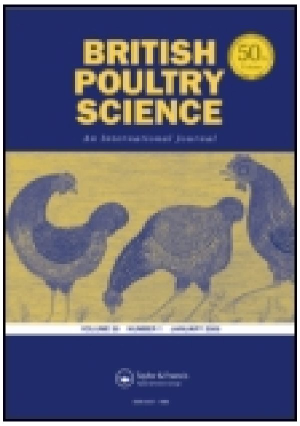

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The effect of dietary calcium concentration and particle size on performance, eggshell quality, bone mechanical properties and tibia mineral contents in moulted laying hens

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Abstract 1. A total of 72 H&N Brown Nick laying hens, 76 weeks of age (moulted at 60 weeks of age), were randomly assigned into a 3×3 factorial arrangement with three limestone particle sizes and three dietary Ca concentrations (30, 36 and 42 g/kg Ca); with 8 replicates per treatments, and one hen (individual) per experimental unit.

2. Particle sizes of the limestone were: distributions smaller than 2 mm (Fine), between 2 to 5 mm (Medium), and larger than 5 mm (Large). The fine, medium and large limestone particles were all obtained from the same source, and sieve sizes used had screen sizes 2 mm and 5 mm.

3. The different concentrations of dietary Ca, particle size or interactions had no significant effect on egg production, egg weight, egg mass, feed intake or feed conversion ratio.

4. The concentrations of dietary Ca and particle sizes had no significant effect on egg breaking strength, eggshell as % by weight of whole egg or eggshell thickness. The interactions between Ca concentrations and particle sizes had a significant effect on eggshell breaking strength, but not on other parameters.

5. Different dietary particle sizes had no significant effect on the Ca, P and Mg mineral contents of eggshell. While the different concentrations of Ca in diets had a significant effect on the P content of eggshell, they had no significant effect on Ca and Mg contents.

6. Different dietary concentrations of Ca had a significant effect on shear stress, and Ca and Mg contents of tibiae, but not other parameters. Also, dietary particle sizes had a significant effect on shear stress and Ca contents of tibiae. The interaction between Ca concentrations and particle sizes had a significant effect on tibia shear force and Ca content.

7. According to the results of this study, moulted brown laying hens should be fed 36 g/kg Ca and a medium limestone particle size (2–5 mm) in the diet to maintain performance, eggshell and bone quality.

INTRODUCTION

Despite the numerous studies of calcium (Ca) requirements in laying hens, egg breakage caused by poor shell quality still remains high in old laying hens (Bar *et al.*, 2002). The quality of the eggshells has a major influence on the economics of egg production. Damaged or broken shells account for 6 to 8% of all the eggs laid (Bain, 1997). The intake of inadequate amounts of Ca may increase skeletal abnormalities, reduce the size and the number of eggs produced, and give poor eggshell quality, leading to high rates of cracked eggs (Maynard *et al.*, 1984). In addition, the larger size of the eggs produced by older laying hens also results in lower eggshell quality, as the degree of egg size increase is higher than the eggshell weight increase (Adams and Bell, 1998).

In commercial layers, there is great interest in Ca metabolism because, as the hens age, eggshell quality decreases. Older layers also have

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a diminished capacity for replacing the Ca lost during hypocalcemic periods compared with young layers (Elaroussi *et al.*, 1994). When there is adequate Ca intake, the hen's needs can be supplied by increasing its intestinal absorption. However, if feed intake is low, increasing intestinal absorption will not be sufficient to ensure Ca supply. The NRC (1994) estimated the Ca requirement of brown laying hens to be 36 g/kg for 110 g/hen/d feed intake. Limited research information is available on the nutrient requirements of moulted hens; therefore the committee has assumed that requirements are similar to those of hens during the first cycle of production. Also, this committee reported that the requirement may be higher for maximum eggshell thickness. In brown laying hens at 58–73 weeks of age, 40 g/kg Ca in diets was required to maintain egg production and eggshell quality (Safaa *et al.*, 2008).

