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Intestinal Calcium Uptake, Shell Quality and Reproductive Hormones Levels of Three Laying Hen Varieties after Prolonged Egg Production

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Abstract: Hy-Line W98, W36, and Brown hens are among the most popular strains for commercial egg production. It is of interest to evaluate the differences in performance of old hens of each variety. As part of a larger study, hens of the three varieties were maintained under production longer than 100 weeks of age and hens of each strain were randomly selected for *in vitro* calcium uptake (CaT) by duodenal cells. Blood samples were collected 4-6 hours prior to oviposition via brachial vein cannulation for plasma estrogen (E_2), Luteinizing hormone (LH), and progesterone (P_4) determination. Hens were then euthanatized by cervical dislocation and CaT determined. The data were analyzed as a completely randomized design, using the General Linear Model procedure (SAS, Proc GLM, 2001), with strain as a fixed treatment and hen as the experimental unit nested with strain. For W36 hens, CaT was significantly greater than that found in Brown hens (P=0.05) while for W98 did not differ from W36 and Brown strains. P_4, E_2 and LH concentrations were not significantly different in the three varieties of laying hens. Based on the higher CaT by duodenal cells, these results suggest a higher efficiency of the W36 hens in calcium regulation during the terminal phase of egg production (more than 100 weeks of age) compared to the Brown and W98 hens. However, when specific gravity and thickness of shells were compared, the W36 appeared to have no advantage over W98 (P=0.460 and 0.684) or Brown (P=0.197 and 0.941) hens, respectively.

Key words: Calcium uptake, laying hens, reproductive hormones, shell quality

Introduction

It is widely acknowledged that eggshell quality is affected by many factors, such as diseases, nutritional status of the flock, heat stress and age (Roberts, 2004). Economic losses because of poor shell quality around the world are estimated at approximately $500 million per year (Etches, 1996).The deposition of calcium carbonate into the shell requires concentrations of calcium in the shell gland fluid 4 to 12 times higher than in blood; the greatest amounts of calcium are obtained from blood, bones and the gastrointestinal tract (Etches, 1995, 1996). In the domestic hen, the shell gland extracts 2-2.5g of calcium from the blood and transfers the element, without accumulation, to the egg over a period of 15 hours (Eastin and Spaziani, 1978). Intestinal calcium uptake plays an important role in providing the amount of calcium required to perform this task and in some instances have been observed a lower efficiency in calcium metabolism as the hen ages (Al-Batshan *et al*., 1994). Estrogens (E_2) have a complex relationship with calcium metabolism and have been shown to increase serum calcium by increasing the density of parathyroid hormone (PTH) receptors in kidneys and indirectly increasing the renal activity of 1-α-hydroxylase (Castillo *et al*., 1977). The 1-α-hydroxylase is believed to be responsible for formation of 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] (Martz *et al*., 1985), which is able to mediate intestinal absorption of calcium and phosphorus (Bar *et al*., 1978) by a saturable trans-cellular route and increase the concentration of calcium binding protein in the target cells. As the hen ages, the number of eggs produced declines along with a reduction in eggshell quality, estrogen levels, and estrogen receptor population (Hansen *et al*., 2003). The same response has been observed in laying hens exposed to heat stress. Also, it has been reported that E_2 implants improve calcium uptake throughout the intestine (Forman *et al*., 1996; Hansen, 1998; Hansen *et al*., 2003; Beck and Hansen, 2004). The increase in egg size that age brings along with insufficient calcium carbonate secretion consequently results in a reduced thickness of the eggshell (Etches, 1996). The effect of genetic factors on calcium metabolisms have been reported by differences in bone development and bone characteristics (Whitehead, 2004). Numerous studies have been conducted to address poor eggshell quality but few studies have compared differences in intestinal calcium uptake and eggshell quality relative to hormone profiles in older laying hens of different strains. The objective of this study was to compare hens of the Hy-Line strains W36, Brown, and W98 for intestinal calcium uptake rate, eggshell quality (specific gravity and eggshell thickness), and physiological levels of reproductive

hormones after prolonged egg production (more than 100 weeks of age).

