intake and solubility. The larger particle Ca stays in the gizzard longer and thus more Ca from the intestine is utilized and less Ca is mobilized from the skeletal system for egg shell formation. If new cortical bone is not produced after sexual maturity at least the bone of the layer may be maintained during the laying cycle with appropriate solubility and amount of calcium carbonate.

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