The particle size of Ca sources may influence its availability to the hens. As eggshell is usually formed during the night, when hens do not eat, the advantage of the use of larger particles is their slower passage through the gastrointestinal tract. This makes Ca available for eggshell formation, with consequent lower mobilisation of bone Ca by the hen (Harms, 1982). Zhang and Coon (1997) concluded that larger particle size limestone with lower *in vitro* solubility was retained in the gizzard for a longer time, which may increase Ca retention. Rao *et al.* (1992) reported that a minimum particle size of less than 1·0 mm did not sustain retention in the gizzard. Scheideler (2004) reported that the large particles of the Ca source in the diet of laying hens should constitute at least 25%. Calcium nutrition also plays a significant role in bone quality of the laying hen. Poor bone quality in laying hens can lead to many problems, which include broken or weak bones, osteoporosis, and economic losses (Whitehead and Fleming, 2000; Julian, 2005). The large particle size is more important for older hens and seems to help maintain the quantity and activity of medullary bone (Leeson and Summers, 2005).

While there are many studies on the effects of dietary Ca concentration and limestone particle size on performance and eggshell quality in laying hens during the first production period, there are limited reports on their effects on eggshell mineral content, bone mechanical properties and bone mineralisation in moulted brown laying hens. The purpose of this study was to determine the effects of interactions between dietary Ca concentrations and limestone particle size on performance, eggshell quality, bone biomechanical properties and mineral contents in moulted brown laying hens from 76 to 88 weeks of age.

MATERIALS AND METHODS

A total of 72 H&N Brown Nick hens, 76 weeks of age (moulted at 60 weeks of age), were randomly assigned into a 3×3 factorial arrangement with three limestone particle sizes and three dietary Ca concentrations (30, 36, and 42 g/kg Ca), with 8 replicates per treatment and one hen (individual) per experimental unit. Hens were housed in a layer house equipped with 72 metal battery cages ($50 \times 50 \times 40$ cm). Particle sizes of the limestone were: distributions smaller than 2 mm (Fine); between 2 to 5 mm (Medium); and larger than 5 mm (Large). The fine, medium and large limestone particles were all obtained from the same source, and the sieve sizes used had screen sizes 2 mm and 5 mm.

Experimental diets were balanced to meet or exceed the nutrient requirements for brown laying hens (National Research Council, 1994) and formulated to be isocaloric and isonitrogenous, with only the Ca particle sizes of the limestone in the diets differing (Table 1). Hens were offered feed and water *ad libitum* throughout the experiment (76–88 weeks of age). Lighting was provided for 16 h/d from 05:00 to 21:00 h throughout the experimental period. Hens were housed in individual layer cages in an environmentally controlled room (23–25°C).

Table 1. *Composition of experimental diets*

Ingredients (g/kg)	Calcium concentrations[3]		
	Ca-L	Ca-C	Ca-H
Maize	559	525	491
Barley	50	50	50
Soyabean meal (475 g/kg Crude protein)[1]	212	217·5	223
Sunflower meal (355 g/kg Crude protein)[1]	50	50	50
Sunflower oil (33·89 ME, MJ/kg)	42	54·6	67
Limestone	72	87·9	104
Dicalcium phosphate	8·1	8·1	8·1
Premix[2]	2·5	2·5	2·5
Salt	3·5	3·5	3·5
Methionine	0·9	0·9	0·9
Calculated nutrients			
Metabolisable energy (MJ/kg)	12·14	12·14	12·15
Crude protein (g/kg)	165	165·1	165·3
Calcium (g/kg)	30·04	36·00	42·05
Available phosphorus (g/kg)	2·75	2·74	2·73
Lysine (g/kg)	8·20	8·28	8·37
Methionine (g/kg)	3·41	3·39	3·37
Methionine + Cystine (g/kg)	6·57	6·53	6·50

[1]Analysed value. [2]Premix provided the following per kg of diet: retinyl acetate, 4·0 mg; cholecalciferol, 0·055 mg; DL-α-tocopheryl acetate, 11 mg; nicotinic acid, 44 mg; calcium-D-pantothenate, 8·8 mg; riboflavin sodium phosphate 5·8 mg; thiamine hydrochloride 2·8 mg; cyanocobalamin, 0 ·66 mg; folic acid, 1 mg; biotin, 0·11 mg; choline, 220 mg; Mn, 60 mg; Fe, 30 mg; Zn, 50 mg; Cu, 5 mg; I, 1·1 mg; Se, 0·1 mg. [3]Ca-L: 30 g/kg; Ca-C: 36 g/kg; Ca-H: 42 g/kg.