Materials and Methods

Birds: As part of a larger study, Hy-Line strains Brown, W36 and W98 were maintained in production longer than 100 weeks of age. They were provided water and a layer diet (2,947 ME Kcal/kg feed, 3.8% Ca, 0.5% P, 17.0% protein) ad libitum, and maintained at an average temperature of 22°C in cages of 22"L x 24"H x 16.5"W with 4 hens per cage for each strain (90.75 square inches of space per bird). There were approximately forty cages where the strains were randomly allocated in the hen house. The photoperiod consisted of 16L: 8D. Fifteen hens per strain were randomly selected from the hen house (none hen came from the same cage) for *in vitro* calcium uptake rate (CaT) by intestinal duodenal cells. All animal and experimental procedures were conducted with approval of the University of Nebraska-Lincoln IACUC.

Sampling protocol: Blood samples were collected 4-6 hours before oviposition throughout the brachial vein using 5-ml sterilized syringes with a 22-gauge x 1 inch needle. Blood was transferred into 15 x 85 mm heparinized tubes. Immediately plasma was separated from the red blood cells by centrifugation at 2,000 x g. Saturated Na citrate was added to the plasma samples (20µl/ml) to prevent further clotting (Novero *et al.*, 1991). The samples were stored at -20°C until they were assayed for estrogen (E_2), progesterone (P_4) and LH. Hens were euthatized by cervical dislocation shortly before oviposition (as determined by abdominal palpation) at approximately the same time each day, and immediately a 3-cm segment from the mid-duodenal loop was cut into six thin slices (1.5cm x 2mm wide) and *in vitro* calcium transport was then conducted as described by Al-Batshan *et al.* (1994) with a slight modification of tissue incubation time.

Calcium uptake: Six thin slices of duodenum tissue (approximately 1.5 cmm x 2 mm wide) were taken from the loop and incubated in disposable beakers containing 2.0 ml of calcium transport buffer (CaTB): 140 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES at pH 7.40[3] for 10 min at 37°C (5 mM glucose and 0.5 mM $CaCl_2$ were added the same day of assay). After incubation in CaTB, the assay was begun by transferring the tissues to identical beakers containing CaTB and ^{45}Ca (25,000cpm/100µl).The tissues were incubated for 4 (3 tissue slices per bird) and 9 (3 tissue slices per bird) minutes at 37°C in a shaking water bath. The reaction was terminated by transferring the slices at 10-second intervals to beakers containing 4 ml of 300 mM mannitol. $^{45}Calcium$ was extracted from the tissue in 2 ml of 2.5% trichloroacetic acid (TCA) for 60 min at 37°C in a shaking water bath. The tissue samples were weight after extraction and recorded and the supernatant was poured off into 15 x 85 mm test tubes and centrifuged for 5 min at 500 x g. One ml of the supernatant was then pipetted into a 20-ml scintillation vials; 6 ml of EcoLite[4] scintillation cocktail was added; and the radioactivity of ^{45}Ca was counted in a ß-counter[5]. Data were calculated as the rate of calcium uptake by duodenal tissue (CaT) and was expressed and analyzed as a rate (nmol/g/min) or as total calcium (nmol/g). Calcium uptake at 9 minutes (Ca 9 min) is calculated as follows: [66nM Ca x CPM of sample (from nine minute incubation period) ÷ (Total CPM of isotope buffer) x 2 ÷ mg of tissue]. Rate of calcium uptake was calculated by subtracting the calculate calcium uptake at the four minute incubation period (Ca 4 min) from the calculated calcium uptake at the nine minute incubation period and dividing by five [(Ca 9 min-Ca 4 min) / 5].

Shell quality: For eggshell quality, specific gravity (SG) and eggshell thickness were determined. Specific gravity (SG) is a non-invasive method to determine eggshell thickness and, therefore, eggshell quality. The SG of an egg is equal to the egg's density relative to water. To perform the SG determination, we immersed the eggs in a series of increasingly concentrated salt solutions until the eggs floated on the surface of one of the solutions. The saline solutions with a SG ranging from 1.064 to 1.100 in increments of 0.05 were used following the floatation method described by Hamilton (1982).