Body weight (BW) was obtained by weighing hens at the beginning and end of the experiment. Feed intake (FI) and egg weight (EW) were recorded bi-weekly. Egg production (EP) was recorded daily; and egg mass (EM) was calculated bi-weekly from collected data of EP and EW, using: $EM = (EP \times EW)/\text{period (days)}$. Feed conversion ratio (FCR; g of feed per g of egg) was calculated using: FCR = FI (g of feed/hen/period)/EM (g of egg/hen/period).

The eggs were analysed to determine the characteristics of eggshell quality parameters (shell breaking strength, shell weight and shell thickness) on all collected eggs produced during the last 2 d of each period during the experiment. Eggshell breaking strength was measured using a cantilever system by applying increased pressure to the broad pole of the shell using an instrument (Egg Force Reader, Orka Food Technology, Israel). Eggs were then broken; and eggshell, albumen, and yolk were separated and weighed. Eggshells were rinsed in running water and dried in an oven at 60°C for 12 h; and eggshell thickness (including the membrane) was determined on three points on the eggs (one point on the air cell, and two randomised points on the equator) using a micrometer (Mitutoyo, 0·01 mm, Japan). Eggshells were weighed using a 0·001 g precision scale. Eggshell percentage was calculated using: Eggshell % (g/100 g egg) = [Eggshell weight (g)/Egg weight (g)].

Bone mechanical properties were determined from the load-deformation curve generated from a three point bending test (ASAE Standard S459, 2001) using an Instron Universal Testing Instrument (Model 1122; Instron, Canton, MA) and the TestWorks 4 software package (version 4·02; MTS System Corporation, Eden Prairie, MN). The crosshead speed was constant at 5 mm per min. The full-scale load of the load cell was 5·0 Newtons (N), and none of the bones failed or fractured at or below 5·0 N. Shear tests were performed on the tibiae using a double shear block apparatus. The shear force was exerted over a 6·35 mm (0·25 inch) section located at the centre of the diaphysis. These tests resulted in the ultimate shear force and shear stress being evaluated for each bone. An average wall thickness (cortex thickness) of the tibia was measured at two points on the central axis of the broken tibia used in determining mechanical properties, using digital calipers with a precision of 0·001 mm. These mechanical properties of bone are described by Wilson and Ruszler (1996) and Armstrong *et al.* (2002).

Eggshell and tibia Ca, P or Mg contents were determined by MarsXpress Technology Inside and Inductively Coupled Plasma Atomic Emission Spectrometer (Vista AX CCD Simultaneous ICP-AES). Approximately 0·20 g of dried sample (eggshell without membrane, and bone with marrow removed) was put into a burning cup, and 5 mL nitric acid, 3 mL perchloric acid and 2 mL hydrogen peroxide was added. The sample was incinerated in a MARS 5 Microwave Oven (CEM Corp., USA, 3100 Smith Farm Road, Matthews, NC) at 190°C temperature and 1·207 kPa pressure, and diluted to a certain volume (50 mL) with distilled water. Mineral concentrations were determined by an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Skujins, 1998).

Data were subjected to ANOVA by using General Linear Model procedure (GLM) in Minitab (2000). Duncan's multiple range tests were applied to separate means (Duncan, 1955). Statements of statistical significance are based on probability of $P < 0{\cdot}05$.

RESULTS

The egg production, egg weight, egg mass, feed intake and feed conversion ratio are shown in Table 2. Different concentrations of dietary Ca, particle size and interactions had no significant effect on egg production, egg weight, egg mass, feed intake and feed conversion ratio.

The eggshell breaking strength, eggshell ratio and eggshell thickness are presented in Table 3. The levels of dietary Ca and particle size had no significant effect on egg breaking strength, eggshell percentage weight and eggshell thickness. The interactions between Ca concentrations and particle size had a significant effect on eggshell breaking strength ($P < 0{\cdot}05$), but not on other parameters in Table 3. Eggshell breaking strength was significantly affected by the interactions between Ca concentrations and particle size. Eggshell breaking strength was increased for the groups fed Ca-L∗Large and Ca-H∗Fine.

The eggshell mineral contents are shown in Table 3. Different dietary particle sizes had no significant effect on Ca, P and Mg as mineral contents of eggshell. Different dietary concentrations of Ca had a significant effect on the P content of eggshell, but not on Ca and Mg. Eggshell P content was increased by increasing dietary Ca concentrations.

The bone biomechanical properties and mineral content are shown in Table 4. There were no significant differences in cortex thickness between treatment groups. The shear stress was significantly higher in the group fed with the diet containing high Ca (Ca-H), in comparison with the groups fed with the diets containing Ca-C and Ca-L. Hens fed with the fine particle size had a significantly lower shear stress of tibia than

Table 2. *Effect of different concentrations of calcium and particle size supplementation to diets on performance in moulted H&N brown nick laying hens from 76 to 88 weeks of age (Mean ± standard error)*

Diets	Egg production (eggs/100 birds/d)	Egg weight (g)	Egg mass (g/hen/d)	Feed intake (g/hen/d)	Feed conversion ratio (g feed/g egg)
Ca concentrations					
Ca-L	85·59 ± 1·42	69·12 ± 0·50	59·2 ± 0·77	107·7 ± 1·08	1·82 ± 0·031
Ca-C	85·45 ± 1·40	69·03 ± 0·68	59·0 ± 0·93	109·1 ± 1·55	1·85 ± 0·028
Ca-H	84·09 ± 1·60	69·82 ± 0·76	58·7 ± 0·85	107·0 ± 0·96	1·82 ± 0·028
Particle size					
Fine	86·15 ± 1·20	69·86 ± 0·73	59·9 ± 0·93	107·7 ± 1·12	1·79 ± 0·024
Medium	85·84 ± 1·58	68·68 ± 0·53	59·0 ± 0·92	108·6 ± 1·14	1·84 ± 0·039
Large	83·40 ± 1·47	69·35 ± 0·64	57·8 ± 0·71	107·3 ± 1·34	1·86 ± 0·027
Concentrations * size					
Ca-L * Fine	87·26 ± 1·16	70·03 ± 0·78	61·1 ± 0·77	108·7 ± 2·04	1·78 ± 0·049
Ca-L * Medium	87·13 ± 2·52	68·98 ± 1·05	60·1 ± 1·75	107·2 ± 1·05	1·78 ± 0·066
Ca-L * Large	82·37 ± 3·14	68·23 ± 0·63	56·2 ± 1·29	107·3 ± 2·49	1·90 ± 0·046
Ca-C * Fine	87·50 ± 2·08	69·43 ± 1·51	60·8 ± 2·18	110·0 ± 2·23	1·81 ± 0·048
Ca-C * Medium	84·81 ± 2·46	68·26 ± 0·94	57·8 ± 0·95	110·1 ± 2·98	1·90 ± 0·045
Ca-C * Large	84·24 ± 2·83	69·30 ± 1·03	58·4 ± 1·38	107·2 ± 3·11	1·84 ± 0·051
Ca-H * Fine	82·21 ± 3·07	70·16 ± 1·58	57·7 ± 1·30	105·0 ± 1·36	1·82 ± 0·025
Ca-H * Medium	85·77 ± 2·74	68·75 ± 0·86	59·0 ± 1·17	108·8 ± 2·10	1·84 ± 0·080
Ca-H * Large	83·58 ± 2·55	70·54 ± 1·48	59·0 ± 1·95	107·5 ± 1·16	1·82 ± 0·042

Ca-L: 30 g/kg; Ca-C: 36 g/kg; Ca-H: 42 g/kg; Fine: 0–2 mm limestone particle size; Medium: 2–5 mm limestone particle size; Large: >5 mm limestone particle size.

Table 3. *Effect of different concentrations of calcium and particle size supplementation to diets on eggshell quality and mineral contents in moulted H&N brown nick laying hens from 76 to 88 weeks of age (Mean ± standard error)*