Hormone analysis

P_4 Radioimmunoassay: Progesterone was assayed by radioimmunoassay (RIA) validated for the chicken at the Animal Science Physiology Laboratory, University of Nebraska-Lincoln. The methods of the assay to determine plasma progesterone concentrations have been described by Roberson *et al.* (1989). For validation of the assay with chicken plasma, recovery of added mass (7.8 and 15.6pg) from 10 µl of plasma from four independent samples, averaged 115 ± 4.7%. Assay determination of 10, 12.5, and 15 µl of sample from each of nine independent samples were highly correlated (10 and 12.5 µl, t = .986; 10 and 15 µl, t = .960; and 12.5 and 15 µl, t = .983). The intra - and interassay coefficients of variations were 4.3 and 6%, respectively.

E_2 Radioimmunoassay: Estrogen was assayed by RIA validated for the chicken at the Animal Science Physiology Laboratory, University of Nebraska-Lincoln. Radioimmunoassay for E_2 was validated as follows for chicken plasma at the Animal Sciences Physiology Laboratory, University of Nebraska. Duplicate aliquots (6.6 µl) of sample were extracted twice with 2 ml of

diethyl-ether, and extract residues were subjected to E_2 RIA as described by Kojima *et al.* (1992). The assay utilized an antiserum to E_2 at a dilution of 1:1,600.000 (Lilly lot #022367) provided by N. R. Mason[6]. Pooled avian plasma samples (n = 4) were assayed at 100, 50, 25, and 12.5 µl. Four pools of avian plasma were used to determine recovery of added E_2 (0.2, 1.6, and 12.8 pg). Recovery ranged from 76.1 to 111.8%, averaging 87.85±10.65%. Parallelism was determined by using the Allfit program (DeLean *et al.*, 1978). Slopes of the dilutions of plasma and the standard curve were not different as determined by the Allfit program (P = 0.2610). The intra- and interassay coefficients of variation were 5.4 and 8.2%, respectively.

LH radioimmunoassay: LH RIA assay was conducted as previously described (Johnson *et al.*, 1985). This assay protocol was provided by Dr. John S. Proudman[7]. The correlation coefficient was 0.999653 for volumes of 300 µl, 200 µl, 100 µl, 50 µl, and 25 µl. Slopes of dilutions of plasma and the standard curve were not different (P>0.1). The intra- and interassay coefficients of variation were 3.9 and 5.1%, respectively.

Statistical analysis: The data were analyzed as a completely randomized design, using the General Linear Model procedure (SAS, Proc GLM, 2001), with strain as a fixed treatment and hen as the experimental unit nested with strain. The model for the design is:

$$Yij = \mu + \alpha i + \Sigma ij$$

I = 1,2,3 (strain) j = 1,2,3..15. (replications).

Where: Yij is the variable of interest for the j observation strain I, µ is the overall mean, ai is the strain effect, and Σij is the experimental error. To use this model, we must assume that $\Sigma ij \sim N(0, \sigma^2)$. The differences among means were determined by Fisher's protected least significant difference (LSD) with a level of significance of $\alpha = 0.05$.

Results

Both rate and absolute amounts of intestinal calcium taken up were greater in the W36 hens compared to the other two strains (P<0.05; Table 1). Plasma concentrations of P_4 and LH were the same among the three strains (P>0.10; Table 1). Essentially the same pattern was observed for E_2 plasma concentrations (P>0.10), however, there was one lost sample for the W36 birds. Both specific gravity of eggs and eggshell thickness were essentially the same in all three strains (P>0.10; Table 1).