Diets	Eggshell breaking strength (kg)	Eggshell weight % (g/100 g egg)	Eggshell thickness ($mm10^{-2}$)	Eggshell mineral content		
				Ca (mg/g)	P (mg/g)	Mg (mg/g)
Ca concentrations						
Ca-L	3·63 ± 0·066	8·83 ± 0·15	35·67 ± 0·56	325·3 ± 2·4	1·32 ± 0·04^{b}	3·58 ± 0·09
Ca-C	3·77 ± 0·080	9·07 ± 0·10	36·88 ± 0·34	323·9 ± 1·7	1·42 ± 0·04^{ab}	3·47 ± 0·11
Ca-H	3·72 ± 0·088	8·97 ± 0·17	36·88 ± 0·36	328·9 ± 1·8	1·46 ± 0·04^{a}	3·39 ± 0·09
Particle size						
Fine	3·71 ± 0·075	9·01 ± 0·11	36·25 ± 0·39	327·6 ± 2·0	1·43 ± 0·04	3·48 ± 0·10
Medium	3·62 ± 0·083	9·04 ± 0·12	36·38 ± 0·47	322·9 ± 1·7	1·41 ± 0·04	3·44 ± 0·11
Large	3·79 ± 0·076	8·82 ± 0·19	36·79 ± 0·47	327·7 ± 2·1	1·36 ± 0·04	3·52 ± 0·09
Concentrations * size						
Ca-L * Fine	3·49 ± 0·077^{bc}	8·93 ± 0·26	35·25 ± 0·92	328·0 ± 4·9	1·31 ± 0·05	3·68 ± 0·15
Ca-L * Medium	3·43 ± 0·071^{c}	8·78 ± 0·20	35·25 ± 1·01	321·0 ± 3·6	1·38 ± 0·08	3·42 ± 0·15
Ca-L * Large	3·99 ± 0·056^{a}	8·77 ± 0·35	36·50 ± 1·04	327·0 ± 3·7	1·27 ± 0·06	3·65 ± 0·16
Ca-C * Fine	3·74 ± 0·125^{abc}	8·92 ± 0·11	36·38 ± 0·42	329·8 ± 2·9	1·48 ± 0·08	3·38 ± 0·18
Ca-C * Medium	3·83 ± 0·157^{ab}	9·25 ± 0·19	37·25 ± 0·67	318·9 ± 2·3	1·35 ± 0·07	3·46 ± 0·23
Ca-C * Large	3·74 ± 0·149^{abc}	9·02 ± 0·19	37·00 ± 0·65	323·1 ± 2·3	1·43 ± 0·06	3·57 ± 0·18
Ca-H * Fine	3·91 ± 0·146^{a}	9·18 ± 0·18	37·13 ± 0·48	324·9 ± 2·5	1·49 ± 0·04	3·38 ± 0·17
Ca-H * Medium	3·60 ± 0·150^{abc}	9·08 ± 0·21	36·63 ± 0·63	328·8 ± 1·9	1·50 ± 0·08	3·44 ± 0·20
Ca-H * Large	3·63 ± 0·152^{abc}	8·66 ± 0·43	36·88 ± 0·81	333·0 ± 4·0	1·39 ± 0·08	3·35 ± 0·08

^{a,b,c}Within a column, values not sharing a common superscript are statistically different; $P < 0{\cdot}01$. Ca-L: 30 g/kg; Ca-C: 36 g/kg; Ca-H: 42 g/kg; Fine: 0–2 mm limestone particle size; Medium: 2–5 mm limestone particle size; Large: >5 mm limestone particle size.

those fed with medium or large particle sizes. The interaction groups between Ca concentrations and particle size had a significant effect on shear force. The shear force was significantly lower in the interaction groups fed with the diet containing Ca-L*Fine than in the other groups. A significant increase was observed for the shear force of tibia in the Ca-L groups fed medium or large particle sizes. Ca-C and Ca-H groups with large particle size had no significant effect on shear force of the tibia, but a tendency was observed.

Dietary Ca concentrations and particle size as main factors had a significant effect on Ca and

Table 4. *Effect of different concentrations of calcium and particle size supplementation to diets on tibia mechanical properties and mineral contents in moulted H&N brown nick laying hens from 76 to 88 weeks of age (Mean ± standard error)*

Diets	Cortex thickness (mm)	Shear force (N)	Shear stress (N/mm^2)	Tibia mineral content		
				Ca (mg/g)	P (mg/g)	Mg (mg/g)
Ca concentrations						
Ca-L	0·705 ± 0·034	519·7 ± 20·9	$14·54 \pm 0·56^{b}$	$216·5 \pm 2·2^{A}$	100·2 ± 0·9	$3·11 \pm 0·05^{a}$
Ca-C	0·746 ± 0·028	528·4 ± 17·6	$14·71 \pm 0·58^{b}$	$208·7 \pm 1·5^{B}$	97·4 ± 0·7	$3·01 \pm 0·03^{ab}$
Ca-H	0·713 ± 0·026	557·9 ± 16·0	$16·41 \pm 0·53^{a}$	$204·0 \pm 2·5^{B}$	96·9 ± 0·9	$2·96 \pm 0·05^{b}$
Particle size						
Fine	0·687 ± 0·034	477·1 ± 14·3	$13·68 \pm 0·53^{B}$	$203·7 \pm 2·6^{B}$	97·1 ± 1·0	$2·92 \pm 0·05^{B}$
Medium	0·755 ± 0·024	564·6 ± 18·2	$16·06 \pm 0·50^{A}$	$214·3 \pm 1·9^{A}$	98·4 ± 0·7	$3·13 \pm 0·04^{A}$
Large	0·723 ± 0·029	564·3 ± 16·8	$15·92 \pm 0·59^{A}$	$211·1 \pm 2·0^{A}$	98·9 ± 0·9	$3·03 \pm 0·04^{AB}$
Concentrations* size						
Ca-L * Fine	0·705 ± 0·092	$419·2 \pm 18·2^{C}$	12·00 ± 0·90	212·5 ± 4·5	$102·2 \pm 1·5^{A}$	3·00 ± 0·08
Ca-L * Medium	0·728 ± 0·047	$615·3 \pm 33·2^{A}$	16·36 ± 0·79	218·4 ± 2·5	$100·0 \pm 1·1^{AB}$	3·21 ± 0·06
Ca-L * Large	0·684 ± 0·026	$524·5 \pm 11·3^{AB}$	15·26 ± 0·70	218·5 ± 4·0	$98·5 \pm 2·0^{ABC}$	3·13 ± 0·09
Ca-C * Fine	0·715 ± 0·038	$517·3 \pm 22·9^{ABC}$	13·90 ± 0·70	204·2 ± 2·9	$95·7 \pm 0·9^{BC}$	2·96 ± 0·07
Ca-C * Medium	0·800 ± 0·037	$500·4 \pm 21·5^{BC}$	14·88 ± 0·97	212·3 ± 2·3	$97·2 \pm 1·1^{ABC}$	3·07 ± 0·06
Ca-C * Large	0·724 ± 0·065	$567·6 \pm 41·4^{AB}$	15·36 ± 1·32	209·5 ± 1·9	$99·3 \pm 1·1^{AB}$	3·01 ± 0·05
Ca-H * Fine	0·641 ± 0·034	$494·8 \pm 19·8^{BC}$	15·15 ± 0·88	194·4 ± 3·5	$93·4 \pm 0·6^{C}$	2·78 ± 0·10
Ca-H * Medium	0·738 ± 0·041	$578·1 \pm 26·9^{AB}$	16·95 ± 0·76	212·3 ± 4·4	$98·2 \pm 1·3^{ABC}$	3·11 ± 0·08
Ca-H * Large	0·723 ± 0·053	$600·8 \pm 23·0^{AB}$	17·12 ± 1·05	205·2 ± 2·5	$99·0 \pm 1·8^{AB}$	2·97 ± 0·04

[A,B,C,D,E,F]Within a column, values not sharing a common superscript are statistically different; $P < 0·01$. [a,b]Within a column, values not sharing a common superscript are statistically different; $P < 0·05$. Ca-L: 30 g/kg; Ca-C: 36 g/kg; Ca-H: 42 g/kg; Fine: 0–2 mm limestone particle size; Medium: 2–5 mm limestone particle size; Large: > 5 mm limestone particle size.

Mg content of the tibia. The highest Ca and Mg contents in tibiae were obtained for the group fed with Ca-L. Also, the lowest Ca and Mg contents of tibiae were obtained for the group fed with the fine particle size. The interaction between Ca concentrations and particle size had a significant effect on P content of the tibia. The P content of the tibia was found to be lower for the hens fed with the diet containing Ca-H*Fine, in comparison with Ca-L*Fine, Ca-L*Medium, Ca-C*Large or Ca-H*Large.