Discussion

Genetic strain has a direct effect on egg size and eggshell quality; in this case, eggs of Brown hens were larger than those of W36 but not detectably larger than those of W98. Regardless, each hen has a physiological limit to the amount of calcium she can absorb from intestine. For any strain, as hen age, the ability to build skeletal mass and to absorb calcium decreases (Hansen *et al.*, 2003). In this study, all three strains were well past the typical laying age of commercial hens, though all were still in production, laying at a rate of about 40%. The hens had not been molted, though it is likely that many of them had experienced at least a partial spontaneous molt. Based on the calcium uptake data obtained, the W36 hens retained a higher capacity for calcium absorption at the cellular level than the other two strains, even though circulating E_2 did not differ. However, it is of interest that the apparent advantage with regard to calcium transport did not transfer an advantage to the shell, since the specific gravities and shell thicknesses were all equivalent. The mechanism behind this observation warrants further investigation, with focus on estrogen induced enzymatic (1α-hydroxylase) activity in the kidney and on 1α-hydroxylase induced estrogen receptor regulation at the gut level. The skeletal integrity of hens of this age should also be examined to determine whether there is an interaction between bone calcium and the ability of the gut to transport calcium. It is possible that the Brown hens because of its greater size and body weight have an strong bone structure and higher amount of calcium stored or accumulated in bone, which allows these birds to have an adequate source of calcium for eggshell formation without having a higher intestinal calcium uptake rate. This statement agree with the results reported by Riczu *et al.* (2004), who found that Brown strains at the end of cycle had a higher bone breaking strengths than the white strains, however, in this study Brown strains had also a higher specific gravity than the white strains. Shell formation occurs during the night when supply of calcium from the digestive tract is usually low, so it is possible that a high proportion of shell calcium comes from medullary bone. The difference in intestinal calcium uptake observed in this study could be also related to the higher reduction in egg production with age that have been reported in the Brown hens (Renema *et al.*, 2001) and that reduction may allow the Brown hens to build up a better calcium reserves and improve calcium utilization from medullary bone to sustain eggshell quality. In general, the values obtained for egg shell quality (specific gravity and egg shell thickness) correspond or are in relation with the values found in old layers (Riczu *et al.*, 2004). Both calcium uptake rate and total calcium uptake data were similar to those reported by Hansen *et al.* (2004) at thermoneutral condition for W36 birds at 40 to 46 wk of age, which may indicates a lower decline on calcium uptake rate over time (age) for this particular strain. Plasma concentration of luteinizing hormone, progesterone, and estrogen levels were similar to those reported by Etches (1990), 4-6 hours before oviposition. In conclusion, the

Table 1: Intestinal calcium uptake, reproductive hormone levels, and eggshell quality

Variable[1]	Browns	W36	W98
Calcium uptake rate (nMol/g/min)	(N=15) 38.13±7.85^{B}	(N=15) 81.95±8.12^{A}	(N=15) 48.00±7.85^{B}
Total calcium uptake (nMol/g)	869.7±44.7^{B}	1060.97±46.3^{A}	967.13±44.7A^{B}
Progesterone (ng/ml)[2]	(N=15) 1.53±0.2^{A}	(N=15) 1.69±0.2^{A}	(N=15) 1.81±0.2^{A}
LH (ng/mL)[3]	(N=15) 3.62±0.5^{A}	(N=15) 3.66±0.5^{A}	(N=15) 3.61±0.5^{A}
Estrogen (pg/mL)[4]	(N=15) 462.39±30.3^{A}	(N=14) 458.73±31.4^{A}	(N=15) 453.07±30.3^{A}
Specific gravity[5]	(N=71) 1.07514 ± 0.003^{A}	(N=71) 1.0757 ± 0.003^{A}	(N=71) 1.07521 ± 0.003^{A}
Eggshell thickness (mm)[5]	(N=63) 0.33 ± 0.004^{A}	(N=63) 0.3285 ± 0.004^{A}	(N=63) 0.3346 ± 0.004^{A}

[1]The values are expressed as a mean plus/ minus standard error of mean; row means followed by same capital letters are not significantly different (P=0.05) based on Fisher's protected LSD test. [2]Plasma progesterone concentrations in laying hens. [3]Plasma luteinizing hormone concentrations in laying hens. [4]Plasma estradiol concentrations in laying hens. [5]Specific gravity of the egg. [6]Egg shell thickness in millimeters.

W36 hen, at >100 weeks of age and still in lay, appears to be more efficient with regard to the ability to sustain calcium uptake by the cells of the duodenum even though circulating E_2 and eggshell thickness are about the same as in the other two strains (W98 and Brown).

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