DISCUSSION

In the present study, dietary Ca concentrations and particle size had no effect on performance parameters. Castillo *et al.* (2004) reported that egg production, egg mass and feed conversion ratio were not affected when Ca intake increased from 3·68 to 4·26 g/hen/d in laying hens from 55 to 70 week of age. Similarly, Keshavarz *et al.* (1993) observed no difference in egg production, egg weight, egg mass and feed conversion ratio of laying hens from 22 to 62 weeks of age when the Ca intake of the diet was increased from 3·29 to 4·31 g/hen/d. Bar *et al.* (2002) reported that increasing dietary Ca from 48 to 50 g/kg did not affect egg production in moulted Lohmann Brown hens from 80 to 92 weeks of age. Narvarez-Solarte *et al.* (2006) observed that an increase in Ca intake from 3·86 to 4·70 g/hen/d had no effect on egg production and egg weight in white laying hens from 46 to 62 weeks of age. Also, the increase in Ca intake from 4·26 to 4·70 g/hen/d had no effect on feed conversion ratio and egg mass. In this study, an average feed intake of 108 g/hen/d was observed, and the daily Ca intake may be calculated as 3·25 g/hen for the lowest dietary Ca concentration (Ca-L), which was apparently sufficient to supply the requirements for the performance parameters. Accordingly, it appears that the Ca requirement for second cycle production in brown laying hens is lower than the NRC recommendations of 3·96 g/hen/d, for 110 g/hen/d feed intake.

Particle size did not significantly affect the performance parameters. Oliveira *et al.* (2002) found that limestone particle size did not influence the performance of laying hens. The substitution of 40% fine limestone with large limestone did not affect performance in brown laying hens in the late production period (Safaa *et al.*, 2008). Cheng and Coon (1990) reported that 6 different limestone particle sizes had no effect on egg production and egg weight. The study carried out by Faria (2002) also did not observe significant effects of dietary Ca concentrations or particle size on egg weight. Ito *et al.* (2006) stated that feed intake was not affected by limestone particle size from 0·5 to 3·0 mm. De Witt *et al.* (2009*a*) reported that different particle sizes (<1·0 mm, 1·0–2·0 mm and 2·0–2·8 mm) had no effect on egg production. Similar results have also been reported by Scheideler (1998). The results of the aforementioned studies all confirm the results of this study. Rao *et al*. (1992) reported that the substitution of 2/3 fine limestone by large limestone particles has been applied for several years in the poultry industry, but the partial substitution of fine limestone by larger particles in layers may not promote better performance or eggshell quality under optimal conditions. The authors suggest that large limestone particles may be advantageous under adverse conditions, in which Ca intake or availability is reduced.

The results of the present study showed that different Ca concentrations, particle sizes and their interactions had no significant effect on eggshell weight and eggshell thickness, but interaction between Ca concentrations and particle size had a significant effect on eggshell breaking strength ($P < 0{\cdot}01$). With increased particle size, a positive effect was observed on eggshell breaking strength for the groups fed with the Ca-L diet (30 g/kg). Roland (1986) reported that the replacement of a portion of the ground limestone with oyster shell was more likely to increase eggshell quality when diets were low in Ca, than for those that had adequate concentrations of Ca. Pelicia *et al.* (2009) reported that interaction between Ca concentrations and particle size had no significant effect on eggshell percentage and eggshell thickness. Rao *et al.* (2003) observed that eggshell thickness was not affected by increasing Ca intake from 32·5 to 45 g/kg of the diet. Safaa *et al*. (2008) reported that brown laying hens in the late phase of production required more than 35 g/kg Ca in the diet, and that the substitution of 40% fine limestone with coarse limestone or oyster shell had little impact on eggshell quality. Particle sizes have to be greater than 1·0 mm for selective retention in the gizzard (Zhang and Coon, 1997) and have to be between 1–4 mm to positively influence eggshell quality (Nys, 1999). According to Dekalb (1998), one third of laying hens' dietary Ca should be supplied in large particle form (2–5 mm). Similarly, Lichovnikova (2007) recommended that at least two-thirds of the Ca should be included as large particles to maintain eggshell quality in the last third of the laying period. Skrivan *et al.* (2010) reported that limestone particle size alone had no effect on shell breaking strength. According to some researchers, larger limestone particles had generally an ameliorating effect on eggshell strength (Pavlovski *et al.*, 2003; Koreleski and Swiatkiewitz, 2004; Lichonikova, 2007). In the present study, the positive effect of large particle size on the eggshell breaking strength was more pronounced when the dietary Ca concentration was low. However, the positive effect of fine particle size on the eggshell breaking strength was more pronounced when the dietary Ca concentration was high. As a result, if the dietary calcium concentration is low, the particle size of limestone should be large; but if the dietary calcium concentration is high, the particle size of limestone should be fine. Nys (1999) stated that large particles of Ca improved eggshell quality especially when given to hens towards the end of the laying period, when they were fed low to medium concentrations of dietary calcium. Some researchers stated that the usage of a large particle size for nutrition of older laying hens may have a positive effect on eggshell quality (Scott *et al.*, 1971; Roland, 1986; Zhang and Coon, 1997).

According to the results of the present study, the different Ca concentrations, particle sizes and their interactions had no significant effect on eggshell Ca or Mg contents; but dietary Ca concentrations had a significant effect on P content. De Witt *et al.* (2009*a*) found no differences in eggshell total Ca content due to limestone particle size in diets. Lichovnikova (2007) reported that eggshell Ca content was not influenced by the different dietary particle sizes. According to the results of the present study, the increase in dietary Ca concentration was accompanied by an increase in eggshell P content (1·32–1·46 mg/g). There are limited studies on the effects of dietary Ca concentrations on eggshell P content. Phosphorus and Mg are known to be very important minerals during the formation of eggshell. Cusack *et al.* (2003) reported that there is a greater increase in Mg concentration in the outer region of eggshells from older hens. Phosphorus occurs in the outer quarter of the eggshell and rises to termination.

In the present study, shear stress was found to be lower in groups fed with fine particle size than in the other groups. In this case, fine particle size had a negative effect on this parameter. The group fed with Ca-H (42 g/kg Ca) showed a positive effect on shear stress. The highest shear force was observed for the group fed with Ca-L*Medium in the diet. Also, the lowest shear force was observed for the group fed with Ca-L*Fine in the diet. According to this result, when the Ca concentration in the diet is low, the particle size should preferably be medium or large. When the Ca concentration of the diet is normal or high, the particle size was found to have no effect on the shear force. The results of the present study showed a negative relationship between dietary Ca concentrations and tibia Ca, P or Mg content. However, a positive relationship was found between increasing particle size, and tibia Ca or Mg content. There was an interaction between Ca concentrations and particle size that affected tibia P content. As a result, if the dietary calcium concentration is low, the particle size of limestone should be fine; but if the dietary calcium concentration is normal or high, the particle size of limestone should be medium or large. Koutoulis *et al.* (2009) reported that increasing Ca concentrations from 35 to 40 mg/kg significantly increased tibia breaking strength in brown laying hens at 72 weeks of age. Increasing the dietary Ca concentration resulted in bone strength increasing from 8·27 to 12·52 kg-f/cm^2 (Narvaez-Solarte *et al.* 2006). This result was similar to that of Roland *et al.* (1996), who reported that increasing dietary Ca concentration linearly increased bone strength. This is because hens can use dietary Ca to save Ca from bones when Ca intake is adequate (Farmer *et al.*, 1986). Cheng and Coon (1990) and Guinotte and Nys (1991) reported that larger limestone particles resulted in a higher tibia bone breaking strength than ground limestone, especially during the later (66) weeks of age. De Witt *et al.* (2009*b*) reported that an increase in limestone particle size resulted in an increased tibia breaking strength and stress in laying hens at 70 weeks of age.

As a result, the dietary Ca concentrations and particle sizes had no significant effect on performance parameters. The medium or large particle sizes of limestone had a positive effect on eggshell breaking strength and shear force of the tibia when the diet was low in Ca (30 g/kg). However, these positive effects of medium or large particle sizes on eggshell breaking strength and shear force of the tibia were not observed in hens fed with normal (36 g/kg) or high (42 g/kg) Ca concentrations. In conclusion, the moulted brown laying hens should be fed with 36 g/kg Ca and a medium limestone particle size (2–5 mm) in the diet to maintain performance, eggshell and bone quality.

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